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MONITORING THE LOCATION PROFILE OF FLUOROPHORES IN PHOSPHATIDYLCHOLINE BILAYERS BY THE USE OF PARAMAGNETIC QUENCHING

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Summary

Spin probes differing in the position of their paramagnetic centre are used to quench the fluorescence of pyrene derivatives and chlorophylls incorporated into dimyristoyl phosphatidylcholine membranes. Pyrene butyric acid and pyrene decanoic acid with known orientation relative to the membrane surface are investigated. The quenching efficiency of fatty acid spin probes is dependent on the position of the nitroxide radical group in the fatty acid chain. Using this short range interaction we developed a spectroscopic method to characterize the molecular arrangement within the lipid membrane. Applied to chlorophyll-containing vesicles, we were able to characterize the orientation of the porphyrin ring within the membrane. Moreover, the chlorophyll fluorescence is also quenched by a water-soluble spin label. Therefore the porphyrin ring appears to be orientated in the polar head group region of the lipid layer, but not to be protruding out into the water phase.

This conclusion is confirmed by the use of pyrene derivatives. Fluorescence quenching by a water-soluble spin label within the lipid matrix is observed even in the rigid state of the membrane. Fluorescence lifetime measurements suggest the existence of two different quenching mechanisms: (1) a static quenching occurring below the lipid phase transition temperature, and (2) an additional dynamic quenching taking place in the fluid state of the lipid bilayer.

Introduction

Paramagnetic molecules are able to quench the fluorescence of fluorophores and thus to reduce their lifetimes [1]. This paramagnetic quenching requires

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Abbreviations: TEMPOL, 2,2,6,6-tetramethyl-4-piperidinol-1-oxide; Chl, chlorophyll.

interaction distances of about 4—6 Å [2] and its measurement might therefore give an indication of the distance between nitroxide radicals and the fluorophores in question [3]. The validity of this method has been demonstrated in artificial lipid bilayers [4] and in the erythrocyte membrane [5—7] where quenching of the internal protein fluorescence is investigated to yield information on the lipid-protein interaction. The location of different spin probes in in the lipid layer on the other hand is well known from the EPR isotropic splittung factor [8] and from permeability measurements of spinlabel reducing ascorbate ions [9]. Therefore paramagnetic quenching experiments using these well-defined spin probes should give information on the position of the fluorophore within a membrane.

In a preceding short communication we reported paramagnetic quenching of chlorophyll fluorescence to study the orientation of the porphyrin ring within a lipid bilayer membrane [10]. This artificial system can be considered a good model for the chloroplast thylakoid membrane [11] containing the light-harvesting chlorophyll molecules. The knowledge of the molecular environment and the orientation of the chlorophyll molecules within the membrane is important for investigations of the primary processes in photosynthesis.

In this paper we will describe the experiments in more detail with respect to the physical background of our earlier results [10]. We used pyrene derivatives to verify the method of determining a fluorophore position in lipid bilayer membranes by paramagnetic quenching with different spin labels. Furthermore measurements using a water-soluble quencher yielded information on chlorophyll aggregation and on the mechanisms of the quenching process. Two mechanisms, a static and a dynamic one, were observed.

Materials and Methods

Lipids and probe molecules. Dimyristoyl phosphatidylcholine from Fluka was checked by thin-layer chromatography and used without further purification. Fatty acid spin labels were obtained from Syva, Palo Alto. 2,2,6,6,-Tetramethyl-4-piperidinol-1-oxide (TEMPOL) was synthesized according to Rozantsev [12]. Pyrene decanoic acid was syntehsized as described elsewhere [13] and pyrene butyric acid was a gift of Drs. H.G. Scholz and W. Kühnle from the Max-Planck-Institut in GOttingen. Chlorophyll *a* was isolated from spinach and purified to highest degree on powdered sugar according to Smith and Benitz [14]. The probes were prepared as described earlier [10]. The structures of the probes and the relative locations of their functional groups are shown in Fig. 1. The diagram indicates the way in which they intercalate into the lipid bilayer membrane as revealed from isotropic hyperfine splitting factor measurements in the case of spin labels and from the present results in the case of pyrene derivatives.

Fluorescence spectra and quenching processes. Fluorescence spectra were taken with a Schoeffel M 460 photometer equipped with a cooled red-sensitive photomultiplier and a thermostated sample holder.

