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INTERACTION OF LYSOPHOSPHATIDYLCHOLINE WITH PHOSPHATIDYLCHOLINE BILAYERS

A PHOTO-PHYSICAL AND NMR STUDY

DAVID A.N. MORRIS, ROBERT McNEIL, FRANCIS J. CASTELLINO and J. KERRY THOMAS

Department of Chemistry, University of Notre Dame, Notre Dame, IN 46556 (U.S.A.)

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Summary

Several photo-physical methods together with ^{31}P-NMR have been used to investigate the effect of lysophosphatidylcholine on phosphatidylcholine bilayers. ^{31}P-NMR shows that the permeability of the vesicle to Eu^{3+} increases sharply above approx. 40% lysophosphatidylcholine: fluorescence-quenching studies also show this type of behavior. Similar sharp changes in vesicle properties are observed via the photo-physical technique at this lysophosphatidylcholine/phosphatidylcholine composition. Fluorescence spectra of pyrene and pyrene carboxaldehyde show that increasing lysophosphatidylcholine composition increases the polarity of the environments of these probes up to 40% lyso-compound. Above this composition the photo-physical properties of the probes slowly revert to those characteristic of the micellar lyso-compound. The pyrene fluorescence lifetime, the fine structure of the fluorescence, and the case of formation of pyrene excimer in these bilayer mixtures suggest that pyrene complexes weakly with the charged nitrogen of the choline group of the phosphatidylcholine and that the physical state of the system has a striking effect on this complexation process. Similar experiments with simple quaternary compounds lend strong support to this suggestion. The studies monitor in several ways the effect of bilayer composition on movement of molecules in these systems. The degree or site of solubilization of carcinogens is also uniquely affected by composition.

Introduction

The aggregation or self-assembly of certain amphiphilic molecules is of great interest to biology [1,2], physical sciences [3,4] and industry [5]. Single-stranded amphiphiles, such as common surfactants and lysophosphatidylcholine, tend to form roughtly spherical structures such as micelles, whilst double-stranded structures such as phosphatidylcholines form bilayer vesicles [6]. Various factors, such as the geometric shape of the amphiphile and the charge created by dissociation of the head groups, play important roles in determining the exact nature of the structure, whether micellar or vesicular. It is of some interest at this stage to inquire into the interaction of micelle-forming amphiphiles with bilayer structures.

It is generally accepted that the lyso-compound content of membranes has implications for the stability of the cell interface [7]. Increased lysophosphatidylcholine concentrations form conditions for cell fusion [8,9] and an excess of exogenously added lysophosphatidylcholine causes lysis. The lytic process has been described in terms of subtle changes in the bilayer orientation causing an increase in cation permeability. The disturbance of the Donnan equilibrium then causes entry of water into the cell which bursts by osmotic force [10,11]. An alternative explanation of the lytic effect proposes that the thickness of the alkyl core is critical in order to act as an effective permeability barrier. X-ray studies have demonstrated a decrease in bilayer thickness of egg phosphatidylcholine with increasing lysophosphatidylcholine content. It has been suggested that decreasing thickness of the alkyl core causes a decrease in interaction between the lipid molecules. If the bilayer thickness decreases below a critical value a stable association of the lipid molecules is no longer warranted [8,12,13]. Physically, in mixed lysophosphatidylcholine/phosphatidylcholine vesicles, the majority of the lysophosphatidylcholine exists on the outer bilayer surface [14,15].

This paper presents data from several different physical methods which reflect on the interaction of lysophosphatidylcholine or lysoplasmologen with phosphatidylcholine vesicles. The techniques used have proved themselves in other studies on micelles, vesicles, and membranes, and the present data are interpreted in the light of these earlier works.

Materials and Methods

Materials. Egg phosphatidylcholine, dipalmitoyl phosphatidylcholine and egg lysophosphatidylcholine were purchased from the Serdary Research Corporation. Thin-layer chromatographic analysis [16] revealed a single spot for each. Therefore, they were used without further purification. $Eu(Cl)_3$ was purchased from Alfa Ventron.

Preparation of vesicles. Individual small vesicles were prepared by co-sonication of a suspension of the desired lipid components in a Bronson model 200 R probe sonicator, for 1.5—2 h, under a constant stream of N_2, at a temperature above the phase transition temperature of the highest melting-point component of the mixture. The vesicle preparation was centrifuged to remove probe particles.

