Biochimica et Biophysica Acta 940 (1988) 149–157
Elsevier

BBA 73968

Interaction of the fluorescent probe *N*-(lissamine Rhodamine B sulfonyl)dipalmitoylphosphatidylethanolamine with phosphatidylcholine bilayers

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(Received 26 October 1987)

Key words: Fluorescent probe; Lipid miscibility; Phosphatidylcholine vesicle

The surface density of the fluorescent probe *N*-(lissamine Rhodamine B sulfonyl)dipalmitoylphosphatidylcholine is the same in the two lipid leaflets of phosphatidylcholine bilayers containing the probe. In the liquid-crystalline state, the probe molecules aggregate above a threshold amount, approximately 0.2 mol/mol phospholipids. Above this threshold value, the surface density of the free probe molecules is constant, and all probe molecules added are incorporated in the aggregated form. The aggregation of the probe increases by approximately 20% when the medium pH is lowered to 4. In the gel state, the probe aggregation is higher than that in the liquid-crystalline state, and the free probe molecules distribute unevenly in the bilayer surface. Even though the results obtained in our model system cannot be directly extrapolated to all model systems, we point out that care is to be taken in the use of the probe. In fact, only in membranes in the liquid-crystalline state in which the amount of probe molecules to phospholipid molecules is lower than 1:7 the fluorescence response of the probe is independent of the pH changes and of the molecular aggregation.

Introduction

The fluorescent properties of phospholipids labeled in their headgroup have been recently used to investigate different features of lipid bilayers: lipid phase transition [1], lipid translational diffusion and packing [2,3], lipid phase separation [4], ion permeation [5], pH measurements of the internal vesicle compartment [6], fusion between vesicles [7,8], lipid transfer between vesicles [9] and surface density determination [10]. Even if the structure of these probes is better related to naturally occurring membrane constituents than other extrinsic fluorescent probes, the interpretation of the fluorescence data can often be difficult, and requires a precise and complete physico-chemical characterization of the interactions between the probe molecules and the lipid bilayer.

N-(Lissamine Rhodamine B sulfonyl)phosphatidylethanolamine has been widely employed in studies of membrane fusion [4,7,11–13], ion permeation [5] and lipid transfer between vesicles [10,14]. In spite of the numerous applications of this probe, a detailed analysis of its behaviour in simple model bilayers is still lacking. In this study, we used the lipid probe containing saturated hy-

Abbreviations: N-Rh-DPPE, *N*-(lissamine Rhodamine B sulfonyl)dipalmitoylphosphatidylethanolamine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; PC, phosphatidylcholine; SUV, small unilamellar vesicles; MLV, multilamellar vesicles.

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drocarbon chains to avoid its degradation under the drastic sonication procedures. We find that the probe molecules aggregate in phosphatidylcholine bilayers even in the liquid-crystalline state, and show that the temperature-, pH-, vesicle size- and concentration-dependent fluorescence changes can be attributed to variations in the aggregation properties of the probe in the lipid bilayers.

Materials and Methods

Saturated phosphatidylcholines and dioleoylphosphatidylcholine (DOPC) were supplied by Sigma and egg phosphatidylcholine (egg PC) by Supelco. All lipids were used without purification. *N*-(Lissamine Rhodamine B sulfonyl)dipalmitoylphosphatidylethanolamine (N-Rh-DPPE) was synthesized in the laboratory of Dr. Montecucco (this Institute). Time-dependent hydrolysis products were virtually absent, as assessed by thin-layer chromatography of the probe.

Small unilamellar vesicles (SUV) were prepared as described previously [15]. Multilamellar vesicles (MLV) were produced by addition of a buffered aqueous solution to a thin film of dry lipids and probe. The suspension was gently shaked for 1 h, at a temperature above that of the lipid phase transition. The suspension medium for SUV and MLV was 0.1 M KCl and 0.01 M acetate-Tris (pH 7.2).