The quenching process is expected to follow the Stern-Vollmer relation

$$\frac{I_0}{I} - 1 = k_Q \cdot \tau_0 \cdot [Q]$$

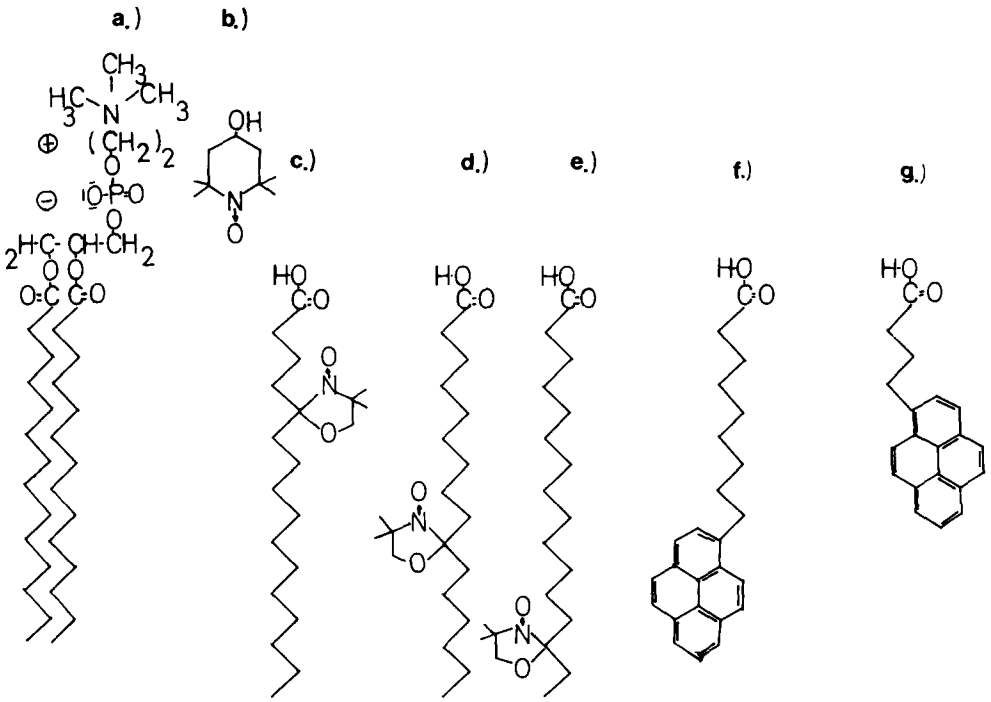

Fig. 1. Molecular structure of probes that are employed in the experiments and their location relative to the membrane surface. (a) Dimyristoyl phosphatidylcholine; (b) TEMPOL; (c) 5-nitroxide stearate; (d) 12-nitroxide stearate; (e) 16-nitroxide stearate; (f) pyrene decanoic acid, and (g) pyrene butyric acid.

where I_0 is the fluorescence intensity in the absence of quencher molecules, I is the emission intensity in the presence of quencher molecules, k_Q is the quenching constant, τ_0 the lifetime of the excited state in the absence of quencher and [Q] is the concentration of quencher molecules. Two types of quenching processes are possible: (1) dynamic quenching which is diffusion controlled and therefore most efficient in fluid solutions. In that case the lifetime of the excited state depends on the encounter probability and thus on the diffusion coefficient. (2) Static quenching requires an active sphere which is a volume of interaction around the quencher molecule. A fluorescent molecule excited within this volume is quenched instantaneously while molecules excited outside this volume remain unquenched.

Irradiation was performed at $\lambda = 337$ nm, $\lambda = 345$ nm and $\lambda = 345$ nm for pyrene, pyrene butyric acid and pyrene decanoic acid, respectively. Chlorophyll *a* was irradiated into its red absorption band at $\lambda = 550$ nm. The absorption of TEMPOL is negligible at these wavelengths *.

Lifetime measurements. Fluorescence lifetimes of the pyrene monomeres are measured with a Lambda Physics N_2 laser flash spectrometer M 100 A. The fluorescence decrease after a flash pulse of 4 ns halfwidth is followed over a

* In our earlier paper [10] chlorophyll *a* was irradiated at its Soret-band at $\lambda = 420$ nm. At this wavelength some light is absorbed by the highly concentrated TEMPOL solution. This led to a somewhat too large quenching constant. The correct values can be derived from Fig. 4.

time period of about 500 ns. All probes were freed of oxygen by a vigourous nitrogen stream.

