Microimmiscibility and Three-Dimensional Dynamic Structures of Phosphatidylcholine–Cholesterol Membranes: Translational Diffusion of a Copper Complex in the Membrane†

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ABSTRACT: Saturated and unsaturated phosphatidylcholine (PC)–cholesterol membranes have been studied, with a special attention paid to fluid-phase immiscibility in cis-unsaturated phosphatidylcholine (PC)–cholesterol membranes as previously proposed and to the three-dimensional structure of the membrane. The investigation was carried out with dual probes: a membrane-soluble, square-planar copper complex, (3-ethoxy-2-oxobutyraldehyde bis(N^4,N^4-dimethylthiosemicarbazonato))copper(II) ($CuKTSM_2$), and one of several nitroxide radical lipid-type spin-labels. Bimolecular collision rates between metal ion and spin-label were determined by measuring the nitroxide spin–lattice relaxation times (T_1's) in the presence and absence of $CuKTSM_2$ by use of saturation–recovery ESR techniques, and from these measured rates, translational diffusion coefficients of $CuKTSM_2$ were estimated. Profiles of the collision rate across the membrane bilayer were obtained with Tempocholine phosphatidic acid ester, 5-doxylstearic acid, 16-doxylstearic acid, and cholesterol-type spin-labels as a function of cholesterol mole fraction, length and unsaturation of acyl chains, and temperature. In the liquid-crystalline phase of saturated PC membranes, incorporation of cholesterol decreases the collision rate at all depths in the membrane, and the effect of cholesterol is smallest in the middle of the bilayer. In trans-unsaturated PC membranes, a cholesterol-induced decrease of the collision rate was also observed, except in the head-group regions. In cis-unsaturated PC membranes, virtually no effect of cholesterol was observed on the collision rate, either with phospholipid-type spin-labels or with cholesterol-type spin-labels. This result is in clear contrast with our previous observation, in which the effect of cholesterol in cis-unsaturated PC membranes is small on the alkyl-chain motion of phospholipid-type spin-labels but large on the wobbling rotational diffusion of cholesterol-type spin-labels [Pasenkiewicz-Gierula, M., Subczynski, W. K., & Kusumi, A. (1990) *Biochemistry 29*, 4059–4069]. A model is proposed to explain these results in which the fluid-phase immiscibility is prevalent in cis-unsaturated PC–cholesterol membranes, but where cholesterol-rich (cholesterol oligomeric) domains are small (several lipids) and/or of short lifetime (10^{-9} s to $<10^{-7}$ s). It is suggested that this microimmiscibility arises from the structural nonconformability between the rigid cholesterol ring structure and the rigid bend at the cis double bonds in PC alkyl chains. Our results also suggest that free volume is created in the central part of the bilayer by intercalation of cholesterol in the membrane due to the short bulky tetracyclic ring of cholesterol, which is enhanced by the mismatch in the hydrophobic length due to the longer PC acyl chains (than cholesterol) and the structural nonconformability between cholesterol and cis-unsaturated PC.

Phospholipids and cholesterol are major constituents of eukaryotic plasma membranes, and the interaction between the two has been studied extensively [for reviews, see Demel and de Kruyff (1976), Schroeder (1984), Yeagle (1985, 1988), and Presti (1985)]. Previously, we have systematically studied phosphatidylcholine (PC)[1]–cholesterol membranes as a function of chain length and unsaturation of alkyl chains, cholesterol mole fraction, and temperature by using spin-labeling techniques (Kusumi et al., 1986; Subczynski & Kusumi, 1986; Merkle et al., 1987; Kusumi & Pasenkiewicz-Gierula, 1988; Pasenkiewicz-Gierula et al., 1990). The chemical structures of the spin-labels are shown in Figure 1. One of the most important findings in this series of work is that unsaturation of PC alkyl chains profoundly affects PC–cholesterol interaction in the membrane: cis-unsaturated PC is less miscible with cholesterol at physiological temperatures than saturated PC [which is in agreement with the results by Shin and Freed (1989)], while the major effect of cholesterol on saturated PC is to mix at certain ratios and to enhance the trans configuration of the saturated chain (the ordering effect of cholesterol) (Pasenkiewicz-Gierula et al., 1990). We proposed that the nonconformability of the molecular shapes of dioleoyl-PC (DOPC, 18 carbon atoms and a C9–C10 cis double bond in a chain) and cholesterol in the membrane is

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[1] Abbreviations: ASL, androstane spin-label; CSL, cholestane spin-label; $CuKTSM_2$, (3-ethoxy-2-oxobutyraldehyde bis(N^4,N^4-dimethylthiosemicarbazonato))copper(II); DEPC, L-α-dielaidoylphosphatidylcholine; DMPC, L-α-dimyristoylphosphatidylcholine; DOPC, L-α-dioleoylphosphatidylcholine; DSPC, L-α-distearoylphosphatidylcholine; EYPC, egg yolk phosphatidylcholine; PC, phosphatidylcholine; SASL, stearic acid spin-label; 5-SASL, 5-doxylstearic acid spin-label; 16-SASL, 16-doxylstearic acid spin-label; T-PC, Tempocholine phosphatidic acid ester; Tempone, 1-oxy-2,2,6,6-tetramethyl-4-piperidone; T_1, spin–lattice relaxation time.

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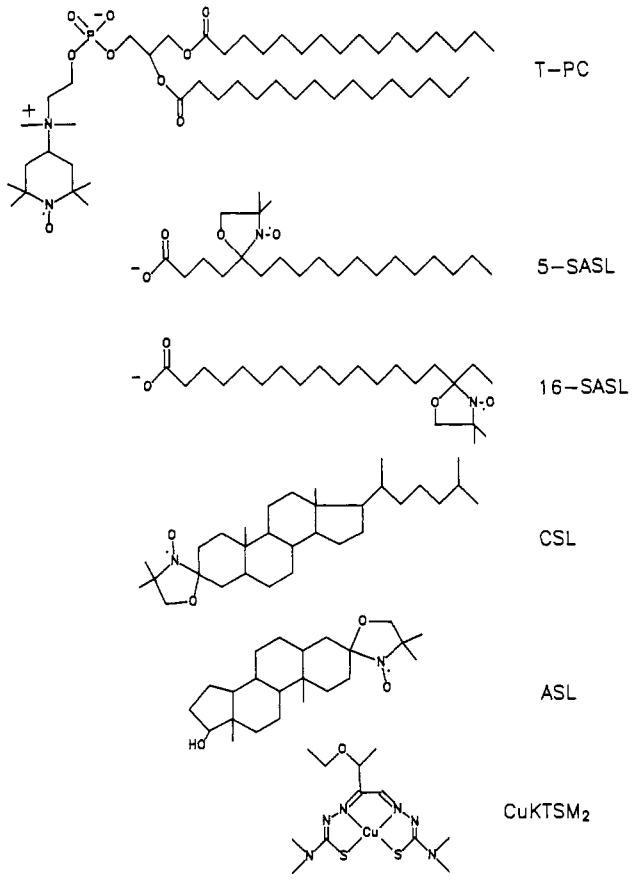

