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FLUORESCENCE QUENCHING IN LECITHIN AND LECITHIN/CHOLESTEROL LIPOSOMES BY PARAMAGNETIC LIPID ANALOGUES

INTRODUCTION OF A NEW PROBE APPROACH

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SUMMARY

1. Perylene, whether incorporated into lecithin or lecithin/cholesterol (1:1) liposomes, exhibits identical fluorescence spectra, but fluorescence in the presence of cholesterol is enhanced by 30–50 %.

2. The fluorescence of perylene in pure dipalmitoyllecithin vesicles increases sharply at the transition temperature (T_t = 41 °C). No such fluorescence jump is observed in lecithin/cholesterol (1:1) micelles.

3. In lecithin liposomes maximal quenching of perylene fluorescence at 25 °C is effected by cholestane spin label (80 %) followed by androstane spin label (70 %), 5-nitroxide stearate (60 %) and 16-nitroxide stearate (50 %).

4. In liposomes containing 50 mol % cholesterol these differences are reduced; however, the sequence of quenching efficiencies is the same except for the nitroxide stearates, which interchange their positions.

5. Paramagnetic quenching of perylene fluorescence is stable below 35 °C and above 45 °C, but decreases sharply about the phase-transition temperature of dipalmitoyllecithin.

6. In lecithin/cholesterol (1:1, molar ratio) liposomes fluorescence quenching diminishes linearly, but only slightly, with increasing temperature.

7. Cholestane spin label and androstane spin label at concentrations of > 20 mol % themselves suppress the quenching discontinuity at T_t, indicating a cholesterol-like structural effect.

8. The quenching phenomena observed are attributed to a non-random accommodation of fluorophore and quencher molecules (co-clustering) below the phase transition and a statistical distribution of both impurities above T_t.

9. In the presence of cholesterol the clustering tendencies are reduced or even eliminated; this is compatible with the concept that cholesterol fluidizes the phosphatide acyl chains below the transition temperature.

INTRODUCTION

Paramagnetic substances, such as nitroxides, can reduce the excited-state lifetimes of fluorophores and can thereby quench fluorescence [1–3]. Such paramagnetic quenching involves interaction distances of only 4–6 Å [4] and Jost et al. [5] have accordingly suggested that evaluation of this process in the cases of macromolecules and membranes might give an indication of the relative positions of bound nitroxide radicals and fluorophores. The validity of this concept has now been demonstrated. Thus, Koblin et al. [6] have shown that the fluorescence of 1-anilino-8-naphthalene sulfonate bound to erythrocyte membrane can be quenched by the subsequent absorption of nitroxide-labelled anesthetics and we have demonstrated quenching of the tryptophan fluorescence of erythrocyte ghosts and bovine serum albumin by nitroxide-stearates [7, 8].

In order to apply the technique of paramagnetic quenching of fluorescence rigorously to the study of biomembranes, we have examined the quenching of the fluorophore, perylene, incorporated into dipalmitoyllecithin or dipalmitoyl/lecithin/cholesterol liposomes, utilizing several nitroxide-labelled lipid derivatives thought to probe different "depths" of membrane hydrophobic regions [9, 10]. In a subsequent report, we will describe the use of the paramagnetic quenching technique for the study of lipid-protein interactions in biomembranes.

EXPERIMENTAL

Chemicals: 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl ,5-nitroxide stearate, 2-(3-carobyxpropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl-methylester (5-nitroxide stearate-methyl ester), 2-(14-carboxytetradecyl)-2-ethyl-4, 4-dimethyl-3-oxazolidinyloxyl (16-nitroxide stearate), 17β-hydroxyl-4′,4′-dimethyl-spiro [5α-androstane-3,2′-oxazolidin]-3′-yloxyl (androstane spin label) and 4′, 4′-dimethylspiro [5α-cholestane-3,2′-oxazolidin]-3′-yloxyl (cholestane spin label) were purchased from Synvar (Palo Alto, California).

Perylene and Δ^5-cholestene-3-ol (cholesterol) were obtained from Sigma (St. Louis, Mo.) and L-α-dipalmitoyllecithin from Calbiochem (San Diego, California).

Preparation of liposomes

Lecithin/perylene, lecithin/cholesterol/perylene and lecithin/cholestane spin label/perylene liposomes were prepared by mixing suitable volumes of chloroformic stock solutions of known concentration in 16×100 mm glass test tubes. To produce a thin, uniform lipid film, the solvent was evaporated by slowly rotating the tube under gentle vacuum. The vacuum-dried mixtures (0.5 mm Hg, 10 min, room temperature) were sonicated in 0.01 M phosphate, pH 7.2, at 50 °C with the microtip of a Quigley dismembrator (Rochester, N.Y.) for 10 min, at power step 20. The sonicates were then centrifuged at $4.7 \cdot 10^6$ g $\cdot$ min^{-1} (Spinco L2 65 B ultracentrifuge; rotor SW 56) and the supernatant fluids used for fluorescence studies. Typically 10^{-6} mol lecithin were mixed with 10^{-9} mol perylene and, after drying, sonicated in 10 ml buffer.

Titration with nitroxides

5-Nitroxide stearate, 16-nitroxide stearate and androstane spin label solutions were dissolved in 0.01 M phosphate, pH 7.2, as in ref. 11 and the solutions used to titrate perylene fluorescence. Dilution effects were eliminated by titration of reference samples with buffer. Perylene fluorescence became stable within 10 min after changing nitroxide stearate concentration; in androstane spin label experiments, equilibrium was reached after 20 min.

