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AN OPTICAL STUDY OF THE EXCHANGE KINETICS OF MEMBRANE BOUND MOLECULES

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SUMMARY

The kinetics of molecular exchange between lipid bilayers are studied using a special fluorescence technique. Pyrene and pyrene decanoic acid are chosen as typical examples of an apolar and amphiphilic molecule. Their property of forming dimers in the excited state (excimer) is exploited. The time dependencies of monomer and excimer intensities after rapid mixing of vesicles doped with fluorescent probe with undoped ones are studied by stopped-flow technique. The transient curves reveal the information on the exchange kinetics. A theoretical analysis shows that the molecular exchange follows a first order kinetics. Surprisingly short half life-times t_{ex} for this exchange process are obtained (for dipalmitoyl phosphatidylcholine $t_{ex} = 3.3$ s for $T = 23$ °C, $t_{ex} = 0.5$ s for $T = 68$ °C). Multilamellar systems (onion like structure) show much slower exchange rates. The exchange rates are nearly equal for polar and unpolar molecules. Addition of cholesterol has a strong reducing effect on this rate. Charging of dipalmitoyl phosphatidylcholine vesicle surfaces by the addition of (a) $EuCl_3$ to the aqueous phase and (b) dipalmitoyl phosphatidic acid to the lipid phase reduces the exchange rate by about an order of magnitude above the phase transition.

In a separate experiment it is shown that the lipid exchange or fusion for two different lipids is a much slower process compared to the label exchange. In fact vesicles kept below the phase transition temperature T_{tr} for both lipids, do not fuse even after 70 h. Noticeable fusion occurs after 10 h when the mixture stays above T_{tr}. Experiment shows that the fusion of pure lipid vesicles is not very much affected by the presence of a charged lipid.

Change in concentration of the monovalent ions in the aqueous solution by two orders of magnitude does not have an appreciable effect on the exchange rate of phospholipids.

INTRODUCTION

The fusion of membranes and the exchange of membrane bound molecules between lipid bilayers is an event of primary importance in biology [1, 2]. In the

present study we report a simple method for the direct measurement of the kinetics of molecular exchange processes.

Excimer forming fluorescent probes, namely pyrene or pyrene-substituted fatty acids, such as pyrene decanoic acid may be used as reporter molecules to measure dynamic parameters of lipid lamellae, such as the coefficients of the lipid lateral diffusion [3, 4], and also the rate of exchange of molecules between lipid bilayers. The crucial physical parameter in the application of excimer probes is the rate of formation of (short lived) excited dimers (excimers). In fluid systems, such as membranes above the lipid phase transition, the excimer formation is a diffusion controlled process. The formation of excited complexes is indicated by the appearance of a new emission band (with a maximum I' at $\lambda' = 485$ nm) well separated from the monomer band (maximum I at $\lambda = 397$ nm). The second order rate constant of association k_a is related to the intensity ratio I'/I according to

$$\frac{I'}{I} = \frac{k'_f}{k_f} \kappa \tau'_0 k_a c \qquad (1)$$

In this equation c is the concentration of the optical probe per unit area. k'_f/k_f is the ratio of the monomer and the excimer radiative decay constant ($k'_f/k_f \approx 0.1$ for pyrene and pyrene-decanoic acid). κ is a correction factor ($\kappa = 0.7$ for pyrene; $\kappa = 0.75$ for pyrene-decanoic acid), τ'_0 is the life-time of the excimer.

Eqn. 1 suggests the following applications of the excimer forming probe: (1) The product $k_a c$ is equal to the collision frequency of the excimer probe in the membrane and thus a measure for the rate of lateral diffusion [3, 4]. (2) The concentration dependence of I'/I may be used (a) to evaluate the lipid organization in mixed membranes that exhibit phase separation [4] and (b) to study the exchange kinetics.

Consider a vesicle labelled with excimer probes that come in contact with an unlabelled vesicle. If there is an exchange of label molecules, the intensity ratio I'/I should then decrease with respect to the original intensity ratio of the doped vesicle. The rate of exchange may be observed simply by recording the increase in the monomer intensity I and the decrease in the excimer emission I' as a function of time after mixing the vesicles. The decrease in the intensity ratio is a direct measure for the extent of exchange whereas its temperature dependence indicates the phase transition temperature, characteristic of the lipid.

