LATERAL DIFFUSION RATES OF PHOSPHATIDYLCHOLINE IN VESICLE MEMBRANES: EFFECTS OF CHOLESTEROL AND HYDROCARBON PHASE TRANSITIONS

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1 . Introduction

Lateral diffusion rates of lipids in biological membranes are important parameters relating to possible heterogeneous distributions of lipids and proteins in the plane of the membrane, and the local 'microviscosity' experienced by proteins embedded in the lipid matrix. Two factors which may influence these diffusion rates are the gel–liquid crystalline nature of the membrane lipids and the presence of cholesterol. Thus in certain bacterial cells it is found that a fraction of the lipid may be in the ordered gel phase at growth temperatures [1], whereas in many mammalian cells high concentrations of cholesterol are obtained [2]. An understanding of the effects of lipid phase transitions and cholesterol content on the lateral mobility of phosphalipids is therefore of fundamental importance to the understanding of lipid mediated membrane processes.

Direct measures of the lateral diffusion rates of membrane components have, however, proved difficult to obtain, and the most reliable measurements have been obtained employing extrinsic probes introduced into the membrane. In particular, rapid lateral diffusion rates ($D_t \approx 10^{-7}$–10^{-8} cm^2/s) have been inferred for liquid crystalline phospholipids in model [3,4], and biological [5] membranes by monitoring lateral diffusion rates of electron spin resonance (ESR) 'spin label' probes introduced into such systems. The primary objections to such probe techniques include the fact that the lateral diffusion rates of the spin labelled impurities are being measured, which may not necessarily reflect the diffusion rates of intrinsic membrane lipids, and that the bulky spin label may perturb the native membrane system [6]. Such difficulties are of course avoided for intrinsic membrane probes. It is shown in this work that the lateral diffusion rates of phospholipids in sonicated 'vesicle' systems may be obtained by employing the intrinsic phosphate phosphorus as a ^{31}P-NMR probe of phospholipid motion. This technique is then employed to examine the effects of temperature, cholesterol content and hydrocarbon phase transitions on the lateral diffusion rates of egg-yolk lecithin and dipalmitoyl lecithin.

1.1. *Theoretical considerations*

The small (~ 250 Å diameter), spherical vesicles obtained by sonication of aqueous dispersions of phospholipid (liposomes) consist of a single bilayer separating inner and outer aqueous phases [7]. They are attractive systems for the application of ^{31}P-NMR techniques as the rapid re-orientations of the phospholipid molecules in such systems give rise to high resolution spectra [8–11]. This isotropic motion arises from two sources, namely rapid rotation (tumbling) of the entire vesicle, or lateral diffusion of the lipids around the inner and outer monolayers of the vesicle [12]. In the case of ^{31}P-NMR these mechanisms both modulate the two line broadening interactions experienced by the phosphate phosphorus, which are the chemical shift anisotropy [9] and the dipolar interactions with the two nearest neighbour methylene groups. These interactions scale according to $P_2(\cos(\theta))$ where P_2 is the second Legendre polynomial and θ is some polar angle appropriate to each interaction. Thus the rotational correlation time τ_c is obtained as a

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solution to the rotational diffusion equation [13] as

$$\frac{1}{\tau_c} = \frac{6 D_{diff}}{a^2} \quad (1)$$

where a is the radius of the vesicle. As indicated above, the diffusion rate D_{diff} arises from two sources. The rotational diffusion arising from the tumbling of the entire vesicle is given by the Stokes-Einstein relation as $D_t = kT/8\pi a\eta$ (where η is the viscosity of the aqueous medium). When the lateral diffusion rate D_t is also included, eq. (1) becomes

$$\frac{1}{\tau_c} = \frac{6}{a^2}\left[\frac{kT}{8\pi a\eta} + D_t\right] \quad (2)$$

In the fast correlation time limit such as obtained in vesicles the linewidth varies as $1/T_2 = M_2\tau_c + C$, where M_2 is the appropriate residual second moment obtained after the averaging due to the restricted anisotropic motion of the lipid in the plane of the membrane is taken into account, and C is a portion of the linewidth which is τ_c independent [9]. Thus eq. (2) may be employed to give

$$\frac{1}{T_2} = \frac{M_2 a^2}{6\left[\frac{kT}{8\pi\eta a} + D_t\right]} + C \quad (3)$$

In favourable circumstances, eq. (3) provides a method of obtaining the lateral diffusion rate D_t. If the viscosity η of the aqueous medium is increased, the linewidth will increase until the lateral diffusion is providing the dominant averaging mechanism, and the linewidth will then approach some limiting value. In this work the viscosity is increased by adding appropriate quantities of glycerol to the aqueous medium. It should be noted that glycerol is appropriate to this technique, as phospholipid membranes are very permeable to glycerol [14] and transition behaviour is not significantly affected.