Absorbance and fluorescence measurements

Absorbance and fluorescence measurements were performed using a Lambda 5 Perkin-Elmer spectrophotometer and a 650-40 Perkin-Elmer spectrofluorimeter, respectively. In both instruments the sample chamber was equipped with a magnetic stirrer, and the temperature was controlled with a thermostatted, circulating water bath. The emission wavelength of sample containing N-Rh-DPPE incorporated into lipid bilayers was 590 nm, the maximum of fluorescence intensity emission. Although the absorbance maximum of N-Rh-DPPE is at 575 nm, the excitation wavelength was set at 550 nm, to minimize light scattering. The absorbance values were lower than 0.03, so that trivial reabsorption of the emitted light and inner filter effect were negligible. Excess of Triton X-100, added to DPPC SUV containing the lipid probe, gave fluorescence intensities about 1.16 higher than those obtained by treatment with excess of *n*-octyl glucoside. The higher fluorescence intensity obtained by Triton X-100 was probably due to a slightly more apolar environment surrounding the fluorophore. In fact, the absorbance and emission maxima were blue-shifted about 2 nm when the probe was incorporated in Triton X-100 as compared with *n*-octyl glucoside (see Table I and Results).

Fluorescence anisotropy was measured with the spectrofluorimeter equipped with plastic polarizers in the excitation and emission beams. Anisotropy values were calculated according to the formula

$$r = (I_{\parallel} - CI_{\perp})/(I_{\parallel} + 2CI_{\perp})$$

where $I_{\parallel}$ and $I_{\perp}$ are the emission intensities detected through a polarizer oriented parallel ($I_{\parallel}$) or perpendicular ($I_{\perp}$) to the direction of polarization of the excitation light, and C is the instrumental correction factor. Under our experimental conditions the light scattering contribution to the anisotropy values is negligible since extrapolation at zero SUV concentration gives only a 6% change of the anisotropy values.

Lifetime measurements

Lifetime measurements were performed with a Greg 200 I.S.S. multifrequency phase and modulation fluorimeter (La Spezia, Italy) interfaced with an IBM-PC for data acquisition and analysis. The excitation source was a Liconix 4207 NB He-Cd laser, which was tuned for output at 442 nm. The emission was observed at 590 nm through a cut-off filter. A set of 10 modulation frequencies was used in the range from 30 to 200 MHz: for each frequency and temperature, the phase and modulation of the fluorescence were measured relatively to a reference of known lifetime. Data were fitted to a sum of exponential terms, and χ^2 value was used to judge the correctness of the fit.

Results and Discussion

Spectral characteristics

The spectral properties of N-Rh-DPPE dissolved in different media are summarized in the Table I. (i) When the chromophore is surrounded by environments of increasing dielectric constant,

TABLE I

SPECTRAL PARAMETERS OF N-Rh-DPPE DISSOLVED IN VARIOUS MEDIA

The concentration of the lipid probe in ethanol was 2 μM. DPPC SUV containing 0.95 mol N-Rh-DPPE/mol DPPC were used to obtain the parameters of the lipid probe in vesicles and in excess of detergents. $(\lambda_{max})_{abs}$ and $(\lambda_{max})_{em}$ are the wavelengths of the maximum absorbance and fluorescence emission, respectively. A_m/A_s is the ratio between the absorbance values of the maximum and of the shoulder at 540 nm.

	$(\lambda_{max})_{abs}$ (nm)	$(\lambda_{max})_{em}$ (nm)	A_m/A_s
Ethanol	560	575	2.9
Ethanol/water (87:13)	563	581	2.9
Triton X-100	570	587	2.9
n-Octyl glucoside	572	589	2.9
DPPC SUV	575	591	2.6

the absorbance and fluorescence emission maxima shift towards the red. The position of the spectral maxima of the probe incorporated into DPPC vesicles indicates that the chromophoric group is localized in an aqueous environment. The fluorescence quantum yield of N-Rh-DPPE in ethanol/water (87:13, v/v) is 1.45-times that obtained when the probe is incorporated in DPPC vesicles at concentrations at which the probe does not aggregate in the bilayer; (ii) the ratio between the absorbance value of the main band and of the shoulder at the shorter wavelengths, A_m/A_s, is about 2.9 in alcohol or in detergent micelles, where the probe molecules are monodispersed, while it decreases when the probe is incorporated into the vesicles, suggesting that a concentration-dependent aggregation of N-Rh-DPPE occurs in the lipid bilayer. In fact, the absorbance spectrum of *N*-lissamine Rhodamine B sulfonate, like those of other metachromatic dyes [16], is sensitive to the aggregation when the dye concentration is enhanced in aqueous media: A_m/A_s decreases from 2.7 to 2.3 when the dye concentration increases from 10 μM to 100 μM.