In the present work the kinetics of label exchange between small vesicles composed of either phosphatidylcholine or mixtures of phosphatidylcholine with (a) cholesterol, (b) phosphatidic acid have been studied. In order to find out whether fusion of vesicles is the primary cause of label exchange, the following experiment is performed. The temperature dependence of I'/I for two different lipid bilayer dispersions is recorded separately. The transition curves are compared at different time intervals with that for the mixture of these two solutions. Results show that label exchange is a much faster process than lipid exchange.

METHODS

Vesicles were prepared by sonication of oxygen free aqueous (2 mM CsCl) lipid dispersions under nitrogen atmosphere. The sonication was performed above 45 °C and was continued until a clear solution was obtained (about 10 min). The

final lipid concentration was 10^{-3} M. For the doped vesicles, the chloroformic solution of label and lipid were mixed prior to sonication*. Oxygen free multilamellar systems were prepared as described previously [3]. The lipids were obtained from Fluka and the purity was checked by thin-layer chromatography. The labels, both pyrene decanoic acid and pyrene were purified by repeated recrystallisation. We have performed two different kinds of experiments:

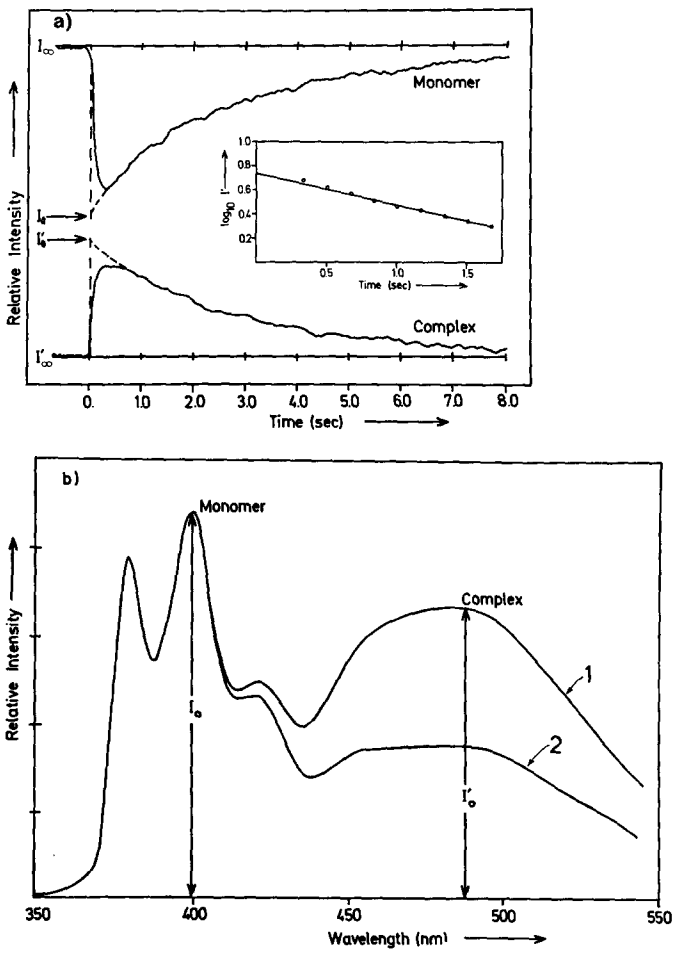

Fig. 1. (a) Typical time dependencies of the monomer and the excimer fluorescence intensities (I and I′ respectively) after mixing of doped (probe: pyrene decanoic acid) and undoped dipalmitoyl phosphatidylcholine vesicles (in 10^{-3} M NaCl) of equal lipid concentration. Temperature 23 °C. Excitation wave = length for both curves: 320 nm; bandwidth: 10 nm. Emission wavelength of monomer, 397 nm; emission wavelength of complex, 485 nm; bandwidth 10 nm. Insert: Theoretical values of log I′ versus time from Eqn 5 indicate a first order process characterized by the rate constant k_{ex} = 0.15 s^{-1}. Experimental points at temperature 23 °C. (b) Total emission spectra of initially doped vesicle preparation (curve 1) is compared with the spectrum obtained after mixing at time $t > 1/k_{ex}$ (curve 2). Excitation wavelength 320 nm; bandwidth 10 nm.

