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Chemistry

Lateral diffusion in binary mixtures of cholesterol and phosphatidylcholines

(lipid bilayer/photobleaching/phase equilibria/model membrane)

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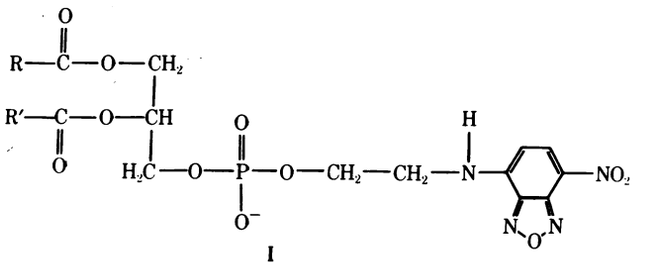
ABSTRACT **The lateral diffusion of a fluorescent-labeled phospholipid, phosphatidyl-*N*-(4-nitrobenzo-2-oxa-1,3,-diazole)ethanolamine, has been measured in binary mixtures of cholesterol and dimyristoyl phosphatidylcholine at temperatures both above and below 23.8°C, the chain-melting transition temperature of this phosphatidylcholine. There is a temperature–composition region, approximately temperature less than 23°C and mole fraction of cholesterol (X) less than 0.20, in which the lateral diffusion coefficient of the fluorescent probe is at least an order of magnitude smaller than it is at points outside of this temperature–composition region. At temperatures above ≈23°C there is a significant increasing cholesterol concentration, for $X > 0.2$.**

The physical properties of bilayer membranes containing cholesterol and phosphatidylcholines have been the subject of extensive investigations employing a large number of experimental techniques (1–16). From these studies certain generalizations emerge. The addition of cholesterol to phosphatidylcholine bilayers in the fluid state (above the chain-melting temperatures of the phosphatidylcholines—i.e., above 23.8°C for dimyristoyl phosphatidylcholine), leads to a decrease in the "fluidity" of the bilayer membrane. On the other hand, inclusion of cholesterol in phosphatidylcholine membranes at temperatures below the chain-melting transition temperatures leads to "fluidization" of these membranes (1–16). These generalizations have been based in part on magnetic resonance studies (spin-label paramagnetic resonance and nuclear magnetic resonance) in which the spectra are sensitive to the rotational motions of the molecules. Another interesting property of binary mixtures of dimyristoyl phosphatidylcholine or dipalmitoyl phosphatidylcholine with cholesterol is that a phosphatidylcholinelike phase transition (at ≈23°C or ≈42°C) can be detected in the presence of mole fraction $X < 0.20$ cholesterol in the binary mixture (1, 3, 6, 7, 9, 10, 12, 16). The chain-melting transition temperature of dimyristoyl phosphatidylcholine is 23.8°C, and that of dipalmitoyl phosphatidylcholine is 41.4°C. Although reliable phase diagrams have been established for a number of binary mixtures of phospholipids (17–20), it has been difficult to use the published phase diagrams for binary mixtures of cholesterol and phosphatidylcholines (3, 4, 7) to account for all of the observed physical properties of these mixtures. Some of this difficulty may originate in sample heterogeneity (4).

In the present paper we give the results of determinations of the lateral diffusion coefficient of the fluorescent probe phosphatidyl-*N*-(4-nitrobenzo-2-oxa-1,3-diazole)ethanolamine (structure I; derived from egg phosphatidylcholine) in binary



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mixtures of cholesterol and dimyristoyl phosphatidylcholine as a function of temperature and composition. Some diffusion coefficients for this probe in binary mixtures of dipalmitoyl phosphatidylcholine and cholesterol are also given. As will be seen, such data shed considerable light on the nature of the phases present in these mixtures. Additional motivation for the present work is provided by current studies of the interactions of hapten- and antigen-sensitized bilayer membranes with various cellular and humoral components of the immune system (21–28). There is already substantial evidence that immunochemical reactions at the surfaces of model membranes depend on the physical state of the membrane, and possibly on lateral diffusion rates (21–28).