Localization of the probe in the lipid bilayer

Using cobalt ions as a collisional quencher to probe surface density of *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine incorporated into large unilamellar phosphatidylserine/phosphatidylethanolamine vesicles, Morris et al. [5] found that the probe was distributed with the same surface density in both leaflets of the bilayer. We used the impermeant solute I^- as fluorescence quencher: after addition of KI to a suspension of N-Rh-DPPE-containing SUV, the fluorescence intensity suddenly decreases to a value which remains then constant for at least 1 h. The ratio of the moles of the lipid probe present in the inner and outer bilayer leaflet, n_i/n_e, is calculated by measuring the fluorescence intensity at infinite quencher concentration, according to a modified form of the Stern-Vollmer equation [17]

$$F/(F-F') = (n_i + n_e)(1+1/kc)/n_e$$

where F' and F are the fluorescence intensities in the presence and in the absence of the quencher, k is the quenching coefficient and c the concentration of the quencher. This equation describes the quenching of two populations of fluorophores, one of which is inaccessible to the quencher. It is deduced under the following assumptions: (i) the fluorescence quantum yield of N-Rh-DPPE is the same in both leaflets of the bilayer; (ii) the KI addition does not cause aggregation of the lipid probe in the bilayer; and (iii) no static quenching occurs between the fluorophore and the quencher molecules. Fig. 1 shows that a linear relationship exists between $F/(F-F')$ and $1/c$, and that the intercept on the ordinate, $(n_i + n_e)/n_e$, is 1.65. Since the external surface of the sonicated unfractionated vesicles is about 60% of the total vesicle surface [18], it may be easily calculated that the surface density of the lipid probe in the inner layer (mol probe/mol phospholipids in the inner layer) is approximately the same as that in the outer layer.

Lifetime measurements

The lower curve of Fig. 2A shows the fluorescence intensity of the probe incorporated in DPPC SUV as a function of the temperature. Two breaks in the curve delimitate a temperature region, 33.5–40.5 °C, which roughly coincides with the lipid phase transition of the SUV. With DPPC MLV, the temperature range was 39–41.5 °C (not shown). When DPPC SUV were dissolved in ex-

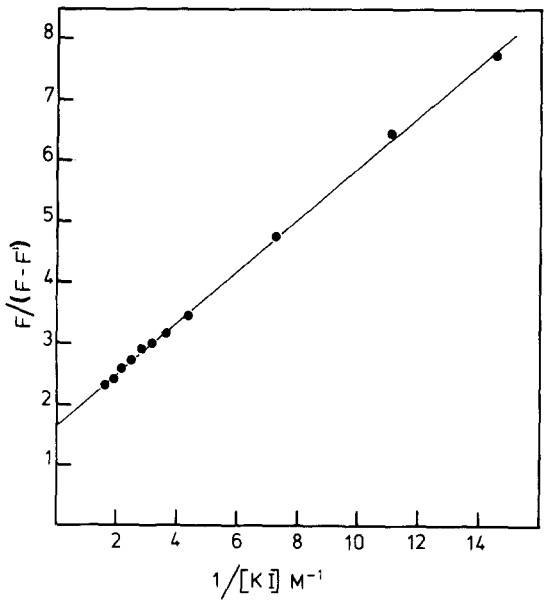

Fig. 1. Fluorescence quenching of N-Rh-DPPE incorporated in DPPC SUV. The sample consisted of DPPC SUV (10 μM lipids) containing 0.07 mol N-Rh-DPPE/mol DPPC, and was incubated at 50 °C. The quenching curve was obtained by adding to each sample a constant volume of a mixture of KCl and KI: the KCl concentration was varied, and the total salt concentration was kept constant. The fluorescence intensity change was corrected for the dilution effect by using a pure KCl solution.

cess *n*-octyl glucoside at 50 °C, the fluorescence intensity increased, and the relation between fluorescence and temperature became linear (upper part of the Fig. 2A).