Results

Paramagnetic quenching of pyrene fluorescence by fatty acid spin probes

Pyrene butyric acid and pyrene decanoic acid (molecules g and f in Fig. 1) were used to demonstrate the validity of the concept that quenching can be used to determine the location of fluorophores within lipid bilayer membranes. Stern-Vollmer plots are given for the 5-, 12- and 16-nitroxide stearate incorporated into dimyristoyl phosphatidylcholine vesicles at $T = 30°C$, a temperature which is above the lipid phase transition temperature. Pyrene butyric acid intercalated with the centre of its aromatic ring near carbon C-6 of the fatty acid chain is most efficiently quenched by the 5-nitroxide stearate. But there is still some quenching using 12-nitroxide stearate and 16-nitroxide stearate. The results are shown in Fig. 2a. The quenching of pyrene decanoic acid is given in Fig. 2b. Again most efficient quenching is obtained by the spin label having nearly the same distance from the membrane surface as the chromophore group which is now centered at carbon C-12 of the fatty acid chain. The 5-nitroxide stearate is less efficient but a better quencher than the 16-nitroxide stearate. Note that a Stern-Vollmer relationship of $I_0/I - 1 = 11$ implies an intensity reduction by a factor of 10.

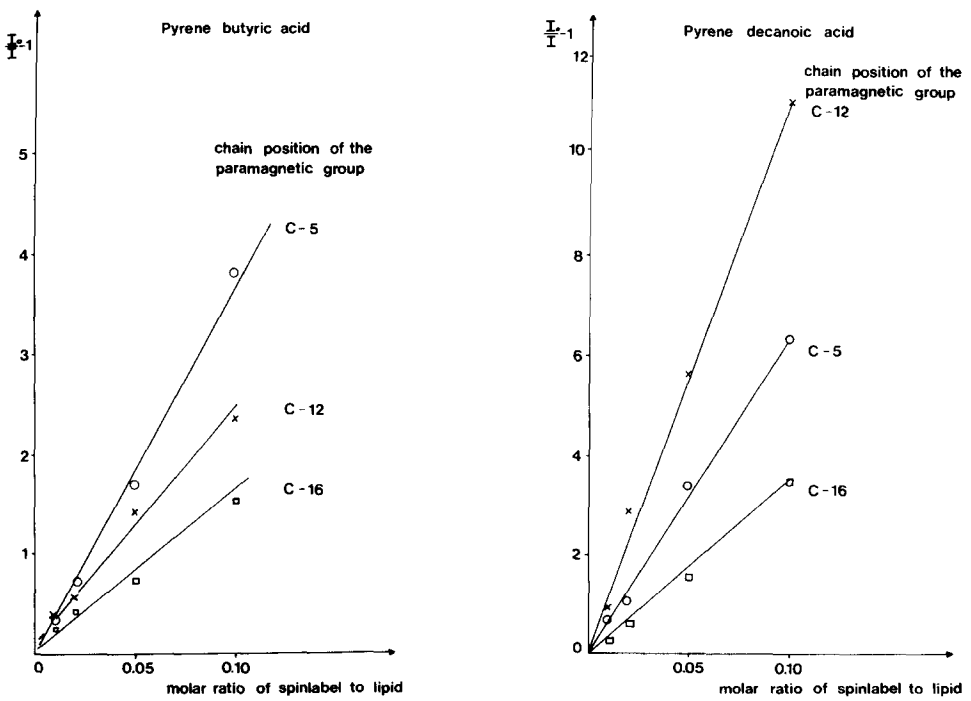

Fig. 2. Stern-Vollmer plots of fatty acid spin label quenching in dimyristoyl phosphatidylcholine membranes at $T = 32°C$. Pyrene butyric acid and pyrene decanoic acid are used as fluorophores. Lipid concentration: 10^{-3} M in $2 \cdot 10^{-3}$ M CsCl solution.

Quenching of chlorophyll a fluorescence by fatty acid spin labels

The Stern-Vollmer plots of paramagnetic quenching of the Chl *a* fluorescence in dimyristroyl phosphatidylcholine bilayer vesicles are given in Fig. 3. The chlorophyll content amounts to 1 mol% with respect to the lipid whereas the membrane is in the fluid state at the given temperature of $T = 32°C$. Strong quenching is observed by the 5-nitroxide stearate which is oriented near the polar surface of the lipid layer. The quenching effect of the 12-nitroxide stearate is much smaller whereas nearly no change in the fluorescence intensity is observed by addition of 16-nitroxide stearate. Therefore a location of the porphyrin ring near the polar head-group region must be assumed.

