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LATERAL DIFFUSION IN THE HYDROPHOBIC REGION OF MEMBRANES: USE OF PYRENE EXCIMERS AS OPTICAL PROBES

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SUMMARY

A new (optical) method of diffusion measurement is described which allows the determination of the coefficient of lateral diffusion, D_{diff}, of aromatic molecules in the hydrophobic region of lipid bilayers. In the present work pyrene is used as a fluorescence probe. The method is based on the finding that the formation of excited pyrene dimers (excimers) in fluid membranes is a diffusion controlled process. The value of D_{diff} is obtained from the second order rate constant of excimer formation which is determined from the ratio of the excimer to the monomer fluorescence quantum yields.

The method has been applied to dipalmitoyllecithin membranes and mixed dipalmitoyllecithin–cholesterol bilayers. The diffusion coefficient for pyrene in dipalmitoyllecithin membranes at 50 °C (that is above the lipid phase transition) is $D_{diff} = 1.4 \cdot 10^{-7}$ cm^2/s. This value is by a factor of 10 larger than the coefficient of the lipid self diffusion. The activation energy for the pyrene diffusion is $\Delta E = 8.8$ kcal/mole.

Below the lipid phase transition pyrene aggregates into small clusters embedded in the lipid matrix. For bilayers of unsonicated lipid dispersions the cluster formation is observed at very low pyrene concentration of 0.1 mole %, showing that the lipid matrix forms a rather regular crystal structure.

Above the lipid phase transition cholesterol reduces the lateral mobility of pyrene considerably (at 30 mole cholesterol: $D_{diff} = 0.64 \cdot 10^{-7}$ cm^2/s). Below the phase transition cholesterol suppresses the cluster formation.

The excimer formation of pyrene is also sensitive to the pretransition of the lipid matrix occurring about 10 °C below the main lipid phase transition at $T_t = 41$ °C.

INTRODUCTION

There is ample evidence now available showing that artificial and biological membranes may often be understood as two-dimensional liquids which are characterized by a high lateral mobility of the phospholipid molecules. Indirect evidence for the high fluidity within the lipid matrix of biological membranes has been provided (1) by the observation of a rapid mobility of surface antigens parallel to the surface of cells [1] and (2) by the finding that newly synthesized lipid molecules randomize

rapidly in fatty acid auxotrophs of *Escherichia coli* bacteria [2–4]. A direct proof of the fluid membrane model was the observation of a rapid lateral diffusion of spin labelled androstane, fatty acids and phospholipid molecules in artificial and biological membranes [5–8].

A direct consequence of the rapid lipid self diffusion is a considerable lateral and rotational mobility of proteins tightly bound within membranes. The above-mentioned motion of surface antigens is an example of the lateral mobility of membrane bound proteins. Very recently, Cone [9] and Brown [10] showed that rhodopsin rotates rapidly about an axis normal to the visual receptor membrane: a rotational relaxation time of 20 μs was measured. As shown previously [8] the coefficients of both lateral and rotational diffusion of macromolecules, that are tightly bound within a two-dimensional lipid matrix, can be estimated from the hopping frequency of the lipid molecules. This shows that the lateral diffusion coefficient is a most important physical parameter for the characterization of the "fluidity" (or the internal viscosity η) of membranes.

Up to the present the spin label technique provided the only method which allows a direct measurement of the lipid self-diffusion. Very recently Lee et al. [11] estimated the self-diffusion coefficient of lipid molecules from the transverse proton spin relaxation time. Good agreement with the spin label results was obtained. Provided a membrane can be understood as a two-dimensional (continuous) liquid, the Stokes–Einstein relation predicts that the coefficients of both rotational and translational diffusion are inversely proportional to the viscosity η. Thus the lipid self diffusion coefficient can, in principle, be estimated from the rotational correlation time of molecules (of known diameter) performing a rotational tumbling within the lipid matrix. Such an indirect approach towards the measurement of the diffusion coefficients has been applied by Seelig and Limacher [12] to smectic phases of soaps.

A totally different method of diffusion measurement in membranes based on the use of radioactive tracers (e.g. benzene) has been introduced by Rigaud et al. [13]. Radda and Vanderkooi [14] suggested to estimate the diffusion of aromatic molecules in membranes from the Stern–Volmer plots of the fluorescence quenching.