NMR spectrometry. ^{31}P-NMR spectra were obtained at 30°C, utilizing a Varian XL-100-15 spectrometer, locked on ^{2}H, and operating at 40.48 MHz, in the pulsed Fourier Transform mode. In these studies we used gated proton decoupling (decoupler on during data acquisition, and off during the pulse delay) to eliminate spectral enhancements due to the nuclear Overhauser effect [17]. At least 200 scans, utilizing 4000 data points, were observed at a spectral width of 5000 Hz. A pulse width of 65 ms and a pulse delay of 14 s were used to ensure that complete relaxation occurred between successive pulses.

Fluorescence polarization analysis. A sufficient volume of $1 \cdot 10^{-3}$ M 1,6-diphenyl-1,3,5-hexatriene in benzene to make the concentration of 1,6-diphenyl-1,3,5-hexatriene 8 μM in surfactant solution was placed in a vessel, and the solvent was evaporated under a stream of N_2. Then sufficient solid phospholipid was added to make the total surfactant concentration 2 mM. A small amount of chloroform was added in order to produce a homogeneous solution, and then the solvent was evaporated under N_2, the object being to deposit the surfactant/probe mixture uniformly on the walls of the vessel. Water was added, and the mixture was sonicated for 2 h at a temperature of 50°C under a stream of N_2.

The polarization measurements were performed as previously described [18].

Pulse laser excitation. Fluid samples were excited with 20-ns pulses of light of wavelength λ = 347.1 nm from a Q-switched frequency-doubled ruby laser, and the rate of decay of the fluorescence observed via fast spectrofluorimetry [19]. The decay rate constant, k_o, respresents a first-order decay. In the presence of quenchers at concentrations far greater than that of the excited state the decay rate increases, the rate constant being k_q. The observed rate constant, k for the total decay rate is given by $k = k_o + k[Q]$ from which k can be calculated [18].

Steady-state fluorescence was observed with a Perkin Elmer II spectrofluorimeter. The fine structure of the pyrene fluorescence is a measure of the environment of the pyrene, the ratio of peak 3 (λ = 390 nm) to peak 1 (λ = 370 nm) varying with the nature of the solvent [21]. With pyrene carboxaldehyde the position of the fluorescence spectral maximum is related to the local dieletric constant of the probe environment [22]. Both probes were used to observe changes in the membrane systems with composition and temperature change.

Experimental Data and Discussion

Fig. 1 shows the variation in two properties of the excited state of pyrene, the vibrational fine structure and the fluorescence lifetime, with lysophosphatidylcholine concentration. The fluorescence lifetime of pyrene is quite sensitive to its environment and varies from 120 ns in water to greater than 280 ns in less-polar media such as alcohols and alkanes. In the experiments shown in Fig. 1, a small amount of pyrene (approx. 0.5 μM) in water and lysophosphatidylcholine solutions was excited by a 20 ns pulse of 3471 Å light from a Q-switched ruby laser. The excited state p^* is formed, which subsequently decays back to the ground state, emitting characteristic fluorescence or giving

excited triplets, p^T:

$$p \xrightarrow{h\nu} p^* \rightarrow p + h\nu \text{ (fluorescence)}$$
$$\searrow$$
$$p^T$$

The state of decay of p^* was monitored by fast spectrofluorimetry. Fig. 1A shows that the lifetime of p^*, τ_0, decreases on addition of small amounts of lysophosphatidylcholine up to $0.8 \cdot 10^{-4}$ M after which it sharply increases to $\tau_0 > 280$ ns. The latter value is typical of pyrene in a non-aqueous environment and we suggest that this reflects the situation where micelles of lysophosphatidylcholine are formed at $0.8 \cdot 10^{-4}$ M with pyrene solubilized in these hydrophobic entities. The onset of this process occurs at the critical micelle concentration of $0.8 \cdot 10^{-4}$ M where micellization commences. The initial decrease in τ_0 with lysophosphatidylcholine up to the critical micelle concentration may be due to complex formation.