FIGURE 1: Structures of $CuKTSM_2$, T-PC, 5-SASL, 16-SASL, CSL, and ASL.

the key feature of the DOPC–cholesterol interaction by which these results can be explained. The rigid tetracyclic ring structure of cholesterol [and steroid-type spin-labels such as androstane spin-label (ASL) and cholestane spin-label (CSL)] and the rigid bend at the cis double bonds in the oleoyl chains do not conform to each other when they are in direct contact in the membrane (Franks, 1976; Worcester & Franks, 1976; Huang, 1977; Pace & Chan, 1982a,b; Presti & Chan, 1982). Due to this structural nonconformability, (1) cholesterol molecules tend to be excluded from DOPC domains and segregated out, and (2) the ordering effect of cholesterol is much weaker in DOPC membranes compared with that in saturated PC membranes. [The ordering effect of cholesterol can be defined as a physical interaction of cholesterol with saturated acyl chains that enhances the extended conformation of PC alkyl chains (more trans bonds) next to cholesterol.]

In the present investigation, we have further studied the PC–cholesterol interaction with particular attention paid to (1) differences between saturated PC and unsaturated PC and (2) the size and the lifetime of the cholesterol-rich (oligomeric) domains. In order to gain more knowledge on the domain structures of PC–cholesterol membranes, we used an observable which is more sensitive to longer time–space scales than the reorientational correlation time of lipid-type spin-labels: instead of the latter, translational diffusion of a small membrane-soluble solute, a copper square-planar complex, (3-ethoxy-2-oxobutyraldehyde bis(N^4,N^4-dimethylthiosemicarbazonato))copper(II) called $CuKTSM_2$ (Figure 1, MW = 393.5, 8 × 8 Å square-plane) has been examined. Since $CuKTSM_2$ is paramagnetic, has a short spin–lattice relaxation time ($T_1 \approx 10^{-8}$ s; Hyde & Sarna, 1978), and is hydrophobic, it works as an effective relaxing agent for the nitroxide spin-labels in the membrane (Subczynski et al., 1987). The translational diffusion of $CuKTSM_2$ in the membrane was estimated from the rate of bimolecular collision between $CuKTSM_2$ and the nitroxide radicals on the basis of T_1's measured in the presence and absence of $CuKTSM_2$. Since the time scale of the spin-label T_1 (10^{-6}–10^{-5} s) is much longer than the reorientational correlation time of the nitroxide attached to lipids (10^{-10}–10^{-9} s), membrane dynamics in longer time–space scales can be observed, and thereby, it is possible to obtain more information on the size and the lifetime of lipid domains such as cholesterol-rich (or cholesterol oligomeric) domains. Thus, we also intend to further develop a spin-label T_1 method that is sensitive to membrane dynamic structures on longer time–space ranges.

On the basis of the relationship between the mean square distance and time, $\langle x^2 \rangle = 4Dt$, where D is the translational diffusion coefficient, an observation method with a longer time scale allows one to observe membrane processes with longer correlation lengths. The basic clock for the measurement of the bimolecular collision rate is T_1 of the nitroxide radical spin-labels (10^{-6}–10^{-5} s), while that for reorientational diffusion is the magnetic anisotropy (10^{-8}–10^{-7} s). Assuming that the translational diffusion coefficient of lipid in the membrane is 3×10^{-8} cm^2/s, a lipid can approximately cover an area of 50 lipids during T_1 and an area of only 1 lipid during the time scale of magnetic anisotropy.

We have previously studied translational diffusion of molecular oxygen in the membrane by observing the effect of bimolecular collision of oxygen (as a relaxing reagent) with the nitroxide group of lipid-type spin-labels using the saturation–recovery ESR technique (Kusumi et al., 1982a; Subczynski et al., 1989, 1990; Hyde & Subczynski, 1989). The profiles of the oxygen transport parameter across various membranes have been obtained, and the oxygen permeability coefficient of the membrane has been estimated (Subczynski et al., 1989, 1990). In the present study, the role of oxygen has been replaced with $CuKTSM_2$. Diffusion of small molecules such as oxygen in the membrane has been related to the kink conformation of phospholipid alkyl chains in the membrane. Since $CuKTSM_2$ is substantially larger than molecular oxygen, it is interesting to compare the diffusive properties of these molecules in the membrane.

$CuKTSM_2$ belongs to a class of potent antitumor metallo drugs (Petering & Petering, 1975). This study is also intended to contribute to understanding of pharmacological functions of $CuKTSM_2$.

We have investigated $CuKTSM_2$ transport at various "depths" in the membrane using Tempocholine phosphatidic acid ester (T-PC, to probe the head-group region), 5-doxylstearic acid spin-label (5-SASL, to probe the hydrocarbon region near the membrane surface), and 16-doxylstearic acid spin-label (16-SASL, to probe the central region of the membrane), which together enabled us to study three-dimensional organization of the membrane. We also utilized steroid-type spin probes, CSL and ASL, which are likely to partition into the cholesterol-rich domains, with the nitroxide radical of CSL in the membrane surface region and that of ASL in the central part of the bilayer. Utilization of all these probes is important because we are interested in the mismatch between the fused-ring backbone of cholesterol and PC acyl chains both in length and in conformation.

Outline of Theory

Bimolecular collision of $CuKTSM_2$ (a fast relaxing species) and the nitroxide (a slow relaxing species) induces spin exchange, which leads to a faster effective spin–lattice relaxation of the nitroxide (Subczynski et al., 1987). According to Hyde

and Sarna (1978), the exchange process is a strong-encounter type, in which every collision causes spin exchange.

