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TRANSVERSAL MOBILITY IN BILAYER MEMBRANE VESICLES: USE OF PYRENE LECITHIN AS OPTICAL PROBE

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Pyrene lecithin, a new excimer-forming lipid molecule, has been synthesized to examine the transversal mobility of probe molecules in lecithin bilayer vesicles. The rate of the lipid exchange is obtained by following the excimer yield as a function of time after mixing of fluorescence doped and undoped vesicles. A rapid exchange ($\tau_{1/2}$ = 11 s) is followed by a slow transfer ($t_{1/2}$ = 8 h). Above the lipid phase transition the fast transfer can be attributed to an exchange of lipid molecules from the outer layer of one vesicle to the outer layer of another one. The slow exchange is interpreted in terms of the 'flip-flop' process between the two layers of a single bilayer vesicle.

Using pyrene and pyrene decanoic acid as probe molecules only the fast transfer through the water phase is observed ($\tau_{1/2}$ = 4 s for pyrene and $\tau_{1/2}$ = 7 s for pyrene decanoic acid). This indicates that molecules like fatty acids or apolar membrane constituents must equilibrate very rapidly in a single bilayer vesicle.

The water solubility or the critical micelle concentration of the probe molecules is determined and related to the transfer rates. An exchange process through the water phase via a monomeric state can be excluded.

I. Introduction

Transport of membrane bound molecules parallel (lateral) or perpendicular (transversal) to the membrane surface is a problem of current interest in membrane biology. Spin label experiments [1–3] have been performed to measure the lateral diffusion of probe molecules in a bilayer membrane as well as in natural membranes [4, 5]. These results are in good agreement with values obtained from proton spin-lattice relaxation time measurements [6]. The method of fluorescence recovery after photobleaching of fluorescent lipid analogues [7] comfirmed the earlier results. A fluorescence method for the determination of the lateral diffusion coefficient based on the excimer formation of pyrene has been developed some years ago [8,9].

Another important aspect of phospholipid movement has to be taken into account: the movement or exchange between membrane structures. In biological membrane systems the transfer of phospholipids is performed by so called exchange proteins [10, 11] whereas the inside-outside transposition in phosphatidylcholine bilayers [12] and in excitable membrane vesicles [13] has been interpreted in terms of a 'flip-flop' mechanism. Very recently it was shown that the inter-vesicle lipid mixing

between vesicles consisting of phospholipid extract from *E. coli* occurs via lipid monomers or small micelles in the water phase [14, 15].

In the present study we used the pyrene phosphatidylcholine derivative to investigate the phospholipid transfer in artificial membranes. A two step transfer was obtained and compared with earlier experiments using pyrene and pyrene decanoic acid as probe molecules [16]. From our experiments performed with the pyrene lecithin label we are able to distinguish between a rapid inter vesicle transfer from the outer layers of different vesicles and a slow rate of flip-flop in one bilayer vesicle.

II. Materials and methods

A. Synthesis of 1-palmitoyl-2-ω-pyrenedecanoyl-phosphatidylcholine

Pyrene decanoic acid was prepared by a well known Friedel-Crafts-Reaction of pyrene and sebacic acid chloride monoethylester [17]. The reaction product was selectively reduced and hydrolyzed to the pyrene decanoic acid [18]. This way of synthesis was proposed by H.G. Scholz [19]. Because of the great interest in this substance the synthesis will be described. More relevant is the lipid derivative of this fatty acid. Therefore we transformed in two steps the pyrene decanoic acid to the acid chloride with oxalylchloride and condensed it to the 1-palmitoyl-lyso-lecithin.

1. ω-Pyrenoyl nonanoic acid ethylester

24. 1 g of sebacic acid chloride monoethylester (Fluka) were solved in 150 ml nitrobenzene. After cooling the solution in an ice bath 24.3 g of $AlCl_3$ were added in small portions under magnetic stirring. The temperature should not exceed 283 K. The solution was combined with 18.4 g of pyrene in small quantities so that the temperature was kept below 293 K. After magnetic stirring for 18 h under a dry nitrogen atmosphere the solution was poured to about 300 g of ice and acidified with conc. HCl. The dark red and viscous solution turned to a yellow colour.

