PULSE FLUORIMETRY OF 1,6-DIPHENYL-1,3,5-HEXATRIENE INCORPORATED IN MEMBRANES OF MOUSE LEUKEMIC L 1210 CELLS

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1. Introduction

During the past few years, a very large number of investigations was devoted to the dynamics properties of cell membranes. Dynamic studies of lipid bilayers were done either by the use of spin label or fluorescence depolarization techniques. Amongst various fluorescent probes, 1,6-diphenyl-1,3,5-hexatriene [1] was the most widely used. DPH is easily introduced into membrane bilayers from aqueous dispersions, in which it is not fluorescent [2]. On the basis of phase fluorometric measurements in a homogeneous reference oil, this probe was reported to exhibit a single exponential decay of the emission and a linear Perrin plot [3] e.g., fluorescence intensity and lifetime are in a constant ratio when temperature changes [4]. These results were confirmed by direct measurements of the decay of both intensity and emission anisotropy of DPH in a different oil, using a pulse fluorimeter [5]. However, very recently, nanosecond pulse fluorescence studies of DPH incorporated into L-α-dimyristoyl lecithin vesicles [6] and into DL-α-dipalmitoylphosphatidyl choline vesicles [7] showed that the decay of the total fluorescent emission and the decay of the emission anisotropy could not be described adequately in terms of single exponential decay laws. Therefore, when DPH is embedded in vesicles, the Perrin relationship is no longer valid, and steady state polarization measurements cannot be interpreted in terms of microviscosity.

The complex decay could be interpreted either in terms of microheterogeneity of sites for the probe in the bilayer or in terms of anisotropic rotation of DPH in the bilayer. One possible interpretation is that the probe undergoes rapid but restricted rotation in a cage formed by the fatty acid chains, as was suggested for spin labels in lipid bilayers [8]. So, in contrast with steady state depolarization data, nanosecond time dependent depolarization measurements reveal the complex rotational motion of fluorescent probes in lipid bilayer vesicles and provide information on both their fluidity and structure. We report here the first study of the emission anisotropy of DPH embedded in membranes of intact cells using nanosecond pulse fluorimeters.

2. Materials and methods

2.1. *Cell culture*

Mouse leukemic L 1210 cells, adapted to grow in suspension culture, were grown at 37°C with a humid atmosphere of 95% air and 5% CO_2 in Eagle's minimum essential medium (MEM). Cell density was never allowed to exceed 10^6 cells/ml.

2.2. *Fluorescence labelling of cells*

Cells (10^6 cells/ml) were labelled in the conditions of [3]. A tetrahydrofuran solution of 2×10^{-3} M 1,6-diphenyl-1,3,5-hexatriene DPH (Koch-Light Ltd, England) was diluted 1000-fold by injection in phosphate buffered saline (PBS), pH 7.4, with vigorous shaking. The DPH solution was stirred for 15 min at 25°C and was added to 1 vol. cell suspension (10^6 cells/ml). Maximum DPH incorporation was reached within 30 min at 25°C. Cells were sedimented by

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centrifugation (1200 × g, 10 min) and suspended in PBS.

2.3. *Fluorescence steady state measurements*

Excitation and emission spectra were recorded with a double-beam FICA spectrofluorimeter. Steady state depolarization measurements were recorded with an Elscint MV-1 fluoropolarimeter equipped with Glan-Thompson polarizers, a 366 nm band-pass filter on the excitation beam, and cut-off filters for wavelength below 390 nm on the emission beams. The steady state emission polarizations (P) were calculated according to the formula:

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

with an accuracy of ± 0.003.

2.4. *Nanosecond time-resolved emission measurements*

The time courses of total emission and of the emission anisotropy were obtained in a single photon counting fluorimeter [9]. An MTO 336 nm band pass interference filter (bandwidth 4 nm) selected the excitation wavelength from a hydrogen lamp; an MTO 463 nm band pass interference filter (bandwidth 11 nm) was used on the emission beam. The excitation beam was vertically polarized by a Polaroid polarizer. The emission beam passed through a Polaroid polarizer which alternatively selected the vertical and horizontal components of the fluorescence emission. Each of the 2 components was recorded in 2 separate parts of a multichannel analyzer. From the horizontal and vertical components the decay curves