Paramagnetic quenching of chlorophyll a fluorescence by a water-soluble spin label

TEMPOL is used to quench the fluorescence of the chromophore being localized near the lipid-water interphase. Up to a spin probe concentration in

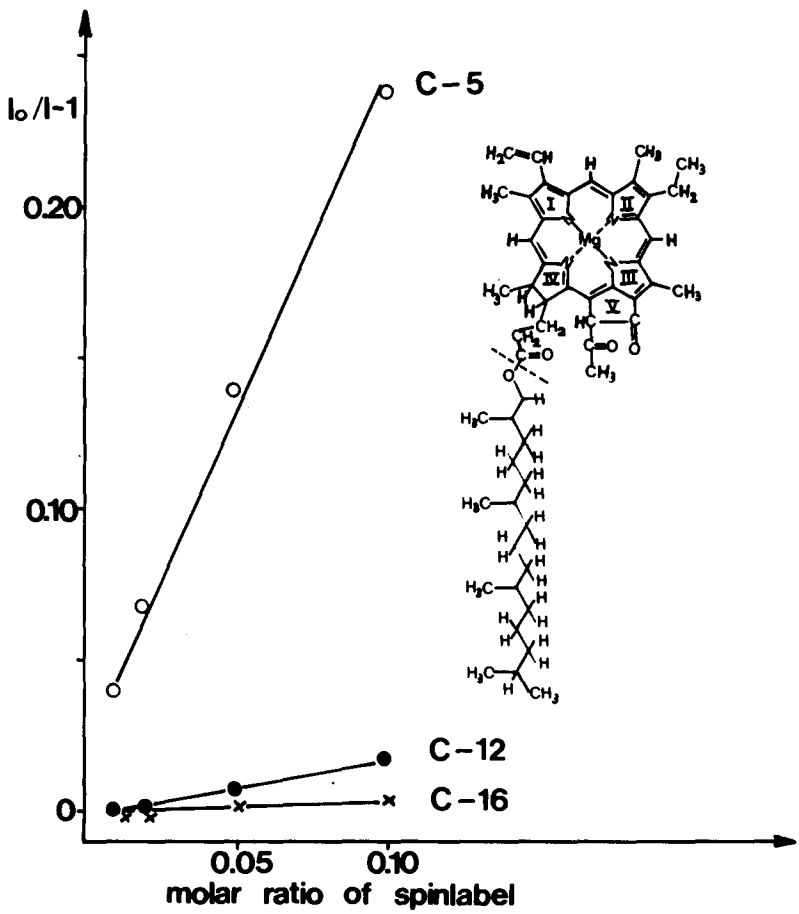

Fig. 3. Stern-Vollmer plot of paramagnetic quenching of the chlorophyll *a* fluorescence in dimyristoyl phosphatidylcholine membranes at $T = 32°C$. The chlorophyll *a* concentration is 1 mol% according to the lipid. C-5, C-12 and C-16 nitroxy stearate were used as quencher.

the water phase of about 0.01 M the Chl *a* fluorescence remains nearly uneffected. At concentrations up to 0.1 M a linear Stern-Vollmer relationship is observed. Fig. 4 represents a probe containing 1 mol% of Chl *a* in dimyristoyl phosphatidylcholine vesicles at $T = 8°C$, which is below the lipid phase transition temperature. Addition of spin label reducing ascorbate solution yields a 100% fluorescence recovery. The insert in Fig. 4 shows the increase in the fluorescence intensity of a 500 µl probe containing 0.1 M TEMPOL as function of ascorbate addition.

Formation of chlorophyll aggregates as determined by paramagnetic quenching

From Eqn. 1 we see that the quenching process depends on the concentration of quencher molecules and on the lifetime, but not on the concentration of the fluorophore. Fig. 5 shows the Stern-Vollmer plots for dimyristoyl phosphatidylcholine vesicles containing Chl *a* in different amounts relative to the lipid. The slope in the straight lines remains constant up to a chlorophyll content of about 1 mol%. Increasing the chlorophyll concentration leads to a decrease of the slope of the Stern-Vollmer plot reflecting a decrease in the lifetime τ_0 of the fluorophore. This result will be interpreted in terms of the formation of chlorophyll aggregates.