The fluorescence decay of N-Rh-DPPE in ethanol is monoexponential, while a sum of two exponential functions has to be applied to fit the fluorescence decay when the probe is incorporated in DPPC SUV or in mixed *n*-octyl glucoside-DPPC micelles (not shown). However, when the light scattering contribution is taken into account, a monoexponential curve fits the experimental decay with the same χ^2 as that found for the two component decay. The light scattering correction has been performed by setting $\tau = 0$ for the scattering component and by varying the preexponential factor and the lifetime of the lipid probe until χ^2 reached its minimum.

Fig. 2B shows the temperature-dependence of the lifetime of N-Rh-DPPE incorporated in DPPC SUV and in mixed *n*-octyl glucoside-DPPC micelles: in DPPC SUV the lifetime at 40 °C is 2.10 ± 0.05, and the standard error 0.05 is estimated from four different SUV preparations. The

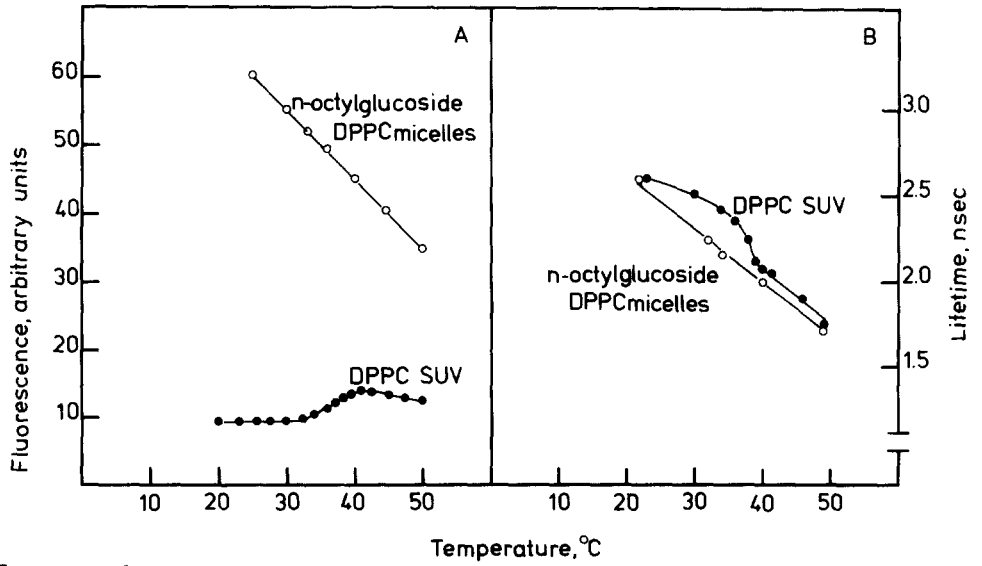

Fig. 2. (A) Temperature-dependent fluorescence intensity of DPPC SUV and mixed *n*-octyl glucoside-DPPC micelles containing N-Rh-DPPE. DPPC SUV (20 μM lipids) containing 0.47 mol N-Rh-DPPE/mol DPPC were incubated at 50 °C, and the fluorescence intensity was measured by lowering the temperature at a rate of 0.5 °C/min. The temperature-dependent fluorescence intensity curve obtained by heating the sample from 20 °C to 50 °C was identical (not shown). The fluorescence intensity of *n*-octyl glucoside-DPPC micelles was measured by incubating the SUV and the detergent (final concentration 0.75% (w/w) at 50 °C and then by lowering the temperature. (B) Temperature-dependent fluorescence lifetime measurements of DPPC SUV and mixed *n*-octyl glucoside-DPPC micelles containing N-Rh-DPPE. The DPPC SUV (0.2 mM lipids) contained 0.07 mol N-Rh-DPPE/mol DPPC.

lifetime values in the two systems are approximately the same in the range of temperatures tested. The curve obtained with DPPC SUV is sensitive to the gel to liquid-crystalline phase transition of the phospholipids. The lifetime values remained unchanged by increasing the amount of N-Rh-DPPE incorporated into the lipid bilayer, and thus by changing the aggregational state of the lipid probe. The aggregated form indeed does not emit, as reported in the next section, and fluorescence energy transfer processes between identical molecules should not, in principle, modify the observed lifetime.