2. Materials and methods

Vesicles were prepared from 50 mg dry phospholipid, or, in the case of cholesterol containing samples, from the mixed lipids dried down under nitrogen from a chloroform solution. The phospholipid was dispersed in ^2H_2O in the presence of 100 mM Tris–HCl (p^2H = 7.2) and 10 mM EDTA. This mixture was then sonicated to clearness (2–3 min) using a Bronson sonicating system (power setting 2) where the tip of the sonicator was immersed in the sample, which in turn was in a vial immersed in a water bath. This water bath was held at 0°C for the egg-yolk lecithin, and at 45°C for the dipalmitoyl lecithin samples. The lipids used were a kind gift of Dr B. de Kruijff.

The desired aqueous viscosity was obtained by adding appropriate quantities of glycerol to small aliquots of the sample. The viscosity of the glycerol–^2H_2O medium was obtained from standard tables and in some cases was checked using an Ostwald viscometer.

A 129 MHz ^{31}P-NMR Fourier transform spectrometer constructed in this laboratory [15] was employed in this investigation. Spectra were obtained (in the absence of proton decoupling) from up to 1000 scans, employing a 60° pulse and an interpulse time of 15 s.

3. Results and discussion

Close agreement is found between the viscosity dependence of the ^{31}P-NMR vesicle linewidth predicted by eq. (3) and that obtained experimentally. This agreement is illustrated in fig.1 for egg-yolk lecithin vesicles at 50°C. The solid line describes the best fit of eq. (3) to the data, using a least squares technique and employing the known values of a [8] to give $D_t = 2.6 \times 10^{-8}$ cm^2/s. It should be noted that

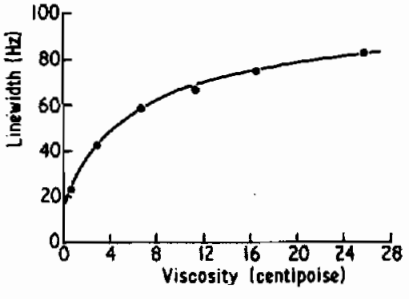

Fig.1. 129 MHz. ^{31}P-NMR linewidths observed for sonicated egg yolk lecithin vesicles at 50°C as a function of the viscosity of the aqueous medium. The solid line illustrates the best fit obtained using eq. 3, where $D_t = 2.6 \times 10^{-8}$ cm^{-2}/s.

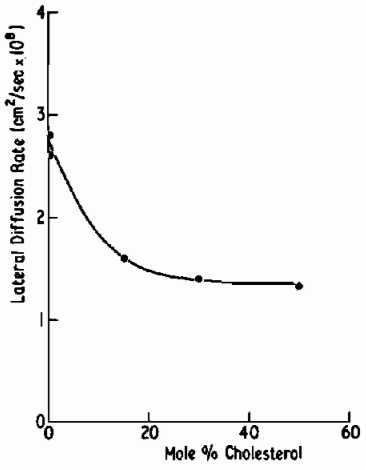

Fig.2. Lateral diffusion rates of egg-yolk lecithin in the presence of various concentrations of cholesterol at 50°C.

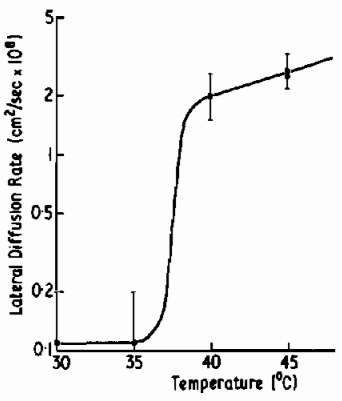

Fig.3. Lateral diffusion rates of dipalmitoyl lecithin as a function of temperature.

the fit is particularly sensitive to D_t, as adjustment of D_t directly affects the characteristic shape of the curve. Thus in the case of fig.1 adjustment of D_t by as little as 10% caused a 10-fold increase in the r.m.s. error.

These measurements were performed over the temperature interval 10–60°C. An Arrhenius plot of the lateral diffusion rates obtained produces a straight line and an activation energy for diffusion of 11.2 kcal/mol.