The $CuKTSM_2$ transport parameter

$$W(x) = T_1^{-1}(2 \text{ mol \% } CuKTSM_2, x) - T_1^{-1}(\text{no } CuKTSM_2, x) \quad (1)$$

has been defined by Subczynski et al. (1987). The value of 2 mol % was chosen due to the optimal time range for the sensitivity of the instrument and the extrapolatability of $W(x)$ to 2 mol % as shown in Figure 2 (inset). This parameter has been introduced in analogy to the oxygen transport parameter (Kusumi et al., 1982a):

$$W_O(x) = T_1^{-1}(\text{air}, x) - T_1^{-1}(N_2, x) \quad (2)$$

$W(x)$ is a function of both the concentration $C(x)$ and the translational diffusion constant $D(x)$ of $CuKTSM_2$, x indicating that these parameters are functions of depth (distance from the membrane surface). On the basis of the Smoluchowski equation for isotropic diffusion, $W(x)$ can be expressed as

$$W(x) = AD(x)C(x) \quad (3)$$

$$A = 8\pi p r_0 \quad (4)$$

where p is the probability that an exchange event occurs when a collision does take place and r_0 is the interaction distance (6 Å; Subczynski et al., 1987). According to Salikhov et al. (1971) and Molin et al. (1980), p equals 1/2 if T_1 of the colliding paramagnetic metal ion ($S = {}^1/_2$) is longer than the duration of collision. Copper complexes have been shown to undergo strong Heisenberg exchange when they collide with the nitroxide spin-label, the duration of which is estimated to be 10^{-10}–10^{-9} s. The T_1 of copper ion is approximately 10^{-8} s (Hyde & Sarna, 1978). Thus, taking p as 1/2, eq 4 is simplified to

$$A = 4\pi r_0 \quad (5)$$

Equation 3 is based on two assumptions: (1) the translational diffusion constant for $CuKTSM_2$ is much larger than that for spin-labeled lipids; (2) $CuKTSM_2$ diffuses isotropically in the membrane. Since the mutual diffusion constant (a sum of diffusion constants for $CuKTSM_2$ and the spin-label) in DMPC membranes is (7–24) × 10^{-8} cm^2/s between 25 and 45 °C [Table I in Subczynski et al. (1987)][2] and the diffusion constant for CSL is (1.5–2.7) × 10^{-8} cm^2/s between 27 and 37 °C (Yin et al., 1988), the first assumption is largely satisfied. The second assumption needs attention in interpretation of results; the thickness of the membrane is 30–40 Å and the in-plane dimensions of $CuKTSM_2$ are ca. 8 × 8 Å.

Experimental Procedures

Materials. $CuKTSM_2$ was generously supplied by Dr. D. H. Petering at the University of Wisconsin—Milwaukee. All phospholipids were purchased from Sigma (St. Louis, MO), cholesterol (crystallized) was from Boehringer (Indianapolis, IN), and spin-labels were from Molecular Probes (Eugene, OR). Tempocholine phosphatidic acid ester (T-PC) was a generous gift from Dr. S. Ohnishi at Kyoto University.

Methods. The membranes used in this work were multilamellar dispersions of phospholipids and cholesterol, containing 1 mol % spin-label and various amounts of $CuKTSM_2$ (from 0 to 2 mol %). Briefly, membranes were prepared in the following method (Kusumi et al., 1986): chloroform solutions of the lipids and probes were mixed (containing 10^{-5} mol of total lipids), and chloroform was evaporated with a stream of nitrogen gas and then under a reduced pressure (≈0.1 mmHg) for at least 12 h. A buffer solution (0.1 mL) was added to the dried lipid at about 20 °C above the phase transition temperature of the phospholipid membranes and vortexed vigorously. All samples were prepared in 0.1 M borate at pH 9.5 to ensure that all carboxyl groups of SASL were ionized in phosphatidylcholine membranes (Sanson et al., 1976; Egret-Charlier et al., 1978; Kusumi et al., 1982a,b; Subczynski et al., 1989). The lipid dispersion was centrifuged briefly, and the loose pellet (≈20% lipid, w/w) was placed into a gas-permeable capillary (0.5-mm i.d.) made of a methylpentene polymer called TPX (Hyde & Subczynski, 1989). This plastic is permeable to nitrogen, oxygen, and carbon dioxide and is substantially impermeable to water. The TPX sample tube was placed inside the loop–gap resonator (Froncisz & Hyde, 1982), and a flow of temperature-regulated nitrogen gas over the capillary was used to remove oxygen and to control the sample temperature, which enables us to obtain correct T_1's of spin-labels at various temperatures (Kusumi et al., 1982a; Subczynski et al., 1989).

The concentration of $CuKTSM_2$ in the aqueous phase of the membrane suspension was determined by optical absorption. The membrane suspension (1 mL) containing 2 × 10^{-7} mol of $CuKTSM_2$ and 10^{-5} mol of DMPC (±cholesterol) was centrifuged at 4 °C, and the optical density of the supernatant was measured (ϵ_{469nm} = 6300 M^{-1} cm^{-1}; Petering, 1972).

T_1's of the spin-labels were measured at X-band with the long-pulse saturation–recovery technique. With long and intense microwave pulses, the spin system approaches a steady state, at which the populations of spins at each energy level tend to be equalized. After the saturating pulse is turned off, the recovery of the spin system to Boltzmann equilibrium is observed with a weak observing power. In general, the nitrogen nuclear spin–lattice relaxation time is much shorter than the electron spin–lattice relaxation time for spin-labels in membranes (Yin et al., 1987; Yin & Hyde, 1987), and single-exponential decays were found. The duration of the saturating pulse is then not critical. However, if the electron spin–lattice relaxation becomes sufficiently short by introduction of high concentrations of $CuKTSM_2$, multiple-exponential signals are expected, and the long-pulse techniques should be used. The saturation–recovery spectrometer is based on the design of Huisjen and Hyde (1974) and is interfaced with a loop–gap resonator. A field-effect transistor microwave amplifier has been recently introduced. Typically, 2 × 10^7 accumulations of the decay signal were carried out with 128–512 data points on each dacay. Accumulation time was usually between 5 and 15 min.

All measurements of T_1 were made on the central line (m_I = 0) of the ESR spectrum of the spin-label. The saturation–recovery of copper was undetectable with the present setting of the instrument.