* Formation of small bilayer vesicle is ensured by electron microscopy.

(a) Kinetic experiment

The exchange kinetic was studied by a stopped-flow technique. A mixing chamber with a round cell of inner diameter 3 mm was used. The details of the apparatus will be published elsewhere. The time dependence of the monomer and the excimer emission peaks after mixing of doped and undoped vesicles was recorded with a Schoeffel Instrument fluorescence spectrometer. The kinetic experiment was performed in the following way. Before each new measurement the cell was allowed to be filled with already fused vesicles. Immediately after the simultaneous influx of the doped and undoped vesicles, I′ increases and I decreases abruptly since the label concentration, c, is equal to that of the doped vesicle preparation. Now, if a label exchange process between the two types of vesicles is followed, it will be expected that with time there will be an increase in I and decrease in I′ to their equilibrium values I'_∞ and I_∞, respectively. Such a behaviour is indeed found as shown in Fig. 1a. In the insert of Fig. 1a a logarithmic plot of I′ versus time is given. A straight line is obtained suggesting that the label molecule exchange proceeds according to a first order process characterized by a rate constant k_{ex} (and a half life-time t_{ex}).

(b) Static experiments

In the first experiment the fluorescence spectra showing the excimer and the monomer emission were recorded at different times. Fig. 1b shows the complete emission spectra before mixing (curve 1) and at time $t > t_{ex}$ after mixing of doped and undoped vesicles (curve 2). The intensity ratio for the doped vesicle is I'_o/I_o. When two equal portions of doped and undoped vesicles of equal lipid concentration are mixed, the intensity ratio decreases by a factor of 2 ($I'/I = \frac{1}{2} I'_o/I_o$).

In the second experiment the temperature dependencies of I'/I are recorded after mixing equal amounts of vesicle preparations of two different lipids (dipalmitoyl phosphatidylcholine and distearoyl phosphatidylcholine, respectively) doped with pyrene decanoic acid. The curves define the phase transition temperature characteristic of each lipid. The experiment is repeated at different time intervals. The two phase transition points are distinctly shown when there is only label exchange, and they merge to show one phase transition after completion of fusion of these two lipids.

RESULTS

Dipalmitoyl lecithin vesicles. The exchange of molecules between dipalmitoyl phosphatidylcholine vesicles has been studied as a function of temperature between 18 and 65 °C using both pyrene decanoic acid and pyrene as optical probes. At all temperatures exponential decay curves are observed indicating a first order process. The rate constants k_{ex} are summarized in Fig. 2. A sharp increase in the rate constant k_{ex} by a factor of 5 is observed between 40 and 50 °C. The transition temperature of dipalmitoyl phosphatidylcholine fits well within this range. The lower value of $k_{ex} = 0.2\ s^{-1}$ and the higher value of $k_{ex} = 1.0\ s^{-1}$ characterize the exchange rates in the crystalline phase and in the liquid crystalline states of the lipid lamellae, respectively.

Mixed dipalmitoyl phosphatidylcholine/cholesterol bilayers. In Fig. 2 the exchange rates k_{ex} of mixed dipalmitoyl phosphatidylcholine/cholesterol vesicles are presented for different temperatures. The result indicates that the addition of cholesterol reduces the exchange rate between fluid dipalmitoyl phosphatidylcholine

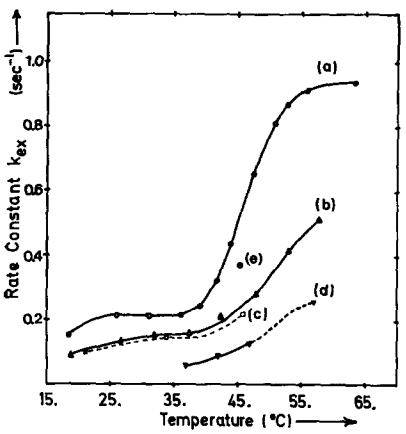

Fig. 2. Rate constant of pyrene decanoic acid exchange (k_{ex}) between vesicles measured as a function of temperature by stopped-flow method. (a) (○), dipalmitoyl phosphatidylcholine (in 10^{-3} M CsCl); (b) (△), dipalmitoyl phosphatidylcholine with 5 mol % cholesterol; (c) (□), 80 mol % dipalmitoyl phosphatidylcholine with 20 mol % dipalmitoyl phosphatidic acid in 10^{-3} M CsCl; (d) (▼), effect of $EuCl_3$ on k_{ex} of dipalmitoyl phosphatidylcholine vesicles; (e) (●), value of exchange rate of pyrene between vesicles of dipalmitoyl phosphatidylcholine at 45 °C.