The fine structure of pyrene fluorescence varies quite markedly with environment [21], the ratio of band 3 at 390 nm to band 1 at 370 nm varying from 1.68 in hexane to 0.62 in water. This property also reflects on the onset of micellization in Fig. 1B, I_3/I_1 changing from 0.62 in water to 0.85 at $0.8 \cdot 10^{-4}$ M lysophosphatidylcholine and remaining constant at high concentration. These two photo-physical properties reflect quite markedly on the medium surrounding excited pyrene and were used to investigate the effect of lysophosphatidylcholine in pyrene solubilized in phosphatidylcholine bilayers (Fig. 2).

Fig. 2A shows the variation in the pyrene fluorescence lifetime, τ_0, and the fluorescence fin structure (I_3/I_1) in phosphatidylcholine vesicles on addition of lysophosphatidylcholine (Fig. 2B). The τ_0 value of 280 ns in the phosphatidylcholine bilayer is indicative of a non-aqueous and hydrophobic-like environment for pyrene, the environment being similar to that at high lysophosphatidylcholine concentration. Similar information is also provided by the I_3/I_1 data which indicate a hydrophobic environment for pyrene in phosphatidyl-

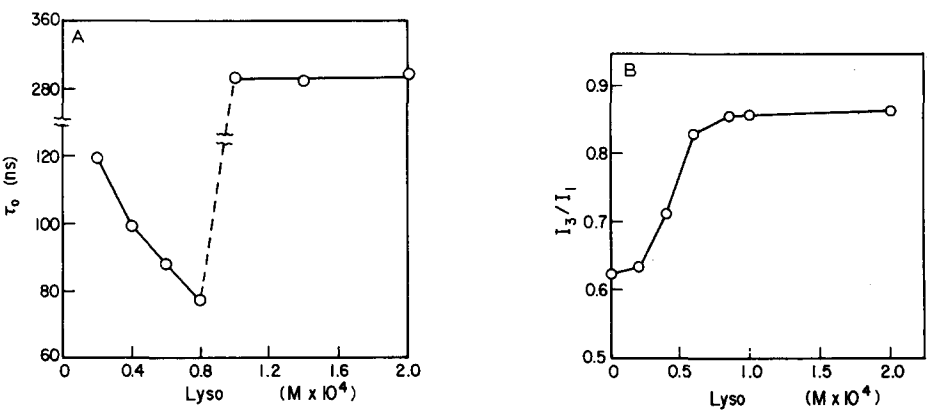

Fig. 1. (A) Dependence of pyrene monomer fluorescence lifetime (τ_0) on [lysophosphatidylcholine] (lyso). (B) Dependence of pyrene vibrational fine structure (I_3/I_1) band intensities on [lysophosphatidylcholine].

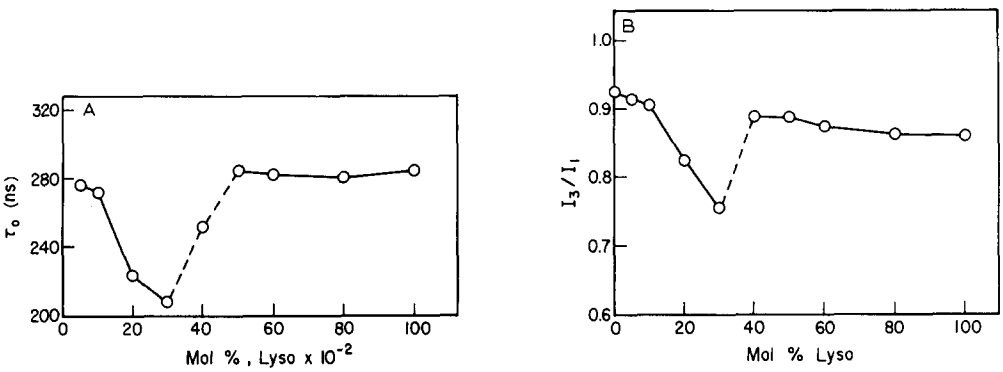

Fig. 2. Variation in pyrene fluorescence lifetime, τ_0 (A), and the fluorescence I_3/I_1 (B), in phosphatidylcholine vesicles on addition of lysophosphatidylcholine.

