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Fluorescence decay of pyrene in small and large unilamellar L,α-Dipalmitoylphosphatidylcholine vesicles above and below the phase transition temperature*

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Abstract. The fluorescence decays of pyrene in small and large unilamellar *L*,α-dipalmitoylphosphatidylcholine vesicles have been investigated as a function of probe concentration and temperature. When the molar ratio of pyrene to phospholipid equals 1:3000, no excimer emission is observed and the fluorescence decays are mono-exponential. When this ratio is equal to or higher than 1:120, excimer formation is observed.

Above the phase transition temperature the observed fluorescence decays of monomer and excimer can be adequately described by a bi-exponential function. The monomer decays can be equally well fitted to a decay law which takes into account a time-dependence in the probe diffusion rate constant. The fluorescence decay kinetics are compatible with the excimer formation scheme which is valid in an isotropic medium. The excimer lifetime and the (apparent) rate constant of excimer formation have been determined as a function of probe concentration at different temperatures above the phase transition temperature. The activation energy of excimer formation is found to be 29.4 ± 1.3 kJ/mol. In small unilamellar vesicles the diffusion constant associated with the pyrene excimer formation process varies from 8.0×10^{-7} cm^2/s at 40 °C to 2.2×10^{-6} cm^2/s at 70 °C.

Below the phase transition temperature the monomer decays can be described by a decay law which takes into account a time dependence of the rate constant of excimer formation. The lateral diffusion coefficient of pyrene calculated from the decay fitting parameters of the monomer region varies from 4.0×10^{-9} cm^2/s at 20 °C to 7.9×10^{-8} cm^2/s at 35 °C. No significant difference could be observed between the pyrene fluorescence decay kinetics in small and large unilamellar vesicles.

Key words: Fluorescence decay, pyrene, unilamellar vesicles, *L*,α-dipalmitoylphosphatidylcholine, excimer kinetics

Introduction

Excimer formation is an attractive tool for studying the dynamic behavior of biological and synthetic phospholipid membranes. The excimer forming probe, pyrene, is entirely solubilized in the bilayer phase (Vanderkooi et al. 1975). The lateral diffusion of pyrene dispersed in multilamellar dipalmitoylphosphatidylcholine vesicles above the phase transition temperature (50 °C) was first studied by Galla and Sackmann (1974a and b) by measurements of the monomer and excimer fluorescence intensities and "the single excimer lifetime". They assumed that the classic scheme (Birks et al. 1963, 1964) of excimer formation was valid in fluid membranes and that excimer formation was diffusion controlled. In their scheme, excimer dissociation was neglected. Vanderkooi and Callis (1974) described the fluorescence decays of pyrene in multilamellar dimyristoylphosphatidylcholine vesicles above the phase transition temperature (30 °C) by a model based on the general time dependent diffusion theory of Smoluchowski. They could not fit the results below the phase transition temperature to the three-dimensional time-dependent diffusion model. In a subsequent paper, Vanderkooi and coworkers (Vanderkooi et al. 1975) used a two-dimensional time-dependent diffusion theory (Owen 1975) to describe their experimental data (Vanderkooi and Callis 1974) and this gave a more reasonable value (3×10^{-8} cm^2/s at 30 °C) for the diffusion constant. Liu et al. (1980) studied the fluor-

Abbreviations: SUV, small unilamellar vesicles; LUV, large unilamellar vesicles; DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; FRAP, fluorescence recovery after photobleaching

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escence decay kinetics of pyrene in biological and synthetic membranes at one temperature above and one below the phase transition temperature. They found that the monomer decay could be adequately described by three exponential terms and not by the time-dependent diffusion theory. They proposed a complex reaction scheme involving excited state interaction but they did not prove its validity. Very recently, the excimer formation of 1-methylpyrene in dimyristoylphosphatidylcholine (DMPC) vesicles was studied above the phase transition temperature (Van den Zegel et al. 1984). The fluorescence decay kinetics at four different molar ratios of 1-methylpyrene to DMPC was studied in the 25°–70°C temperature range. It was shown that the classic scheme of excimer formation was valid above the phase transition temperature in these small unilamellar vesicles.

The present paper reports the results of a study of the concentration dependence of the fluorescence decay of pyrene in small and large unilamellar *L*,*α*-dipalmitoylphosphatidylcholine (DPPC) vesicles above and below the phase transition temperature.

Materials and methods

Chemicals

DPPC was obtained from Sigma Chemicals Co. Thin layer chromatography (solvent, chloroform: methanol: water 65:25:4; I_2 staining) showed a single spot. Pyrene was purchased from Aldrich and was purified by repeated recrystallization from ethanol. The purity was checked by chromatography and a fluorescence lifetime measurement in iso-octane (450 ns at room temperature). KCl (Aldrich Gold Label) was used as received. Chloroform (Fluka reagent grade), iso-octane (Aldrich Gold Label) and bi-distilled deionized water were used as solvents.