Concentration-dependent fluorescence quenching

Fig. 3A shows the dependence of the percentage of fluorescence quenching on the temperature and on the amount of the probe incorporated into DPPC SUV. The quenching largely increases as the amount of incorporated probe is increased, suggesting that low-fluorescent aggregates are formed in the lipid bilayer. Since the fluorescence quenching is almost complete at 1.2 mol N-Rh-DPPE/mol DPPC, the fluorescence intensity of the N-Rh-DPPE aggregates is practically zero. The gel to liquid-crystalline lipid phase transition causes a large decrease of the fluorescence quenching.

Fig. 3B shows the dependence of F°/F on the probe concentration in DPPC bilayers. $F^\circ/F = Q^\circ n_t/Qn_f$, where n_t and n_f are the moles of total and free probe, and Q° and Q are the fluorescence quantum yields of the probe in mixed *n*-octyl glucoside-DPPC micelles and DPPC bilayers, respectively. In lipid bilayers in the liquid-crystalline state, $Q^\circ = Q$, so that $F^\circ/F = n_t/n_f$. When the amount of lipid probe is lower than a threshold value, about 0.2 mol N-Rh-DPPE/mol DPPC, $F^\circ/F = 1$. Then $n_t = n_f$, and no aggregates are formed. When the amount of probe is higher than this threshold value, F°/F shows a linear relationship according to the equation: $F^\circ/F = kn_t$. Hence the amount of free probe is constant and all added probe molecules are incorporated in the aggre-