Perylene fluorescence was measured with a Perkin-Elmer MPF 3A spectrophotometer with excitation at 412 nm (slit width 6–8 nm) and emission at 473 nm (slit width 7–9 nm).

INTRODUCTORY THEORETICAL CONSIDERATIONS

In solution, the quenching process is expected to follow the Stern-Volmer relationship

$$(I_0/I)-1 = K_Q \cdot \tau \cdot [Q] \tag{1}$$

where I_0 = intensity of fluorescence emission without quencher, I = the emission intensity in the presence of quencher, K_Q is the Stern-Volmer quenching constant, τ is the lifetime of the excited state and $[Q]$ is the concentration of the quenching agent. In the present case, however, we are concerned only with those molecules of quencher which are located in the perylene-containing liposomes. In the case of cholestane spin label, essentially all of the nitroxide will be within the lipid bilayers. However, androstane spin label, 5-nitroxide stearate and 16-nitroxide stearate possess appreciable water solubilities and will thus be distributed between the bulk phase and the liposomes according to the partition coefficient, $K = Q_{li} \cdot V_w/Q_w \cdot V_{li}$, where Q_{li} and Q_w are the numbers of quencher molecules in the liposome and water compartments, respectively, $Q_{li}/Q_w = D$, the mass distribution coefficient and V_{li} and V_w are the volumes of the lipid and water compartments. K has been reported to lie near 10^5 for 12-nitroxide stearate and dipalmitoyllecithin below the transition temperature [12], T_t, but approaches 10^6 for egg lecithin above T_t. At our phospholipid concentrations, 10^{-4} M, i.e. $V_w/V_{li} \geqslant 10^4$, we might expect to find as much as 10 % of the quencher in the aqueous phase. Defining Q as equal to the total number of quenching molecules

$$(I_0/I)-1 = K_Q \cdot \tau \cdot Q\ (D/1+D) \tag{2}$$

In the liposome case, both fluorophore and quencher are constrained within an essentially two-dimensional domain, the lipid bilayers. The quencher concentration is thus more appropriately expressed in terms of surface density. Proceeding according to Träuble and Sackmann [13], we define ρ as the total number of molecules (phospholipid+quencher+fluorophore) per unit area of bilayer surface and A as the area/molecule, $\rho = 1/A$. As an approximation one can assume a single value of A for the phospholipids, nitroxides and perylene. If ρ_{li}, ρ_Q and ρ_f are the number of phospholipid, quencher and fluorophore molecules per unit surface, respectively, $\rho = \rho_{li}+\rho_Q+\rho_f$ and the molar ratio [quencher: other lipid] is

$$c = \frac{\rho_Q}{\rho_{li}+\rho_f} \tag{3}$$

then

$$\rho_Q = \rho \cdot \frac{c}{1+c} = \frac{1}{A} \cdot \frac{c}{1+c} \tag{4}$$

accordingly

$$(I_0/I)-1 = K_Q \cdot \tau \cdot \frac{1}{A} \cdot \frac{c}{1+c} \tag{5}$$

When the quencher and fluorophore molecules are distributed statistically in the lipid phase but unable to diffuse, Eqns 2 and 5 should hold; i.e. one should observe a linear increase of quenching with either Q (Eqn 2) or the "density" $c/(1+c)$ of the quencher in the bilayer (Eqn 5). The same would be true if the quencher and fluorophore molecules were free to diffuse laterally within the bilayers. However, if the quencher and fluorophore molecules are distributed in "clusters", $(I_0/I)-1$ cannot be directly proportional to $c/(1+c)$ but should vary linearly with $((1+c)/c)^{1/2}$ over a wide range of c. The term $((1+c)/c)^{1/2}$ is proportional to the average nearest-neighbor-distance between the interacting molecules.

RESULTS

Perylene fluorescence in lecithin and lecithin/cholesterol micelles as a function of temperature

The fluorescence spectra of perylene incorporated into lecithin liposomes are identical to those found with lecithin/cholesterol liposomes. However, vesicles containing 1–40 mol % cholesterol exhibit 30–50 % higher fluorescence intensities than pure lecithin vesicles.

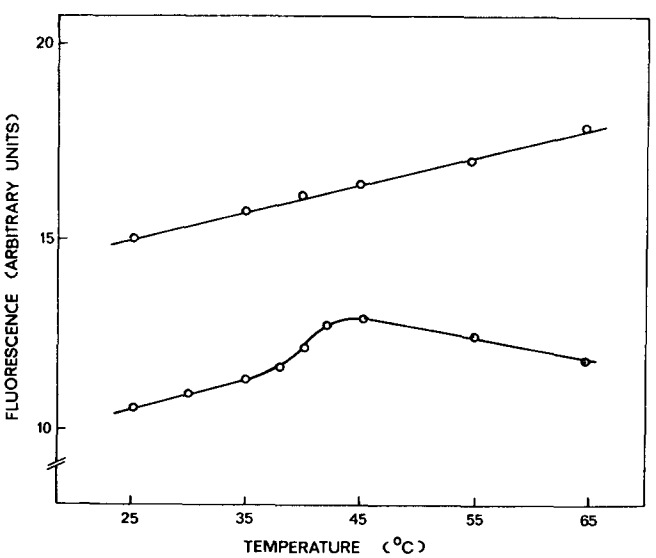

Fig. 1. Temperature dependence of perylene fluorescence in lecithin and lecithin/cholesterol (molar ratio, 1 : 1) liposomes. See text for details.