Results and Discussion

Saturation–Recovery Measurements. Typical saturation–recovery curves for 5-SASL in the liquid-crystalline egg yolk PC (EYPC) membranes in the presence and absence of $CuKTSM_2$ are displayed in Figure 2. The recovery followed a single-exponential curve under all our experimental condi-

[2] The diffusion coefficient of $CuKTSM_2$ was calculated on the basis of the average concentration of $CuKTSM_2$ in the membrane and the collision rate. Since $CuKTSM_2$ is mostly located in the membrane (Subczynski et al., 1987; also see Results and Discussion), the average $CuKTSM_2$ concentration in the membrane was estimated to be 0.03 M for 2 mol % $CuKTSM_2$ (vs DMPC), on the basis of a density of DMPC membranes of 1.003 g/mL (Cornell & Separovic, 1983). In Subczynski et al. (1987), the diffusion coefficient was overestimated by a factor of 4.5.

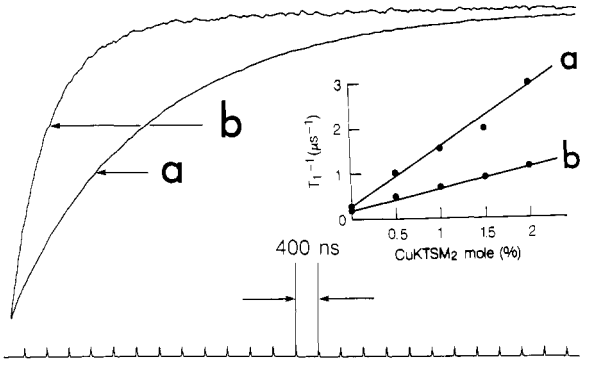

FIGURE 2: Typical saturation–recovery signal of 5-SASL in EYPC membranes containing 27.5 mol % cholesterol at 60 °C. Samples were equilibrated with nitrogen. (a) In the absence of $CuKTSM_2$. T_1 = 2.61 μs. Conditions: pump power = 60 mW (microwave field = 1.2 G in the rotating frame); pump width = 9 μs. 4×10^6 decays were accumulated during 2.7 min with 256 data points/decay. (b) In the presence of 0.5 mol % $CuKTSM_2$. T_1 = 0.78 μs. Conditions were the same as above except that the pump width used was 5 μs. (Inset) T_1^{-1} of 5-SASL plotted as a function of the mole fraction of $CuKTSM_2$ in DMPC membranes measured at 45 °C (a) and 25 °C (b).

tions and instrumental settings used in this work, and T_1 was measured with an accuracy of ≈5%. Figure 2 shows that the collision of $CuKTSM_2$ with the spin-label decreases T_1 of the nitroxide.

In the inset of Figure 2, T_1^{-1}'s for 5-SASL in DMPC membranes are shown as a function of mole fraction of $CuKTSM_2$ (vs DMPC), indicating that T_1^{-1} increases linearly with the $CuKTSM_2$ mole fraction. The linear relationship between T_1^{-1} and the mole fraction of $CuKTSM_2$ was also observed for 16-SASL (not shown). This proportionality allows us to extrapolate all our data to 2 mol % $CuKTSM_2$ (vs total lipid) to obtain $W(x)$, as introduced under Outline of Theory.

Due to the hydrophobicity of $CuKTSM_2$, more than 99% partitioned into the membrane even in the gel phase of DMPC membranes, as determined by the optical absorption measurement (see Methods). As an additional check on the solubility of the complex, W in the aqueous phase was determined with 1-oxy-2,2,6,6-tetramethyl-4-piperidone (Tempone). No effect of $CuKTSM_2$ on T_1 was detected ($W = 0$), indicating that the concentration of $CuKTSM_2$ in water is very low.

Previously, we have also shown from the cupric ESR signal that $CuKTSM_2$ preferentially partitions into the membrane, orients its plane of the skeletal ring parallel to the bilayer normal, and undergoes anisotropic rotational diffusion in the membrane. The presence of 1 mol % $CuKTSM_2$ in DMPC membranes lowers the phase transition temperature of DMPC membranes by 1.4 °C (Subczynski et al., 1987). This observation indicates that due caution must be exercised in interpretation of our data. However, incorporation of 1 mol % $CuKTSM_2$ in DMPC membranes does not alter the Tempo partition coefficient in the liquid-crystalline phase, showing that perturbation on the membrane structure with $CuKTSM_2$ may not be serious above the phase transition temperature.

Saturation–recovery measurements were carried out systematically as a function of temperature (0–60 °C), cholesterol mole fraction, and alkyl-chain length and unsaturation in the presence and absence of $CuKTSM_2$. PC's used were DMPC (14 carbons in a saturated alkyl chain), L-α-distearoyl-PC (DSPC, 18 carbons, saturated), DOPC (18 carbons, 9–10 cis unsaturated), L-α-dielaidoyl-PC (DEPC, 18 carbons, 9–10 trans unsaturated), and EYPC (mixed PC containing both saturated and unsaturated alkyl chains). Five spin probes, T-PC, 5-SASL, 16-SASL, CSL, and ASL, were utilized to observe the collision rate at various membrane depths and in the membrane domains containing various mole fractions of cholesterol.

Table I: Activation Energy for Translational Diffusion of $CuKTSM_2$ in Various PC–Cholesterol Membranes

host lipid	cholesterol (mol %)	activation energy (kcal/mol) T-PC	5-SASL	16-SASL	temp range (°C)
DMPC	0	9.2	8.4	8.4	25–60
DMPC	30	14.2	16.1[a]	18.3	25–60
EYPC	0	11.0	9.8	8.9	10–60
EYPC	30	11.0	9.8	8.9	10–60

[a] The temperature range is between 30 and 60 °C.

In spin-label studies of membrane lipids, two classes of labels have generally been used: phospholipid spin-labels and SASL to study alkyl chain motion and CSL and ASL to study cholesterol motion. It must be remembered that experiments carried out with these probe molecules necessitate due caution in interpretation of the results and that the labeled molecules cannot be expected to mimic all properties of phospholipid alkyl chains and cholesterol. Nevertheless, interaction of ASL (or CSL or SASL) with phospholipid and cholesterol should, to a certain degree, approximate cholesterol–PC and cholesterol–cholesterol interactions in the membrane because of the overall similarity of the molecular structure (Müller-Landau & Cadenhead, 1979a,b; Cadenhead & Müller-Landau, 1979; Presti & Chan, 1982; Kusumi et al., 1986).