choline bilayers and lysophosphatidylcholine micelles; both techniques show that the environment becomes more aqueous-like or polar lysophosphatidylcholine is added to phosphatidylcholine bilayers up to 30 mol%, τ_0 decreasing as well as I_3/I_1. This is in agreement with the suggestion that the up to 30 mol% interaction incorporation of lysophosphatidylcholine with phosphatidylcholine vesicles leads to a penetration of water into the bilayer, thus causing an increasingly aqueous-like environment for pyrene. Above 30 mol% lysophosphatidylcholine the bilayer breaks up, and it is suggested that mixed micelles of lysophosphatidylcholine and phosphatidylcholine are formed. The environment of pyrene in these entities is again hydrophobic-like. The data may also be explained in terms of various complexes formed between pyrene and the aggregates.

Two other experiments are in agreement with the mechanism for the interaction of lysophosphatidylcholine with bilayers. Fig. 3A shows the effect of the mole percent of lysophosphatidylcholine on pyrene carboxaldehyde fluorescence, and the quenching by CH_3NO_2 of pyrene fluorescence in bilayers (Fig. 3B). Pyrene, excited by laser excitation, is quenched by CH_3NO_2:

$$p^* + CH_3NO_2 \rightarrow p + CH_3NO_2^*$$

the process being diffusion-controlled in homogeneous media [23]. The quenching rate constant (k_Q) varies from approx. $1 \cdot 10^{10}$ in non-viscous homogeneous media to approx. $1 \cdot 10^9$ in restricted media such as micelles and phosphatidylcholine bilayers. Addition of lysophosphatidylcholine up to approx. 40% tends to increase k_Q due to increased case of penetration of the bilayer by CH_3NO_2. However, on disruption of the bilayer by lysophosphatidylcholine and formation of mixed micelles, k_Q drops and increases again towards the limit observed in lysophosphatidylcholine micelles.

The fluorescence of pyrene carboxaldehyde is quite solvent-dependent [22] and the maximum of the fluorescence, λ_{max}, may be related to the local dielectric constant of the probe molecule. λ_{max} of pyrene carboxaldehyde increases (Fig. 3) with increasing lysophosphatidylcholine as the penetration of H_2O into the bilayer increases. The local dielectric constant of pyrene carboxaldehyde also increases. On disruption of the bilayer and formation of mixed

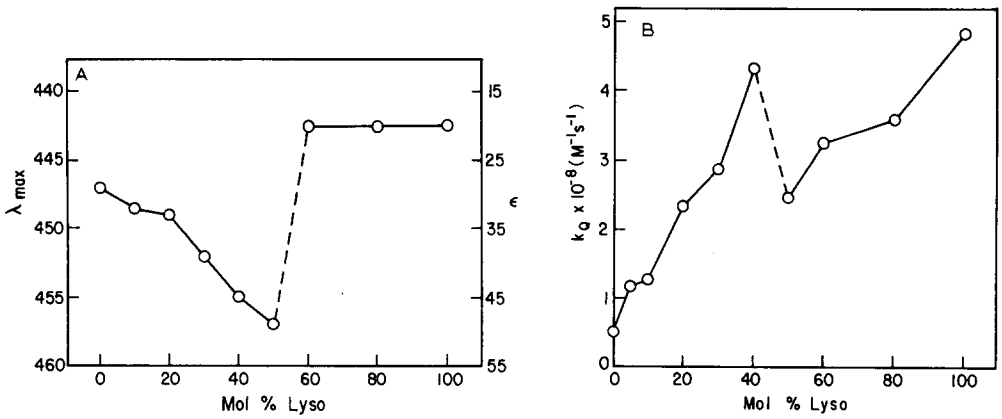

Fig. 3. (A) Effect of lysophosphatidylcholine on pyrene carboxaldehyde fluorescence. (B) Effect of lysophosphatidylcholine on the CH_3NO_2 quenching of pyrene fluorescence, in phosphatidylcholine bilayers.

micelles, the probe fluorescence becomes indicative of non-aqueous hydrophobic-like character.

The increased rate of reaction of CH_3NO_2 with the excited state of pyrene may be also due in part to a decrease in the rigidity of the bilayer on addition of lysophosphatidylcholine. The decrease in the microviscosity of the bilayer is indicated by the decrease in fluorescence polarization of diphenylhexatriene in these systems on addition of lysophosphatidylcholine.