Fig. 1 depicts the temperature dependence of perylene fluorescence between 20 and 60° C. In lecithin liposomes, fluorescence rises linearly up to 38 °C; between 38 and 42 °C; (i.e. at the transition temperature T_t, of dipalmitoyllecithin) a sharp increase is manifest, and above 45 °C fluorescence again declines. In contrast, the fluorescence of perylene in lecithin/cholesterol liposomes (molar ratio 1:1) shows a continuous slight linear increase over the whole temperature range.

Quenching of perylene fluorescence by cholestane spin label

Fig. 2 shows how increasing proportions of the cholesterol analogue cholestane spin label incorporated into lecithin liposomes containing 0.1 mol % perylene influence perylene fluorescence at various temperatures. The fluorescence of cholestane spin label-containing liposomes is expressed as percentage of liposomes containing identical molar ratios of cholesterol instead of cholestane spin label. Fluorescence quenching is stable with temperature below 35 °C and above 45 °C. However, between these temperatures (mid point 41 °C) quenching decreases abruptly at cholestane spin label levels up to 10 mol %. At increasing proportions of cholestane spin label the discontinuity about T_t becomes progressively less pronounced. The

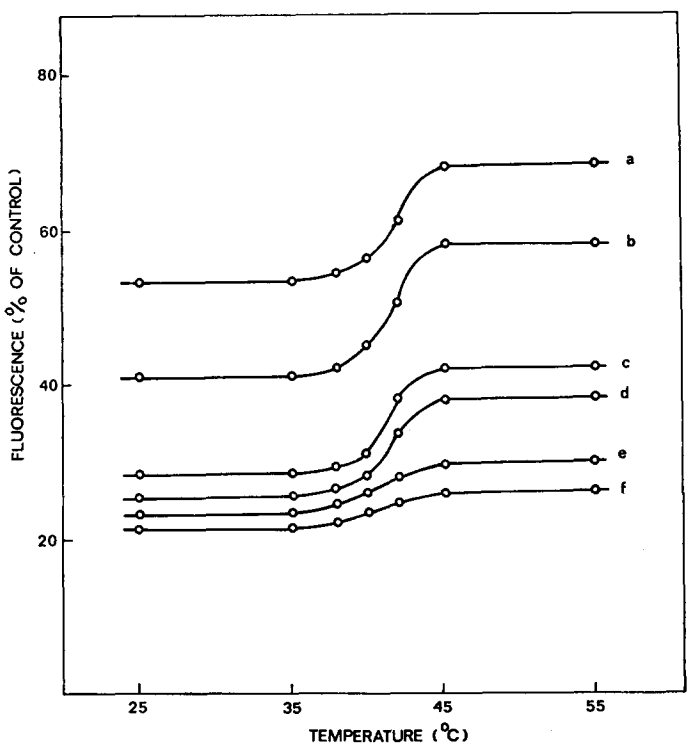

Fig. 2. Paramagnetic fluorescence quenching caused by cholestane spin label in a 10^{-4} M dipalmitoyllecithin dispersion containing 0.1 mol % perylene as a function of temperature: a, 1.0 mol %; b, 2.5 mol %; c, 5 mol %; d, 10 mol %; e, 20 mol %; f, 40 mol % cholestane spin label. Fluorescence intensity is compared to that of lecithin/perylene liposomes containing the appropriate amount of cholesterol.

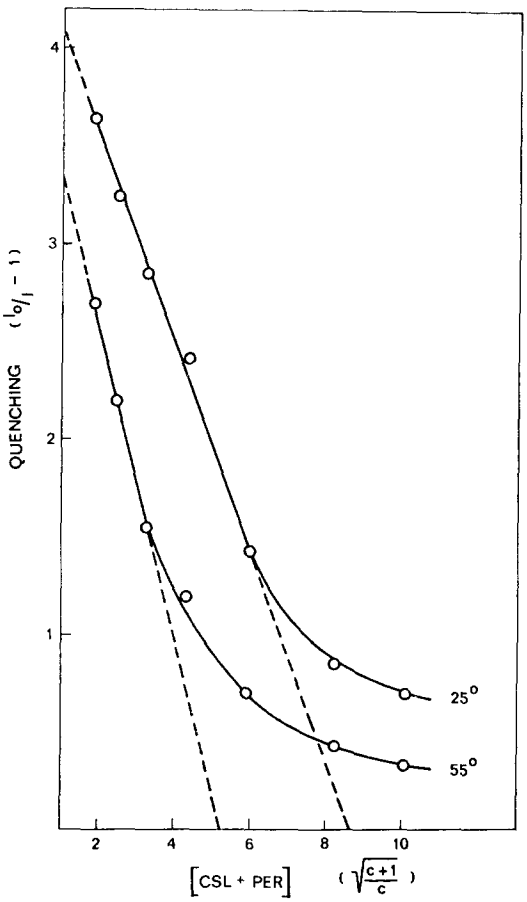

Fig. 3. Modified Stern-Volmer plots of cholestane spin label quenching in lecithin liposomes containing 0.05 mol % perylene above and below the phospholipid phase transition. Details in text.

thermotropic effects described are fully reversible. The data thus indicate that quenching becomes abruptly less effective about T_t, but that cholestane spin label, like cholesterol, suppresses the thermotropic gel → liquid crystalline transition.