Membrane preparations

Small unilamellar vesicles (SUV) (Huang 1969; Suurkuusk et al. 1976; Barenholz et al. 1977; Wong and Thompson 1982) were prepared from DPPC as reported earlier (Van den Zegel et al. 1984). During the whole preparation the phospholipid sample was kept above the phase transition temperature. Large unilamellar vesicles (LUV) were obtained from the unilamellar vesicle dispersions by storing them for 35 days at 4°C (Wong and Thompson 1982).

Fluorescence decay experiments

Fluorescence decay curves were measured using a Spectra-Physics mode-locked, cavity-dumped, frequency-doubled, synchronously-pumped, R6G dye laser system (Spears et al. 1978; Koester and Dowben 1978) with time-correlated single photon counting detection (Demas 1983; O'Connor and Phillips 1984). The fluorescence lifetime apparatus and the associated optical and electronic components are described in detail elsewhere (Boens et al. 1984; Van den Zegel et al. 1984). Samples containing SUV were measured within 12 h after vesicle preparation. Before the samples were measured they were deoxygenated by flushing argon for 15 min. The vesicle preparations were contained in 1 cm optical pathlength quartz cuvettes closed with a two-way teflon valve. Experiments performed as a function of temperature were always done from high to low temperatures. The temperature of the samples was kept constant by circulating water through the cell holder. The temperature stability was better than 0.2°C.

Theoretical decay functions used to analyze the fluorescence decay data

To analyze the data, the experimental decays were fitted to several theoretical decay functions using a non-linear least-squares (McKinnon et al. 1977; O'Connor et al. 1979) decay fitting program based on Marquardt's algorithm (Marquardt 1963). The criteria used to judge the goodness of fit (Roberts et al. 1981) are the reduced chi-square (χ^2_ν), the Durbin-Watson test statistic, *d*, (Durbin and Watson 1950, 1951, 1971), the plot of the weighted residuals (R_i) versus channel number, and the autocorrelation function (C_n) (Grinvald and Steinberg 1974). The experimental fluorescence decays were analyzed using trial decay functions (Eq. (1)) containing one, two or three exponentially decaying terms.

$$i(t) = \sum_{i=1}^{n} A_i \exp(-\lambda_i t) \quad n = 1, 2, 3, \tag{1}$$

where $i(t)$ is the time-dependent fluorescence intensity. A_i are pre-exponential factors and $1/\lambda_i$ are decay times. The decays of the monomer were also analyzed according to Eq. (2) which takes into account a time-dependence in the diffusion controlled formation of excimers. This equation is based on the Smoluchowski diffusion theory (Smoluchowski 1917).

$$i_m(t) = C \times \exp(-At - B\sqrt{t}). \tag{2}$$

Recursion formulas derived by Liu et al. (1980) were used to determine A, B and C. The meaning of A and B is explained further in the text.

The experimental fluorescence decay curve, $I(t)$, is a convolution of the true decay, $i(t)$, with the measured pulse shape of the excitation, $E(t)$ (the instrument response function), i.e.,

$$I(t) = \int_0^t E(u)\, i(t-u)\, du. \tag{3}$$

When $I(t)$ and $E(t)$ are known, $i(t)$ may be determined by a variety of techniques (McKinnon et al. 1977; O'Connor et al. 1979).

Results

The fluorescence decay kinetics of pyrene in small (SUV) and large (LUV) unilamellar DPPC vesicles were studied in the temperature range 20°–70°C. Four different molar rations of pyrene to DPPC were examined, namely 1:50, 1:80, 1:120 and 1:3000. For the sample with pyrene/DPPC = 1:3000, no excimer emission was observed (Fig. 1a). At all temperatures, the fluorescence decays could be described adequately by a mono-exponential decay law. The F test (Ameloot and Hendrickx 1982; Jennrich and Ralston 1979; Draper and Smith 1966) indicated that no extra terms were required to adequately fit the data. The lifetimes as a function of temperature are shown in Table 1. All the other samples investigated showed excimer emission (Fig. 1b). At the same temperature a higher yield of excimer fluorescence was detected with increasing pyrene/DPPC ratios.

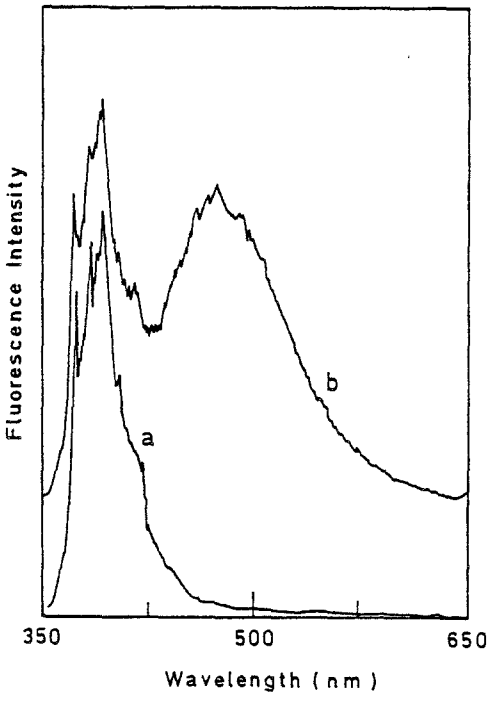


Fig. 1. Fluorescence spectra of pyrene in DPPC SUV.
a: pyrene/DPPC molar ratio 1:3000, λ_{exc} = 343 nm, 71°C.
b: pyrene/DPPC molar ratio 1:80, λ_{exc} = 343 nm, 50°C