In the present paper we report a new spectroscopic method of diffusion measurement using pyrene as spectroscopic probe. The rate of formation of excited complexes (excimers) between a pyrene molecule in the ground state (A) and an excited pyrene molecule (A^*) is used as basic physical parameter in order to measure the hopping frequency of the diffusing pyrene molecules. Pyrene is an apolar molecule and is completely buried within the hydrophobic region of the membrane. The present method therefore allows diffusion measurements in the interior of membranes. An excimer probe has recently been used as a "volume indicator" for the internal membrane binding region [15].

EXPERIMENTAL

Dipalmitoyllecithin and cholesterol were purchased from Fluka and were used without further purification. Pyrene was zone refined. Oxygen-free lipid dispersions were prepared as follows: A thin lipid film was deposited on the wall of a 100-ml glass flask by evaporating a chloroform solution containing 50 mg of the lipid and an appropriate amount of pyrene. The evaporation was effected with a stream of puri-

fied, oxygen free nitrogen. After addition of 50 ml of (oxygen free) 2 mM CsCl aqueous solution, the solution was shaken for about 40 min on a Vortex mixer under a nitrogen atmosphere. During agitation the temperature of the flask was kept at 45 °C that is above the crystalline–liquid crystalline transition temperature of the lipid. For the measurement the solutions were sealed in quartz tubes of 5 mm outer diameter. The fluorescence spectra were taken with a Baird Atomic fluorescence spectrometer. Spectra were recorded at increasing and decreasing temperatures. At each measurement the temperature was kept constant to an accuracy of 0.2 °C. A typical fluorescence spectrum of a pyrene-containing membrane preparation (cf. Fig. 1) exhibits a superposition of the monomer and the excimer emission. The solubility of pyrene in water is extremely low (about 10^{-6} mole/l). Therefore, a saturated aqueous solution of pyrene does not show an appreciable excimer emission. The pyrene in an aqueous lipid dispersion is therefore expected to be completely dissolved in the lipid phase. For the evaluation of our experiments we need the ratio, ϕ'/ϕ, of the excimer fluorescence quantum yield (ϕ') to the monomer quantum yield (ϕ). The quantum yields is determined by the area under the fluorescence intensity distribution $I(\nu):\phi = \int_0^\infty I(\nu)\mathrm{d}\nu$. Due to the overlap of the monomer and the excimer emission, the intensity distribution $I(\nu)$ and $I'(\nu)$ are not known in our experiments. However, the ratio I'/I of the fluorescence intensities measure at the maxima of the monomer (I) and the excimer (I') band, respectively, (see Fig. 1) is proportional to the ratio of the fluorescence quantum yields ϕ'/ϕ ($I'/I = \kappa\, \phi'/\phi$). The proportionality coefficient κ depends only on the spectral distribution of the monomer and the excimer emission, respectively. By measuring the ratios I/ϕ and I'/ϕ' κ was determined in a separate experiment as $\kappa = 0.8$. The ratio I/ϕ was obtained from the fluorescence spectrum of a membrane preparation which showed only a monomer band (limit of very low pyrene concentration). I'/ϕ' was obtained from the fluorescence spectrum of a membrane preparation with high pyrene content which showed the dimer emission only. The decay times

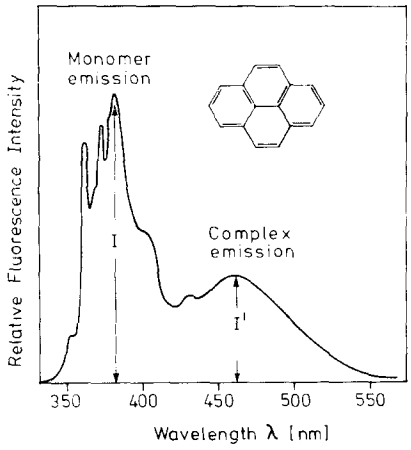


Fig. 1. Fluorescence spectrum of pyrene embedded in dipalmitoyllecithin bilayers. Irradiation at 320 nm. The spectrum was taken at a temperature (45 °C) above the lipid phase transition. The aqueous dispersion contained 1 mg lecithin in 1 ml 2 mM CsCl solution. The pyrene-to-lipid-molar ratio was $R = 0.0073$.

τ'_0 of the excimer fluorescence were measured with a laser flash spectrometer. τ'_0 was measured for each membrane preparation as a function of the temperature.