Fig. 3 represents a modified Stern-Vollmer plot for cholestane spin label quenching at 25 and 55 °C. Here $(I_0/I)-1$ is plotted against $((c+1)/c)^{1/2}$, where c is the molar ratio (cholestane spin label+perylene): phospholipid. For both temperatures the quenching curves are linear at high quencher concentrations, but deviate at very low c values: at 25 °C the plot is linear between $c = 0.4$ and $c = 0.03$, but at 55 °C the linear range extends only to $c = 0.1$. Linear extrapolation to $((c+1)/c)^{1/2} = 1$ yields maximal quenching efficiencies, Q_{max}, of 80% at 25 °C and 77% at 55 °C. The "critical" c values, c_c, obtained by linear extrapolation to $(I_0/I)-1 = 0$ are 0.014 at 25 °C and 0.040 at 55 °C.

Influence of cholesterol on paramagnetic quenching by cholestane spin label

In lecithin liposomes containing 50 mol % cholesterol, paramagnetic quenching due to cholestane spin label is reduced by about 30 % at 25 °C (Fig, 4). Fluores-

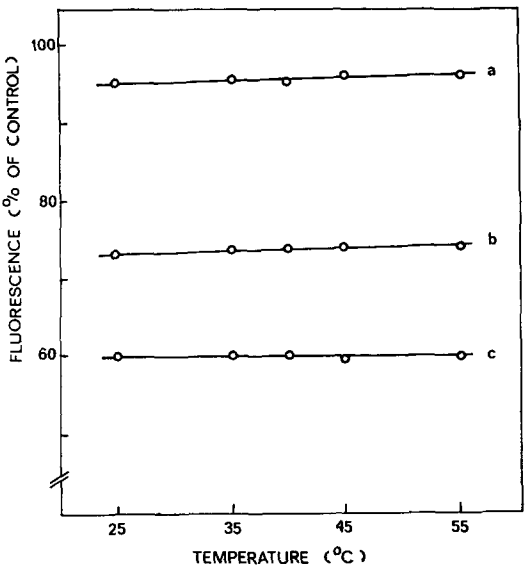

Fig. 4. Temperature dependence of fluorescence suppression by cholestane spin label in lecithin/cholesterol liposomes (molar ratio, 1 : 1): a, 1.0 mol %; b, 2.5 mol %; c, 5 mol % cholestane spin label. Perylene concentration was 0.1 mol % of total lipid.

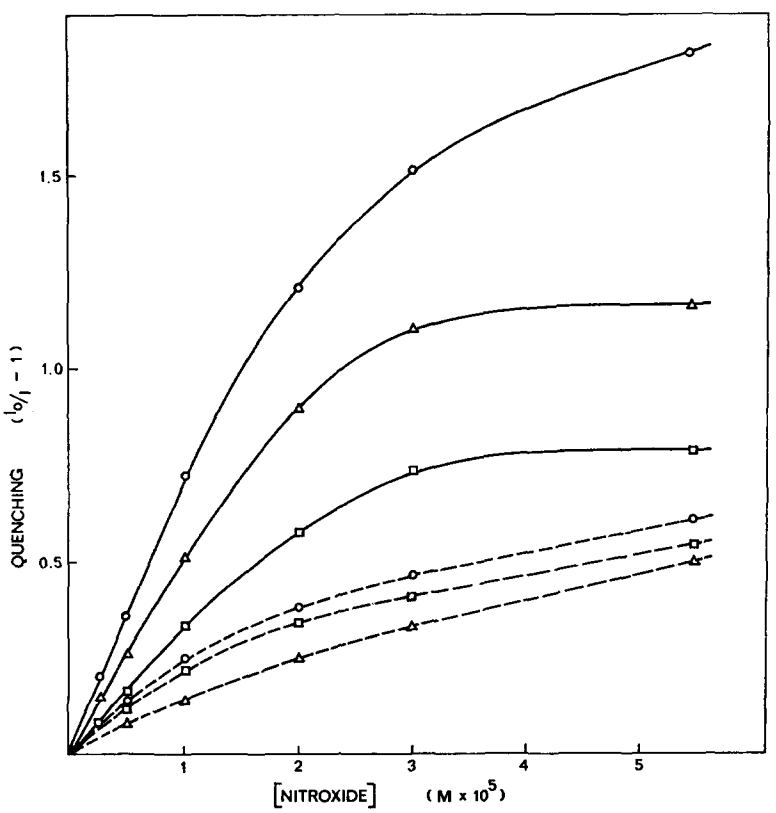

Fig. 5. Stern-Volmer plots of paramagnetic quenching in lecithin (—) and lecithin/cholesterol (1 : 1) (- - -) liposomes containing 0.05 mol % perylene at 25 °C with androstane spin label (○), 5-nitroxide stearate (△) and 16-nitroxide stearate (□); 2 ml of 10^{-4} M lipid dispersions were titrated with 10^{-4} M nitroxide solutions.

cence quenching diminishes only slightly with temperature and the decrement is linear up to 65 °C. No discontinuity is detected between 35 and 45 °C, whereas this is prominent in the absence of cholesterol at cholestane nitroxide proportions < 20 mol %. The fact that cholestane spin label itself can suppress the discontinuity at T_t at concentrations > 20 mol % indicates that this substance simulates cholesterol in its structural effects.