Quenching of pyrene fluorescence by TEMPOL

TEMPOL quenching is also effective for chromophores incorporated in the apolar part of the lipid bilayer. The accessibility of the chromophore depends

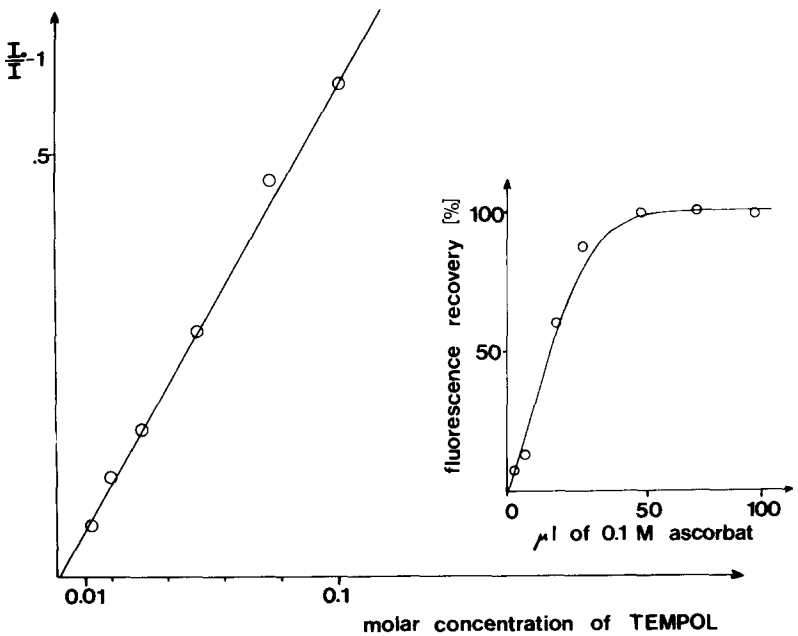

Fig. 4. Chlorophyll *a* fluorescence quenched by the water-soluble spin probe TEMPOL. The chlorophyll *a* concentration amounts to 1 mol% with respect to the lipid. Irradiation was performed at $\lambda = 550$ nm. The insert shows the fluorescence recovery obtained after partially reduction of spin label molecules by addition of ascorbic acid. Probe volume: 500 µl dispersion in a 0.1 M TEMPOL solution.

on the way in which it intercalates into the phospholipid bilayer. The Stern-Vollmer plots for pyrene, pyrene butyric acid and pyrene decanoic acid are given in Fig. 6 for dimyristoyl phosphatidylcholine membranes in the rigid state ($T = 8°C$). Quenching efficiency decreases with increasing distance of the chromophore from the membrane surface. Pyrene which even at low concentrations is reported to form clusters in the apolar part of the membrane [14] at a temperature below the lipid phase transition temperature is quenched least of all.

Lifetime measurements

An example of lifetime measurements with (0.1 M TEMPOL) and without quencher is given in Fig. 7 for pyrene incorporated into a dimyristoyl phosphatidylcholine bilayer.

The fluorescence decay versus time is shown on a semilogarithmic plot. Equivalent plots were obtained for pyrene butyric acid and pyrene decanoic acid in dimyristoyl phosphatidylcholine membranes. The measurements were