Table 1. Fluorescence lifetime of pyrene in small unilamellar vesicles of dipalmitoylphosphatidylcholine as a function of temperature (Pyrene/phospholipid 1:3000), $0.7 < \chi_\nu^2 < 1.3$ for all experiments. The standard errors on the lifetimes are about 2%

T[°C]	τ_0[ns]	T[°C]	τ_0[ns]
20	370	50	333
25	368	55	324
30	368	60	314
35	356	65	304
40	350	70	287
45	342		

Above the phase transition temperature the fluorescence decays of the locally excited state could be fitted to a bi-exponential decay function and to Eq. (2). The excimer fluorescence showed a rise and a decay and could be fitted to a difference of two exponential terms. Below the phase transition temperature the decay of the locally excited state could be described by Eq. (2). Examples of monomer and excimer decay fitting results are given in Fig. 2.

Above the phase transition temperature the experimental decay curves in the monomer and excimer region for the samples with pyrene/DPPC 1:50, 1:80, 1:120 were fitted to a double – exponential decay law. The measured decay times in SUV are shown in Fig. 3. The decay times, $1/\lambda_1$ and $1/\lambda_2$, of the monomer region were within experimental error equal to those of the excimer region. For the molar ratios pyrene/DPPC 1:50. 1:80 and 1:120 the average ratios of the pre-exponentials A_3/A_4 (Eqs. (6) and (7)) were −1.0, −1.1 and −1.1 respectively. The standard errors on the pre-exponential factors were of the order of 15%. The decay times $1/\lambda_1$ and $1/\lambda_2$ decreased with increasing temperature in both the monomer and excimer region (Fig. 3). The long decay time, $1/\lambda_1$, decreased strongly with increasing probe concentration (Fig. 3). The contribution of the short decay time, $1/\lambda_2$, to the two-exponential decay in the monomer region was small compared with the relative contribution of the long decay time, $1/\lambda_1$ (Fig. 4). The pre-exponential factor of the short decay time, $1/\lambda_2$, increased with increasing probe/DPPC ratio and with increasing temperature (Fig. 4). The pre-exponential factors of the monomer region in SUV are given in Fig. 4. The decay times and pre-exponential factors for the sample pyrene/DPPC