Paramagnetic quenching by androstane spin label, 5-nitroxide stearate and 16-nitroxide stearate

Androstane spin label, 5-nitroxide stearate and 16-nitroxide stearate also quench perylene fluorescence. As in the case of cholestane spin label there is a sharp decrease in quenching between 35 and 45 °C. The discontinuity is equally prominent at high and low concentrations of nitroxide stearate. However, androstane spin label acts like cholestane spin label when present at concentrations > 20 mol % and suppresses

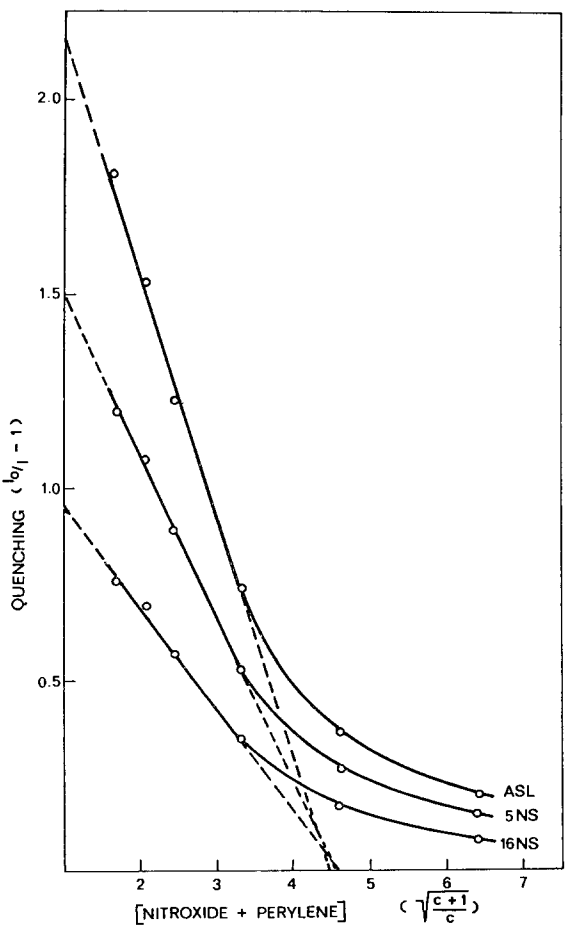

Fig. 6. Modified Stern-Volmer plots of fluorescence quenching with androstane spin label, 5-nitroxide stearate and 16-nitroxide stearate at 25 °C in lecithin liposomes (10^{-4} M) containing 0.05 mol % perylene. Details in text.

TABLE I

Stern-Volmer constants K_Q at 25 and 55 °C for androstane spin label, 5 and 16-nitroxide stearate in lecithin and lecithin/cholesterol (molar ratio, 1 : 1) liposomes. Perylene concentration was 0.05 mol % of total lipid.

Quencher	$K_Q \times 10^{-4}$			
	Lecithin		Lecithin/cholesterol	
	25 °C	55 °C	25 °C	55 °C
Androstane spin label	7.2	2.5	2.6	3.5
5-Nitroxide stearate	5.1	2.2	1.5	3.1
16-Nitroxide stearate	3.3	2.4	2.2	2.8

the discontinuity between 35 and 45 °C. This quenching discontinuity is also not seen in micelles containing 50 mol % cholesterol.

Fig. 5 depicts the quenching properties of androstane spin label, 5-nitroxide stearate and 16-nitroxide stearate at 25 °C in micelles of dipalmitoyllecithin with or without cholesterol in the usual Stern-Volmer manner. In lecithin liposomes the order of quenching efficiency is androstane spin label > 5-nitroxide stearate > 16-nitroxide stearate. Although 5-nitroxide stearate shows 30 % higher quenching than 16-nitroxide stearate, saturation is reached at about the same total concentration; this indicates a lower quenching efficiency for 16-nitroxide stearate. The fluorescence suppression by androstane spin label, although greater than that observed with both stearates, continues to increase even at bulk concentrations where quenching by 5-nitroxide stearate and 16-nitroxide stearate is maximal. In lecithin/cholesterol vesicles (molar ratio, 1:1) the quenching curves do not show saturation character. Moreover, the differences between 5-nitroxide stearate, 16-nitroxide stearate and androstane spin label become reduced; indeed, 16-nitroxide stearate is a slightly more effective quencher under these conditions than 5-nitroxide stearate.

At 55 °C 5-nitroxide stearate and 16-nitroxide stearate, yield virtually identical Stern-Volmer plots whether cholesterol is present or not. However, androstane spin label again deviates and acts as a far more efficient quencher than the nitroxide stearates. This point is illustrated in Table I which lists the Stern-Volmer constants, K_Q, calculated from the slopes of the Stern-Volmer plots at low quencher concentrations.

At 25 °C, the modified Stern-Volmer plot of the quenching of perylene fluorescence by androstane spin label, 5-nitroxide stearate and 16-nitroxide stearate for lecithin vesicles are linear down to $c = 0.01$ (Fig. 6). Extrapolations of the linear segments to $(I_0/I)-1 = 0$ yield a single "critical" c value of 0.046. However, extrapolation to $((c+1)/c)^{1/2} = 1$ suggests that the maximal quenching efficiencies are all less than that of cholestane spin label (80 %) and range in the order androstane spin label > 5-nitroxide stearate > 16-nitroxide stearate. At 55 °C, none of the $(I_0/I)-1$ vs $((c+1)/c)^{1/2}$ plots for androstane spin label, 5-nitroxide stearate or 16-nitroxide stearate are linear (Fig. 7). Lecithin/cholesterol vesicles (1:1, molar ratio) do not yield linear $(I_0/I)-1$ vs $((c+1)/c)^{1/2}$ plots for androstane spin label, 5-nitroxide stearate or 16-nitroxide stearate. On the contrary, quenching varies linearly with Q over a wide range of quencher concentration.