lamellae ($T > 45$ °C) to a large extent. Below the phase transition this reduction is much smaller.

Activation energy. By plotting the logarithm of k_{ex} for the mixed phosphatidylcholine/cholesterol system (curve b in Fig. 2) as a function of the reciprocal temperature, a straight line is obtained between 40 and 58 °C. Since the lipid phase transition of the mixed membrane is completed at 40 °C, the slope of the straight line is a measure for the activation energy ΔE_{ex} of the molecular exchange process. A value of $\Delta E_{ex} = 13$ kcal/mol is obtained.

Influence of surface charge. In order to evaluate the influence of the surface charge on k_{ex}, experiments were performed (a) with mixed dipalmitoyl phosphatidylcholine/dipalmitoyl phosphatidic acid vesicles at pH 7 and (b) with dipalmitoyl phosphatidylcholine vesicles dispersed in Eu^{3+} solution at pH 7.

(a) At neutral pH, phosphatidic acid carries one negative charge. Addition of 20 % of the charged lipid reduces k_{ex} by a factor of 3 above the phase transition (at 46 °C, cf. Fig. 2). Below the phase transition (at 21 °C) the influence of the charged lipid is very small.

(b) The presence of Eu^{3+} in the aqueous phase has the similar reducing effect on k_{ex} as charged lipids (cf. Fig. 2). But the effect is also very prominent at temperatures below the phase transition.

Multilamellar systems. (Onion like structure). By comparing the intensity ratio I'/I (after mixing of doped and undoped vesicles) with the value of I'_o/I_o (of the doped vesicle preparation), the extent of exchange may be estimated. Complete mixing yields $I'/I = \frac{1}{2}(I'_o/I_o)$. In contrast to sonicated vesicles (cf. Fig. 1b) the mixing of doped and undoped multilamellar systems does not lead to a rapid and complete exchange of the label molecules between the lipid bilayers. I'/I remains larger than $\frac{1}{2}(I'_o/I_o)$ for several hours. In Fig. 3 the time dependencies of the intensity ratios after

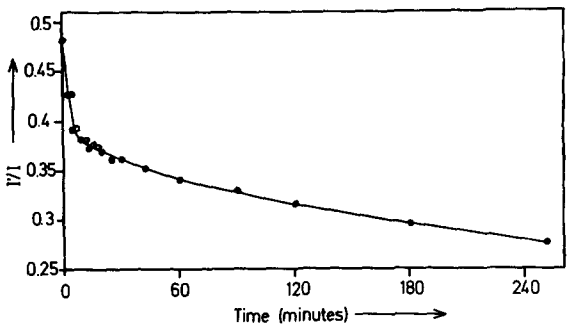

Fig. 3. Time dependence of I'/I after mixing of doped and undoped multilamellar systems of dipalmitoyl phosphatidylcholine. Note: I'/I does not reduce to $\frac{1}{2}$ I'_o/I_o even within 4 h.

mixing of multilamellar systems are shown (static experiment). The exchange process is composed of a rapid initial step followed by a slower second process. This initial rapid process is due to the transfer of label molecules from the outer bilayer whereas the slow process characterizes the mixing of the inner bilayer of the onion like structure. The half life-time of the slow process is about 5 h showing that the label molecules in the interior of the lyposomes exchange rather slowly. In order to follow the initial fast process, we repeated the kinetic experiment with the multilamellar system. The transient curves of I and I' show an exponential decay. Above the phase transition, k_{ex} for multilamellar system is about 10-times smaller ($\approx 0.09\ s^{-1}$) than k_{ex} for sonicated vesicles.