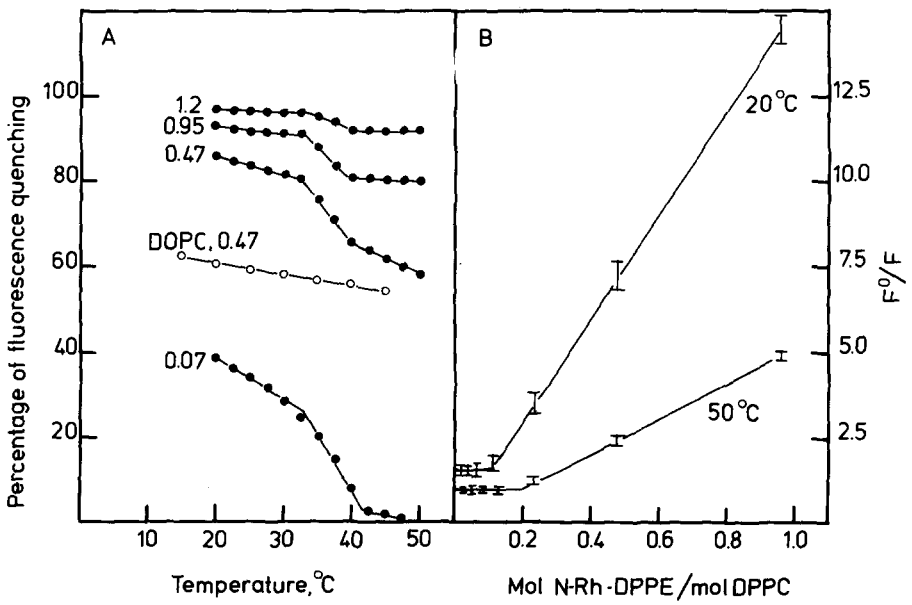

Fig. 3. (A) Temperature-dependent percentage of fluorescence quenching of N-Rh-DPPE incorporated in phosphatidylcholine SUV *at various probe concentrations. The lipid concentration was 20 μM.* ●———● DPPC SUV: the ratio of mol N-Rh-DPPE/mol DPPC is reported in the figure; ○———○ DOPC SUV. The percentage of fluorescence quenching was calculated as $100(F^\circ - F)/F^\circ$, where F° and F are the fluorescence intensities of the probe dispersed in excess *n*-octyl glucoside and of the probe incorporated in the vesicles, respectively. (B) Dependence of F°/F on the amount of N-Rh-DPPE incorporated in DPPC SUV. The points were calculated from the values of the percentage of fluorescence quenching reported in the Fig. 3A. The bars indicate the standard error estimated from three different preparations of SUV.

gated form. In lipid bilayers in the gel state, $F^{\circ}/F = 1.6$ when the amount of probe is lower than its threshold value. In this case, if $Q^{\circ} = 1.6Q$, no aggregation occurs. Alternatively, if $Q^{\circ} = Q$, about 63% of the probe molecules incorporated into the bilayer are in the free form, while the remaining molecules aggregate even at very low concentrations. The threshold value of the probe molecules in DPPC SUV in the gel state is 0.11 mol N-Rh-DPPE/mol DPPC, about half of that found in the liquid-crystalline state. Preferential partition in one phase has been widely ascertained for many fatty acids and lipid probes incorporated into lipid vesicles [19–25].

Besides the formation of non-fluorescent aggregates of lipid probe molecules, fluorescence quenching could be due to energy transfer between neighbouring free and aggregated molecules or to dynamic encounter among N-Rh-DPPE molecules. (i) Fluorescence energy transfer does not influence the threshold value of the lipid probe calculated from the experiments of Fig. 3B. (ii) Dynamic quenching predicts an increase of fluorescence quenching when the temperature is enhanced in bilayers in liquid-crystalline state. On the contrary, the experiments of Fig. 3A show that the fluorescence quenching decreases as the temperature is increased. Moreover, in the gel state, where lipid diffusion is two or more orders of magnitude smaller than that in the liquid-crystalline state, the dynamic quenching should be much lower than in the liquid-crystalline state. On the contrary, the experiments of Fig. 3A show that the fluorescence quenching in the gel state is higher. Finally, if the lifetime of the lipid probe in the liquid-crystalline state is about 2 ns, and if the diffusion coefficient is about $5 \cdot 10^{-8}$ cm^2/s, during the lifetime the lipid probe can be displaced only over a distance of 1.4 Å, insufficient to allow appreciable dynamic encounters. These considerations indicate that the dynamic quenching contribution, if anything, is small.

Table II shows the values of the percentage of fluorescence quenching when N-Rh-DPPE is incorporated in bilayers composed of different phosphatidylcholines. Temperatures were chosen so that the fluid lipid bilayers display the same viscosity [26]. The quenching is the same irrespective of the length and the degree of unsaturation of the acyl chain. However, in MLV the percentage of quenching is about one half of the quenching occurring in SUV. The higher aggregation of N-Rh-DPPE in small vesicles may be due to the higher constraints imposed to the lipid probe molecules by bilayers of lower radius of curvature.

TABLE II

PERCENTAGE OF FLUORESCENCE QUENCHING OF N-Rh-DPPE INCORPORATED IN BILAYERS COMPOSED OF DIFFERENT PHOSPHATIDYLCHOLINES

The amount of the lipid probe in the vesicles was 0.47 mol/mol phospholipids. The quenching was calculated at temperatures where the lipid bilayers display the same viscosity [26].

	Fluorescence quenching (%)
DOPC SUV at 34 °C	58
EYPC SUV at 34 °C	58
DMPC SUV at 40 °C	57
DPPC SUV at 50 °C	59
DSPC SUV at 58 °C	60
DPPC MLV at 50 °C	30