$CuKTSM_2$ Transport Parameters in DMPC–Cholesterol Membranes. Figure 3 shows T_1^{-1} and W plotted as a function of inverse temperature in DMPC–cholesterol membranes. In the absence of $CuKTSM_2$, overall variation in T_1 is approximately a factor of 10. The overall variation in W is approximately a factor of 800, indicating that W is a sensitive parameter that reports on membrane fluidity.

At the main phase transition of DMPC membranes, W increases by a factor of 3–5 in the absence of cholesterol. This sudden change was detected by all three spin-labels. Addition of cholesterol abolishes discontinuous changes of T_1 and W at the phase transition. The presence of cholesterol decreases W in both liquid-crystalline and solid phases.[3]

W for DMPC membranes is replotted as a function of the location of the nitroxide across the bilayer membrane (Figure 4) to display the membrane profiles of W. A decrease of W induced by the addition of cholesterol is seen at all depths in the membrane.

$CuKTSM_2$ Transport Parameters in EYPC–Cholesterol Membranes. In Figure 5, values of T_1^{-1} and W in EYPC–cholesterol membranes are plotted as a function of inverse temperature. In contrast to the results with DMPC membranes, no influence of cholesterol on W was detected at any temperature at any depth in EYPC membranes. The effect of cholesterol on W is remarkably absent.

W's for EYPC (–cholesterol) membranes are linearly dependent on T^{-1} as shown in Figure 5. The activation energy for translational diffusion of $CuKTSM_2$ was obtained, assuming that the concentration profile of $CuKTSM_2$ across the membrane is independent of temperature. The activation energy is listed in Table I. For the sake of comparison, activation energy for DMPC–cholesterol membranes is esti-

[3] Note that very small amounts of cholesterol (1/30000 to 1/100 mole ratios of cholesterol in L-α-dipalmitoyl-PC) increase the motional freedom of 5-SASL below the phase transition temperature (Subczynski & Kusumi, 1986).

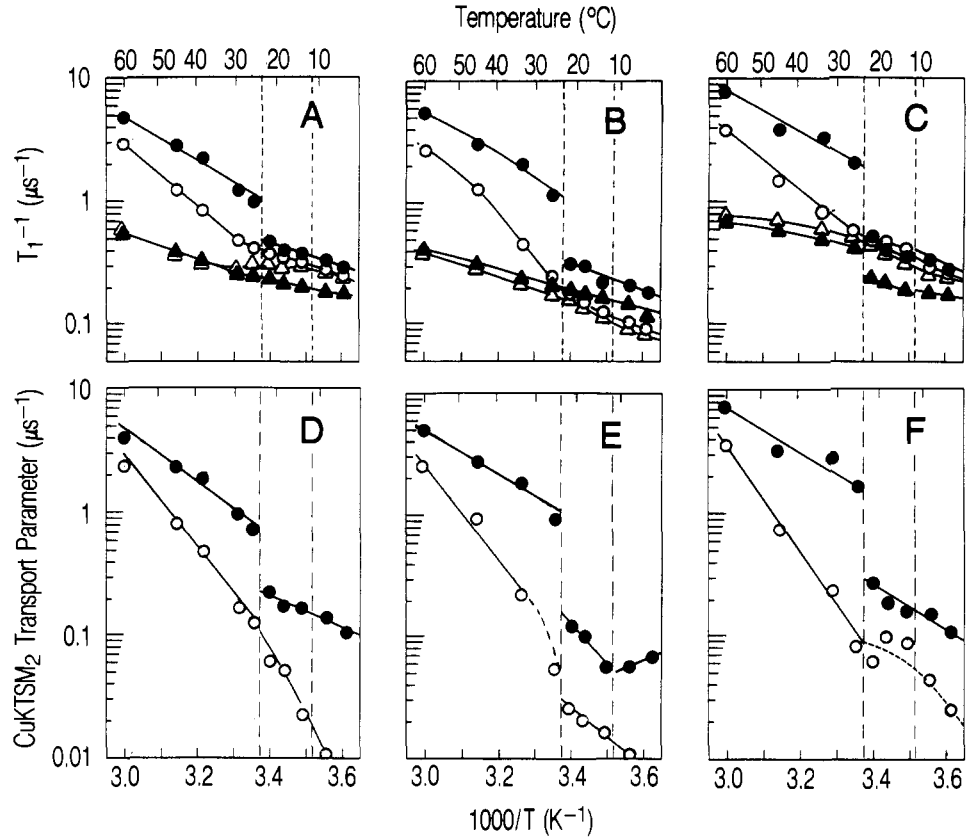

FIGURE 3: T_1^{-1} and $W(CuKTSM_2)$ as a function of reciprocal temperature for T-PC (A, D), 5-SASL (B, E), and 16-SASL (C, F) in DMPC–cholesterol (0 and 27.5 mol % cholesterol) membranes in the presence and absence of 2 mol % $CuKTSM_2$. The samples were saturated with nitrogen gas. Dashed vertical lines show the main transition and pretransition temperatures of DMPC membranes. Closed and open keys indicate the absence and presence of 27.5 mol % cholesterol, respectively. (A–C) In the presence of $CuKTSM_2$ (●, ○) and in the absence of $CuKTSM_2$ (▲, △).

mated between 25 and 60 °C. In the absence of cholesterol, the activation energy for EYPC membranes is slightly larger than that for DMPC membranes. In the presence of 30 mol % cholesterol, the activation energy for DMPC membranes is larger than that for EYPC membranes. These values of activation energy for $CuKTSM_2$ diffusion should be compared with those for lateral diffusion of phospholipids (Table II; Kuo & Wade, 1979; Rubenstein et al., 1979; Vaz et al., 1985). The activation energies for translational diffusion of $CuKTSM_2$ and for lateral diffusion of phospholipids are similar.

Cholesterol Effect on W Profiles. (A) Influence of Alkyl-Chain Length. The membrane profiles of W for DSPC bilayers are shown in Figure 6A. The acyl chains of DSPC contain 18 carbons, 4 more than those of DMPC. In the liquid-crystalline phase of DSPC membranes in and near the head-group regions (T-PC and 5-SASL), the effect of cholesterol on W is large and comparable to that for DMPC membranes when these membranes are compared at 60 °C. However, in the central region of the DSPC bilayer, as monitored with 16-SASL, the cholesterol-induced decrease in W is only 20% as compared to 50% for DMPC membranes (Figures 3 and 4) at 60 °C. (Notice that, in the absence of cholesterol, W for DMPC membranes is larger than W for DSPC membranes. This is probably because the effective concentration of $CuKTSM_2$ in DSPC membranes is 20% lower than that in DMPC membranes due to the longer alkyl chains.)