PRINCIPLE OF THE METHOD

The diffusion coefficient, D_{diff}, of molecules performing a lateral diffusion parallel to the membrane surface may be related to the number of collisions per second (ν_{col}) between the diffusing particles as follows:

The number of collisions per second experienced by one molecule may be expresed as [5]:

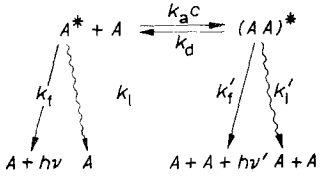
$$\nu_{col} = 2\, d_c l c \tag{1}$$

In this equation l is the total distance of travel per s or the average velocity of the diffusing particle, d_c is its Van der Waals diameter and c is the concentration of the diffusing particles measured in units of molecules per Å^2. If the diffusion of a molecule can be considered as a random jump process, D_{diff} is related to the total number of jumps, ν, per s by $D_{diff} = 1/2\, \nu\lambda^2$, where λ is the length of one diffusional jump. Since $l = \lambda\nu = 2\, D_{diff}/\lambda$ one obtains:

$$D_{diff} = \frac{\nu_{col}\, \lambda}{4\, d_c c} \tag{2}$$

In principle ν_{col} can be determined if the diffusing molecules undergo a reversible physical (or chemical) reaction upon the encounters. ν_{col} is then directly related to the rate constant of the second order reaction. In the spin label method [5, 6] the spin exchange interaction between the radicals represents the (reversible) physical reaction. In this method the reaction rate constant was determined from the broadening of the esr spectra.

The formation of short lived excited complexes between an aromatic molecule in the ground state A and an excited molecule A^* provides another reversible physical reaction which may be exploited for measuring the coefficient of lateral diffusion. The excimer formation which has been reported for the first time by Förster and Kasper [13] is described by the following process [14]:



(3)

The excimer formation is a bimolecular process characterized by the second order rate constant k_a. k_f and k'_f are the transition probabilities (in s^{-1}) for the radiative decay of the excited monomer and the excimer, respectively. k_l and k'_l are the corresponding radiationless transition probabilities. k_d is the rate constant characterizing the dissociation of the excited complex into A^* and A.

Since the pioneering work of Förster and Kasper [13] the excimer formation of many aromatic molecules has been studied in different organic solvents [17–20].

The results of these studies, which are important in connection with the diffusion measurement, may be summarized as follows:

(1) The ratio of the fluorescence quantum yields of the excimer (ϕ') and the monomer (ϕ) are directly related to the second order rate constant k_a by [17–19]:

$$\phi'/\phi = \frac{I'}{\kappa I} = \frac{k_f' \, \tau'_0 \, c}{k_f \, (1+k_d\tau'_0)} \, k_a \tag{4}$$

(2) At temperatures up to about 60 °C the dissociation constant, k_d, of the pyrene excimer is small compared to the transition rate $1/\tau'_0$ of the complex into two unexcited molecules ($k_d\tau'_0 << 1$) [17]. Accordingly the ratio I'/I (or ϕ'/ϕ) does not depend on k_d at low enough temperatures.

(3) The excimer formation in fluid media is a diffusion controlled process. Accordingly, one would expect that the rate of excimer formation, $k_a c$, is proportional to the collision rate ν_{col}. Birks et al. [21] have shown that in ordinary organic solvents (such as cyclohexane) every collision between A and A^* is effective and leads to excimer formation.
Therefore

$$k_a c = \nu_{col} \tag{5}$$

Obviously this must also be valid if pyrene is incorporated into a fluid lipid matrix. By combining Eqns 2, 4 and 5 one obtains:

$$D_{diff} = \frac{\lambda k_a}{4 \, d_c} = \frac{I'}{\kappa I} \cdot \frac{k_f}{k_f'} \frac{\lambda}{4 d_c \tau'_0 c} \tag{6}$$

(4) The ratio k_f/k_f', does not depend on the temperature or the solvent but is rather an intrinsic property of the (pyrene) molecule forming the excimer. In the following the value $k_f/k_f' \approx 0.1$, measured by Seidel and Förster for nonane will be used.