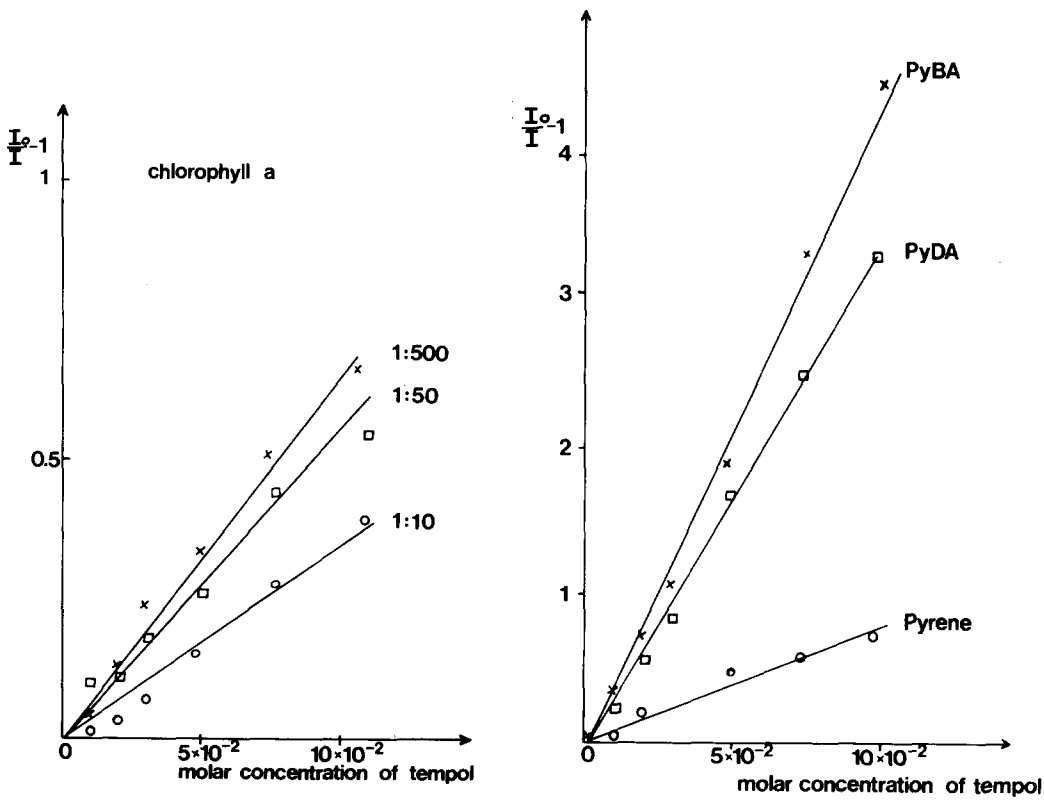

Fig. 5. Stern-Vollmer plots of TEMPOL quenching of dimyristoyl phosphatidylcholine dispersions containing 0.2, 2 and 10 mol% chlorophyll *a*.

Fig. 6. Stern-Vollmer plots of fluorescence quenching by TEMPOL for pyrene, pyrene decanoic acid and pyrene butyric acid. The lipid dispersions (10^{-3} M in $2 \cdot 10^{-3}$ M CsCl solution). Contain 1 mol% of fluorescent probe according to the lipid.

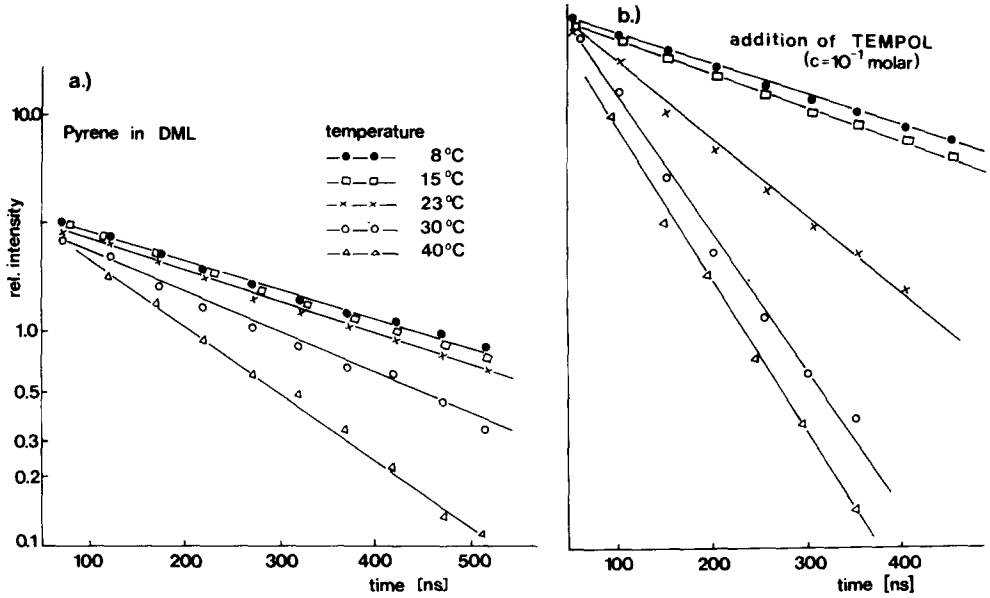

Fig. 7. Semilogarithmic plot of the fluorescence decay of pyrene after pulsed excitation. The corresponding lifetimes are given in Table I. (a) Pyrene in dimyristoyl phosphatidylcholine in the absence of quencher molecules; (b) addition of TEMPOL to come to 0.1 M concentration.

performed at temperatures below (8 and 15°C) and above (30 and 40°C) the lipid phase transition temperature of $T = 23°C$. The lifetimes obtained for the excited states of the pyrene derivatives are given in Table I. Although upon addition of paramagnetic quencher molecules the fluorescence is reduced drastically, only minor changes of the lifetime are observed at low temperatures. Above the lipid phase transition temperature the lifetime is reduced drastically. This can be understood as resulting from dynamical quenching in the fluid state of the lipid matrix.

