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Effect of Cholesterol in Membranes. Pulsed Nuclear Magnetic Resonance Measurements of Lipid Lateral Diffusion†

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ABSTRACT: Lateral diffusion coefficients of lipids in a bilayer can be measured directly in a macroscopically aligned sample by use of a pulsed NMR method with pulsed magnetic field gradients [Lindblom, G., & Wennerström, H. (1977) *Biophys. Chem. 6*, 167]. This technique has been utilized to investigate the influence of cholesterol on the lipid diffusion of egg yolk lecithin, palmitoyloleoyllecithin, and dioleoyllecithin. It is found that cholesterol has a very small effect on the phospholipid diffusion. On the other hand, cholesterol has a great influence on the molecular ordering in the bilayer and on the lipid phase structure. It is therefore suggested that cholesterol exerts its dominant effect on the lipid membrane stability.

The effects of cholesterol on the physicochemical properties of lipid bilayers have been studied for many years by various physical methods, including, in particular, different forms of spectroscopy. Investigations of this kind should increase our understanding of the role that membrane-bound cholesterol might play in such important biological phenomena as endo- and exocytosis, membrane fusion, permeability regulation, etc. A possible function for cholesterol in these processes is indeed indicated by many studies. Exactly how cholesterol influences the properties of membranes at a molecular level is, however, still a matter under debate. The purpose of the present paper is to show the fundamental importance of making a distinction between static and dynamic parameters when discussing the interactions of cholesterol with phospholipid molecules in bilayers. We also want to draw attention to a hitherto somewhat neglected aspect on cholesterol function in membranes, viz., its effects on the stability of the lamellar phase structure itself and the relevance of such effects for the possible occurrence of phase transitions in biological membranes.

Material and Methods

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOL) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POL) were synthesized according to the methods described by Gupta et al. (1977). The egg yolk lecithin (EYL) was prepared from freshly extracted egg yolk lipids by column chromatography on alumina. The lipids were then purified by silic acid column chromatography until the purity was ≥99% as judged by thin-layer chromatography. The macroscopic alignment of the lamellar samples was performed as described in previous papers (deVries & Berendsen, 1969; Lindblom, 1972). The diffusion coefficients were measured at about 61 MHz on a Bruker 322s spin–echo NMR spectrometer by using the pulsed magnetic field gradient technique developed by Stejskal & Tanner (1965). The spectrometer was supplemented with a home-built pulsed magnetic field gradient unit, permitting digital settings for all parameters used, i.e., magnetic field gradient width, δ, the distance between gradient pulses, Δ, and the amplitude of the gradients, g. The diffusion experiment was done as follows: a spin–echo was produced by the common two-pulse sequence $\pi/2-\tau-\pi$ and the magnetic field gradient was also applied as two pulses, one before and one after the π-rf pulse. The advantage of this method is that it is possible to use much larger magnetic field gradients than can be used in the static gradient technique and thus smaller diffusion coefficients can be measured. The translational diffusion of the molecules will attenuate the spin–echo amplitude, E, at 2τ accordingly:

$$\ln (E_g/E_0) = -(\gamma\delta g)^2 D[\Delta - (\delta/3)]$$

where E_g/E_0 is the echo attenuation. In a typical experiment, Δ and δ were kept constant at about 30 and 5 ms, respectively, and g was varied between 0 and 3.5 T m^{-1}. The echo amplitude was measured repeatedly several times on an oscilloscope. The diffusion coefficient, D, was calculated from a least-squares determination of the slope in a plot of ln E_g vs.

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Table I: Effect of Cholesterol on the Lateral Diffusion Coefficient, D_L, of Lecithin[a]

mol % cholesterol in lipid bilayer	$10^{12}D_L$ (m^2 s^{-1})		
	EYL	POL	DOL
0	5 ± 2	6 ± 2	5 ± 1
2.5	5 ± 2		5 ± 1
5.0	6 ± 2		5 ± 1
10.0	6 ± 2		6 ± 2
15.0	6 ± 2		7 ± 2
20.0	6 ± 2	6 ± 2	7 ± 2
25.0	6 ± 2	7 ± 2	6 ± 2
33.0	7 ± 2	7 ± 2	8 ± 2

[a] Temperature 35 °C. The ^2H_2O concentration was 20% w/w.