After removing the nitrobenzene by hot water vapour the remaining solution was cooled to room temperature. The precipitate was dispersed in 500 ml $CHCl_3$ and filtered. The filtrate was dried over anhydrous sodium sulfate and evaporated under reduced pressure to 34 g of a yellow solid residue. Chromatography on a dry Al_2O_3-column (Merck, Activity Grade III) developed in a 1 : 1 mixture of cyclohexane and diethylether led to 15 g of pure product eluted with chloroform from the second zone behind the solvent front. The purity of the product was checked by an Al_2O_3-thin layer chromatogram developed in the above used solvent mixture with an R_F-value of $R_F = 0.58$. The melting point is found to be at $t = 320$ K. The infrared spectrum shows the ester band at 1735 cm^{-1}.

2. Pyrene decanoic acid

A mixture of 8.3 g -pyrenoyl nonanoic acid ethylester, 3.25 hydrazine hydrate

(N_2H_5OH 80%) and 5.6 g of powdered potassium hydroxide in 30 ml tri-ethylene-glycol was refluxed for 2 h in a bath thermostated at 413 K. Upon increasing the temperature within 2 h to 473 K a mixture of hydrazine, water and ethanol was distilled. The remaining solution was kept overnight at this temperature. After cooling to room temperature the solution was diluted with two volumes of water and acidified with conc. HCl. The light yellow precipitate was filtered and washed several times with water. The crude product was dried over P_4O_{10} under reduced pressure and recrystallized three times from *n*-hexane. The light yellow crystals were dried overnight to 6 g at 343 K under vacuum. The product had a melting point at t = 383 K. It appeared a single spot on a silicic acid thin layer chromatogram developed in cyclohexane/acetic acid ethylester (1:1, v/v) with an R_F = 0.46. The infrared spectrum showed the carbonyl band at 1700 cm^{-1}.

3. Pyrene decanoic acid chloride

674 mg of pyrene decanoic acid were solved in 80 ml of dry benzene. After addition of 1.2 ml freshly-distilled oxalyl chloride (Fluka) the solution was refluxed for 5 h under a dry nitrogen atmosphere. The excess of oxalyl chloride was removed by repeated evaporation under vacuum from a benzene solution. The infrared spectrum showed the characteristic acid chloride band at 1790 cm^{-1}.

4. 1-Palmitoyl-2-pyrenyl decanoyl lecithin

The decanoic acid chloride was solved in 40 ml of dry chloroform. 400 mg of lysolecithin (Fluka) dried over P_4O_{10} under reduced pressure was added and the solution was stirred for two days at room temperature under a nitrogen atmosphere. The acylated lecithin was purified on a preparative silicic acid chromatogram (Merck, 2 mm thick, 20 × 20 cm) developed in chloroform/acetic acid/methanol/water (17 : 15 : 25 : 6 v/v).

For detection of the lipid band the main part of the chromatogram was covered with a glass plate and the free part was stained with Dittmer spray [20]. The stained edges were charred and descarded and the lipid band was scraped out of the unstained part. Elution was performed with 20 ml of a 1 : 1 mixture of chloroform and methanol containing 0.1% glacial acetic acid. After sonication for approx. 5 min the dispersion was filtered and the filter cake was extracted again. Addition of 50 ml of water to the combined filtrates and centrifugation at approx. 6000 *g* led to a phase separation. Remaining gel particles congregated at the interphase. The chloroform phase was dried over sodium sulfate and evaporated to dryness. Typical yields were of the order of 40% of the original lysolecithin. The pyrene-labelled lipid showed a single spot on silica TLC corresponding to dipalmitoyl-lecithin. The absorption and the fluorescence spectrum are identical to that one of pyrene decanoic acid.

B. Preparation of phospholipid dispersions

All phospholipids were obtained from Fluka and checked for purity by TLC.

Chloroform solutions of both the lipid and the label were evaporated to dryness and dispersed for 5 min by ultra sonication under a nitrogen atmosphere. A Branson sonifier type B12 at $P = 30$ W was applied. The final lipid concentration was 1 mg/ml of buffer solution. All buffer solutions were deoxygenated by bubbling pure nitrogen through the solutions for at least 2 h. The vesicles were checked by freeze etch electron microscopy and found to be single walled.