Eqn 6 provides the basis for the measurement of the pyrene diffusion coefficient. The intensity ratio I'/I and the life time τ'_0 can be measured rather accurately. The concentration c is defined as the number of molecules per Å^2. At the low pyrene concentrations used in this work the lateral expansion of the lipid matrix by the solute is negligibly small. Accordingly in pure lecithin membranes the pyrene concentration is given by $c = R/F$, where F is the area per lipid molecule and where R is molar ratio pyrene-to-lipid .Above the lipid phase transition the area per lipid molecule is F $\approx$ 58Å^2 [5]. The label concentration in the membranes containing cholesterol is given by

$$c = R/(F+\alpha F_C),$$

where α is the molar ratio of cholesterol-to-lipid and where F_C is the area that one cholesterol molecule occupies in the lipid matrix. F_C is assumed equal to the area per cholesterol molecule in condensed cholesterol monolayers: $F_C = 40$ Å^2 [22]. The condensing effect of cholesterol is neglected. A small reduction in the area per molecule of about 7 Å^2 has been reported for mixed egg lecithin–cholesterol bilayers [23]. The parameter λ and d_c occurring in Eqn 6 can only be estimated: In Eqn 6 d_c is defined as the critical interaction distance for the onset of the excimer formation: k_a = constant if the distance d between two molecules A^* and A is smaller or equal to d_c and

$k_a = 0$ if $d > d_c$. Pyrene is an elongated molecule (length about 10 Å, diameter in the direction of the short in-plane axis about 7 Å) which is expected to orient with its long axis preferentially parallel to the normal of the membrane. We therefore assume for d_c a value of $d_c \approx 8$ Å. Since pyrene is a rather large molecule it is reasonable to assume that each pyrene molecule occupies the site of one lipid molecule in the lipid matrix. Accordingly one would expect that the length of one diffusional jump of pyrene is about equal to the average distance between two lipid molecules, i.e. we assume $\lambda \approx 8$ Å*.

With the values $k_f/k_f' \approx 0.1$ and $d_c \approx \lambda \approx 8$ Å one obtains from Eqn 6:

$$D_{diff} = \frac{k_o}{4} = \frac{I'}{\kappa I} \frac{1}{40\tau'_0 c} \tag{7}$$

RESULTS

Pure dipalmitoyllecithin dispersions and mixed lecithin cholesterol model membranes with cholesterol contents ranging from 1 mole % to 50 mole % were investigated in this work. Spectra were recorded between 18 and 60 °C. This temperature range covers the crystal line–liquid crystalline phase transition of the dipalmitoyllecithin which had a characteristic transition temperature of $T_t = 41$ °C.

Dipalmitoyllecithin membranes

Typical results are shown in Fig. 2. The fluorescence intensity ratio I'/I (cf. Fig. 1) is plotted as a function of the temperature for several membrane preparations containing different label concentrations. At temperatures well below the phase transition ($T < T_t$) the intensity ratio I'/I increases with increasing temperature. The same is found for temperatures above the phase transition, $T > 42$ °C. In the region of the lipid phase transition, that is between 30 and 42 °C, the ratio I'/I decreases, however, with increasing temperature. It is well known from spin label studies [8] that the fluidity of the hydrophobic region of lipid bilayers increases with increasing temperature in the whole temperature range, while the lipid phase transition manifests itself in an abrupt increase in the membrane fluidity in going from $T < T_t$ to $T > T_t$. Provided the excimer formation in the lipid matrix was diffusion controlled in the whole temperature range, one would expect that the rate of excimer formation $k_a c$ (or the intensity ratio I'/I) increases with increasing temperature between 18 and 60 °C. Accordingly Fig. 2 shows that the excimer formation is only completely diffusion controlled if the lipid matrix is in a fluid (that is liquid–crystalline) state. In Fig. 3 we plotted the intensity ratio I'/I as a function of the pyrene concentration for temperatures above T_t. I'/I increases linearly with increasing pyrene concentration c as predicted by the diffusion model (cf. Eqn 4). The excimer formation in fluid membranes is therefore completely diffusion controlled. This is a necessary condition for the diffusion measurement. From the slopes of the straight line in Fig. 3 we determined the value of k_a given in Table I. With these values we obtained the diffusion coefficients given in Table II.

* A lower limit of λ would be the average distance between the two CH_2-chains of a lipid molecule ($\lambda \approx 5$ Å).