Fig. 4 shows the effect of temperature on the fluorescence polarization ratio of diphenylhextriene as a function of temperature for solutions containing dipalmitoyl phosphatidylcholine (DPPC, a vesicle-forming compound) and various amounts of lysophosphatidylcholine which forms micelles. The phase transition of the pure bilayer is well-defined, but the sharpness of the transition of the transition decreases with increasing amounts of lysophosphatidylcholine, finally reaching the point, at 40% additive, at which there is a question as to

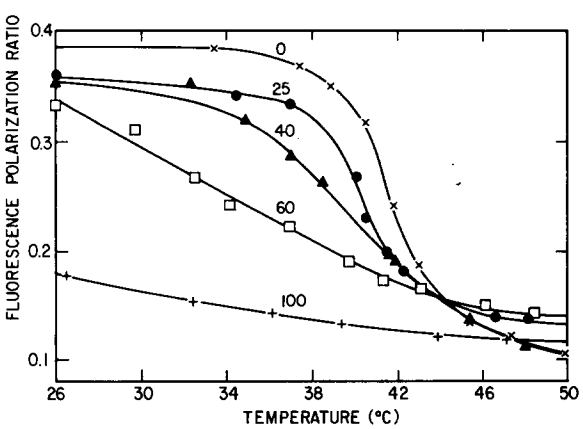

Fig. 4. The effect of incorporated lysophosphatidylcholine on the fluoresence polarization of 1,6-diphenyl-1,3,5-hexatriene in dipalmitoyl phosphatidylcholine vesicles in water as a function of temperature. Concentrations: diphenylhexatriene, 8 μM; total lipid, 2 mM. Lysoplasmologen (mol%): x, 0; •, 25; ▲, 40; □, 60; +, 100.

whether the bilayer character is maintained. These data show a gradual incorporation of the lysophosphatidylcholine into the structure of the bilayer, leading to increased fluidity. As shown by Fig. 5, addition of lysophosphatidylcholine increase the rate of transport of ions across the bilayer.

The several physical techniques discussed above all point to the same type of structural changes associated with the interaction of lysophosphatidylcholine with lecithin vesicles. Increasing the lysophosphatidylcholine content of the bilayer leads to:

(a) A decrease in the rigidity of the bilayer as indicated by the fluorescence polarization.

(b) Increasing ease of penetration of CH_3NO_2 and H_2O into the structure as illustrated by the increased rate of quenching of pyrene fluorescence by CH_3NO_2, and the effect of H_2O on the pyrene and pyrene carboxaldehyde environments.

(c) Above approx. 40% lyso-compound a sharp change in all properties occurs indicating that the bilayer structure breaks up and that micelllar-type structures may be formed.

(d) Longer term measurements utilizing Eu^{3+} show complete penetration of the bilayer by Eu^{3+} into the aqueous core, in agreement with the above picture.

Effect of temperature

Fig. 6 shows the effect of temperature on the pyrene fluorescence ratio I_3/I_1 in phosphatidylcholine/lysophosphatidylcholine mixtures. In the pure bilayer, I_3/I_1 rises slowly with increasing temperature but shows a sharp and pronounced increase at the phase transition at 40°C. This shows that the pyrene environment becomes dramatically more hydrophobic at the phase transition. This is probably due to the increased fluidity of the bilayer at this temperature which allows greater access of the pyrene to the bilayer lipid interior. The increased fluidity at the phase transition is well established and also illustrated in our data with diphenylhexatriene (Fig. 4). The greater access