C. Excimer-forming fluorescent probes

Excimer-forming probes are well suited to investigate the coefficient of the lateral diffusion in phospholipid bilayers [8,9]. The physical parameter in this application is the formation rate of excited dimers formed upon collision of short living excited monomers and monomers in the grund state. The fluorescence band of the excimer is well separated from the monomer emission (e.g. insert Fig. 5). The collision is diffusion controlled in the fluid state of the membrane and directly related to the intensity ratio excimer-to-monomer emission according to

$$D_{diff} = \kappa \cdot \frac{I'}{I} \cdot \frac{k_f}{k_f'} \cdot \frac{\lambda}{4 \cdot d_c \cdot \tau_0 \cdot c} \qquad (I)$$

κ, the correction factor as described in ref. 8 comes to $\kappa = 2.0$ for our fluorescence spectrometer; k_f and k_f', are the radiative decay constants for the monomer and the excimer state where the ratio $k_f/k_f' = 0.14$ is valid for both pyrene decanoic acid and pyrene lecithin, whereas the ratio $k_f/k_f' = 0.1$ is given for pyrene [8]; λ is the length of one diffusional jump given with $\lambda = 8$ Å and d_c is the critical interaction distance $d_c = 8$ Å; τ'_0 is the life time of the excimer; c is the concentration of label per unit area of the lipid matrix.

The following two cases can be considered: (1) The label concentration is homogeneous in the whole vesicle preparation. The intensity ratio I'/I as function of label concentration combined with the excimer lifetime determined after a 5 ns laser pulse leads to the investigation of the coefficient of the lateral diffusion. (2) Half of the vesicles are doped and the other half is not. The lipid lateral diffusion and the τ'_0 value are assumed to be the same in both. Now the ratio I'/I is only a function of the label concentration. A decrease in I'/I upon mixing the two vesicle preparations is given directly by the decrease of the label concentration in the doped vesicle as a consequence of the probe transfer [21].

D. Fluorescence measurements

All measurements were performed with a 'Schoeffel RRS 1000' instrument equipped with two photomultipliers perpendicular to the radiation light. This allows us to determine I' and I at the same time and to develop the quotient I'/I by an analog computer. The samples were thermostated to an accuracy of 0.2°C. Transfer experiments were performed using a stopped-flow apparatus. The separated vesicle reservoirs, the mixing chamber and the round measuring cell were thermostated to an accuracy of about 0.5°C The ratio I'/I after rapid mixing of doped and undoped vesicles is recorded with time.

III. Results and their interpretation

A. Determination of the critical micelle concentration

Pyrene lecithin (insert Fig. 1) was sonicated in a 2 mM CsCl water solution. The intensity ratio I'/I of the excimer to the monomer fluorescence intensity was measured in a concentration range between $c = 10^{-8}$ M and 10^{-4} M. The results are given in Fig. 1. The intensity ratio is nearly independent of the lipid concentration up to $c = 10^{-7}$ M. Upon increasing the concentration a break is obtained at $c = 1.5 \times 10^{-7}$ M followed by a constant increase in the intensity ratio. The concentration at this point is the critical micelle concentration $c_{CMC} = 1.5 \times 10^{-7}$ M for pyrene lecithin. At $c < c_{CMC}$ the lipid is solved in a monomeric form whereas at $c > c_{CMC}$ micelles or vesicular structures are formed which is indicated by the abrupt increase of I'/I with lipid concentration. This value is about a factor of ten larger than values reported by L.Thilo [15] for lipid extracts from *E. coli* membranes. This difference must be due to the structural difference of the molecules. For example, lipids containing palmitelaidic acid exhibit a monomer concentration of 6.6×10^{-8} mol/l whereas the elaidic derived phospholipids have momomer concentration of 7.7×10^{-9} mol/l [15].

The same method has been applied to pyrene decanoic acid. An increase in I'/I is found at $c = 10^{-5}$ M in the CsCl-solution. This increase is accompanied by the formation of pyrene decanoic acid crystals which precipitate. Therefore this value is not a critical micelle concentration but the limit of the solubility. Pyrene is found to be soluble in water up to concentration of $c = 10^{-6}$ M [22].