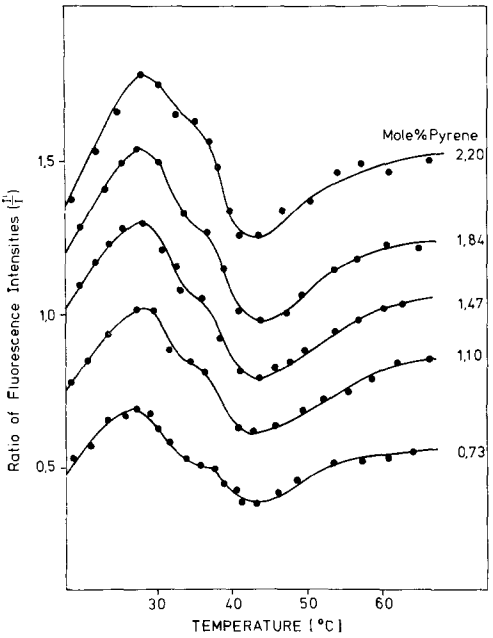


Fig. 2. Temperature dependence of the ratio, I'/I, of the maximum excimer fluorescence intensity (I') to the maximum monomer fluorescence intensity (I) (cf. Fig. 1) of pyrene embedded in dipalmitoyllecithin bilayers. The lipid phase transition is characterized by a sharp decrease in the intensity ratio I'/I. The change in I'/I at the phase transition decreases with decreasing pyrene concentration c. I'/I is a measure of the second order rate constant, k_a, characterizing the bimolecular process of the excimer formation (cf. Eqn 4).

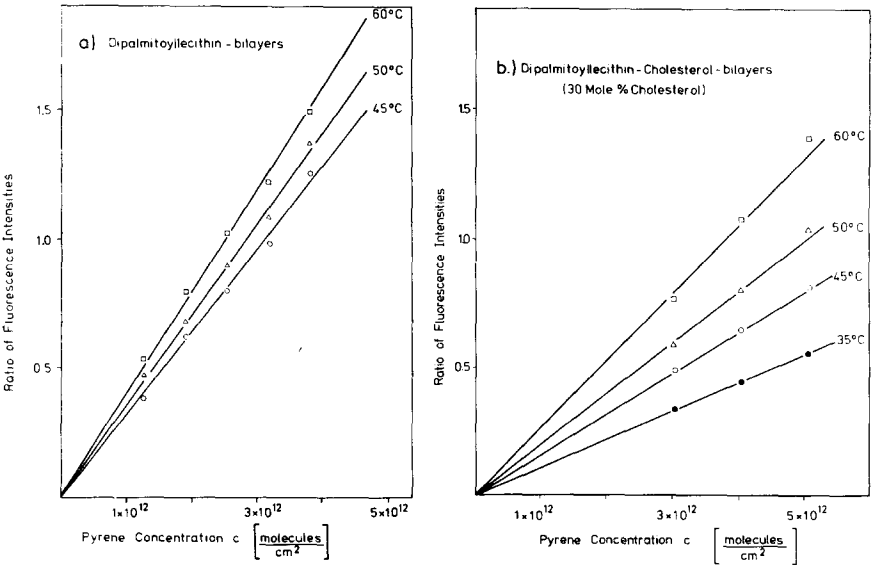


Fig. 3. Concentration dependence of fluorescence intensity ratio I'/I for temperatures above the lipid phase transition. (a) Intensity ratio for pure dipalmitoyllecithin membranes. The I'/I values are those given in Fig. 2. (b) Intensity ratio for a mixed cholesterol–dipalmitoyllecithin membrane containing 30 mole % cholesterol. Note that for all cases I'/I is proportional to the pyrene concentration c as predicted by the diffusion model.

TABLE I

EXCIMER FLUORESCENCE DECAY TIMES, τ'_0, AND SECOND ORDERS REACTION RATE CONSTANTS, k_a, OF PYRENE EXCIMER FORMATION IN BILAYER OF DIPALMITOYLLECITHIN DISPERSIONS

k_a has been determined from the slopes of the straight lines in Fig. 3a using a value of $\kappa = 0.8$.

Temp. (°C)	τ'_0 (ns)	$k_a \times 10^7$ ($cm^2 \cdot molecule^{-1} \cdot s^{-1}$)
45	100	3.1
50	80	4.4
60	65	6.0

The abrupt decrease in the intensity ratio I'/I (or in the rate constant k_a) at the phase transition is not in agreement with the prediction of the diffusion model. The anomalous decrease in k_a is most pronounced at high pyrene concentration and is absent if the pyrene-to-lipid molar ratio is smaller than $R = 0.001$.