Fluorescence anisotropy

Since the fluorescence intensity of the probe aggregated in the vesicles is zero, the fluorescence anisotropy changes are sensitive only to the behaviour of the free form of the lipid probe. Fig. 4 shows the temperature-dependent fluorescence anisotropy of various amounts of N-Rh-DPPE incorporated in DPPC SUV. In the liquid-crystalline state the fluorescence anisotropy decreases steeply as the amount of the incorporated probe is increased, while it decreases slowly beyond a threshold value of 0.2 mol/mol DPPC. The anisotropy decrease may be due to a fluorescence energy transfer between free probe molecules. In fact, the emission and the absorption bands of the chromophore largely overlap [7]. When the amount of probe is lower than its threshold value, the surface density of the free probe increases by increasing the concentration of the added probe molecules. This would lead to a smaller distance between free probe molecules and to an enhanced energy transfer efficiency and fluorescence depolarization. When the amount of probe exceeds the threshold value, the surface density of the free

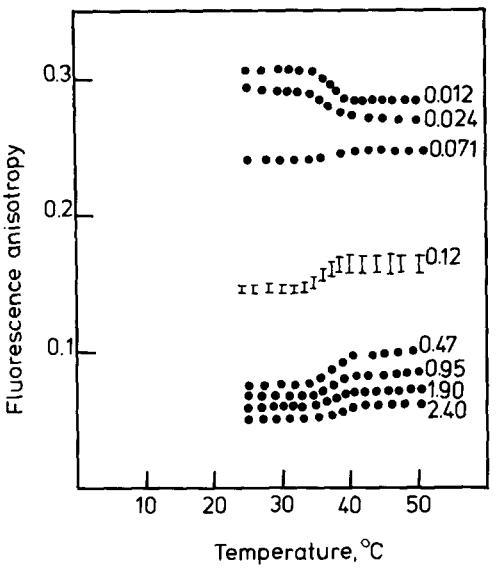

Fig. 4. Temperature-dependent fluorescence anisotropy of N-Rh-DPPE incorporated in DPPC SUV at various concentrations. The experimental conditions were as in Fig. 3A. The bars in the 0.12 curve indicate the standard error estimated from three different preparations of SUV.

probe molecules is constant, leading to a constant energy transfer efficiency and a constant degree of anisotropy.

The temperature-dependent fluorescence anisotropy is sensitive to the gel to liquid-crystalline lipid phase transition. We would expect a higher anisotropy in the gel state for several reasons: (i) the mobility of the fluorophore is lowered, and therefore fluorescence emitted by both the donor and the acceptor molecules is less depolarized. Similar conclusions have been reached for other head group-labeled phosphatidylethanolamines [1,3]; (ii) the probe aggregation is enhanced, and thus the surface density of the free probe molecules and the depolarization are smaller; (iii) the membrane thickness is increased. As a consequence, the average distance between probe molecules located in the opposite bilayer leaflets becomes larger and the transfer efficiency and the depolarization become smaller.

When lipid membranes undergo a transition toward the gel state, the vesicle surface area decreases. This would cause a higher surface density of the probe molecules, and a lower anisotropy value. However, when the amount of probe is higher than the threshold value, a decreased surface area should cause only a further aggregation of the probe molecules, and the surface density is maintained constant.

Fig. 4 shows higher anisotropy values in gel state membranes at low amount of N-Rh-DPPE, and lower anisotropy values at higher amounts. One possible explanation for this unexpected result is that the free probe molecules are not fully dispersed in membranes in the gel state [19]. When a lipid bilayer undergoes a transition toward the gel state, membrane regions of phospholipids in the liquid-crystalline and gel state coexist. N-Rh-DPPE prefers to be partitioned into the fluid membrane regions, whose surface decreases as the process proceeds. At the end of this process, small domains of phosphatidylcholine molecules enriched in probe molecules are formed. In conclusion, the fluorescence response of the probe is to be carefully analyzed when phospholipid domains of heterogeneous fluidity are present, as is the case for many natural membranes.

Effect of $CaCl_2$ and pH

The addition of 10 mM $CaCl_2$ to DPPC SUV containing N-Rh-DPPE at 50 °C does not change the fluorescence intensity of the sample. However, when the SUV are subjected to a pH drop from 7.2 to 4, there is a slow fluorescence quenching that equilibrates after 20 min. The fluorescence intensity, after equilibration at acidic pH, is approximately 20% lower than the initial fluorescence intensity at neutral pH. Fig. 5A shows the temperature-dependent fluorescence intensity of N-Rh-DPPE incorporated in DPPC SUV at pH 7.2 and 4. The curve at pH 4 was obtained by acidification at 50 °C, incubation for 20 min at the same temperature and by slowly lowering the temperature.