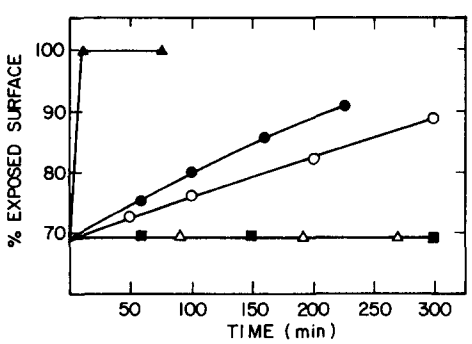

Fig. 5. Rate of Eu^{3+} permeation into egg phosphatidylcholine/lysophosphatidylcholine vesicles. The indicated level of lysophosphatidylcholine was co-sonicated with 44 mM egg phosphatidylcholine. $EuCl_3$ (0.4 mM) was added to the mixed system and the spectra were obtained at various times after addition. The ordinate was calculated by integration of the upfield and downfield peak (^{31}P) areas. △, 44 mM egg phosphatidylcholine; ■, 44 mM egg phosphatidylcholine/8.2 mM lysophosphatidylcholine; ○, 44 mM egg phosphatidylcholine/12.2 mM lysophosphatidylcholine; ●, 44 mM egg phosphatidylcholine/16.3 mM lysophosphatidylcholine; ▲, 44 mM egg phosphatidylcholine/20.4 mM lysophosphatidylcholine.

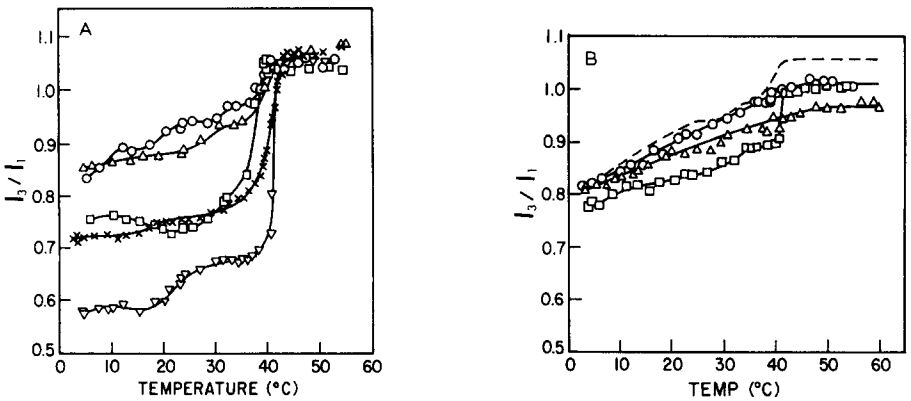

Fig. 6. Effect of temperature on the pyrene fluorescence fine structure ratio, I_3/I_1, in phosphatidylcholine/lysophosphatidylcholine mixture. The mol% of lysophosphatidylcholine in the mixture was varied as follows: (A) (○), 0; (△), 10; (□), 20 (X), 30; (▽), 40. (B) As in A, the mol% of lysophosphatidylcholine was (□), 60; (△), 80; and (○), 100. The dashed line refers to 0% lysophosphatidylcholine (as in A), and is shown in B for reference.

of the probe to the membrane interior has been suggested previously by Galler and Sackman [24] and will be discussed in conjunction with the pyrene excimer data (Fig. 7). The data of Figs. 4 and 6 are in agreement in that bilayer integrity, as exemplified by a precise phase transition at 40°C, is maintained at least up to 40% kysophosphatidylcholine.

The pyrene excimer, p_2^*, formed via interaction of p* and p has been used previously to indicate phase changes in membranes [22]. Fig. 7 shows typical data where, in the pure bilayer the ratio of excited dimer to monomer (D/M) increases with temperature up to the phase transition of 40°C. This is due to

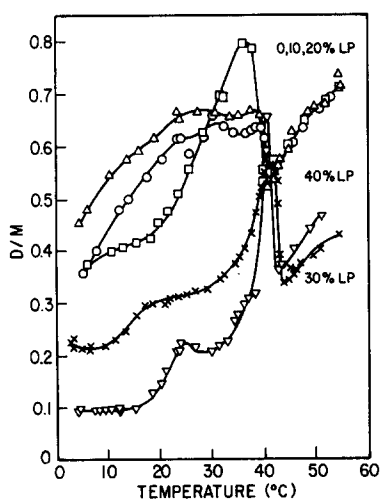

Fig. 7. Effect of temperature on the pyrene dimer:pyrene monomer ratio (D/M) in phosphatidylcholine/lysophosphatidylcholine mixtures. The total lipid concentration is 2 mM, and the total pyrene concentration is 0.05 mM. The mol% of lysophosphatidylcholine was varied in each curve in the following manner: (○), 0; (△), 10; (□), 20; (X), 30; (▽), 40.

increased fluidity of the system with temperature and is also observed in its fluorescence polarization data (Fig. 4). Increased media fluidity leads to an increase in the mobility of p^* and p leading to increased formation of p_2^*. At the phase transition, D/M drops sharply. If as indicated earlier the access of p and p^* to inner regions of the membrane increases at τ_0 then the concentrations of these entities decrease abruptly at this temperature and the extent of the reaction, $p^* + p \rightarrow p_2^*$, decreases leading to a decrease in D/M. At higher temperatures D/M increases again as the medium becomes less rigid.