The effects of cholesterol on the lateral diffusion rates of egg-yolk lecithin (at 50°C) in cosonicated egg-yolk lecithin–cholesterol vesicles are illustrated in fig.2. The vesicle sizes were obtained previously [16]. It may be noted that the lateral diffusion rate of egg-yolk lecithin decreases by approximately a factor of two on incorporating 30 mol% cholesterol, whereas increasing the cholesterol concentration further to 50 mol% does not influence the diffusion rate appreciably. It should be noted, however, that the cholesterol in these large vesicles consisting of 50 mol% cholesterol is asymmetrically distributed across the vesicle bilayer with a greater concentration of cholesterol in the inside monolayer [16]. Thus the value of D_t for 50 mol% cholesterol may be some average of two diffusion rates for egg-yolk lecithin on the inside and outside of the vesicle bilayer. However, the fact that the values of D_t obtained for 50 mol% cholesterol are very similar to those observed in the presence of 30 mol% cholesterol (where the lipids are not asymmetrically distributed) strongly suggests that D_t is not affected on increasing the cholesterol content above 30 mol%.

The activation energy for diffusion for egg-yolk lecithin in the presence of 15 mol% cholesterol was obtained as 10.5 kcal/mol, which is very similar to that observed in the absence of cholesterol. The reduction of D_t on adding cholesterol may be attributed to a reduction of the entropy of the system [17], corresponding to a more ordered hydrocarbon phase in the presence of cholesterol.

The lateral diffusion rates of dipalmitoyl lecithin over the temperature interval 30–50°C are illustrated in fig.3. It may be observed that D_t increases by at least an order of magnitude as the temperature is increased from 35–40°C. Thus the onset of rapid lateral diffusion occurs somewhat below the main hydrocarbon phase transition T_c, which occurs at 42°C in similarly prepared sonicated vesicle systems [8]. In this regard recent studies [18–20] have shown that there is an onset of rapid axial rotation of the phospholipids at a temperature approximately 5°C below T_c, which has been associated [20] with the 'pre-transition' observed [21] in calorimetric studies. It would therefore appear, as would be expected, that lateral diffusion and axial rotation are associated phenomena. More importantly, however, these results imply that the onset of rapid lateral diffusion may occur well below the main gel–liquid crystalline hydrocarbon phase transition temperature.

The above analysis assumes homogeneous vesicle

sizes. If there were a large size distribution it would be expected that the larger vesicles would contribute appreciably broader ^{31}P-NMR signals than the smaller vesicles, leading to a hybrid (non-Lorentzian) lineshape with broad 'wings' and a narrow central component. Such effects would be particularly marked at higher viscosities and would result in a progressive loss of intensity from the narrow component as the viscosity is increased. Control experiments (with egg-yolk lecithin vesicles) did not reveal such effects, however, as the lineshape remained closely Lorentzian and the intensity constant (within 5%) for glycerol concentrations in the range 0–80% by wt, thus justifying the assumption of a homogeneous vesicle population.

Criticisms which may be directed at the technique employed include the fact that in order to obtain the highest viscosities the aqueous medium contained up to 85% glycerol by weight, which may perturb the vesicle system and the dynamic behaviour of the phospholipids. Control experiments with broadening reagents revealed, however, that the vesicles still maintained a permeability barrier to cations with 85% glycerol present, and outside/inside ratios very similar to those obtained [8] in the absence of glycerol were obtained. Similarly, the ^{31}P-NMR spectra obtained from unsonicated dispersions of the phospholipid in the absence and presence of up to 85% glycerol were identical, showing that the motion in the phosphate region of the polar headgroup at least is not affected by the presence of glycerol. A further criticism is that the glycerol may not necessarily be affecting the viscosity experienced by the vesicle, in that the layer of bound water associated with the phospholipid polar headgroup may determine the local viscosity for the vesicle. If this were the case, however, similar increases of linewidth for a given increase in glycerol concentration would be expected for vesicles composed of phospholipids with different lateral diffusion rates. Such behaviour is not observed. At 30°C, for example, the τ_c dependent part of the ^{31}P-NMR linewidth of egg-yolk lecithin vesicles increases by a factor of 3.5 when the glycerol concentration is increased from 0–60% by wt. This may be compared with the τ_c dependent part of the linewidths of dipalmitoyl lecithin vesicles (which are of a similar size as egg-yolk lecithin vesicles) which increases by a factor of 9 for a similar increase in glycerol concentration, at 30°C.