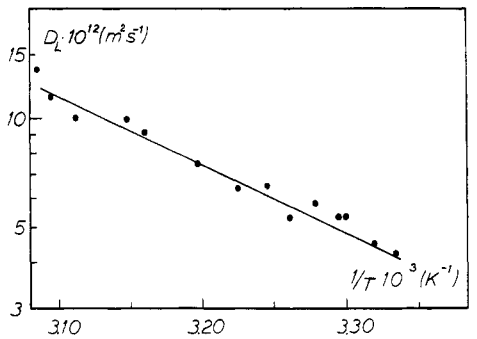

FIGURE 1: Temperature dependence of the lateral diffusion coefficient of dioleoyllecithin, DOL, in a lipid bilayer.

g^2 for both the aligned samples and a glycerol reference. The diffusion coefficient of glycerol was obtained from the work of Tomlinson (1973). Thus the diffusion coefficient of the lipid was calculated. Note that, although the sample is heterogeneous, the protons of the cholesterol molecule will not contribute to the echo amplitude since T_2 is too short [see the study by Kroon et al. (1975)].

Results

The lateral diffusion coefficients obtained for palmitoyloleoyllecithin (POL), dioleoyllecithin (DOL), and egg yolk lecithin (EYL) in bilayers at various cholesterol contents are summarized in Table I. The water content was 20% w/w for all samples studied. The temperature dependence of the lateral diffusion coefficient of DOL is shown in Figure 1, and the apparent energy of activation for the diffusional motion was calculated to be 38 kJ/mol.

Discussion

Molecular Dynamics in Bilayers. We have studied the effect of cholesterol on the translational motion of POL, DOL, and EYL molecules in bilayers. Such investigations have now become feasible with a quite recent method (Lindblom & Wennerström, 1977) with which the lateral diffusion coefficient of lipids in a bilayer can be measured *directly*, i.e., without probe molecules. Recently also Kuo & Wade (1979) determined the lateral diffusion of various lecithins, using the same method. The technique is based on a standard nuclear magnetic resonance (NMR) method for studies of diffusion (Stejskal & Tanner, 1965) in systems where high resolution NMR spectra are observed. Normally such spectra are not obtained for a lamellar liquid crystalline system. However, by a macroscopical alignment of the multilamellar system, narrow spectral peaks can be observed by orienting the sample at the so-called "magic angle" in the magnetic field. With such an oriented bilayer sample, the conventional pulsed NMR technique with pulsed magnetic field gradients has been used to determine the diffusion coefficients. The NMR experiment then directly measures the translational diffusion along the bilayer if the lipid diffusion perpendicular to the lamellae can be neglected. This assumption is strongly supported by previous studies (Kornberg & McConnell, 1971; Lindblom & Wennerström, 1977).

In Table I it can be seen that changes in the concentration of cholesterol have a very small effect on the lipid diffusion of all the lecithins studied. The translational motion of lecithin *increases* slightly upon addition of cholesterol up to about 30 mol %. (Unfortunately we were not able to get good enough alignment of samples containing more than 30% cholesterol so that diffusion could be measured with acceptable accuracy.) These experimental findings disagree with most other measurements reported in the literature, where the translational diffusion of *probe molecules* have been studied (Fahey et al., 1977; Rubenstein et al., 1979; Wu et al., 1977). These investigations showed a slight *decrease* in the probe translational diffusion with increasing cholesterol content. In these investigations a technique of a fluorescence photobleaching recovery was utilized, and it should be noted that often differing results have been obtained with this method at different laboratories (Barisas, 1980). However, in spite of the discrepancies observed it can be concluded that the effect of cholesterol on the translational motion is very small. This has also been found for the lipids in erythrocyte membranes at temperatures between 30 and 40 °C (Thompson & Axelrod, 1980). In a recent study Kuo & Wade (1979) also studied the effect of cholesterol on the lecithin lateral diffusion, but on the saturated dipalmitoyllecithin (DPL), utilizing the same NMR method as we have. These authors found a large increase of almost 1 order of magnitude in the diffusion rate at low cholesterol contents up to about 5 mol %, and thereafter the diffusion coefficient decreased down to its original value at about 50 mol % cholesterol. We have not succeeded in reproducing their data, since we were unable to get a good macroscopical alignment of DPL–cholesterol samples in large enough quantities for diffusion studies. Even without cholesterol we found the DPL samples very difficult to align compared with DOL and EYL samples. This is very unfortunate, and we are currently studying this problem to see whether cholesterol generally affects the lateral diffusion of saturated lipids much more than that of unsaturated ones.