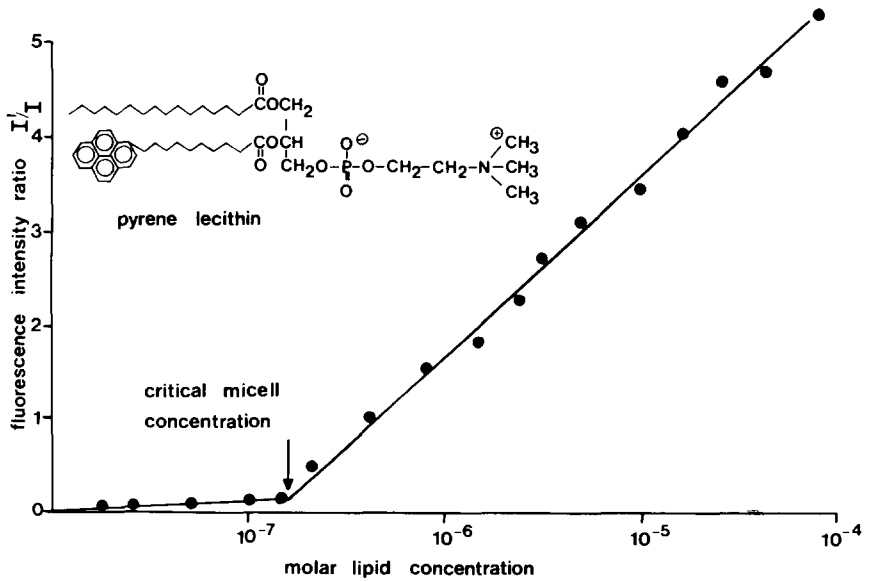

Fig. 1. Fluorescence intensity ratio I'/I for pure pyrene lecithin vesicles as a function of lipid concentration. The break demonstrates the critical micelle concentration $c_{CMC} = 1.5 \times 10^{-7}$ M.

B. Determination of the coefficient of the lateral diffusion in dipalmitoyl lecithin vesicles

The lateral diffusion coefficients are calculated following the method given by Galla and Sackmann [8] (e.g. formula I of this paper). The values for pyrene, pyrene decanoic acid and pyrene lecithin incorporated into dipalmitoyl lecithin bilayers are opposed in Table 1. The excimer lifetime, τ_0', used for the calculation was obtained as described before [8] at a probe concentration of 10 mol % concerning the lipid. In the temperature range of t = 318 K to t = 333 K the excimer lifetime decreases from τ_0', = 90 ns to τ_0' to 70 ns for both the pyrene decanoic acid and the pyrene lecithin. Coming from pyrene a decrease in the diffusion coefficient is obtained in this label series. The diffusion of pyrene decanoic acid compared to pyrene is reduced because of the polar carbonyl group leading to a prevention of the lateral mobility. Pyrene lecithin again shows a slower diffusion compared to the pyrene decanoic acid, although there is no significant change in the polarity of the headgroup. Nevertheless there is a significant change in the diameters of the molecules. Following the Stokes-Einstein equation for the diffusion coefficient of a membrane component, D_L of the pyrene lecithin related to D_D the coefficient of pyrene decanoic acid is given by $D_L = \sqrt{M_L/M_D} \cdot D_D$ [4] where M_L and M_D are the molecular weights of the pyrene lecithin and the pyrene decanoic acid. From the change in size we have to expect a change in the lateral mobility by approx. 40%. This is very well consistent with the obtained values (e.g. Table 1).

C. Determination of the transversal phospholipid exchange

Vesicle preparations containing fluorescent probes (pyrene, pyrene decanoic acid, pyrene lecithin) have been mixed with undoped vesicles of the same lipid in a stopped flow apparatus as described earlier [21]. The time dependence of the excimer-to-monomer ratio as a direct measure of the probe concentration is followed. Typical curves are shown in Fig. 2 for dimyristoyllecithin vesicles at t = 296 K. Using pyrene and pyrene decanoic acid short half lifetimes for the transfer process ($\tau_{1/2}$ = 4 s and $\tau_{1/2}$ = 7 s) are obtained. Within the time scale of seconds the intensity ratio decreases

Table 1

Coefficients of the lateral diffusion of pyrene, pyrene decanoic acid, pyrene lecithin in dipalmitoyl lecithin membranes.

Temp (K)	$10^7 \times D_{diff}$ [cm²/s]		
	pyrene	pyrene decanoic acid	pyrene lecithin
318	1.0 (1.0)[a]	0.67	0.43
323	1.3 (1.4)[a]	0.81	0.53
333	1.7 (1.9)[a]	1.1	0.77

[a]values given by Galla and Sackmann [8].