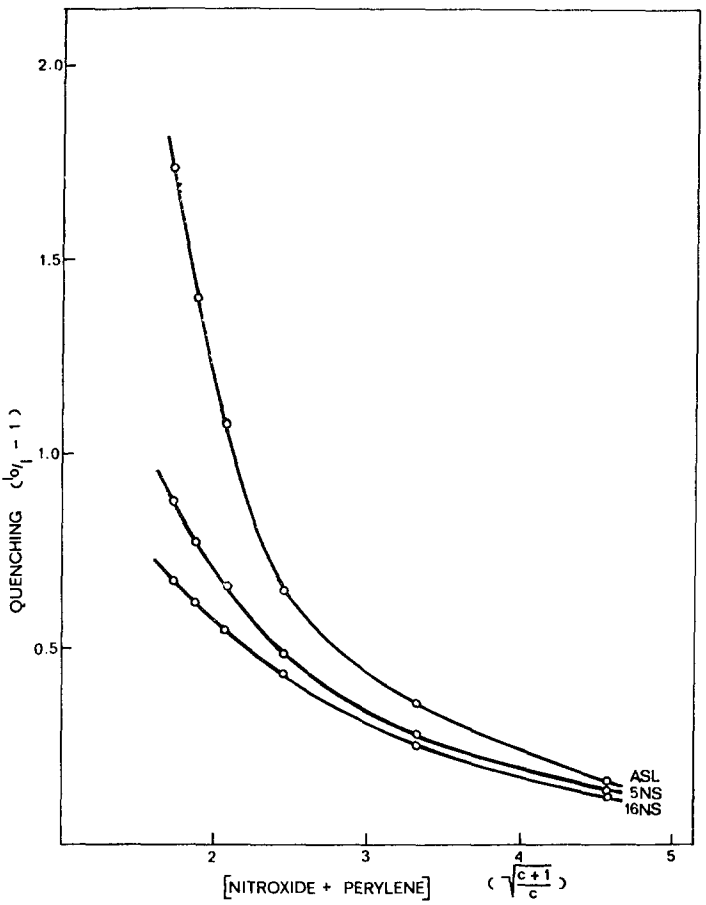

Fig. 7. Modified Stern-Volmer plots for androstane spin label, 5-nitroxide stearate and 16 nitroxide stearate at 55 °C; same conditions as in Fig. 6.

Effects of perylene concentration

It is conceivable that perylene, acting as an impurity within the hydrocarbon cores of the lecithin micelles, creates localized "fluid" regions which are more effective solvents for the nitroxide analogues than the pure lecithin regions. We accordingly evaluated paramagnetic quenching at perylene proportions ranging between 0.025 and 0.5 mol %. We find the same order of quenching efficiencies over the whole range of perylene concentrations. Moreover, extrapolations of the modified Stern-Volmer plots at 0.5 mol % perylene yield a c_c value of 0.058 for androstane spin label, 5-nitroxide stearate and 16-nitroxide stearate which is not significantly different in this system from the value of 0.05 found at 0.05 mol % perylene.

DISCUSSION

Known properties of the system

Dipalmitoyllecithin or dipalmitoyllecithin/cholesterol (1:1, molar ratio) form the continuous phases of our system; into these, the fluorescent probe and the

lipid spin probes are inserted. Extensive studies by X-ray diffraction, thermal techniques and NMR [14] indicate that hydrated dipalmitoylphosphatidylcholine with or without cholesterol forms bilayer leaflets. The pure, hydrated phosphatide exhibits a cooperative thermotropic order → disorder (crystal → liquid crystal) transition, centered at 41 °C. The presence of cholesterol above 20 mol % lowers the transition temperature as well as the heat of transition. By 50 mol % cholesterol, thermal techniques no longer reveal a phase transition. However, laser-Raman evidence [15] indicates that the thermotropic transition is shifted over a broad temperature range, rather than abolished. In effect, the cholesterol nucleus increases the fluidity of the palmitate chains below the transition temperature and interferes with chain flexing above the transition temperature, thus buffering the "fluidity" of the hydrocarbon domains at an "intermediate value" which is stable over a wide temperature range.

The fluorescence characteristics of perylene incorporated into dipalmitoyllecithin liposomes has been recently described [16]. A sharp increase of fluorescence emission is observed at the transition temperature of the phosphatide, although the emission of fluorophores generally decreases with temperature. The thermotropic fluorescence jump is not observed in lecithin/cholesterol liposomes (1:1, molar ratio). Our results confirm these findings. Other data on egg lecithin liposomes [16] show that above the transition temperature of such phosphatides, the fluorophore rotates at a rate of $2.75 \cdot 10^8\ s^{-1}$. This is slower than the rate found in *n*-hexadecane, $24 \cdot 10^8\ s^{-1}$, but the activation energy is the same in both cases, suggesting similar processes of rotational diffusion. However, in lecithin cholesterol liposomes (1:1, molar ratio) the rate of rotational diffusion drops to $9.0 \cdot 10^7\ s^{-1}$ with a lower activation energy. These data suggest two classes of perylene binding sites, namely a relatively polar one which allows less fluorescence emission than the second apolar category. The thermal effects can thus be interpreted to signify that the apolar regions can accommodate perylene more readily above the gel → liquid crystalline transition. When the palmitate chains crystallize below the transition temperature, the fluorophore tends to be forced into more polar domains with a concomitant loss of quantity efficiency. A corollary to this argument is that perylene tends to produce localized perturbations in the ordered structure existing below the transition temperature.