MATERIALS AND METHODS

Lipids. Dimyristoyl and dipalmitoyl phosphatidylcholines were purchased from Sigma and found to be pure by thin-layer chromatography on silica gel developed with chloroform/methanol/acetic acid/water (25:15:4:2, vol/vol). Cholesterol was purified by recrystallization from ethanol, was found to be >99% pure by elemental analysis and mass spectroscopy, and was stored under argon. Epoxide oxidation products in the cholesterol were periodically screened for by using 1% tetramethylphenylenediamine in methanol/water (50:50, vol/vol) with 1% glacial acetic acid as a diagnostic stain.

Fluorescent probe I was purchased from Avanti Biochemicals (Birmingham, AL) and analyzed for purity by thin-layer chromatography, as above. Phospholipid concentrations were determined by a spectrophotometric phosphate assay (29). We estimate a maximum error in the multibilayer cholesterol concentration of ±1 mol %.

Oriented Lipid Multilayers. Mixtures of dimyristoyl or dipalmitoyl phosphatidylcholine, cholesterol, and fluorescent probe I (0.5 mol % of total lipid) were evaporated under re-

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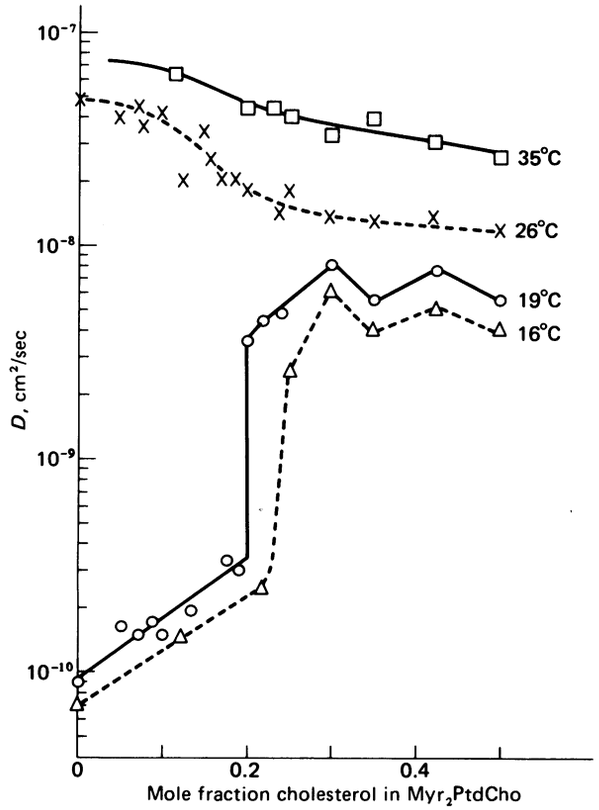

FIG. 1. Diffusion constants for the fluorescent lipid probe I (see text) in binary mixtures of cholesterol and dimyristoyl phosphatidylcholine ($Myr_2PtdCho$), at temperatures above and below 23.8°C, the chain-melting transition temperature of this phosphatidylcholine. There is approximately a 5% error associated with each plotted data point. The sample temperature was measured to an accuracy of ±0.5°C with a thermistor.

duced pressure from ethanol solutions in round-bottomed flasks. One micromole of total lipid was dissolved in 75 μl of chloroform and applied as 5-μl drops over a 0.15-cm^2 region on a cleaned glass microscope slide at 45°C. The chloroform was evaporated for 30 sec after the last drop of lipid solution was added, and the slide was placed in a glass chamber that contained a reservoir of distilled water. The chamber was flushed with argon gas, to decrease the oxidation of cholesterol, and the sealed container was maintained at 40°C (60°C for dipalmitoyl phosphatidylcholine). After 24 hr, the sample was removed and a glass coverslip was rapidly placed over the lipid. The lipid was further oriented and spread out at 45°C by gently pressing on the coverslip with a smooth metal block and sliding it a few

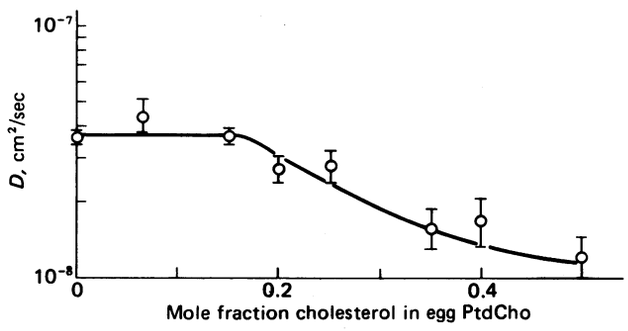

FIG. 2. Lateral diffusion of the fluorescent probe I in mixtures of egg phosphatidylcholine (PtdCho) and cholesterol, at 15°C. The egg phosphatidylcholine was prepared by A. H. Ross (31).