$$s(t) = I_V(t) + 2I_H(t)$$

and

$$d(t) = I_V(t) - I_H(t)$$

were calculated. The response function $g(t)$ of the apparatus was determined with 1,1,4,4-tetraphenyl butadiene in cyclohexane (10 mg/l, τ 1.76 ns, 25°C) [10]. Observed fluorescence decay $f(t)$ is defined as the convolution product (*) of $g(t)$ and of the actual fluorescence decay $F(t)$, [$S(t)$, $D(t)$] as $f(t) = g(t) \circledast F_t$ [10]. This relationship can be solved for $S(t)$ assuming that the decay curve is a sum of exponential terms:

$$S(t) = \sum_i C_i \exp^{-t/\tau_i}$$

where C_i and τ_i are the amplitude and the lifetime respectively of the fluorescence of molecules i. The parameters C_i and τ_i were computed assuming either a mono or a double exponential decay. Calculations were done using the modulation function method [11]. The fit [12] between the experimental decay curve $f(t)$ and the computed curve $fc(t)$ was evaluated using the deviation function [13] and the weighted mean residue defined by:

$$\frac{1}{N}\sum_{i=1}^{N} \frac{(f(i) - fc(i))^2}{f_i}$$

where N is the numbers of points of the curve $f(t)$.

The time course of total emission $S(t)$ and of emission anisotropy $R(t) = D(t)/S(t)$ were determined. Assuming that $R(t) = \Sigma\alpha_i \exp - t/\theta_i$ and trying systematically given values of α_i and θ_i the convolution products $d'(t)$ were calculated:

$$d'(t) = g(t) \circledast (R(t) \cdot S(t))$$

and compared with the experimental data. Values of α_i and θ_i giving the best fit were selected as the actual ones.

Time course fluorescent measurements were carried out at 25°C during 4 h, with 5 × 10^5 cells/ml, every 20 min, the cell suspension was gently mixed. Experimental data were corrected for light-scattering by data obtained with unlabelled cells.

3. Results and discussion

3.1. *Fluorescence spectra of DPH embedded in L 1210 cell membranes*

The fluorescence spectra of DPH embedded in L 1210 cell membranes and of DPH dissolved in tetrahydrofuran and in cyclohexane are shown in fig.1. The structure of the excitation and emission spectra and also the position of maxima and minima are quite identical. Therefore, the fluorescence of DPH incorporated into cells should be located in the hydrocarbon regions

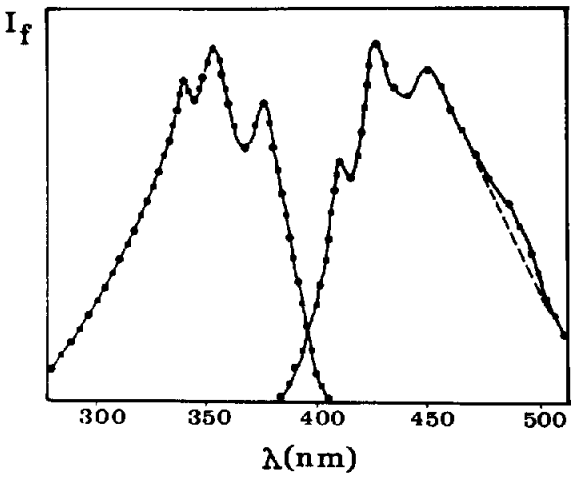

Fig.1. Normalized excitation and emission spectra of DPH incorporated into L 1210 cells (– –) of DPH solution in tetrahydrofuran (-•--•-) and in cyclohexane, at 25°C. Excitation spectra recorded with an emission wavelength of 463 nm; emission spectra were recorded with an excitation wavelength of 336 nm.