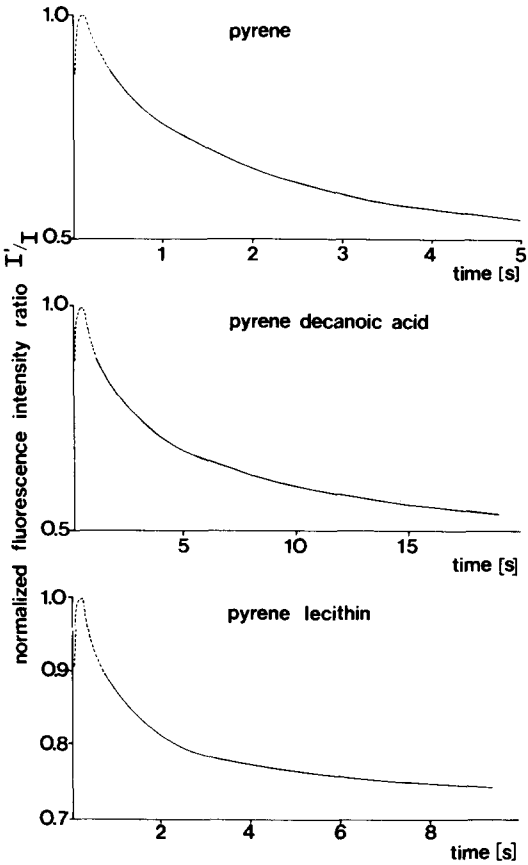

Fig. 2. Time dependence of the intensity ratio I'/I after rapid mixing in a stopped flow apparatus. Note that I'/I goes to an equilibrium state in the case of pyrene and pyrene decanoic acid but not in the case of pyrene lecithin. Because of instabilities in the first second after mixing the dotted part is not used to evaluate the exchange constants.

to half of the initial value. This is not the case using pyrene lecithin as optical probe. The lower curve in Fig. 2 is a typical one for the decrease of the normalized fluorescence intensity. In the above mentioned time scale a decrease to only 75% of the inital value of I'/I is observed. For this process a half lifetime of $\tau_{1/2}$ = 10 s is obtained. The rate constants k_r of this exchange process are calculated from the logarithmic plot given in Fig. 3. N_0 is the intensity ratio I'/I of the doped probe before mixing whereas N_R is the intensity ratio I'/I after the rapid exchange process. This value is taken one minute after the stopped flow. For pyrene and pyrene decanoic acid a value of N_R = 0.5 is obtained. This equals the value of totally equilibrated vesicles. This is not so using pyrene lecithin as optical probe. Within one minute the value N_R = 0.75 is reached being nearly uneffected from τ = 10 s after mixing. The rate constants of this

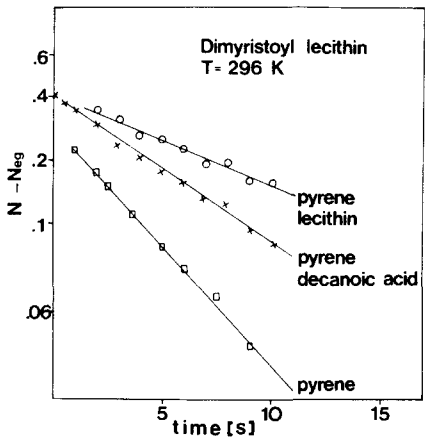

Fig. 3. The decrease of the experimental intensitity ratio $I'/I = N$ related to the equilibrium intensity ratio after mixing. $N - N_{eq}$ is plotted against time on a logarithm scale. The constants of the rapid exchange are given in Table 2.

process are given in Table 2 for dimyristoyl lecithin and dipalmitoyl lecithin at a temperature above the corresponding phase transition. The fastest exchange is obtained for the apolar pyrene molecule ($k_r = 0.25\ s^{-1}$ in DML and $k_r = 0.61\ s^{-1}$ in DPL bilayers) whereas the values given for the pyrene lecithin are reduced by a factor of about three.

Nevertheless the equilibrium distribution corresponding to an intensity ratio $I'/I = N_{eq} = 0.5$ is not reached within one minute after the rapid mixing for the pyrene lecithin. This is only fulfilled for pyrene and pyrene decanoic acid. Therefore the intensity ratio I'/I is followed over a period of about 20 h. The decrease of the intensity ratio I'/I with time at $t = 323$ K for dipalmitoyl lecithin vesicles containing 7 mol % pyrene lecithin is demonstated in Fig. 4. The three significant intensity ratios are obtained from the spectra shown in the insert. The intensity ratio changes from $N_0 = 0.56$ of the doped probe itself, to $N_R = 0.42$ within 10 s (e.g. Fig. 2) and is not

Table 2

Rate constant, K_r, for the rapid exchange between bilayer vesicles of dimyristoyl- or dimyristoyl lecithin. Probe molecules: pyrene, pyrene decanoic acid (PDA), pyrene lecithin (PL).