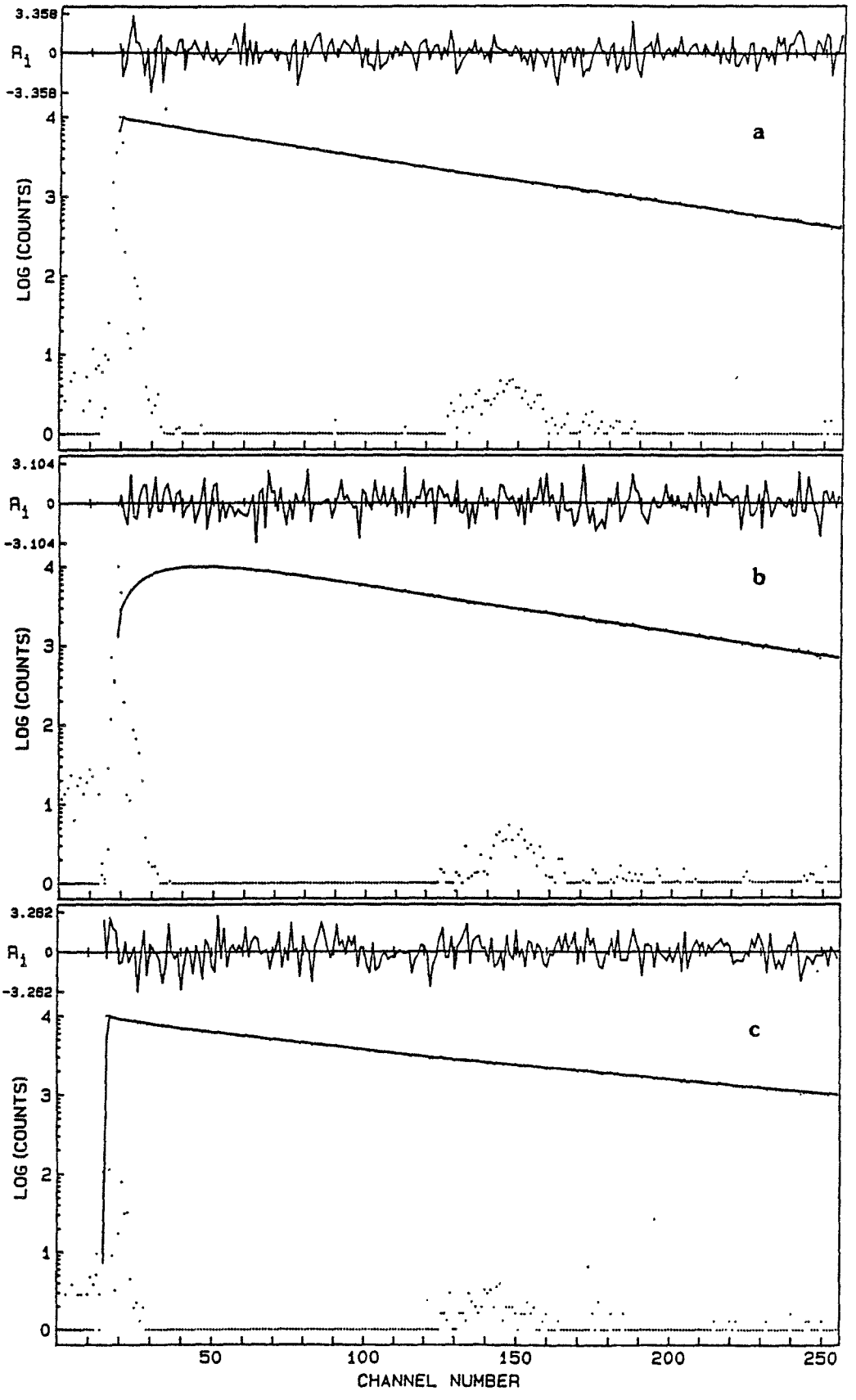


Fig. 2. a Experimental fluorescence decay curve (*point plot*) of the monomer region of pyrene in DPPC SUV. Analysis as a sum of two exponential terms (*solid line*). Pyrene/DPPC = 1:120, T = 55°C, λ_{exc} = 300 nm, λ_{em} = 386 nm, channel width = 1.94 ns, χ^2 = 1.04, serial correlation coefficient d = 1.87. Analysis according to Eq. (2) gave χ^2 = 1.09 and d = 1.79. **b** Experimental fluorescence decay curve (*point plot*) of the excimer region of pyrene in DPPC SUV. Analysis as a bi-exponential (*solid line*). Pyrene/DPPC = 1:120, T = 55°C, λ_{exc} = 300 nm, λ_{em} = 490 nm, channel width = 1.94 ns, χ^2 = 1.22, serial correlation coefficient d = 2.15. **c** Experimental fluorescence decay curve (*point plot*) of the monomer region of pyrene in DPPC SUV. Analysis according to Eq. (2) (*solid line*). Pyrene/DPPC = 1:120, T = 25°C, λ_{exc} = 300 nm, λ_{em} = 382 nm, channel width = 1.94 ns, χ^2 = 1.21, serial correlation coefficient d = 1.95. Analysis as a bi-exponential gave χ^2 = 1.44 and d = 1.70. The instrument response functions (*point plot*) and the residual plots are also shown. The parameters extracted from these decays are given in Figs. 3 and 4, and in Table 3

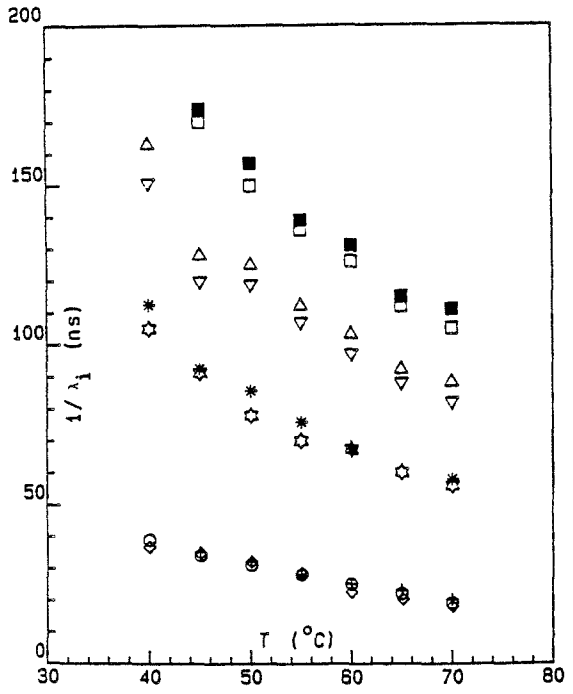


Fig. 3. Experimentally determined decay times of the monomer and excimer region of pyrene in DPPC SUV for different ratios (1:50, 1:80, 1:120) and at different temperatures above the phase transition temperature. Analysis as a bi-exponential. The standard deviations on the decay times are of the order of 10%.
For the monomer region: $i_m(t) = A_1 \exp(-\lambda_{1M} t) + A_2 \exp(-\lambda_{2M} t)$
For the excimer region: $i_e(t) = A_3 \exp(-\lambda_{1E} t) + A_4 \exp(-\lambda_{2E} t)$