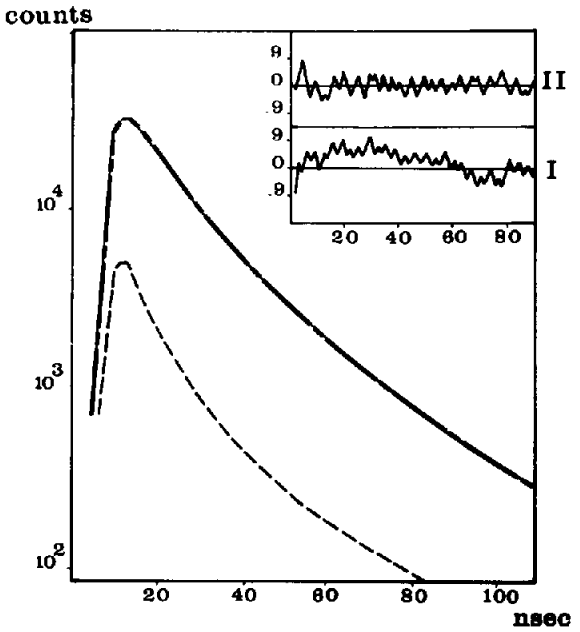

Fig.2. The experimental fluorescence decay curves of the total emission $S(t) = I_V(t) + 2I_H(t)$ (——) and of the difference emission $D(t) = I_V(t) - I_H(t)$ (- - -). Excitation wavelength, 336 nm; emission wavelength, 463 nm; bandwidths, 4 nm and 11 nm, respectively. *Insert*: The deviation function between the experimental curve and the convolved theoretical curve for S(t) assuming a single-(I) or a double-(II) exponential decay law.

[1,3,14]; the actual concentration of DPH in the cells was low enough to avoid any spectrum disturbance, and the measured fluorescence should only come from DPH embedded in lipid bilayers. In these conditions, the light scattering did not preclude fluorescence measurements.

3.2. *Decay of the total fluorescence*

The experimental decay curve for the total fluorescence s(*t*) and the experimental difference curve d(*t*) are shown in fig.2. The residuals between the experimental decay curve and the computed one indicate that the fluorescence is not in agreement with a single exponential decay law (I). A more acceptable fit is obtained in terms of a double-exponential decay law (II).

The fluorescence decay parameters for DPH incorporated into L 1210 cells are summarized in table 1. The data could not be analyzed in terms of a single exponential decay law because the fit parameter was not good (high value). An acceptable analysis was obtained in terms of a double-exponential decay law, the fit parameter was very good (low value). So, DPH molecules have, at least, 2 main types of environment: the major one leads to a lifetime of 9 ns, close to the lifetime of DPH (9.8 ns) in an isotropic medium such as in liquid paraffin [7]; the second one leads to a short lifetime of 4.5 ns, close to the lifetime (3.5 ns) in glycerol. It should be noted that the fraction of the short-lived component is quite high; for comparison, with DPH embedded in dimyristoyl lecithin vesicles only 13% at 37°2 had a short lifetime (3.9 ns) and 22% at 14°8 (5.9 ns) [6].

3.3. *Decay of the fluorescence emission anisotropy*

The experimental decay of the emission anisotropy R(*t*) for DPH incorporated in L 1210 cells is shown in fig.3. The data could not be analyzed in terms of a single-exponential decay law but could be satisfactory represented in terms of a double-exponential decay law

$$R(t) = R_0 [\alpha_1 \exp - t/\theta_1 + \alpha_2 \exp - t/\theta_2]$$

Table 1
Decay of the total fluorescence S(t) for 1,6-diphenyl-1,3,5-hexatriene in membranes of L 1210 cells, at 25°C

Analysis	τ_1 (ns)	τ_2 (ns)	C_1	C_2	$<\tau>$ (ns)	Residue
I[a]	7.66	–	1	–	7.66	91.7
II[b]	4.5	9.0	0.42	0.58	7.09	2.89

[a] I, analysis in terms of a single-exponential decay law:

$$S(t) = C_1 \exp - t/\tau_1, \text{ with } C_1 = 1$$

[b] II, analysis in terms of a double-exponential decay law:

$$S(t) = C_1 \exp - t/\tau_1 + C_2 \exp - t/\tau_2, \text{ with } C_1 + C_2 = 1$$

τ_1, τ_2, lifetime; C_1, C_2, fractional amplitude; $<\tau>$, average lifetime = $\sum_i C_i \tau_i$; residue, average weighted residue

where R_0 is the value of emission anisotropy at $t = 0$; α_1 and α_2 are the relative amplitude with $\alpha_1 + \alpha_2 = 1$, θ_1 and θ_2 correlation times related to the rotational correlation time of the Brownian motion of the fluorophore. θ_1 was found to be 2.6 ns, $\theta_2 = \infty$ and $\alpha_1 = 0.67$, so the emission anisotropy is satisfactorily represented in terms of a single-exponential decay plus a constant: $R_\infty = R_0\ \alpha_2$. The total amplitude R_0 was 0.218, so the amplitude $\alpha_1 R_0$ of the fast-moving component was 0.146 and the amplitude R_∞ of the low moving component was 0.072.