Probe molecule	rate constant K_r [s^{-1}]	
	DML (t = 296 K)	DPL (t = 323 K)
Pyrene	0.25	0.61
PDA	0.15	0.29
PL	0.10	0.17

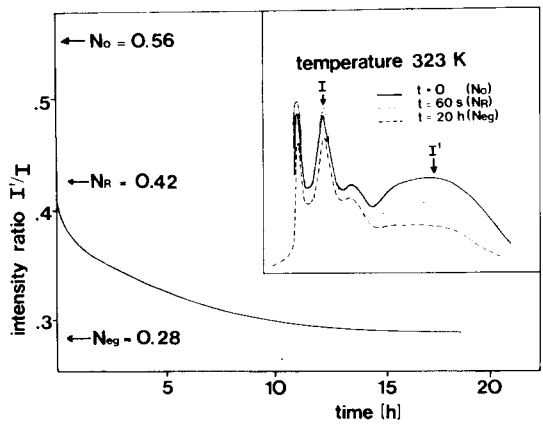

Fig. 4. The fluorescence intensity ratio I'/I is followed over a time period of approx. 20 h for pyrene lecithin-doped vesicles mixed together with undoped vesicles. The intensity ratio N decreases from N_0 to N_R in a rapid exchange process followed by a slow decrease to an equilibrium state N_{eq}. The insert shows the change in the spectral parameter I'/I with time.

further changed within a minute. After 20 h a value for I'/I = 0.28 is reached which we call the equilibrium ratio N_{eq}. This means that the rapid exchange process is followed by a slow one. The time course is given in Fig. 5a. The logarithm of the normalized decrease of the fluorescence intensity ratios $(N - N_{eq})/(N_R - N_{eq})$ gives a straight line with time as demonstrated in Fig. 5b. A half lifetime of $t_{1/2}$ = 8 h is

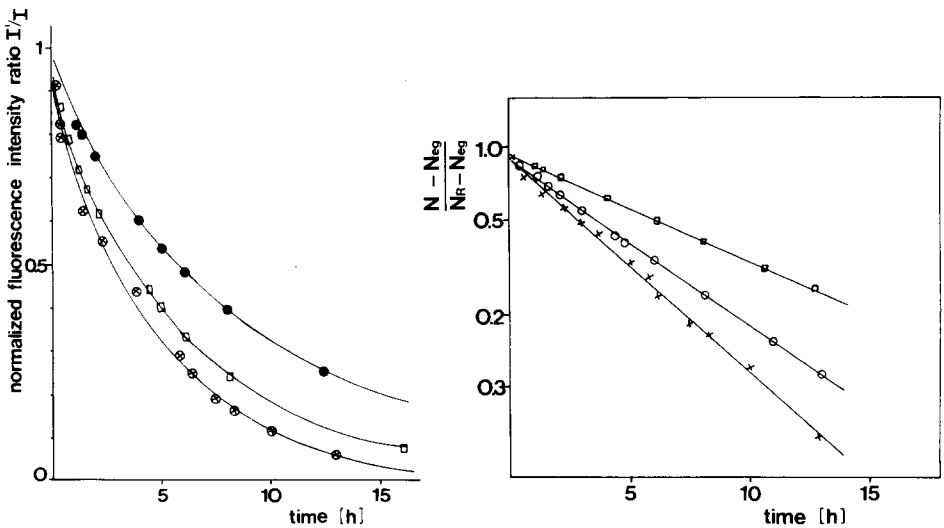

Fig. 5. (a) Time dependence of the normalized fluorescence intensity ratio $(N - N_{eq})/(N_R - N_{eq})$ for dipalmitoyl lecithin probes containing pyrene lecithin as optical probe. Only the slow exchange process is demonstrated for three different temperatures above th lipid phase transition. Temperature: (•) 314 K; (□) 323 K; (⊕) 333 K. (b) The same curves are given in a semi-logarithmic plot. Temperature: (□) 314 K; (○) 323 K; (×) 333 K.