According to Franks and Lieb (1979), the β-OH group on the 17th carbon of cholesterol is located very close to the position of fatty acyl ester groups. Although there is uncertainty of 1.5 carbons due to the difference in the vertical location of α- and β-chains, it follows that the rigid ring structure of cholesterol penetrates the membrane into the 7th to 10th carbons of the alkyl chain and the alkyl-chain tail of cholesterol reaches to the 12th to 15th carbons. On the basis of this estimate, the observation above showing a larger effect of cholesterol on DMPC than on DSPC membranes in the middle of the bilayer raises the possibility that this difference is due to the match in length between cholesterol (the rigid ring system of cholesterol, in particular) and alkyl chains. Insertion of the bulky ring structure of cholesterol in the membrane can create free volume in the central region of the bilayer. This tendency is magnified in DSPC membranes because the stearoyl chain is longer than the total length of cholesterol.

Below the phase transition temperature, cholesterol *increases* W in DSPC membranes (45 °C). This result is to be contrasted with that in DMPC–cholesterol membranes, in which

Table II: Activation Energy for Lateral Diffusion of Phospholipids in Various PC (−Cholesterol) Membranes

host lipid	chol (mol %)	ΔE (kcal/mol)	temp range (°C)
DMPC	0	6.5;[a] 15.2[c]	35–50;[a] 31–51[c]
DMPC	30	17.2[b]	16–35[b]
EYPC	0	9.0[c]	27–56[c]
EYPC	30	8.2[c]	31–57[c]
DLPC	0	6.5;[a] 9.4[c]	10–50;[a] 26–51[c]
DPPC	0	6.5;[a] 15.2[c]	45–71;[a] 49–62[c]
POPC	0	7.5;[a] 6.1;[d] 6.3[e] (CSL)	15–45;[a] 15–62[d,e]
POPC	20	8.9[d]	15–60[d]
POPC	30	9.9[e] (CSL)	15–63[e]

[a] ΔE was obtained from the temperature dependence of the diffusion constant in Rubenstein et al. (1979). [b] ΔE was obtained from the temperature dependence of the diffusion constant in Vaz et al. (1985). [c] According to Kuo and Wade (1979). [d] According to Shin and Freed (1989). [e] ΔE for lateral diffusion of CSL (Shin & Freed, 1989).

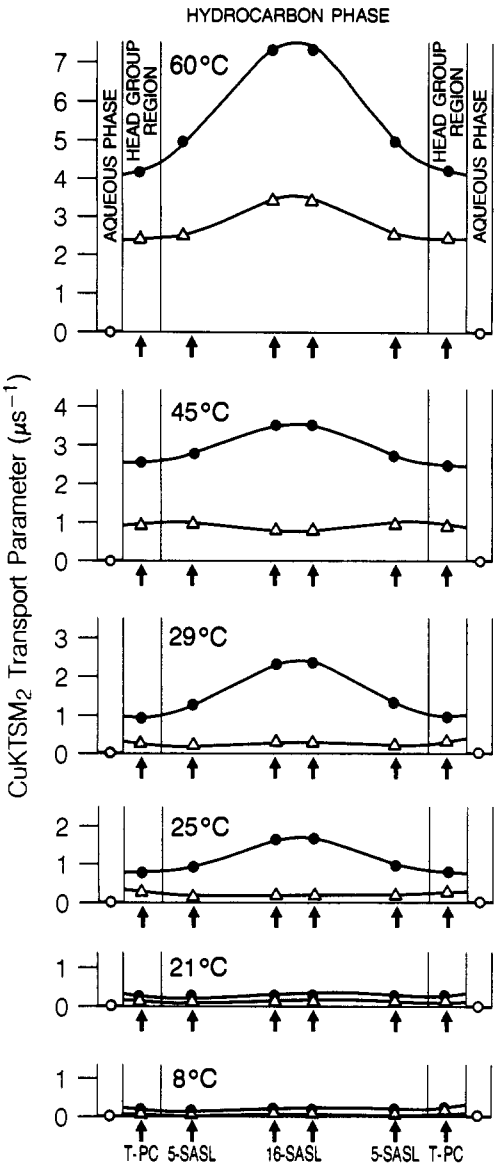

FIGURE 4: Effect of cholesterol on the $W(CuKTSM_2)$ profiles across the DMPC membranes evaluated with T-PC, 5-SASL, and 16-SASL. The estimate of the location of the nitroxide moiety in the membrane was made as previously (Subczynski et al., 1989). W in the aqueous phase is 0 as obtained with Tempone (see text). Closed and open keys indicate the absence and presence of 27.5 mol % cholesterol, respectively. Approximate locations of the nitroxide moieties of the spin-labels are indicated by the arrows at the bottom.

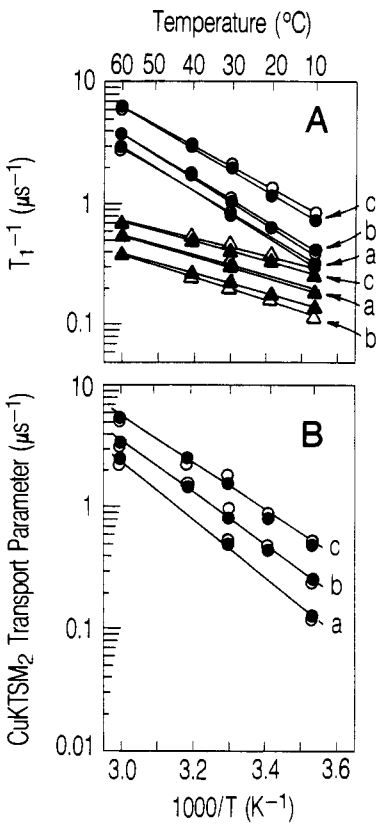

FIGURE 5: T_1^{-1} (A) and $W(CuKTSM_2)$ (B) as a function of reciprocal temperature for T-PC, 5-SASL, and 16-SASL in EYPC–cholesterol (0 and 27.5 mol % cholesterol) membranes in the presence and absence of 2 mol % $CuKTSM_2$. The samples were saturated with nitrogen gas. Closed and open keys indicate the absence and presence of 27.5 mol % cholesterol, respectively. (a) T-PC; (b) 5-SASL; (c) 16-SASL. (A) In the presence of $CuKTSM_2$ (●, ○) and in the absence of $CuKTSM_2$ (▲, △).

cholesterol *decreases* W at all depths (3–23 °C). The cholesterol-induced increase in W in DSPC membranes is particularly marked in the central region of the membrane. This observation can be explained again by the creation of free volume in the central part of the bilayer due to the short length of the bulky cholesterol ring structure.