	$1/\lambda_{1M}$	$1/\lambda_{1E}$	$1/\lambda_{2M}$ and $1/\lambda_{2E}$
1:120	■	□	+
1:80	△	▽	○
1:50	*	☆	◇

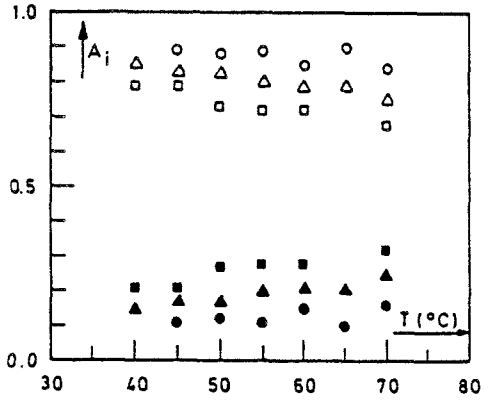


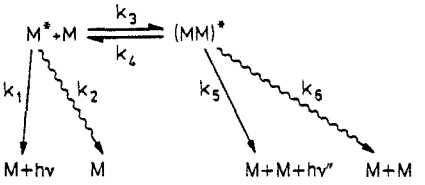
Fig. 4. Experimentally determined pre-exponential factors of the monomer region of pyrene in DPPC SUV for different ratios (1:50, 1:80, 1:120) and at different temperatures above the phase transition temperature

	A_1	A_2
1:120	○	●
1:80	△	▲
1:50	□	■

1:80 in LUV (not shown) are similar to those for the corresponding SUV sample.

The scheme of excimer formation which is valid in most isotropic media (Birks et al. 1963, 1964) has been applied to the excimer formation of pyrene in membrane dispersions above the phase transition temperature (Galla and Sackmann 1974a and b).

Scheme I



where k_3 is the rate constant of excimer formation, k_4 is the rate constant of excimer dissociation, k_1 and k_5 are the rate constants of fluorescence of the monomer and excimer respectively, and k_2 and k_6 are the rate constants of the non-radiative decay of the monomer and excimer respectively.

From this scheme the following eqations are derived:

$$i_m(t) = A_1 \exp(-\lambda_1 t) + A_2 \exp(-\lambda_2 t) \quad (4)$$

$$\text{with} \quad A_1/A_2 = (X - \lambda_2)/(\lambda_1 - X) \quad (5)$$

$$i_e(t) = A_3 \exp(-\lambda_1 t) + A_4 \exp(-\lambda_2 t) \quad (6)$$

$$\text{with} \quad A_3 = -A_4 \quad (7)$$

$$\lambda_{1,2} = 0.5\,\{(X+Y) \mp [(X-Y)^2 + 4k_3k_4[M]]^{1/2}\} \quad (8)$$

$$X = k_1 + k_2 + k_3[M] \quad (9)$$

$$Y = k_5 + k_6 + k_4 \quad (10)$$

where $i_m(t)$ and $i_e(t)$ are the time-dependent intensities of the monomer and excimer fluorescence. This scheme was applied to the decay fitting results of the analysis as a bi-exponential above the transition temperature. The apparent rate constant for excimer formation ($k_3[M]$) and the excimer lifetime ($\tau_e = 1/Y$) are given in Table 2.

Diffusion coefficients of pyrene in the lipid membrane above the transition temperature were calculated from the apparent rate constant of excimer formation ($k_3[M]$) using the time-independent Smoluchowski eqation adapted to a two-dimensional system (Eq. 11) (Kano et al. 1981).

$$k_3 = 1.585\, ZD'N', \quad (11)$$

where Z is the thickness of the two-dimensional system, D' is twice the diffusion constant, D, of pyrene and N' is Avogadro's number per millimole. It is necessary to estimate the effective probe concentration, $[M]$, in order to calculate k_3 from $k_3[M]$. This was

Table 2. Apparent excimer formation constants $k_3[M]$, diffusion constants D and excimer lifetime τ_e above the phase transition temperature at different pyrene/DPPC ratios in SUV and LUV. $k_3[M]$ and τ_e were calculated from Eqs. (5), (8), (9), (10) and the data in table 1. D was calculated from Eq. (11) after calculating the effective pyrene concentration $[M]$ in the membrane. The studied molar ratios pyrene/DPPC were for SUV 1:50, 1:80, 1:120 and for LUV 1:80. The propagation errors on $k_3[M]$ and τ_e are about 25% and 10% respectively