Table 3

Half lifetime and rate constants for the slow exchange of pyrene lecithin in dipalmitoyl lecithin bilayers at temperature above the lipid phase transition.

Temp K	$t_{1/2}$[h]	K_S [$10^{-5} \cdot s^{-1}$]
314	13	5.8
323	8	4.0
333	6.3	3.3

obtained at t = 323 K corresponding to an exchange rate of $k_S = 4 \cdot 10^{-5}$ s^{-1} in dipalmitoyl vesicles. The values for three different temperatures are summarized in Table 3.

The experiments performed should be recapitulated:
(1) Pyrene as well as pyrene decanoic acid perform a rapid exchange from one bilayer membrane to another one. (2) Pyrene lecithin undergoes the same rapid exchange but only part of the probe molecules are transferred. This rapid exchange ($\tau_{1/2}$ = 11 s) is followed by a slow exchange ($t_{1/2}$ = 8 h).

Our interpretation of a rapid inter vesicle transfer through the water phase followed by a slow flip-flop transfer in one bilayer vesicle will be given in the discussion part.

IV. Discussion

A new fluorescence probe, pyrene lecithin, was applied to investigate different model membrane systems. The coefficient of the lateral diffusion of this fluorophore has been determined to $D = 7.7 \times 10^{-8}$ cm^2/s in dipalmitoyl lecithin at t = 333 K. As is expected from the structure of the molecule the mobility is reduced compared to pyrene decanoic acid ($D = 1.1 \times 10^{-7}$ cm^2/s) and pyrene ($D = 1.7 \times 10^{-7}$ cm^2/s). Our result is well consistent with diffusion coefficients obtained by different methods. Scandella et al. [5] reported a value of $D = 7.5 \times 10^{-8}$ cm^2/s for a spin labelled phosphatidylcholine inserted into sarcoplasmic reticulum membrane. Very recently a value of $D = 7 \times 10^{-8}$ cm^2/s was measured by fluorescence recovery after photobleaching using a phosphatidyl ethanolamine label in dipalmitoyl lecithin membranes at t = 318 K [7].

The main importance of our new fluorescence probe molecule is the application to lipid exchange processes in and between bilayer membranes. Using pyrene or pyrene decanoic acid as optical probe a rapid transfer of fluorescent molecules indicated by a decrease in the intensity ratio I'/I is observed. An equilibrium state where the ratio I'/I is half of the initial value is reached within approx 10 s. Using our new optical probe, pyrene lecithin, we determined a two step process for the exchange kinetic. A rapid transfer ($\tau_{1/2}$ = 11 s) only leads to a decrease of the excimer-to-monomer ratio to approx. 0.75 of the initial value. This process is contributed to an exchange of

lipid molecules between the outer layer of one vesicle to the outer layer of a second one. This transfer through the water phase may occur via a monomeric or micellar state, or it may occur upon collision of two vesicles which must not necessarily lead to a fusion process.

A second, long-term exchange is observed only using pyrene lecithin ($t_{1/2}$ = 8 h) corresponding to an exchange constant $k_S = 4 \times 10^{-5}$ s^{-1} in dipalmitoyl lecithin membranes at t = 323 K. This value is in good agreement with measurements of a lipid exchange reported earlier [14, 15, 21, 25]. The rate constants differ with the lipid fatty acid composition but remain always in the range of 10^{-5} s^{-1} and 10^{-6} s^{-1}. On the other side our value for the half time of the slow lipid exchange is also consistent with the value obtained by Kornberg and McConnell [12] for the phospholipid flip-flop ($t_{1/2}$ = 6.5 h for dipalmitoyl lecithin in egg lecithin at t = 303 K).