TABLE I

Lifetimes of the excited states of pyrene, pyrene butyric acid and pyrene decanoic acid in the absence (τ_a) and in the presence (τ_p) of 0.1 M TEMPOL solution. The lipid concentration amounts to $c = 10^{-3}$ M containing 1 mol% of the corresponding pyrene derivatives.

Temp. (°C)	Lifetimes (ns)					
	Pyrene		Pyrene butyric acid		Pyrene decanoic acid	
	τ_a	τ_p	τ_a	τ_p	τ_a	τ_p
8	290	290	260	205	260	230
15	290	290	260	200	260	230
23	270	125	220	160	230	135
30	230	75	200	100	210	90
40	165	60	195	70	180	70

Discussion

In our preliminary paper [10] we have concluded that the porphyrin ring of a chlorophyll molecule in a lipid layer is located near the polar head group region but does not protrude out into the water phase. This is in agreement with experiments of other authors [16,17]. The results of this work allow a better understanding of the earlier experiments because we investigated quenching of the fluorophores pyrene butyric acid and pyrene decanoic acid of known location within the membrane (e.g. Fig. 2). In addition there is also quenching by spin labels whose active centres are localized at some distance from the fluorophore. This must be attributed to the flexibility of the fatty acid chains which allows the functional groups to approach each other more closely. In the case of pyrene decanoic acid with the chromophore being localized at carbon C-12 of the lipid fatty acid chain a more efficient quenching is observed for the 5-nitroxide fatty acid than for the terminal 16-nitroxide spin label. This shows that an upward deflection of the pyrene ring in direction to C-5 is more favoured than an upward deflection of the terminal spin label group directed to the pyrene ring. Pyrene butyric acid which is located closer to the polar water interphase is quenched less efficiently when the nitroxide group is immersed more deeply in the apolar membrane region. Thus we have obtained an accurate method to determine the position of fluorophores being situated near the membrane surface as it is expected for chlorophylls in view of their moelcular structure (see insert Fig. 3). Indeed strong quenching of chlorophyll fluorescence is obtained only for the 5-nitroxy stearate whereas the 12- and the 16-nitroxy spin labels are nearly uneffective. From these results we assume that the porphyrin ring must be located nearer to the membrane surface than the aromatic ring of the pyrene butyric acid.

Now the question arises whether or not the porphyrin ring protrudes out of the membrane into the water phase. We showed that there is fluorescence quenching with the water-soluble spin label TEMPOL even below the lipid phase transition temperature. This indicates that the spin labels are soluble in the rigid lipid matrix to some extent. We were not able to observe this dissolved fraction directly in the EPR spectra. However, an estimated solubility limit of about 1% in the lipid phase would lead to a partitioning coefficient of only 10^{-4} between the lipid and the water phase at a given lipid concentration of 10^{-3} M. Therefore the strong peak arising from spin labels in the water phase hides the signal corresponding to the spin label in the lipid phase (the two signals are only separated by about 1 G from each other). The TEMPOL quenching is completely reversible on addition of spin probe-reducing reagents. This is demonstrated in the insert of Fig. 4 for a spin label reduction by an ascorbate solution.

The decrease in the slopes of the Stern-Vollmer plots of chlorophyll quenching (Fig. 5) with increasing chlorophyll concentration can be understood as being due to a reduction of the fluorescence lifetime. This appears reasonable since for these concentrations we also observed self quenching [18]. The decrease in the slopes can be understood on the assumption that there exist chlorophyll-enriched domains in the lipid matrix. Such domains have been described very recently on a monolayer containing pheophytin [20,21]. Within

these domains aggregates are formed which reduce the fluorescence lifetimes. The observed changes in the slopes of the Stern-Vollmer plots therefore support the monolayer experiments.