Aging of vesicles. As noted earlier, comparison of the intensity ratio before mixing and at time $t > 1/k_{ex}$, after mixing yields a measure of the degree of exchange. Such comparison made at different times after sonication should indicate the degree of transformation of bilayer vesicles to multilamellar system by the process of fusion. Consequently the aging of vesicles was studied. Doped and undoped vesicles were prepared by sonication and aged at 50 °C. Static experiments were then performed at different aging times. Up to 10 h of aging the value of I'/I was $\frac{1}{2}(I'_o/I_o)$ of the initially doped preparation (cf. Table I). This result provides evidence that there is no rapid "aging" of vesicles leading to multilamellar systems.

TABLE I

INTENSITY RATIO I'/I OF SONICATED VESICLE AT DIFFERENT AGING TIME

Static experiment performed at $t > t_{ex}$. Intensity ratio of doped vesicle before mixing $I'_o/I_o = 1.0$.

Aging time (h)	2	4	6	8	10
I'/I	0.50	0.51	0.51	0.51	0.51

Egg lecithin. For comparison, we also performed experiments with vesicles of egg lecithin. Static experiments again indicate complete exchange of label molecules. The kinetic experiments yield values of $k_{ex} = 0.11\ s^{-1}$, $k_{ex} = 0.15\ s^{-1}$ and $k_{ex} = 0.28\ s^{-1}$ for 30, 40 and 50 °C, respectively.

Fusion of vesicles. The above experiments have demonstrated a surprisingly

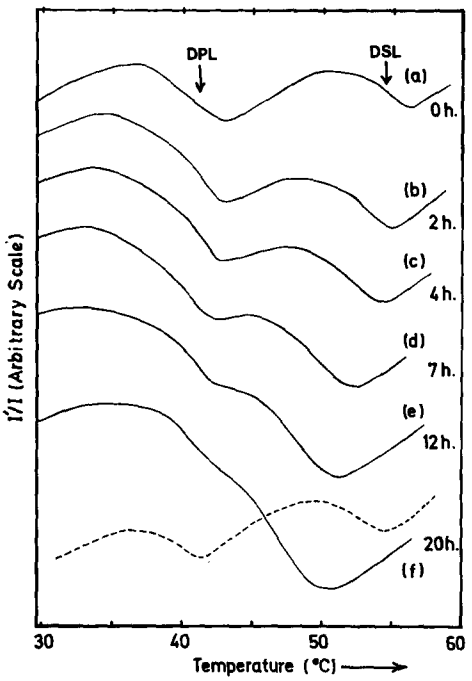

Fig. 4. Demonstration of phospholipid exchange between bilayer vesicles using pyrene decanoic acid as the fluorescent probe. Broken line curve: temperature dependence of I'/I is recorded 90 h after mixing equal portions of vesicles of dipalmitoyl phosphatidylcholine and distearoyl phosphatidylcholine preserved at room temperature. The phase transitions of dipalmitoyl phosphatidylcholine at 40 °C (indicated by DPL) and of distearoyl phosphatidylcholine at 53 °C (indicated by DSL) are visible demonstrating the absence of fusion. Curves (a)–(f): time dependence of transition curve after preserving the mixture above the phase transition temperature of both lipids ($T = 65$ °C). Preservation time in hours is indicated next to each curve. Clearly lipid randomization proceeds with time and is almost completed after 20 h.

rapid exchange of label molecules between lipid bilayers. Now, the question arises whether this molecular exchange is accompanied or caused by vesicle fusion. Consequently, another set of experiments is performed. Using pyrene decanoic acid as the probe, the fusion of dipalmitoyl phosphatidylcholine and distearoyl phosphatidylcholine vesicles is studied by the fluorescence technique as follows. First, the temperature dependencies of I'/I are recorded for each type of vesicles. At a particular temperature, the sudden drop in I'/I indicates the phase transition temperature characteristic of the lipid [9]. The aqueous solution of these two types of vesicles are then mixed in equal proportions and the phase transition curve is recorded as a function of time. In the absence of fusion, this curve clearly exhibits the two distinct transitions, characteristic of the individual lipids. A typical curve is shown in Fig. 4, (curve a). The shape of the transition curve for this mixture changes with time when it is kept above phase transition temperature. As randomization of lipids takes place, the two sharp drops in I'/I merge. Completion of fusion is indicated by the appearance of a single phase transition at an intermediate temperature (curve e in Fig. 4).