(*B*) *Influence of Unsaturation.* The transverse membrane profiles of W for DEPC and DOPC membranes are shown in panels B and C of Figure 6, respectively, for comparison with W for DSPC membranes. All these lipids contain 18 carbons per acyl chain and differ in terms of unsaturation. DOPC contains a 9–10 cis double bond, and DEPC contains a 9–10 trans double bond in each acyl chain. W profiles for these PC–cholesterol membranes are compared in the liquid-crystalline phase at 60 °C.

Cholesterol gives almost no effect on the W profile of DOPC membranes at any depth in the membrane. The profile of W for DEPC membranes in the hydrocarbon phase resembles that for DSPC membranes although the cholesterol effect in the head-group region was not detected. It is concluded that the effect of a trans double bond on the interaction between cholesterol and alkyl chains is much smaller than that of a cis double bond.

W profiles for EYPC membranes at various temperatures are also shown in Figure 6D. As was already described above (see Figure 5), the effect of cholesterol is remarkably absent, in general agreement with the results with DOPC–cholesterol membranes. These results indicate that, in the presence of cis unsaturation, cholesterol does not affect W at any depths in the membrane.

These results should be compared with the effect of cholesterol on oxygen transport parameters in DMPC, DOPC, and EYPC membranes in the liquid-crystalline phase. The presence of cholesterol decreases the oxygen transport parameter in and near the polar head-group regions in all membranes, while it gives minor effect in the central part of the DMPC membranes and *increases* the oxygen transport parameter in the middle of DOPC and EYPC membranes (Subczynski et al., 1989, 1990). The difference between oxygen and $CuKTSM_2$ diffusion may be explained by the difference in size between the two diffusing molecules. Since molecular oxygen is small, creation of small free volume in the membrane could induce considerable increase in local oxygen transport.

$CuKTSM_2$ Transport Parameter As Observed with Cholesterol–Analogue Spin-Labels. Viewing PC–cholesterol membranes from the side of cholesterol as well as from the side of PC is important because the phase separation of the

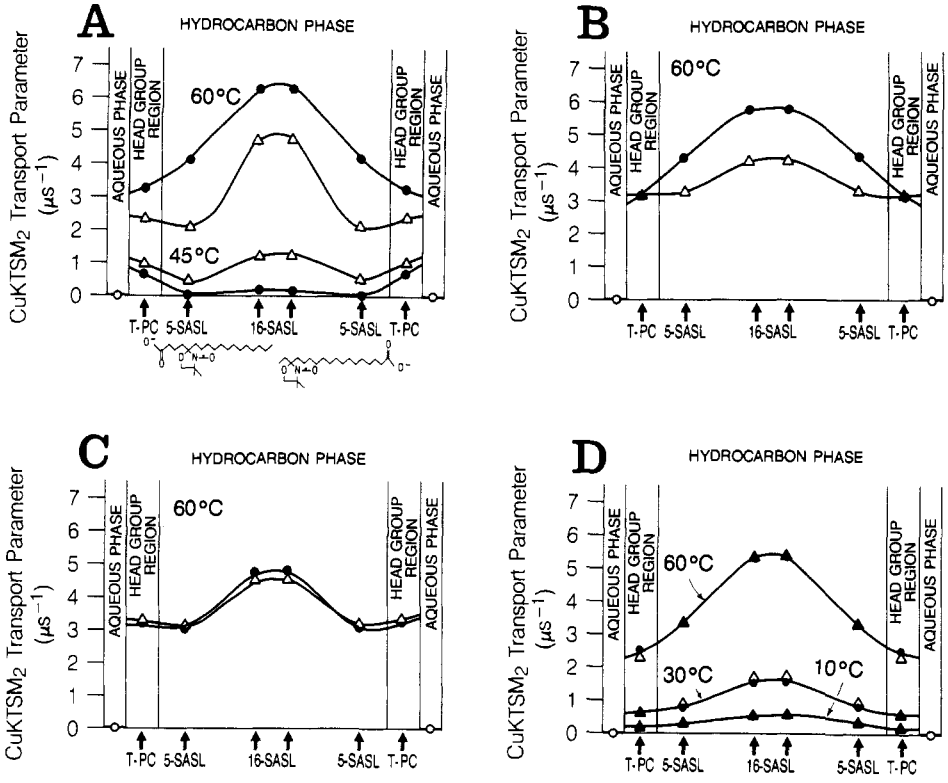

FIGURE 6: Effect of cholesterol on the W(CuKTSM$_2$) profiles across DSPC (A), DEPC (B), DOPC (C), and EYPC (D) membranes, estimated with T-PC, 5-SASL, 16-SASL, and Tempone. Approximate locations of the nitroxide are indicated by arrows as well as by the drawings of SASL. Closed and open keys indicate the absence and presence of 27.5 mol % cholesterol, respectively.

cholesterol-rich domains is proposed to take place even in the liquid-crystalline phase [Lentz et al., 1980; Recktenwald & McConnell, 1981; Presti & Chan, 1982; Kusumi et al., 1986; Pasenkiewicz-Gierula et al., 1990; however, also see Knoll et al. (1985), Pink and MacDonald (1988), and Shin and Freed (1989)]. Taylor et al. (1982), Kusumi et al. (1983), Dufourc et al. (1984), and Dufourc and Smith (1986) have emphasized studies of PC–cholesterol membranes from the viewpoints of both constituents.

In Figure 7, the effect of cholesterol on W in DSPC and DOPC membranes at 60 °C is shown [W(with cholesterol)/W(without cholesterol)]. The steroid-type spin-labels, CSL and ASL, were used as well as T-PC, 5-SASL, and 16-SASL. In terms of the distance of the nitroxide moiety from the membrane surface, the following is the order of the average location from the surface: T-PC, CSL, 5-SASL, ASL, and 16-SASL. In DSPC membranes, the effect of cholesterol on the collision rate is the following: T-PC < CSL ≈ 5-SASL > ASL > 16-SASL (ASL > T-PC > 16-SASL). The smaller effect of cholesterol toward the middle of the bilayer may be explained by the shorter length of the cholesterol backbone than of DSPC alkyl chains as we discussed in the previous sections.