	$k_3[M]$ [1/s] ($\times 10^{-7}$)				D[cm^2/s] ($\times 10^6$)				τ_e[ns]			
	SUV			LUV	SUV			LUV	SUV			LUV
T[°C]	1:50	1:80	1:120	1:80	1:50	1:80	1:120	1:80	1:50	1:80	1:120	1:80
70	2.6	1.8	1.2	1.8	2.1	2.3	2.2	2.3	23	24	23	26
65	–	1.5	0.9	1.4	–	1.9	1.7	1.8	–	26	25	29
60	2.0	1.3	0.9	1.3	1.6	1.6	1.7	1.6	28	30	29	32
55	1.6	1.1	0.7	1.1	1.3	1.4	1.4	1.4	34	33	33	36
50	1.4	0.9	0.6	0.9	1.1	1.2	1.2	1.1	39	36	35	40
45	1.2	0.8	0.5	0.8	0.9	1.1	1.0	0.9	40	38	39	43
40	1.0	0.6	–	0.7	0.7	0.8	–	0.9	43	44	–	46

done as follows: The radius of a small unilamellar vesicle is 12.5 ± 2.5 nm (Huang 1969). A value of 2 nm (Kano et al. 1981) was taken for Z, the thickness of the lipid layer in which the probe can move. The aggregation number of SUV is about 2800 (Huang 1969) and the probe/lipid ratio is known for the different samples. The D-values calculated from Eq. (11) are shown in Table 2. The values for D were of the order of 10^{-6} cm^2/s. The D-values were independent of the pyrene/DPPC ratio and they increased with increasing temperature. The average activation energy for the diffusion controlled excimer formation of pyrene in SUV of DPPC was calculated to be 29.4 ± 1.3 kJ/mol (30.9 kJ/mol, 27.7 kJ/mol and 29.5 kJ/mol for the molar ratios pyrene/DPPC 1:120, 1:80, 1:50 respectively) (Fig. 5). The activation energy for the excimer formation of pyrene in DPPC LUV was found to be 28.9 kJ/mol (Fig. 5) which is comparable with that in SUV. The plots of $\ln(k_3[M])$ versus $1/T$ were parallel and were, within experimental error, separated by the natural logarithm of the concentration ratios, i.e. for the upper pair by 0.39, for the lower pair by 0.42 and for the outer pair by 0.81. The plots for SUV and LUV coincided within experimental error.

At all temperatures, the monomer decays of pyrene in DPPC SUV (1:50, 1:80 and 1:20) and LUV (1:80) could adequately be fitted to Eq. (2) as illustrated in Fig. 2c. The decay fitting parameters of this analysis are shown in Table 3. Below the phase transition temperature, the χ^2_ν values for the bi-exponential fits indicated a lack-of-fit. Equation (2) is derived from a model which takes into account the time-dependence of the rate constant of excimer formation. In this model the following processes are considered (Scheme II) (Yguerabide et al. 1964; Ware and Novros 1966). Excimer dissociation is neglected.

Table 3. Fluorescence decay parameters of pyrene in the monomer region at different pyrene/DPPC molar ratios as a function of temperature. The experimental decay curves were fitted to $i_m(t) = C \exp(-At - B\sqrt{t})$ λ_{exc} = 300 nm, λ_{em} = 378 nm. Below the phase transition temperature, the χ^2_ν values for bi-exponential fits indicated a lack-of-fit. At higher temperatures, the fluorescence decays could be described equally well (using χ^2_ν as criterion) by a double-exponential and by Eq. (2)

Pyrene/ DPPC	SUV 1:50		SUV 1:80		SUV 1:120		LUV 1:80	
T[°C]	$1/A$[ns]	$B(\times 10^3)$	$1/A$[ns]	$B(\times 10^3)$	$1/A$[ns]	$B(\times 10^3)$	$1/A$[ns]	$B(\times 10^3)$
70	75	2.94	101	1.01	–	–	102	1.30
65	–	–	103	0.85	141	0.85	111	1.17
60	82	2.25	114	0.82	155	0.86	120	1.05
55	84	1.67	125	0.76	165	0.66	129	0.92
50	96	1.85	137	0.60	187	0.70	145	0.85
45	105	1.11	150	0.54	205	0.57	161	0.73
40	130	1.26	176	0.51	229	0.47	178	0.66
35	199	2.28	229	1.01	298	1.11	227	1.36
30	248	2.43	285	1.46	310	0.96	266	1.42
25	289	1.99	310	1.20	336	0.79	291	1.39
20	322	1.96	–	–	–	–	320	1.39