When the mixture is preserved at room temperature, the transition curve

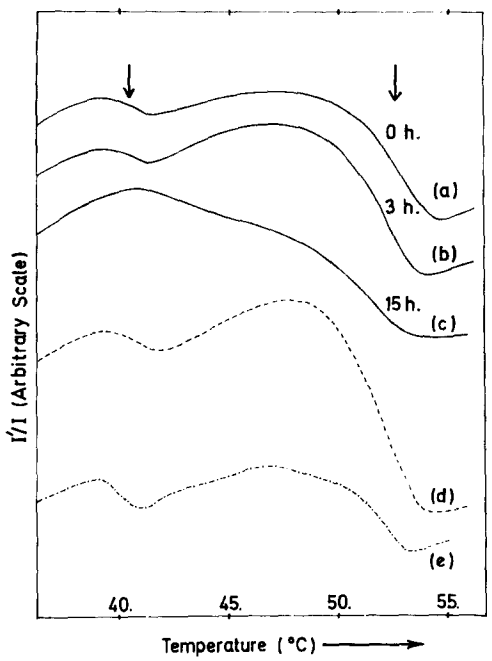

Fig. 5. Influence of charged lipids on phospholipid exchange. Vesicles of dipalmitoyl phosphatidylcholine (transition at 40 °C) are mixed in equal proportions with vesicles composed of equimolar amounts of distearoyl phosphatidylcholine and dipalmitoyl phosphatidic acid (transition at 53 °C). Both types of vesicles are doped with pyrene decanoic acid. Curve (e): transition curve of vesicle mixture preserved at room temperature for 90 h. The transitions of the two types of vesicles are clearly visible. Curve (a)–(c): time dependence of transition curve of vesicle mixture preserved at $T = 65$ °C. Lipid randomization is near completion after 15 h. Curve (d): undoped vesicles of dipalmitoyl phosphatidylcholine are added to vesicles composed of distearoyl phosphatidylcholine and dipalmitoyl phosphatidic acid and probed with pyrene decanoic acid. The transition curve recorded immediately after mixing shows the rapid transfer of the fluorescent probe to the dipalmitoyl phosphatidylcholine vesicles.

indicating both transitions does not change substantially at least up to 90 h after mixing (dashed curve in Fig. 4).

The effect of charge on lipid fusion is studied in a similar set of experiments. Transition curve is recorded for a vesicle preparation composed of distearoyl phosphatidylcholine and dipalmitoyl phosphatidic acid (equal molar portions) in pH = 5 buffer solution (Fig. 5). This solution is then mixed with the aqueous solution of dipalmitoyl phosphatidylcholine vesicle in equal portions. Results show that the fusion of vesicles is not enhanced by the presence of charged lipid. Variation in the CsCl concentration in the aqueous solution (between 2 mM and 0.1 M) also has no pronounced effect on the rate of lipid exchange.

In order to show that the label goes inside the membrane, we prepare a bilayer dipalmitoyl phosphatidylcholine vesicle preparation without any label and mix it with the bilayer vesicle preparation consisting of distereoyl phosphatidylcholine and dipalmitoyl phosphatidic acid which also contains the label pyrene decanoic acid. The spectrum recorded just after mixing shows the transition curve of dipalmitoyl phos-

phatidylcholine thus indicating that the label really has gone into these vesicles (cf. Fig. 5, curve d).

DISCUSSION

In summary, the essential experimental results of the present study are:

(a) after mixing doped phosphatidylcholine vesicles with undoped ones, (1) the fluorescence label is distributed randomly in the two types of vesicles within a very short time t_{ex} (for dipalmitoyl phosphatidylcholine $t_{ex} \approx 3.3$ s at $T < T_t$ and $t_{ex} \approx 0.5\ s^{-1}$ at $T > T_t$, cf. Fig. 2). (2) The value of t_{ex} does not depend appreciably on the polarity of the fluorescence label (cf. Fig. 2, compare (e) and (a)). (3) The randomization obeys a first order kinetic. (4) The rate constant k_{ex} is considerably lower in the presence of surface charges (cf. (c) and (d) in Fig. 2). (5) The rate of exchange does not depend appreciably on the age of the vesicles even up to 24 h after sonication. (6) The exchange in case of onion like systems is much slower than that for sonicated vesicles.