The curves in Fig. 2 show that the decrease in I'/I at the phase transition occurs in two steps. The points of inflexion of these steps define two transition temperatures $T_t^{\star} = 32$ °C and $T_t = 40$ °C. The higher value T_t agrees well with the chain melting temperature of dipalmitoyllecithin (at $T_t = 41$ °C) [25, 26]. The value of $T_t^{\star}$ agrees with the characteristic temperature of the "pretransition" of dipalmitoyllecithin membranes that has been observed previously [8, 26]. It is therefore suggested that the first step in the I'/I versus T plot in Fig. 2 reflects this pretransition.

Membranes containing cholesterol

Fig. 4 shows the effect of cholesterol on the excimer formation in lipid membranes. The intensity ratio I'/I (and thus the rate constant k_a) decreases with increasing cholesterol content at all temperatures. The anomalous decrease in I'/I with increasing temperature at the lipid phase transition is suppressed by the addition of cholesterol. At a cholesterol content larger than 20 mole% the rate of excimer formation increases monotonically at all temperatures. In this case the phase transition is indicated by an increase in the slope of the I'/I versus temperature plot.

Dispersions containing 1, 2, 5, 10, 30 and 50 mole% cholesterol respectively, were studied in this work. The fluorescence spectra were recorded for 5 different pyrene concentrations: (molar ratios pyrene-to-lipid $R = 0.0075$; 0.0147; 0.0220; 0.0294; 0.0367). It is found that for membranes containing cholesterol up to 50 mole% the intensity ratio I'/I increases linearly with increasing pyrene concentration for temperatures for which the mixed membrane is in a fluid state. An example is given in Fig. 4 for a membrane containing 30 mole% cholesterol. Obviously the diffusion model applies also for membranes containing cholesterol. From the slopes of the straight lines obtained by plotting I'/I versus the pyrene concentration c we determined the rate constants k_a. The diffusion coefficients D_{diff} corresponding to these values of k_a are summarized in Table II. The diffusion coefficients could also be measured at 35 °C for the membranes with more than 10 mole% cholesterol.

* In Eqn 4 k_a has the dimension $cm^2 \cdot molecule^{-1} \cdot s^{-1}$. Usually second order rate constants are given in units of $l \cdot mole^{-1} \cdot s^{-1}$. A value of 1.0 $cm^2 \cdot molecule^{-1} \cdot s^{-1}$ corresponds to a value of $1.34 \cdot 10^{14}$ $l \cdot mole^{-1} \cdot s^{-1}$.

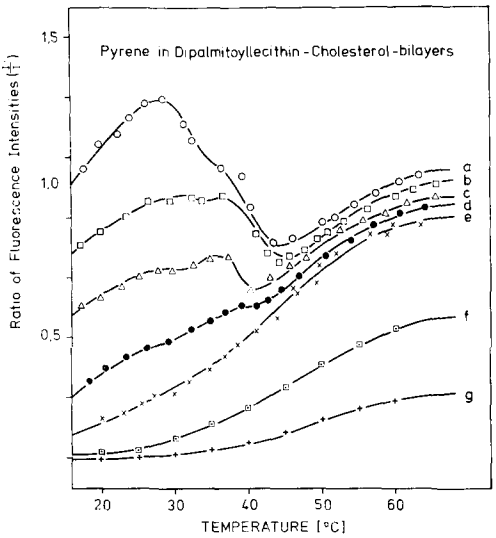


Fig. 4. Intensity ratio I'/I (cf. Fig. 1) for pyrene dissolved in mixed dipalmitoyl–cholesterol bilayers of variable cholesterol content. For all curves a–g the pyrene-to-lipid molar ratio was $R = 0.022$. The molar percentage of cholesterol was for (a) 0 mole %, (b) 1 mole %, (c) 2 mole %, (d) 5 mole %, (e) 10 mole %, (f) 30 mole %, and (g) 50 mole %. Note that the anomalous decrease in I'/I (with increasing temperature) at the lipid phase transition is absent at cholesterol contents higher than 5 mole %.