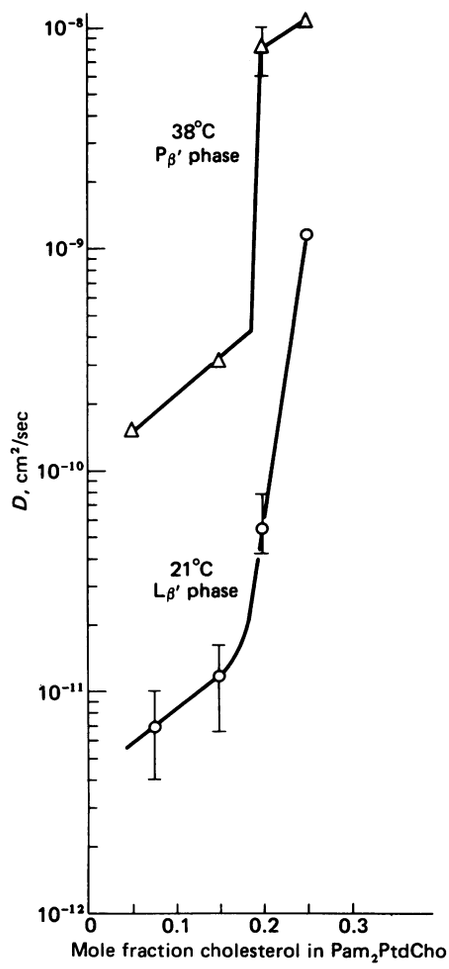

FIG. 3. Lateral diffusion coefficient for the fluorescent lipid probe I in binary mixtures of cholesterol and dipalmitoyl phosphatidylcholine ($Pam_2PtdCho$) at temperatures, 38°C and 21°C, corresponding to the $P_{\beta'}$ and $L_{\beta'}$ phases of the pure phosphatidylcholine.

millimeters back and forth along the long axis of the glass slide. This process was done as rapidly as possible to minimize water evaporation. The sample was replaced in the argon-filled chamber, and maintained for another 24 hr at 40°C (60°C for dipalmitoyl phosphatidylcholine). The sample was inspected by using Nomarski optics to determine whether it had domains of oriented (optically uniaxial) multibilayers large enough to perform a photobleaching experiment. For diffusion coefficients in the range 10^{-12} to 10^{-9} cm^2/sec, domains 90 μm in diameter were employed. For diffusion coefficients in the range 10^{-9} to 5×10^{-8} cm^2/sec, domains 400 to 700 μm in diameter were employed. All domains were scrupulously examined for uniformity. Many types of defect structures found in these samples can seriously affect the measurement of diffusion.

Diffusion Measurements. Diffusion was measured by using the photobleaching technique of Smith and McConnell (30). Briefly, the procedure involves projecting a laser beam, which has been broken up into a stripe pattern by a metal grid, onto the oriented lipid multibilayers through the objective of a microscope. The stripe intensity pattern, which approximates a square wave, causes a photochemical reaction of fluorescent probe I only where the light strikes the sample. The photochemical reaction destroys the fluorescence of the probe. Therefore, the laser-induced photobleaching creates a concentration gradient of fluorescent probe molecules.