There remains an open question as to the mechanisms leading to quenching when water-soluble quenchers are used. Two types of quenching processes may be envisaged [1]: (a) dynamic quenching, and (b) static quenching. Dynamic quenching occurs only when the quencher can diffuse randomly within the solvent which allows collisional encounters with the chromophores. This diffusion is only possible in the fluid state of the membrane and therefore shortens the lifetime of the excited state of the fluorophore only in this case. Static quenching is a viscosity-independent process and can only occur when the quenching partners are in close contact. Then the fluorescence is quenched immediately. Fluorophores having no quencher molecule in their active spheres remain unaffected during their lifetime.

Direct evidence of fluorescence quenching by water-soluble molecules can be drawn from the experiments with pyrene, pyrene butyric acid and pyrene decanoic acid incorporated into dimyristoyl phosphatidylcholine bilayers. Quenching was observed in all cases below the lipid phase transition temperature whereas the quenching efficiency decreases with increasing distance of the fluorophore from the lipid-water interphase. This gives directly a solubility profile of the spin probes within the membrane. Our quenching experiments establish that the spin label is not entirely squeezed out of the rigid membrane. However, free diffusion in the lipid matrix below the lipid phase transition temperature has never been observed. This excludes a dynmaic quenching. From our lifetime measurements we postulate a static quenching process in the rigid state of the membrane.

Consider pyrene incorporated into dimyristoyl phosphatidylcholine bilayers. The lifetime of the excited state remains unaffected upon addition of TEMPOL at temperatures below the lipid phase transition. τ_a, the lifettime in the absence of TEMPOL and τ_p, the lifetime in the presence of TEMPOL, are found to be $\tau_a = \tau_p = 290$ ns. Above the lipid phase transition, however, (at $T = 30°C$) there is a remarkable decrease in the lifetime from $\tau_a = 228$ ns to $\tau_p = 67$ ns. Equivalent results for pyrene butyric acid and pyrene decanoic acid are shown in Table I. At $T = 8°C$ the lifetime decreases slightly upon addition of TEMPOL ($\tau_a = 260$ to $\tau_p = 205$ ns for pyrene butyric acid and $\tau_a = 260$ to $\tau_p = 230$ ns for pyrene decanoic acid). These effects are due to an increased solubility of the spin probes near the more polar membrane region which may allow some dynamic encounteres. But again upon going through the lipid phase transition the lifetime is drastically shortened as discussed for pyrene. At $T = 30°C$ for example $\tau_a = 210$ ns is reduced to $\tau_p = 80$ ns in case of pyrene decanoic acid. This is interpretable in the following way: below the lipid phase transition only static quenching due to solubilized TEMPOL molecules takes place. Within the active sphere a fluorophore is quenched immediately whereas the rest of the molecules remains unaffected. This process requires a high amount of quencher molecules (more than 10% concerning the lipid) within the bilayer membrane. This has never been observed by EPR spectroscopy in pure lipid systems. However, the incorporation of probe molecules such as pyrene derivatives leads to a perturbation of the quasi-crystalline lipid matrix below the phase transition

tempeature because the rigidified lipid hydrocarbon chains cannot frame these molecules. Therefore it seems reasonable to assume that the pyrene derivatives themselves create a pocket within the lipid matrix to give space for a quencher molecule. This quencher-fluorophore 'complex' may explain the static quenching which is clearly shown in the constant values of the lifetime upon TEMPOL addition. This process is viscosity independent. An increase of the temperature from $T = 8°C$ to $T = 15°C$ does not change the lifetime. Starting at the phase transition temperature $T = 23°C$ an additional dynamic quenching process becomes important. As the number of encounters between fluorophores and quencher molecules increases, a strong reduction of the lifetimes is observed. Moreover this process is viscosity dependent, e.g. on going from $T = 30°C$ to $T = 40°C$, the lifetime is decreased by about 20—30%.

Coming back to the problem of the chlorophyll orientation we can postulate that the fluorophore does not need to protrude out into the water phase to be quenched by a water-soluble quencher. Quenching in an isotropic phase is expected to be more efficient by about a factor of ten [19]. Looking at the Stern-Vollmer relationship a value of $I_0/I - 1 = 4.5$ is obtained for pyrene butyric acid whereas the corresponding value for chlorophyll *a* comes to $I_0/I = 0.65$, which is about a factor of seven different. The lifetimes of the excited states of chlorophyll ($\tau = 6$ ns [19]), and of pyrene derivatives however, differ by about a factor of 35. This means that the chlorophyll fluorescence is quenched more efficiently as compared to pyrene butyric acid. This again shows us that the porphyrin ring should be localized near to the polar head group region of the membrane.

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