The molecular dynamics of the lipids in a bilayer are usually discussed in terms of fluidity. However, for such systems the fluidity cannot be directly obtained in an experiment, but one has to measure a physical quantity that, in some way, is related to the mobility. Such quantities can be translational and/or rotational diffusion constants. We will therefore briefly discuss how the measured lateral diffusion coefficient is related to the membrane fluidity (a similar discussion can be done also for rotational diffusion). Let us first consider the diffusion of a free particle in a three-dimensional medium. According to the Einstein relation (Landau & Lifshitz, 1959) the diffusion coefficient depends on the mobility, b, as

$$D = kTb$$

where k is Boltzmann's constant and T is the absolute temperature. The mobility (i.e., the reciprocal friction coefficient) of a particle in a three-dimensional fluid can be written [see, e.g., van Holde (1971)]

$$b = \frac{1}{6\pi\eta P(r)}$$

where η is the viscosity of the medium and $P(r)$ is a function of the size and shape of the particles; r is the axial ratio. Since

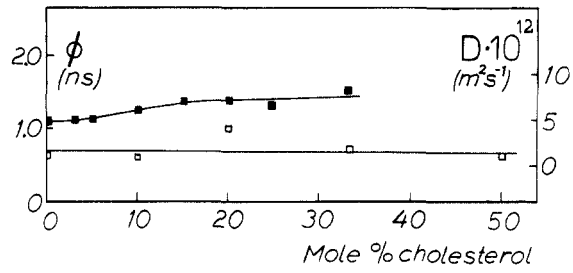

FIGURE 2: Lateral diffusion coefficient, D_L (■), of dioleoyllecithin and rotational relaxation time, ϕ (□), of diphenylhexatriene (DPH) in dipalmitoyllecithin as a function of the cholesterol concentration in bilayers.

$\eta = 1/\phi$, where ϕ is the fluidity [see, e.g., Barrow (1973)], one obtains for the diffusion coefficient

$$D = \frac{kT}{6\pi P(r)}\phi$$

i.e., the diffusion coefficient is directly proportional to the fluidity for a three-dimensional system. Unfortunately, for a two-dimensional fluid like a bilayer such a simple relation between diffusion coefficients and the fluidity does not exist. Recently an approximate expression was derived (Saffman, 1976; Saffman & Delbrück, 1975) for the mobility of a particle restricted to motion in the plane of a thin membrane, and it was found that

$$b \approx \frac{1}{4\pi\eta} \ln \frac{\eta}{\eta_w R}$$

where η_w is the viscosity of the surrounding solution of the membrane and R is the radius of the diffusing particle. However, an estimation of the fluidity from this relation is not very useful. Thus the measured diffusion coefficients themselves should be used when the dynamic behavior of a membrane is described. The rotational diffusion of a molecule in a membrane can be studied by a time-resolved luminescence method [see Johansson & Lindblom (1980)]. Kawato et al. (1978) have measured the rotational relaxation time for 1,3,5-diphenylhexatriene molecules in model membranes by time-resolved fluorescence. This rotational relaxation time plotted in Figure 2 is inversely proportional to an effective rotational diffusion coefficient (Kinosita et al., 1977). It can be inferred from Figure 2 that the effect of cholesterol on the rotational relaxation is also very small. Again it can be concluded that cholesterol has a negligible effect on the dynamic properties of the lipid molecules in a bilayer.

Molecular Ordering. The influence of cholesterol on the molecular ordering in bilayers has been studied with a variety of methods. Especially, several studies have been devoted to the determination of the order parameter of the acyl chains of the lecithin molecules by use of NMR techniques (Seelig, 1977; Oldfield et al., 1971; Mantsch et al., 1977); ESR (Hsia et al., 1970), and polarized light spectroscopy (Hildebrand & Nicolau, 1979; Kinosita et al., 1977; Johansson & Lindblom, 1980). All these studies show that an increase of cholesterol in the lipid bilayer leads to an increase in the order parameter of the acyl chains. The ordering and motion of the choline head group have also been studied as a function of the cholesterol content (Lindblom et al., 1976; Brown & Seelig, 1978; Oldfield et al., 1978; Shepherd & Büldt, 1979). In all these studies a remarkable change in the static parameter measured is found around 20 mol % cholesterol in the bilayer. It is of particular interest to compare our data with those obtained by Oldfield et al. (1978). These authors studied the influence of cholesterol on the deuteron quadrupole splitting of the