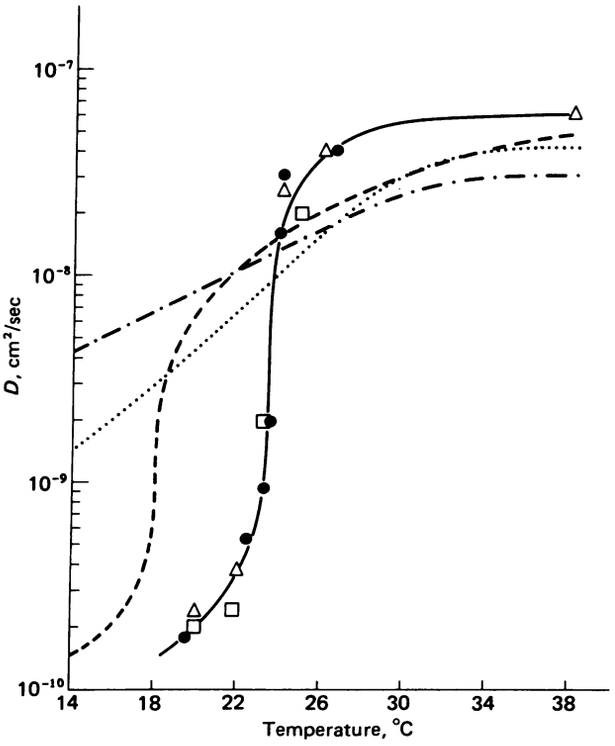

FIG. 4. Temperature dependence of the diffusion coefficient of the fluorescent lipid probe I in binary mixtures of cholesterol and dimyristoyl phosphatidylcholine with cholesterol mole fractions X = 0.05 (●), 0.07 (△), and 0.13 (□) (—). Other curves give diffusion coefficients for X = 0.22 (- - -), X = 0.25 (····), and X = 0.30 (– · – ·).

The rate at which the concentration gradient decays is measured. The time constant of the gradient decay is inversely proportional to the diffusion coefficient. To measure diffusion coefficients on the order of 1×10^{-9} to 5×10^{-8} cm²/sec, periodic stripe patterns were created with a repeat distance of either 36 or 68 μm. Typical time constants for the decay processes were on the order of 30 to 20 sec. For diffusion coefficients between 5×10^{-12} and 1×10^{-9} cm²/sec, stripes with a period of 9 μm were used, and time constants ranging from 2000 to 10 sec were observed.

RESULTS

A surprising and significant result obtained in the present work is given in Fig. 1. It will be seen that in the case of dimyristoyl phosphatidylcholine/cholesterol mixtures at 19°C the diffusion coefficient of I increases by a factor of the order of 10 as the cholesterol mole fraction increases by a few percent around $X \approx 0.20$. Thus, this binary mixture is essentially "fluid" for $X > 0.20$ and "solid" for $X < 0.20$ at temperatures below the pure phospholipid chain-melting transition. At higher temperatures (26–35°C), well above the chain-melting transition temperature of dimyristoyl phosphatidylcholine, cholesterol also has an effect on lateral diffusion at $X \approx 0.2$; in this case the diffusion coefficient decreases with increasing cholesterol concentration. Comparable changes are seen for the diffusion of I in egg phosphatidylcholine/cholesterol mixtures, as shown in Fig. 2.

The marked change in the lateral diffusion of I in dimyristoyl phosphatidylcholine/cholesterol mixtures at 19°C raises the question as to whether this is a special effect of cholesterol on the $P_{\beta'}$ phase of phosphatidylcholines. In order to answer this question we measured the diffusion coefficients for I in dipalmitoyl phosphatidylcholine/cholesterol mixtures at 38°C and 21°C, where the pure phosphatidylcholine is in the $P_{\beta'}$ and $L_{\beta'}$ phases, respectively. At each temperature there are marked order-of-magnitude changes in the lateral diffusion of I at $X \approx 0.2$, as shown in Fig. 3.

The diffusion coefficient of I in pure dimyristoyl phosphatidylcholine multilayers has been measured previously (30, 32) and was found to change sharply by over two orders of magnitude at the chain-melting transition temperature. Previous measurements (32) have shown no sharp transition in the diffusion coefficient of I in an equimolar (X = 0.5) mixture of cholesterol and dimyristoyl phosphatidylcholine. A second major result of the present work is that the sharp transition that is seen in the pure phospholipid persists at approximately the same temperature in the binary mixtures up to X = 0.2, and then abruptly disappears for higher cholesterol concentrations. Illustrative data are given in Fig. 4.