Our direct fluorescence measurements are compatible with the data in refs 16 and 17, but further indicate that the fluidizing action of cholesterol below the transition temperature allows the apolar domains to accommodate perylene more readily even below 41 °C.

Turning to the paramagnetic quenching, this process was originally thought to require that the nitroxide have an absorption band at longer wavelengths than the excited singlet state of the fluorophore [3]. However, a recent study by Green et al. [4] on the quenching by di-*t*-butyl nitroxide of the fluorescence of diverse aromatic hydrocarbons, including perylene, indicates that this is not the case and that the quenching process is either vibrational or due to electron exchange-induced intersystem crossing. Either mechanism is consistent with a 4–6 Å interaction distance. The bimolecular rate constants for quenching varied consistently with diffusion coefficient suggesting that the process occurs very near the diffusion limit. Perylene exhibited one of the highest quenching efficiencies.

The general properties of the four spin probes studied here have been well

characterized [9, 10]. However, recent studies show the stearate nitroxides as well as androstane spin label, act as membrane perturbants and tend to create their own "fluid" micro-environment [11]. Moreover, androstane spin label, 5-nitroxide stearate and 16-nitroxide stearate exhibit appreciable water solubility, increasing in that order [7]. Finally, Butler et al. [12] document that, in the absence of high affinity binding, stearate nitroxides tend to partition preferentially into "fluid" lipid systems.

Concentration dependence of quenching

As pointed out already, if the quencher and fluorophore molecules were fixed within the liposome walls, but were distributed statistically, one would expect $(I_0/I)-1$ to vary linearly with Q or $c/(1+c)$. The same would be true if the quencher and fluorophores could undergo unrestrained lateral diffusion within the bilayers. However, in dipalmitoyllecithin liposomes lacking cholesterol, we do not find a linear increase of $(I_0/I)-1$ with Q or $c/(1+c)$. Instead, at $T < T_t$, $(I_0/I)-1$ is proportional at all but very low values of c, to $((1+c)/c)^{1/2}$. This dependence indicates that at all but very low quencher concentrations, the quenching process takes place within molecular clusters at $T < T_t$.

Further support for this argument comes from the fact that the linear extrapolations of the plot of $(I_0/I)-1$ vs $((1+c)/c)^{1/2}$ to $(I_0/I)-1 = 0$, yields a limiting value of c, the "critical" c value c_c. This represents the gross quencher/total lipid ratio at which the quenching process represented by the linear segments no longer occurs. At c_c, the quencher-fluorophore distances have become too great to allow quenching. One can relate c_c to this "cutoff distance" d_c in the following way. Proceeding as in ref. [13], we assume that quencher, fluorophore and phospholipid occupy similar areas in a bilayer (as an approximation) and that they form triangular lattices, with the average distance d_c between fluorophore and quencher given by

$$d_c = (2F \cdot (3)^{-1/2})^{1/2} \cdot ((1+c)/c)^{1/2} \tag{6}$$

where F = the surface area of the unit cell in the triangular lattice.

Using the c_c data from Figs 3 and 6, and assuming that $F \cong 48$ Å at 25 °C (the area/molecule of dipalmitoyllecithin at $T < T_t$), we compute d_c values of 25 Å for androstane spin label, 5-nitroxide stearate and 16-nitroxide stearate at 25 °C and 63 Å for cholestane spin label. The values of Q_{max} are 70, 60 and 51 % for androstane spin label, 5-nitroxide stearate and 16-nitroxide stearate, respectively, and 80 % for cholestane spin label.

The very high Q_{max} of cholestane spin label confirms the observation of Green et al. [4] that nitroxide radicals can act as efficient fluorescence quenchers. However, our data indicate that, in the liposome model, there is a clear dependence of quenching upon the location of the nitroxide within the membrane. Experiments on liposomes, using ESR spectroscopy, indicate that the OH group of androstane spin label and the carboxyl residues of the nitroxide stearates lie at the membrane-water interfaces. The nitroxide radicals of these probes thus extend to different depths in the apolar regions of the membranes in the sequence 5-nitroxide stearate, androstane spin label, 16-nitroxide stearate, i.e. approximately to carbons 2, 8 and 14 of the phosphatide acyl chains. The situation is different with cholestane spin label where the OH residue of androstane spin label is replaced by a branched 8-carbon acyl chain. ESR experiments

indicate that this sterol intercalates between phosphatide molecules with its long axis parallel to those of the fatty acid chains. If the terminal methylenes lie at the same level as those of the phosphatide acyl chains, the nitroxide radical will be found at approximately carbon 6. This position is consistent with the fact that cholestane spin label cannot be readily reduced by ascorbate. In view of these consideration, we interpret the Q_{max} values to signify that the perylene moledules tend to localize preferentially at a "depth" equivalent to carbon 6 of the phosphatide acyl chains. This suggestion is compatible with other experimentation [17] suggesting that perylene lies in a "relatively polar" region of dipalmitoyllecithin liposomes below T_t.