(b) Vesicles in a fluid (liquid crystalline) state are susceptible to slow fusion characterised by the half life-time of the order of 20 h. In contrast to this, below the phase transition temperature, the fusion time is increased at least by an order of magnitude.

(c) Label exchange takes place at all temperatures independent of lipid exchange.

Analysis of fluorescence transient curves.

We suggest that the label exchange process at any time t may be described by transfer of label molecules between two adjacent vesicles A (doped) and B (undoped). Let us assume that the rate of transfer from A to B is k_{AB} and is equal to k_{BA}, the transfer rate from B to A ($k_{BA} = k_{AB} = k_{ex}$). The exchange process is described by the following coupled equations:

$$\frac{dC_A}{dt} = -k_{ex}C_A + k_{ex}C_B; \quad \frac{dC_B}{dt} = -k_{ex}C_B + k_{ex}C_A \tag{3}$$

C_A and C_B are the molar ratio of label molecules at time t with respect to lipid molecules at time $t = 0$, in vesicles A and B respectively. The trial solutions are:

$$\begin{aligned} C_A &= \tfrac{1}{2}C_A^0 \quad \{1 + e^{-2k}\mathrm{ex}^t\}; \\ C_B &= \tfrac{1}{2}C_A^0 \quad \{1 - e^{-2k}\mathrm{ex}^t\} \end{aligned} \tag{4}$$

According to Förster's theory [8] the quantum yield for excimer (monomer) emission η' (η) is related to C as

$$\eta' = \frac{C}{C + C_h}\,\eta'\ \max; \quad \eta = \frac{C_h}{C + C_h}\,\eta\ \max$$

where C_h is the half value concentration and η'_{max} (η_{max}) is the maximum quantum yield for $C \to \infty$ (for $C \to 0$). Denoting $C_A{}^0$ as the initial value of C_A the quantum yield at any time t after mixing is, for excimer

$$\eta^* = \frac{\eta'}{\eta'_{\max}} = \frac{1}{C_A + C_B}\left[C_A \frac{C_A}{C_A + C_h} + C_B \frac{C_B}{C_B + C_h}\right]$$
$$= C_A^0 \frac{(C_A^0 + 2C_h) - (C_A^0 - 2C_h)e^{-4k}\mathrm{ex}^t}{(C_A^0 + 2C_h)^2 - (C_A^0 e^{-2k}\mathrm{ex}^t)^2} \tag{5}$$

and similarly for monomer:

$$\eta^{**} = \frac{\eta}{\eta_{\max}} = 2C_h \frac{(C_A^0 + 2C_h) - C_A^0 e^{-4k}\mathrm{ex}^t}{(C_A^0 + 2C_h)^2 - (C_A^0 e^{-2k}\mathrm{ex}^t)^2} \tag{6}$$

Provided $C_A/C_h < 1$, η and η' (therefore I and I') vary exponentially to a good approximation. In our case, $C_A \approx 0.35\ C_h$ for $T < T_t$ and $C_A \approx 0.5\ C_h$ for $T > T_t$. In the insert of Fig. 1a the decay curve as obtained from Eqn 5 for $T < T_t$ is compared with the experimental transient curve of the excimer. The good agreement between these two curves shows that the label exchange is indeed a first order process.

Results obtained here are consistent with the work of Papahadjopoulos et al. [2], where the exchange of dipalmitoyl phosphatidylcholine molecules, between small vesicles and multilamellar systems is shown to be a slow process. This experiment clearly demonstrates that fusion is a slow process. Our experiment, however, indicates that other amphiphilic molecules such as fatty acids that are soluble both in water and lipids can transfer very rapidly between two lipid bilayers. The fact that exchange of molecules can take place not necessarily by means of fusion may have some significant implication for biological processes.

Our observation of aging of dipalmitoyl phosphatidylcholine vesicles by an electron microscope has not given much direct evidence of fusion. It is quite likely that pure lipids do not fuse which is also an observation of Kantor and Prestegard [10].

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