A final criticism may be made on the basis of the theoretical considerations of Saffman and Delbrück [22] who point out that the lateral diffusion rate of membrane components may depend on the viscosity of the external aqueous medium. Such a possibility has been ignored in the above treatment. Although there are several conceptual difficulties with the model employed by Saffman and Delbrück [22] as applied to phospholipids (notably the assignment of a cylindrical shape with uniform cross section to the lipid molecule, and the assumption of a uniform viscosity throughout the hydrocarbon region) it is instructive to consider the effects of such a viscosity dependence on the results obtained here. The appropriate form of the diffusion rate is given by [22]

$$D_t = \frac{kT}{4\pi\eta' h}\left[\log\frac{\eta' h}{\eta r} - \gamma\right] \qquad (4)$$

where r and h are the radius and length of the lipid cylinder, and η' is the internal viscosity of the membrane. It may be shown that the inclusion of such a viscosity dependence for reasonable values of h and r (i.e. h = 20 Å and r = 4.7 Å for egg-yolk lecithin [23] still allows excellent fits of linewidth versus viscosity data such as is presented in fig.1. In this case the viscosity η' of the membrane is varied to obtain the best fit. The lateral diffusion rates at particular external aqueous viscosities may then be calculated from eq. (4). It may be noted that at η = 0.01 P lateral diffusion rates are obtained which are approximately 30% smaller than the values obtained assuming D_t to be viscosity independent. The activation energies are not significantly affected.

It may also be suggested that the high curvature of the vesicle bilayer itself may perturb the motional properties of the constituent phospholipids. However, differential scanning calorimetry results [8] strongly suggest that the packing properties in the hydrocarbon chain region of the constituent phospholipids are not markedly different from that obtained in less curved unsonicated (liposome) systems. Similarly, the motion in the phosphate region of the polar headgroup is not appreciably different in the small vesicles as compared to the less perturbed liposome systems [9]. Thus it may be expected that the motional properties of phospholipids are similar in vesicles and liposomes, and, by extrapolation, similar to the motional properties of phospholipids in biological membranes which

are not associated with membrane proteins.

It should be noted that the technique employed is not necessarily restricted to ^{31}P-NMR techniques or lateral diffusion rates of phospholipids. For example, the lateral diffusion rates of specifically deuterated lipids or proteins embedded in the vesicle bilayer should be available, using ^{2}H-NMR and employing exactly similar techniques to those described here for increasing the viscosity of the aqueous medium.

In summary, a technique for obtaining lateral diffusion rates of phospholipids in vesicle bilayers has been demonstrated. Reasonable agreement is obtained between diffusion rates measured by this technique and those obtained by alternative methods. It is shown that cholesterol strongly decreases the lateral diffusion rates of egg-yolk lecithin in concentrations up to 30 mol%. Results obtained at higher cholesterol concentrations strongly suggest that further increase in cholesterol content does not materially affect the phospholipid lateral diffusion rates. Finally the lateral diffusion rates of dipalmitoyl lecithin are at least an order of magnitude slower in the gel phase than in the liquid crystalline phase.

These results have obvious implications for biological membranes. The reduction of D_t on incorporating cholesterol indicates a corresponding increase in the viscosity experienced by the phospholipid. Thus the local microviscosity experienced by a membrane bound protein would be expected to increase in the presence of cholesterol, possibly affecting the protein function – particularly those functions which may occur as a result of protein–protein collisions. The dramatic effects of the phase transition on the lateral diffusion rate for dipalmitoyl lecithin indicate that gel phase phospholipids constitute a very viscous environment in comparison to the liquid crystalline phase. If the Saffman-Delbrück theory [22] is employed, the dipalmitoyl lecithin results indicate a viscosity of less than 2 P above the phase transition, as opposed to a viscosity of at least 30 P below the phase transition. Such a change in viscosity would be expected to strongly influence the properties of membrane bound proteins. In particular, a gel-state environment may be expected to inhibit those properties which rely on the ability of the membrane proteins to diffuse or rotate rapidly in the membrane, or to undergo large, rapid conformational changes. Such effects may account for the inactivation of the Ca^{2+}–Mg^{2+}. ATPase from sarcoplasmic reticulum in the presence of gel-state phosphatidylcholines [24]. Alternatively, very slow lateral diffusion rates in the gel phase provide a relatively static environment with attendant possibilities for long range order. Such an environment would appear to be necessary for the membrane proteins of the purple membrane fragment of *Halobacterium halobium*, which have a rigid gel-state lipid environment [25].

Studies of lateral diffusion rates of other well defined phosphatidylcholines and different phospholipids are in progress and will be published elsewhere.

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