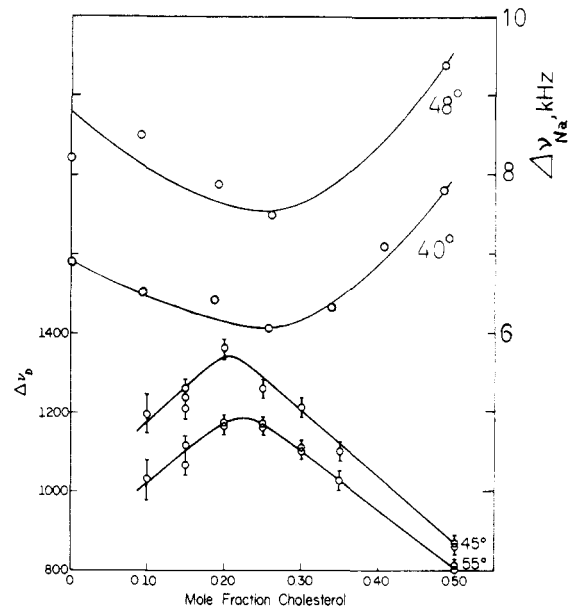

FIGURE 3: Dependence of the deuteron quadrupole splitting, Δ^D, of dimyristoyllecithin (DML) labeled as N–C^2H_3 in the choline head group as a function of cholesterol content in the bilayer [from Oldfield et al. (1978)] and the dependence of the sodium ion quadrupole splitting Δ^{Na} as a function of cholesterol in a DML bilayer. The water layers contained 0.8 M NaCl in ^2H_2O [from Lindblom et al. (1976)]. The various temperatures are indicated.

choline N–C^2H_3 group (see Figure 3). It was found that addition of cholesterol to the membrane results in an increase in the splitting up to a maximum at about 20% cholesterol; then the splitting decreased continuously to 50% cholesterol. We studied the quadrupole splitting of $^{23}Na^+$ ions as a function of cholesterol concentration and found a minimum in the splitting at about 20 mol % cholesterol in a dimyristoyllecithin bilayer (Figure 3). A probable explanation of these data, taken together, is that the steroid causes a rearrangement of the molecular packing in the bilayer (Lindblom et al., 1976). It has been shown that the choline group tends to be oriented parallel to the membrane surface (Büldt et al., 1978; Seelig et al., 1977; Yeagle et al., 1977) and that it has an intermolecular interaction with the phosphate groups (Yeagle et al., 1977). At high cholesterol contents the phospholipid molecules will be further apart, leading to a reduction in the intermolecular choline–phosphate interaction. This in turn will lead to an increasing number of sodium ions binding to the phosphate group and competing with the choline head group. Consequently the sodium quadrupole splitting will increase while the choline deuteron splitting will decrease.

When the dynamic and static behavior of the lipid bilayer is now considered, it can be concluded that although the molecular ordering changes remarkably with increasing cholesterol content, the lateral or rotational diffusion motion are hardly affected at all. Thus, there is no connection between the order parameter (a static quantity) and fluidity (a dynamic parameter). It should be noted that the concept "microviscosity" (Shinitzky & Barenholtz, 1978) as measured by steady-state polarized fluorescence methods usually has no physical meaning and should therefore be avoided.

Lipid Bilayer Stability. To our knowledge, the complete phase equilibria of the system lecithin–cholesterol–water have still not been established. However, the phase behavior of some compositions of phospholipid and cholesterol has been reported (Cullis & deKruijff, 1978). Part of the phase diagram of cholesterol and a mixture of glucolipids from *Acholeplasma laidlawii* membranes has also been investigated (Khan et al.,

1981). It has been found that cholesterol may destablize a lipid bilayer and induce a reversed hexagonal (H_{II}) phase with phosphatidylethanolamine (Cullis & deKruijff, 1978) and that it can form cubic and/or H_{II} liquid crystalline phases with *Acholeplasma* lipids (Khan et al., 1981). Recently, it was shown (Wieslander et al., 1980) that a destabilizing effect of cholesterol also pertains to the membrane of the *Acholeplasma laidlawii*. These findings were explained according to a theory developed by Israelachvili et al. (1976) where the phase structure formed by the membrane lipids strongly depends on the molecular shape. Using this theory it was also possible to qualitatively predict the response of the lipid regulation in the membrane upon addition of cholesterol. It can be expected that cholesterol also in mamalian membranes may have a similar destabilizing effect, inducing cubic or hexagonal phases.

Conclusions

Cholesterol has an ordering influence on the lipids in the bilayer but has almost no effect on the dynamics in the membrane. The greatest importance of cholesterol may instead be on the packing properties and stability of the lipid bilayer.

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