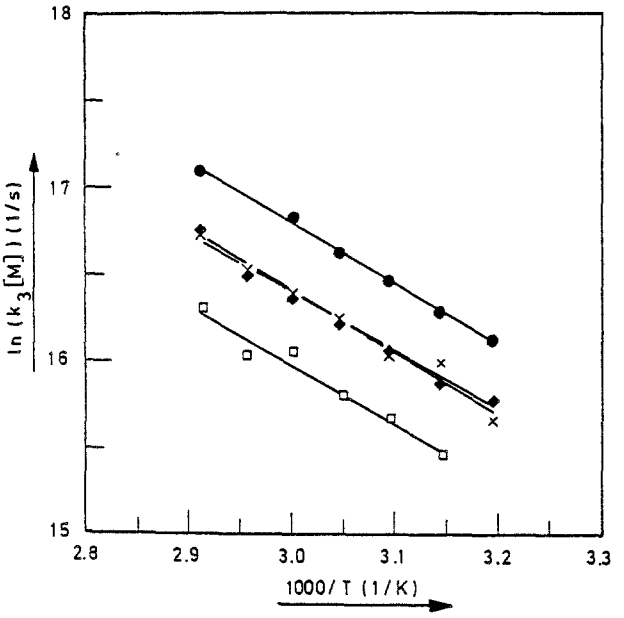


Fig. 5. Determination of the activation energy of the apparent excimer formation rate constant $k_3[M]$ in DPPC SUV (1:50 ●, 1:80 × and 1:120 □) and LUV (1:80 ◆) above the phase transition temperature

Scheme II

process	rate
$M \longrightarrow M^*$	
$M^* \longrightarrow M + h\nu'$	$[M^*]/\tau_0$
$M^* \longrightarrow M$	
$M^* + M \longrightarrow (MM)^*$	$k(t)[M][M^*]$

where M is the monomer, M^* is the excited state of the monomer, $(MM)^*$ is the excimer, $1/\tau_0$ is the fluorescence lifetime of M^* in the abscence of excimer formation, $k(t)$ is a time-dependent rate constant. For a two-dimensional system $k(t)$ is approximated by Eq. (12).

$$k(t) = 0.5\,ZD'N'[M](3.17 + 14.18\,R'/(D't)^{1/2}), \quad (12)$$

where R' is twice the interaction radius of pyrene, $[M]$ is the monomer concentration, N', D' and Z are as defined before. The fluorescence decay of M^* is described by Eq. (13) (Vanderkooi et al. 1975; Owen 1975).

$$i(t) = i_0 \exp\{-[1/\tau_0 + 0.5\,ZD'N'[M](3.17 + 14.18\,R'/(D't)^{1/2})]t\}. \quad (13)$$

This equation is formally equivalent to Eq. (2), A and B are thus given by Eqs. (14) and (15).

$$A = 1/\tau_0 + 1.509\,ZD'N'[M] \quad (14)$$

$$B = 7.09\,ZN'R'[M]D'^{1/2} \quad (15)$$

$$D' = [(A - 1/\tau_0)\,4.47\,R'/B)]^2. \quad (16)$$

Table 4. Diffusion coefficients above and below the phase transition temperature calculated from Eq. (16), using the data of Table 3

	D[cm²/s] (× 10^8)			
T[°C]	SUV 1:50	SUV 1:80	SUV 1:120	LUV 1:80
70	49	178	–	106
65	–	244	86	101
60	71	200	62	98
55	66	184	86	110
50	69	224	48	90
45	154	210	54	88
40	64	136	44	72
35	8.4	10.4	6.6	6.2
30	1.3	1.3	1.2	2.4
25	0.6	0.8	0.6	1.2
20	0.4	–	–	0.4

Equation (16) is derived from Eqs. (14) and (15). D can thus be calculated from the decay parameters A and B. The D-values for $R' = 6.6\,A$ are given in Table 4. Below the phase transition temperature the D values increase with increasing temperature and are nearly independent of the pyrene/DPPC ratio. Above the transition temperature these calculated D values are not correlated with temperature.

Figure 6 shows the variation of the diffusion coefficient as a function of temperature. There is an abrupt change of the diffusion coefficient around the phase transition temperature (~ 37°C), D increases by at least an order of magnitude in the temperature range 35°–40°C.

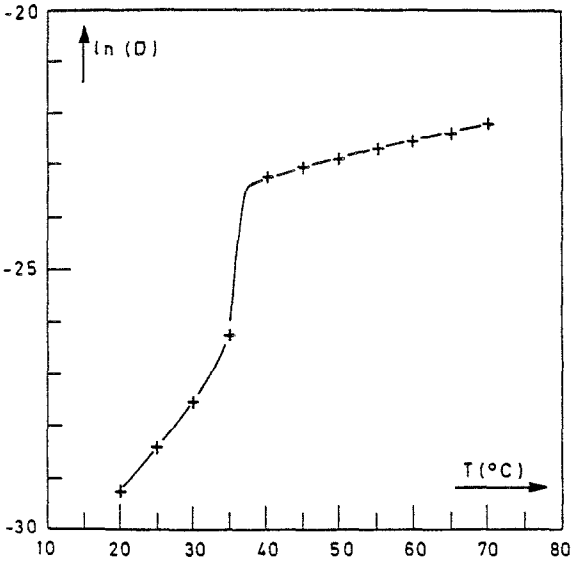


Fig. 6. The logarithm of the diffusion coefficients as a function of temperature. Above the transition temperature (37°C) the coefficients were calculated from Eq. (11). Below the transition temperature the coefficients were obtained from Eq. (16)