The mechanism governing the lipid exchange in bilayer membranes consisting of phospholipid from *E. coli* is discussed by G. Duckwitz-Peterlein et al. [17] and in an accompanying paper by L. Thilo [15]. Calculating the change in free energy, ΔG, upon transfer of a lipid molecule from the monomeric state to the bilayer as function of the effective acyl chain length led to the following conclusion: The difference in ΔG implies a ratio M_1/M_2 (equilibrium monomer concentration of lipid one and two) corresponding to the ratio K_1/K_2 of the rate constants of the lipid exchange. From this consideration a lipid mixing via a monomeric state or of micelles which are small compared to the vesicles is assumed. A rate constant of $K = 10^{-5}$ s is consistent with a half lifetime of approx. one day for a lipid mixing via a monomeric state. Application of the above mentioned considerations to our fluorescent probes led to the following results:

(1) The maximum monomer solubility of our three fluorescent probes is given by: pyrene, $c = 10^{-6}$ M, according to ref. 22; pyrene decanoic acid, $c = 5 \times 10^{-6}$ M; pyrene lecithin, $c = 1.5 \times 10^{-7}$ M. The value for pyrene lecithin is the critical concentration for the onset of micelle formation as demonstrated in fig. 1. The maximum monomer solubility of pyrene decanoic acid has been obtained in the same way. The intensity ratio is constant ($I'/I = 0.05$) up to a concentration of $c = 5 \times 10^{-6}$ M. Only a slow increase in I'/I is observed with further increase in label concentration (I'/I = 0.6 at $c = 10^{-5}$ M). Pyrene lecithin in contrast exhibits an increase in the intensity ratio going up to appox. $I'/I = 5$ at $c = 10^{-4}$ M (compare Fig. 1). Stable micelles are formed in the case of pyrene lecithin whereas pyrene decanoic acid precipitates in micro crystals upon increasing the concentration to $c > 5 \times 10^{-6}$ M. The same is valid for pyrene: no micelle formation is observed. Even at $c > 10^{-6}$ M no excimer formation is observed because of the formation of pyrene crystals which precipitate to the bottom of the measuring cuvette and therefore being lost for the radiation light beam. Nevertheless the ratio of the maximum monomer solubility of our probes is given as pyrene lecithin: pyrene: pyrene decanoic acid = 1 : 7 : 33. This implies the same properties for the rate constants of the exchange process if a transfer via a monomeric state is assumed. Though coming from the probe solubility this possibility must be excluded.

(2) According to L. Thilo [15] the difference in free energy for the transfer of a lipid molecule from the monomeric state to a bilayer is given by:

$$\Delta G = \text{const} - m\, n_c \qquad \text{(II)}$$

whereas the constant term is given by 10.5 kJ/mol and the slope is characterized by m = 2.94 kJ/mol for a zwitterionic molecule as pyrene lecithin and by m = 1.68 kJ/mol for a negatively charged molecule like pyrene decanoic acid. Assuming pyrene decanoic acid to give the same contribution as a CH_2-chain of equal length a value for the sum of affective methylene groups of n_c = 23 for the pyrene lecithin and n_c = 8 for the pyrene decanoic acid is obtained.The difference in ΔG for pyrene decanoic acid and pyrene lecithin is then 54.2 kJ/mol leading to a difference in the momomer solubility and a difference in the transfer rates in the order of 10^8. From the energetically point of view a transfer mechanism for the fluorescence probes via a monomeric state can also be excluded.

(3) Only lipid molecules are known to show a slow bilayer equilibration (flip-flop) with a half value of approx. 10 h [12]. A reduction rate was found for a fatty acid spin label carrying the nitroxide group in an amido bound piperidine ring and thus being oriented to the membrane surface which was 'unmeasurably' fast in multibilayer systems [25]. The permeation of the reducing ascorbic acid, however, is characterized by a $t_{1/2}$ of about 30 min for one monolayer. This fact must be interpreted in terms of a very fast equilibration of this fatty acid head group label between lipid layers. An other argument for the fatty acid flip-flop being faster than the probe transfer is the good agreement of the transfer rates between pyrene and pyrene decanoic acid. Pyrene, an apolar probe, is incorporated into the chain region of the membrane and therefore does not need a flip-flop process to equilibrate. Pyrene lecithin, however, is thought to exhibit a slow flip-flop process.

To summarize our experiments: Probe transfer is carried out through the water phase in a rapid process. It is expected to occur between the surface of different lipid vesicles. Only approx. one quarter of the total pyrene lecithin is exchanged in this short time period. The controlling mechanism for the following process is the diffusion across the bilayer. In the case of pyrene lecithin transversal mobility is exceedingly slow compared to the lateral mobility. Therefore a long term kinetic has to be expected after the outer layers are equilibrated within seconds. This is not so in the case of pyrene and pyrene decanoic acid. The mechanism of the transfer can not be ascertained at the moment but it is possible to exclude a transfer via a monomeric state at least for the fluorescent probes.

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