The rate of increase of the ratio, D/M, up to 40°C appears to be much sharper at higher mole percent of lysophosphatidylcholine. This may be due to expulsion of H_2O from the bilayer/lysophosphatidylcholine assembly with increasing temperature. The rapid rise in I_3/I_1 ratio under identical experimental conditions support this concept. The pyrene monomer fluorescence lifetime also increases as the H_2O content of the assembly decreases. The extent of the reaction, $p^* + p \rightarrow p_2^*$, then increases and D/M increases. No sharp change in fluidity occurs under these experimental conditions as monitored by the diphenylhexatriene fluorescence polarization experiments (Fig. 6).

Conclusions

Addition of lysophosphatidylcholine to bilayer causes the following.

(a) Increased fluidity of membrane (pyrene dimer/monomer ratio, D/M, fluorescence depolarization of diphenylhexatriene).

(b) Increased penetration of H_2O into membrane (pyrene fluorescence ratio I_3/I_1, pyrene D/M).

(c) Increased penetration of polar species, Eu^{3+}, H_2O and CH_3NO_2 into and through the assembly.

(d) Lysis of bilayer at greater than 40% lysophosphatidylcholine to give mixed micelles.

(e) Up to 40% lysophosphatidylcholine physical properties characteristic of bilayer are observed.

Changes in the photophysical properties of the excited pyrene e.g., pyrene/ I_3/I_1, τ_0, quenching rate, etc., have been explained in terms of changes in the lipid structures that lead to greater penetration of polar molecules via H_2O, Eu^{3+} and CH_3NO_2 into and through the bilayer. It is worthwhile commenting in more detail on possible changes in the physical surroundings of pyrene. It is known that many aromatic molecules form weak complexes with quaternary ammonium compounds [25], and it has been shown that pyrene forms complexes with tetraethylammonium chloride in *n*-pentanol solution, I_3/I_1 changing from 1.07 to 0.61 for concentrations of the ammonium compound from 0 to 1.4 M. Formation of pyrene excimers also decreases under these conditions. It is tempting to suggest that similar complexes exist in the pyrene-phosphatidylcholine systems. For example in Fig. 1, the decrease in τ_0 of excited pyrene is then due to complex formation with monomeric lysophosphatidylcholine. However, the formation of a single component micelle or bilayer structure hinders the formation of the complex, and much higher τ_0 and I_3/I_1 values are observed.

Addition of lysophosphatidylcholine to phosphatidylcholine causes a disrup-

tion of the bilayer structure as witnessed by the increased permeability of the structure to polar molecules. The fluorescence polarization of diphenylhexatriene also decreases, indicating that the structure is less ordered and rigid. In disordered structure, pyrene has a more ready access to the head group structure of the phosphatidylcholine forming a complex which decreases I_3/I_1.

The formation of a complex between pyrene and the partially charged nitrogen of the choline group leads to a diminution in the ease of formation of the excimer p_2^* (Fig. 7). Increasing the temperature tends to dissociate the complex and p_2^* and I_3/I_1 increase. However, at approx. 40% the increases are very abrupt at the phase temperature of approx. 40°C. At this temperature the phosphatidylcholine structure suddenly becomes less rigid leading to greater access of pyrene to the lipid interior. I_3/I_1 increases as the pyrene-phosphatidylcholine complex breaks up and p_2^* increases as p^* is now free to move in the structure and form the excimer.

The data show quite clearly that addition of lysophosphatidylcholine to bilayers tends to increase movement of polar molecules across these assemblies due to a break-up of the head group structure. Certain molecules, in particular aromatic (carcinogen-type) molecules, show additional interesting features whereby they have increased complexation by the bilayer head group. These data have a possible bearing on the penetration and solubilization of carcinogenic aromatic compounds in these structures.

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