The pH-dependent fluorescence quenching is not due to direct effect of the acidic environment on the chromophore, since fluorescence emission of N-Rh-DPPE molecules incorporated in mixed *n*-octyl glucoside-DPPC or Triton X-100-DPPC micelles is independent of the medium pH in the range pH 7 to 4. Therefore the pH-dependent fluorescence quenching reflects changes of either the properties of the lipid bilayer, or of the interactions between lipid probe molecules. Since N-

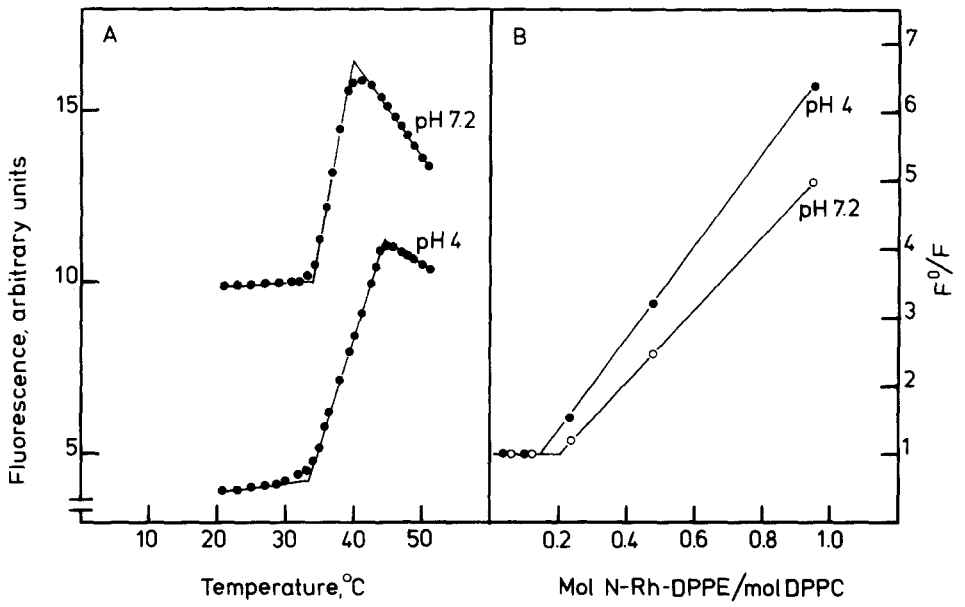

Fig. 5. (A) Temperature-dependent fluorescence intensity of N-Rh-DPPE at pH 7.2 and 4. (B) Dependence of F^0/F on the concentration of N-Rh-DPPE at pH 7.2 and 4. Experimental conditions were as in Figs. 3A and B, respectively.

Rh-DPPE molecules possess pH-dependent charged groups, the degree of aggregation of the lipid probe molecules may be higher at acidic pH in consequence of the decreased electrostatic repulsion. In the liquid-crystalline state the fluorescence quenching due to the acidification is completely reversible. However, in the gel state, either an acidification of DPPC SUV incubated at neutral pH, or the opposite process does not cause any fluorescence change. This indicates that the changes in the properties of the lipid bilayer, or in the aggregational state of the lipid probe require high diffusion rates of the lipid components. Fig. 5B shows that the threshold amount of lipid probe molecules in bilayers in the liquid-crystalline state is lowered by 20% at pH 4 compared to pH 7.2.

The experiments reported in Fig. 5 together with the fluorescence anisotropy results indicate that the probe is homogeneously dispersed in phospholipid SUV only when the probe to phospholipid molecule ratio is lower than 1 : 7, and the lipid components are in the liquid-crystalline state.

Acknowledgement

This research was supported in part by the Ministero della Pubblica Istruzione and by ASSNE (Associazione Sviluppo Scienze Neurologiche).

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