Discussion

Above the phase transition temperature, the fluorescence decays of the locally excited state of pyrene could be described adequately by a bi-exponential decay function or by a decay law which takes into account the time-dependence of the rate constant of excimer formation (Eq. (2)). The decay of the excimer could be fitted excellently to a difference of two exponential terms. The decay parameters of Eq. (2) were used to calculate the diffusion constants D according to Eq. (16). These D-values were nearly independent of the temperature and were dependent on the pyrene/DPPC ratio. From this it could be concluded that the monomer fluorescence decay data above the phase transition temperature were not compatible with reaction Scheme II which takes into account the time-dependence of excimer formation. Fitting the decay curves of the monomer and excimer regions to Eqs. (4) and (6) respectively gave decay times which were in excellent agreement. The ratio of the pre-exponential factors, A_3/A_4, was always close to minus one. These results indicated that the classic scheme of excimer formation (Scheme I) could be applied in small unilamellar vesicles. From Table 2 it can be seen that the excimer lifetime ($\tau_e = 1/Y$) is independent of the pyrene/DPPC ratio, which is predicted by Scheme I. The excimer lifetime is also independent of the size of the liposomes (SUV and LUV). The values vary from 25 ns at 70 °C to 45 ns at 40 °C. These values are shorter than those determined by Galla et al. (1974, 1979, 1980) (100 ns at 45 °C to 65 ns at 60 °C) from excimer decay data only. From our results (Fig. 3 and Table 2) one can conclude that the short decay parameter, $1/\lambda_2$, approximates the excimer lifetime τ_e, and the decay parameter $1/\lambda_1$ approximates the monomer lifetime ($1/X$). In the excimer region, τ_e is approximated by the rise ($1/\lambda_2$) of the excimer fluorescence and not by the decay term with the positive pre-exponential factor ($1/\lambda_1$). It is thus very helpful to monitor both excimer and monomer fluorescence to test if the data can be described by Scheme I.

The D-values calculated from the pseudo-first-order rate constant of excimer formation ($k_3[M]$) are comparable with the D-values of 1-methylpyrene in DMPC SUV above the phase transition temperature (8.5×10^{-7} cm^2/s at 40 °C and 2.1×10^{-6} cm^2/s at 70 °C) (Van den Zegel et al. 1984), and with the D-values for pyrene/*N*,*N*-dimethylaniline in DPPC sonicated vesicles (5.0×10^{7} cm^2/s at 50 °C) (Kano et al. 1981), and are somewhat larger than the D-value of 1.4×10^{-7} cm^2/s for pyrene in DPPC multilamellar vesicles at 50 °C (Galla and Sackmann 1974a and b).

The activation energy of 29.4 kJ/mol for the diffusion controlled excimer formation of pyrene in DPPC SUV is the same as the activation energy determined for the excimer formation of 1-methylpyrene in DMPC SUV and is lower than the value (36.8 kJ/mol) obtained by Galla and Sackmann (1974a and b) for the diffusion of pyrene in DPPC vesicles above the phase transition. The results for the LUV system were identical with these of the SUV systems.

Below the phase transition temperature the classic scheme of excimer formation could no longer be applied. Indeed, the experimental decay curves could not be fitted to a double-exponential decay law. The decay data of the monomer region could be fitted excellently to a decay law which takes into account the time-dependence of the rate constant of excimer formation (Eq. (13)). Similar results were obtained by Kano et al. (1980, 1981) for the fluorescence quenching of pyrene and pyrenedecanoic acid, with *N*,*N*-dimethylaniline and *p*-isopropyl-*N*,*N*-dimethylaniline as quenchers, in DPPC sonicated vesicles below the phase transition temperature. They found a diffusion constant of 1.6×10^{-8} cm^2/s for the pyrene-*N*,*N*-dimethylaniline system at 25 °C which is comparable with our values (Table 4). The diffusion constant for 3,3'-dioctadecyloxocarbocyanine in DPPC vesicles at 25 °C was determined with the FRAP technique and was found to be $< 5 \times 10^{-10}$ cm^2/s (Wu et al. 1977). A fast diffusion in the gel state can be attributed to the linear defects which form quasi-fluid paths for a nearly one-dimensional and thus very effective transport. Since the excimer formation technique measures diffusion over short distances it is understandable that a large mobility is observed. Using the FRAP technique, Kapitza et al. (1984) also observed a fast lateral diffusion for the lipid-probe *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine in giant DMPC vesicles when the concentration of the probe was very small so that it might be dissolved in the few defects.

The change of the diffusion constant with temperature agrees very well with results obtained with other techniques. The lateral diffusion coefficients of phosphatidylcholine in DPPC have been determined with ^{31}P-NMR (Cullis 1976) and with triplet-triplet annihilation (Naqvi et al. 1974). The D-values of the phospholipids are an order of magnitude lower than we found for pyrene, but the change of the diffusion constant at the phase transition temperature is similar.

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