What might be the significance of the "cutoff distance", d_c, calculated from the modified Stern-Volmer plots? The computed values are in considerable excess of the interaction range involved in paramagnetic quenching [4–6 Å) We, therefore, come to a conclusion analogous to that of Träuble and Sackmann [13]. Each cluster consists of a core in which the fluorophores and quencher molecules are so closely associated to make the probability of a quenching encounter very high. This core is surrounded by a shell of width d_c in which the probability of quenching is smaller, due to a lower concentration of interacting molecules. It is of interest that our measured d_c value for androstane spin label of 25 Å lies well below the 51 Å derived by Träuble and Sackmann [13] from their spin exchange measurements. This can be attributed to the fact that the interaction range for paramagnetic quenching (4–6 Å) lies well below that for spin exchange (15 Å). However, our measured value of 25 Å corresponds to the computed shell thickness of Träuble and Sackmann [13]. We, therefore, concur with these authors model: for $c < c_c$, the nitroxide and perylene molecules are dispersed statistically as solutes in the lipid matrix. For $c \geqslant c_c$, the quencher molecules co-aggregate with the perylene into small clusters. The size of the clusters increases with further increase of label concentration.

However, we must extend the model also to 5-nitroxide stearate and 16 nitroxide stearate spin label, which have the same d_c value as androstane spin label and differ only in the slope of the $(I_0/I) - 1$ vs $((1 + c)/c)^{1/2}$ plot.

The behavior of cholestane spin label differs from that of androstane spin label. 5-nitroxide stearate and 16-nitroxide stearate in the very high value of d_c. Although this sterol analogue also forms clusters, these are apparently of larger dimension. Moreover, clustering begins at lower c values than in the case of androstane spin label, 5-nitroxide stearate and 16-nitroxide stearate. Since cholestane spin label is the only molecule not anchored at the aqueous interface, we suggest that cluster dimension, as well as the concentration for cluster initiation are limited by polar interactions.

Effect of temperature on quenching

For all the nitroxides and at all quencher concentrations (I_0/I)–1 is virtually independent of temperature except between 35 and 45 °C, where an increase in temperature produces a sharp decrease in quenching centered at 41 °C, the transition temperature T_t of dipalmitoyl lecithin. This thermal effect is fully reversible. The observed quenching decrease is remarkable for three reasons:

First, the gel → liquid crystalline transition increases the fluidity of the lipid hydrocarbon chains. This should facilitate the diffusional motion of the nitroxides, as well as the fluorophore molecules and should increase the frequency of quenching encounters. Indeed, the temperature dependence of quenching should follow that of the

diffusion coefficient for the system; i.e. quenching should increase exponentially with temperature.

Second, the lifetime, τ, of perylene fluorescence in dipalmitoyllecithin increases sharply from 5.5 to 6.5 nsec at T_t [17]. Since $(I_0/I) - 1$ varies linearly with τ (Eqns 2 and 5), the increase of τ at T_t should also produce an enhancement of quenching at T_t.

Third, the partition coefficient, K, of nitroxides is known to increase with hydrocarbon chain mobility [12]. Moreover, the volume of the lipid phase is expected to increase with temperature more than that of the aqueous phase. Accordingly, $(I_0/I) - 1$ should increase with temperature.

The effects of temperature fit with the cluster model. This can be seen by examining the modified Stern-Volmer plots. In the case of 5-nitroxide stearate and 16-nitroxide stearate these are totally non-linear above $T > T_t$ and androstane spin label shows a linear segment only above $c \cong 0.4$. In the case of cholestane spin label the plot of $(I_0/I)-1$ vs $((1 - c)/c)^{1/2}$ becomes linear only above $c \cong 0.1$ (compared to $c \cong 0.03$ for $T < T_t$). We interpret these data to signify that, above T_t, 5-nitroxide stearate, 16-nitroxide stearate and perylene form homogeneous mixtures at all values of c. Androstane spin label behaves similarly at $c \leqslant 0.4$ and cholestane spin label at $c \leqslant 0.1$. Quenching under these conditions occurs via lateral diffusion of the probe molecules within the membrane plane. The greater clustering tendency of cholestane spin label even above T_t is ascribed to its lack of contact with the membrane-water interface.

Influence of cholesterol

The action of cholesterol (cholesterol/phospholipid, molar ratio = 1 : 1) on the paramagnetic quenching of fluorescence is threefold: First, the sterol eliminates the quenching discontinuity about T_t. Second, cholesterol reduces the differences in quenching efficiency between androstane spin label, 5-nitroxide stearate and 16-nitroxide stearate. Third, cholesterol eliminates the clustering tendencies of the nitroxides.

These findings fit the concept that cholesterol fluidizes the dipalmitoyllecithin below T_t. The unusually high quenching efficiency of androstane spin label can be attributed to the structural similarity of androstane spin label to cholesterol. Indeed high proportions, both androstane spin label and cholestane spin label exert structural effects similar to those of cholesterol.

General comment

Our experiments show that the technique of paramagnetic quenching of fluorescence can accurately and sensitively monitor the known thermotropic properties of phosphatide bilayers, as well as the influence of cholesterol on lecithin bilayer structure. The data furthermore defines some of the association properties of nitroxide-lipid analogues in bilayer systems. We are now applying our approach to the study of lipid-protein interactions in biomembranes, by measuring quenching of tryptophan fluorescence by nitroxide-lipid analogues under various conditions.

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