TABLE II

DIFFUSION COEFFICIENTS, D_{diff}, AND ACTIVATION ENERGY OF THE PYRENE LATERAL DIFFUSION IN DIPALMITOYLLECITHIN BILAYERS AND IN MIXED MEMBRANES CONTAINING 1, 2, 5, 10, 30 AND 50 MOLE % CHOLESTEROL

Up to 5 mole % cholesterol the τ'_0 values given in Table I are applicable. For cholesterol contents between 10 and 50 mole % τ'_0 is somewhat larger. For $T = 35$, 45, 50 and 60 °C, respectively, the values $\tau'_0 = 150$, 120, 100 and 78 ns, respectively, were used.

		$D_{diff} \times 10^7$ (cm^2/s)				Activation energy (kcal/mole)
		35 °C	45 °C	50 °C	60 °C	
Pure dipalmitoyl-lecithin bilayers		–	1.00	1.40	1.88	8.8
Mixed dipalmitoyl-lecithin–cholesterol bilayers	1 mole %	–	1.00	1.35	1.85	8.8
	2 mole %	–	0.91	1.25	1.80	9.0
	5 mole %	–	0.81	1.12	1.53	10.0
	10 mole %	0.38	0.71	0.96	1.37	10.7
	30 mole %	0.23	0.40	0.64	1.12	12.4
	50 mole %	0.18	0.34	0.46	0.78	12.8

In Fig. 5 D_{diff} is plotted as a function of the cholesterol content for several temperatures. It is seen that cholesterol reduces the rate of lateral diffusion of pyrene considerably.

By plotting the D_{diff} values given in Table II on a logarithmic scale as a function of the temperature straight lines are obtained to a good approximation. From

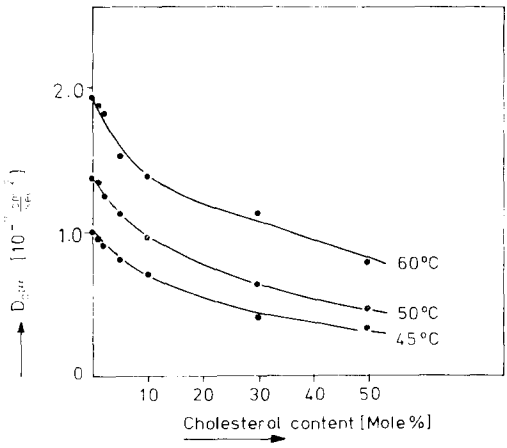


Fig. 5. Lateral diffusion coefficient D_{diff} for pyrene in mixed dipalmitoyllecithin–cholesterol bilayers as a function of the cholesterol content.

the slopes of these straight lines we obtained the activation energy ΔE for the hopping motion of the pyrene molecules in the lipid bilayers. The ΔE values are given in the last column of Table II.

DISCUSSION

Diffusion

The present work shows that the excimer formation in fluid phospholipid membranes is a diffusion controlled process. This finding led to a new method for the measurement of the lateral mobility of aromatic molecules in the hydrophobic region of membranes. In principle the method can also be applied to biological membranes. A great advantage of the present method is that only small concentrations of the spectroscopic probe are needed.

The value of D_{diff} for pyrene in pure lecithin bilayers at 50 °C ($D_{diff} = 1.4 \cdot 10^{-7}$ cm^2/s) corresponds to a hopping frequency of $\nu = 4 \cdot 10^7$ jumps per s or to an average drift velocity of pyrene in a given direction of $\langle v \rangle = 53\,000$ Å/s. The spin label technique yielded a value of $D_{diff} = 3 \cdot 10^{-8}$ cm^2/s for the diffusion coefficient of androstane or fatty acids in dipalmitoyllecithin bilayers or monolayers at 50 °C [5]. These values were found to be about equal to the coefficient of the phospholipid self-diffusion ($D_{diff} \approx 1.8 \cdot 10^{-8}$ cm^2/s) [6]. The near equality of the diffusion coefficients of androstane or fatty acids with the phospholipid self-diffusion coefficient is most probably due to the fact that all these molecules are anchored with their polar groups rather rigidly in the semipolar region of the membrane. Thus the lateral mobility of molecules which are completely buried in the hydrophobic region of the membranes is expected to be greater than the mobility of polar molecules.