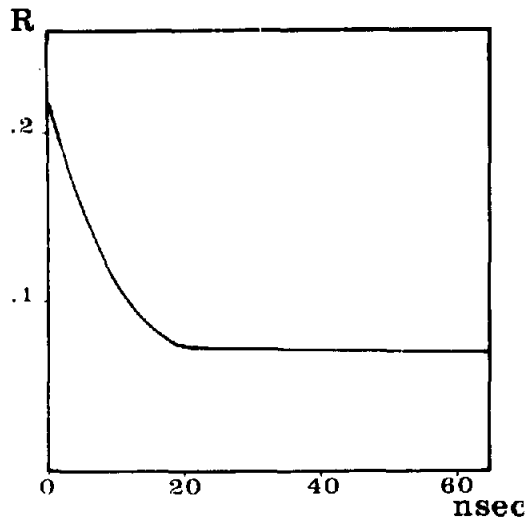

Fig.3. Decay of the emission anisotropy R(t) of DPH in L 1210 cells at 25°C.

From the values of S(t) and D(t), the mean steady state emission anisotropy $<r> = \int D(t)\ dt / \int S(t)\ dt$ was 0.114 and the polarization $P = 3 <r>/(2 + <r>)$ was 0.162. This last value is quite close to the steady state polarization: 0.160 ± 0.003.

Two different correlation times for DPH embedded in pure lecithin vesicles [6,7] have been interpreted in terms of microheterogeneity of sites for the probe or of anisotropic rotation of the probe in the bilayer [6]. Therefore, in a simple system such as lecithin vesicles or in a very complex one such as cell membranes, the orientation distribution of DPH incorporated in the hydrocarbon region is anisotropic even at equilibrium state: θ_1 represents the correlation time of the excited DPH molecules to approach the anisotropic equilibrium distribution, and R_∞ represents the degree of anisotropy in equilibrium distribution of DPH. The equilibrium anisotropy ratio R_∞/R_0 should correspond to the order parameter used in spin label studies [7]; and could be related to the cone angle ω as:

$$R_\infty/R_0 = [1/2 \cos \omega\ (1 + \cos \omega)]^2$$

related to a rapid tumbling of DPH within a cone [8]. The cone angle ω of DPH incorporated in L 1210 cells is about 47° indicating a restricted tumbling.

The mean orientation of the large molecular axis

of a probe like DPH is assumed to be perpendicular to the plane of the bilayer [15–17] and therefore DPH would be allowed to tumble in the lipid bilayer around a direction parallel to that of the lipid chains. The mean depolarization of DPH fluorescence depends upon this cone angle and also upon the correlation time θ and cannot be taken as a measure of membrane microviscosity.

4. Concluding remarks

The decays of the total fluorescence and of the fluorescence emission anisotropy for 1,6-diphenyl-1,3,5-hexatriene incorporated into membranes of mouse leukemic L 1210 cells are complex. Although DPH incorporated into cells is not only located in the plasma membranes but also in intercellular membranes [18,19], and although cell membranes are made of a large number of very different molecules (phospholipids, cholesterol, glycolipids, proteins, glycoproteins) the nanosecond time-dependent fluorescence depolarization is very similar to that of DPH in pure phospholipids vesicles [6,7].

DPH incorporated into cell membranes is characterized by 2 different lifetimes, and by 2 correlation times, a short one and a very long one. These findings should be interpreted either:

1. In terms of microheterogeneity of sites for the probe in a given bilayer [20].
2. In terms of anisotropic rotation of DPH in the bilayer [6,7], as in the case of lecithin vesicles.
3. In terms of preferential interactions with specific component of membranes or with specific domains.
4. In terms of microheterogeneity of sites for the probe in the various membranes of the cells.
5. In terms of excited state cis-trans isomerizations which occur with polymers such as trans-stilbene [21] or DPH [22,23]. This proposal may be thought unlikely because the fluorescence spectra of DPH embedded in cell membranes and of DPH dissolved in cyclohexane are identical.

So, in the case of DPH embedded in cell membranes as in the case of DPH embedded in pure lecithin vesicles, the average polarization obtained under continuous excitation cannot be simply taken as a measure of the membrane viscosity.

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