DISCUSSION

In Fig. 5 we have mapped out a temperature–composition region that separates comparatively slow and rapid lateral diffusion for the fluorescent probe I in binary mixtures of dimyristoyl phosphatidylcholine and cholesterol. The lateral diffusion coefficient of I increases by at least an order of magnitude as one passes across the boundary from the S (slow, or "solid")

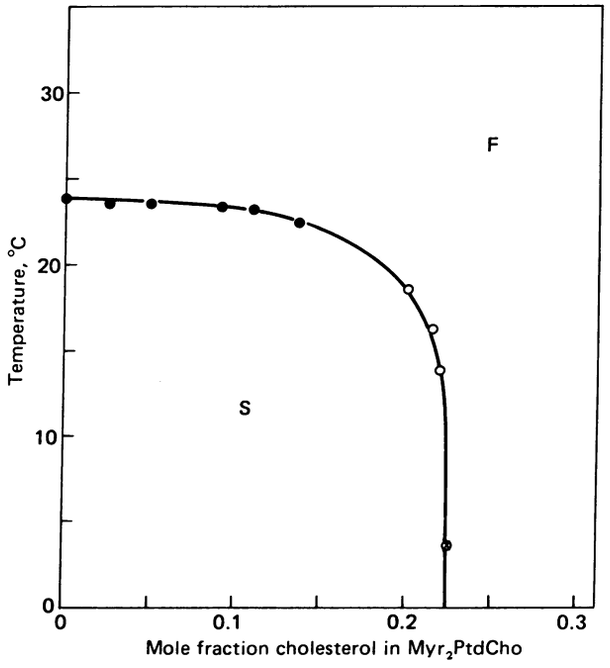

FIG. 5. Temperature–composition regions corresponding to slow (S) and fast (F) diffusion in binary mixtures of cholesterol and dimyristoyl phosphatidylcholine ($Myr_2PtdCho$). The solid line has the significance that as one passes across this line by increasing temperature and/or the concentration of cholesterol, the lateral diffusion of fluorescent probe I increases by an order of magnitude or more. Data points from lateral diffusion measurements are indicated by Os. The point ⊗ is extrapolated from a point at $0.20 \leq X \leq 0.25$ and 21°C obtained in our study of dipalmitoyl phosphatidylcholine. The temperature at ⊗ is 3.4°C, which is 17.6°C below 21°C; 17.6°C is the difference in the chain-melting temperatures of the two phospholipids, 41.4°C – 23.8°C. The ● in the composition region $0 \leq X < 0.15$ are data points obtained from sharp changes in spin-label paramagnetic resonance spectra that are believed to arise at least in part from the abrupt changes in lateral diffusion (J. Rubenstein and H. McConnell, unpublished). There are corroborating diffusion data in the $0 \leq X < 0.15$ region (see Fig. 4), but the temperature control was not sensitive enough to detect the subtle melting point depressions that the spin-label spectra give.

region to the F (fast, or "fluid") region. It is interesting that roughly this same temperature–composition region is outlined by the phase diagram of Shimschick and McConnell (3) based on spin-label data. For each point in the temperature–composition region S we have observed only a single diffusion coefficient, suggesting that S is a one-phase region. The corresponding temperature–composition region described by Shimshick and McConnell (3) or Kleemann and McConnell (7) is a two-phase region. This apparent discrepancy might be resolved if the S region in Fig. 5 were a two-phase region but with solid–fluid domains having sizes significantly smaller than the pattern spacing ($\approx 10\ \mu m$) used in the photobleaching experiments. Published (7, 10) and unpublished (B. Copeland and H. McConnell) freeze-fracture electron microscope studies show that the S region is uniform in appearance on the distance scale of 1 μm, but do not eliminate the possibility of submicroscopic solid–fluid domains. The banding pattern seen by electron microscopy in the $P_{\beta'}$ phase remains regular but becomes less distinct with increasing cholesterol concentration and disappears at approximately $X = 0.20$ (7, 10).

A biological role for cholesterol-modulated membrane "fluidity" is a tantalizing possibility that is reinforced by the present work. Studies of antibody binding to hapten-sensitized liposomes containing phosphatidylcholines and cholesterol show that the cholesterol concentration in these membranes plays a significant role in antibody binding: an enhancement in specific antibody binding at $X \geq 0.2$ has been observed (24). Also, complement depletion by hapten-sensitized model membranes in the presence of specific antibody depends strongly on cholesterol concentration in the model membrane (25, 28). Thus, cholesterol-modulated physical properties of membranes almost certainly have significant biological effects.

Note Added in Proof. C. G. Wade and colleagues have used spin-echo nuclear resonance in a field gradient to measure lipid diffusion in mixtures of cholesterol and dipalmitoyl phosphatidylcholine (personal communication). Their results differ significantly from ours for mole fractions of cholesterol between 0.1 and 0.2.

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