Using the radioactive tracer method, Rigaud et al. [13] measured a diffusion coefficient of $D_{diff} \approx 2 \cdot 10^{-6}$ cm^2/s for benzene in egg-lecithin films. Due to the high degree of unsaturation present in the hydrocarbon chains of natural lipids one expects a considerably higher diffusion rate of aromatic molecules in egg lecithin than in dipalmitoyllecithin. This is confirmed by a previous study [32] where we found that

mobility of fatty acids is about 5 times faster in microsomal membranes ($D_{diff} \approx 14 \cdot 10^{-8}$ cm^2/s) than in dipalmitoyllecithin bilayers at 50 °C (D_{diff} $3 \cdot 10^{-8}$ cm^2/s) [32]. Accordingly, the diffusion coefficients obtained with the three completely different methods, namely the spin label method and the two optical methods, seem to be in a good agreement.

A striking result of this work is the strong reduction in the lateral mobility of pyrene by cholesterol. A cholesterol content of 30 mole% reduces D_{diff} by a factor of two. This result is expected from recent spin label studies of Marsh and Smith [20] showing that cholesterol may strongly reduce the membrane fluidity at temperatures above the lipid phase transition, that is if the membrane is in a fluid state.

Cluster formation

Another important result of the present study is the strong reduction in the rate of excimer formation upon going from the crystalline to the liquid crystalline state of the membrane. This "anomalous" behavior shows that the excimer formation is not a diffusion controlled process in the range of the lipid phase transition or in the crystalline state of the lipid matrix. The only reasonable explanation for the anomalous decrease in I'/I (or k_a) at T_t is the low solubility of pyrene in the crystalline lipid matrix. Obviously, the pyrene molecules aggregate into small clusters of closely packed molecules (or submicroscopic crystallites) upon lowering the temperature from $T > T_t$ to $T < T_t$. It is well known that microcrystals of pyrene or many other aromatic molecules exhibit a very high excimer yield. This is due to a sandwich-like arrangement of the molecules in the crystallites which favors the excimer formation (cf. ref. 20, p. 317).

The two-dimensional solutions of pyrene in lipid bilayers closely resemble the mixed steroid–dipalmitoyllecithin membranes, studied previously [5]. At temperatures below T_t the organization of such membranes may be described as a mosaic-like structure with small clusters of steroids embedded in the lipid matrix. It should be emphasized that pyrene starts to form clusters at very low concentrations. For the lipid dispersions studied in this work, which contain large membrane fragments, the lower limit in the pyrene-to-lipid molar ratio for the onset of cluster formation is $R_s \approx 0.001$. This shows that the lipid matrix of the dispersions forms a rather regular crystal structure below T_t containing only a very small number of free pockets. In small vesicles of about 300 Å diameter obtained by sonication, the solubility R_s of pyrene in the crystalline state is much higher, $R_s \approx 0.01$. Obviously the strain present in small vesicles prevents the formation of a regular crystal structure.

According to Fig. 3 the addition of cholesterol to lecithin membranes greatly increases the solubility of pyrene in the crystalline phase, that is at temperatures below T_t. In bilayers containing 5 mole% cholesterol the solubility of pyrene increases to about $R_s \approx 0.015$ at 30 °C. This dramatic increase in the solubility of pyrene at $T < T_t$ reflects the strong fluidizing effect of cholesterol on membranes in the quasicrystalline state [27–29].

Pretransition

The curves in Fig. 2 suggested that the excimer formation is also sensitive to the pretransition occurring about 10 °C below the chain melting temperature at T_t [8, 26]. In a previous study of lecithin membranes and *E. coli* membranes [8] we found that spin labels which probe the semipolar region of the membrane are prima-

rily sensitive to the pretransition. We concluded therefore that the pretransition mainly affects the polar groups of the lipids. The sensitivity of the pyrene excimer formation to the pretransition shows that the transition has also a pronounced effect on the arrangement of the alkyl chains. According to Fig. 2 (e.g. upper curve) the clusters partially dissolve at the pretransition. This indicates that the pretransition leads also to a considerable disorder of the hydrophobic membrane region. This is in agreement with results reported recently by Shimshick and McConnell [30].

APPENDIX

In deriving Eqn 7 the membrane was considered as a true two-dimensional system. However, the length of pyrene (about 10 Å) is small compared with the thickness of the hydrophobic region of the membranes which is of the order of 33 Å [28].

The apolar pyrene molecule can migrate from one lipid monolayer to the other. The values of the lateral diffusion coefficients determined in this work have to be corrected if the rate of pyrene diffusion, D_{norm}, in a direction normal to the membrane is comparable to the rate of lateral diffusion. In the extreme limit $D_{norm} >> D_{lat}$ the values of D_{diff} given in Table II would be by a factor of two too large.

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