In DOPC membranes, the effect of cholesterol on W is slight for either phospholipid- or steroid-type spin-labels. This result is in clear contrast with our previous observation: incorporation of cholesterol does decrease mobilities of cholesterol-type spin-labels but not those of phospholipid-type spin-labels, which indicates that (1) CSL and ASL preferentially partition into cholesterol-rich (or oligomeric) domains and T-PC, 5-SASL, and 16-SASL into DOPC-rich domains and that (2) the cone angle (reorientational potential) of wobbling diffusion and the wobbling rotational diffusion coefficient of steroid-type spin-labels are smaller in the cholesterol-rich domains than in DOPC membranes (Kusumi et al., 1986; Merkle et al., 1987; Kusumi & Pasenkiewicz-Gierula, 1988; Pasenkiewicz-Gierula et al., 1990). These previous observations suggest that, if the cholesterol-rich domains that are large and stable existed, W in these hypothetical cholesterol-rich domains as monitored with cholesterol-type spin-labels would be small. The present data, thus, are indicative of the absence of stable large cholesterol-rich domains in DOPC–cholesterol membranes and suggest the presence of small and/or unstable cholesterol-rich (oligomeric) domains that are forming and dispersing continually. It is possible that, in the case of ASL, CuKTSM$_2$ collides with the nitroxide radical from the other side of the membrane even if ASL is incorporated in a cholesterol-rich domain. However, this type of collision cannot explain the almost total absence of cholesterol effect on W(CSL) in DOPC–cholesterol membranes. We think it likely, as we

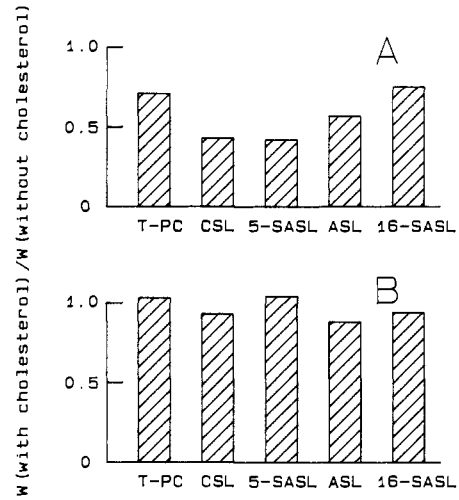

FIGURE 7: Effect of cholesterol (27.5 mol %) on W in DSPC (A) and DOPC (B) membranes as observed with steroid-type spin-labels (CSL and ASL) as well as phospholipid-type spin-labels (T-PC, 5-SASL, and 16-SASL). The ordinate shown is W(with cholesterol)/W(without cholesterol).

discuss in detail in the next section, that the lifetime of the cholesterol-rich domain is short compared with T_1 (2×10^{-6} s) of the spin-label and/or that the size of the cholesterol-rich domain is so small that the nitroxide is exposed to outside of the domain.

We would point out here that the reorientational correlation time of the nitroxide radical of lipid-type spin-labels in liquid-crystalline phase is in the range of 10^{-10}–10^{-9} s and is much shorter than T_1 and the lipid diffusion rate (exchange rate between two lattice points at 10^{-7} s). The shorter time scales of the reorientational diffusion rate, in addition to the distribution of the spin density over the radical ring structure, warrants that the probability of occurrence of Heisenberg exchange is close to 1 when the spin-label and $CuKTSM_2$ are adjacent to each other in the membrane.

General Discussion

Fluid-Phase Microimmiscibility of Unsaturated PC and Cholesterol. In our previous studies, we investigated the rotational diffusion of CSL and ASL, cholesterol analogue spin probes, and the motional freedom of phospholipid alkyl chains using SASL's. The following are the conclusions of these studies (Kusumi et al., 1986; Merkle et al., 1987; Kusumi & Pasenkiewicz-Gierula, 1988; Pasenkiewicz-Gierula et al., 1990).

(1) Cholesterol effects on saturated and unsaturated PC membranes in the fluid phase are different. In unsaturated PC–cholesterol membranes, fluid-phase immiscibility (cholesterol-rich domains) takes place. Cholesterol-rich domains in unsaturated PC membranes contain a much higher fraction of cholesterol (perhaps cholesterol oligomers) than those proposed for saturated PC membranes, as evidenced in the measurements of the nitroxide dynamics of SASL, CSL, and ASL.

(2) The major factor that causes segregation out of cholesterol in unsaturated PC membranes is probably the structural nonconformability of the bend at the cis double bond in unsaturated alkyl chains and the rigid tetracyclic fused ring of cholesterol.

(3) Cholesterol molecules tend to mix with saturated PC, inducing a more ordered state of the saturated alkyl chains. Cholesterol decreases reorientational mobilities of both cholesterol-type and phospholipid-type spin probes in saturated PC membranes.

The most important previous observation that showed anomaly in mixing of unsaturated PC and cholesterol is that in unsaturated PC–cholesterol membranes *incorporation of cholesterol decreases mobilities of cholesterol-type spin-labels but causes little effect on phospholipid-type spin probes.* These results indicate that cholesterol-rich and unsaturated PC-rich domains coexist in the membrane, the former being detected with cholesterol-type spin-labels and the latter with phospholipid-type spin-labels, and that the reorientational mobility of cholesterol is more restricted in cholesterol-rich domains. In contrast, in the present work, virtually *no effect* of cholesterol on the collision rate between the nitroxide and $CuKTSM_2$ was observed in unsaturated PC membranes *either with cholesterol-type spin-labels or with phospholipid-type spin-labels.*

If we assume on the basis of our observation above that the product of the local concentration and the local diffusion coefficient of $CuKTSM_2$ is smaller in the cholesterol-rich domains than in DOPC-rich domains, these contrasting results suggest that the lifetime of the cholesterol-rich domain (or cholesterol oligomers) is shorter than 2×10^{-6} s, T_1 of CSL and ASL in DOPC–cholesterol membranes in the liquid-crystalline phase, and/or the size of the domain is so small that all molecules in the domain may be in contact with the bulk phase (see the model displayed in Figure 8). Since the effect of cholesterol on the reorientational mobility of the cholesterol-type spin-labels has been detected, the lifetime would be in the range of 10^{-9}–10^{-7} s.

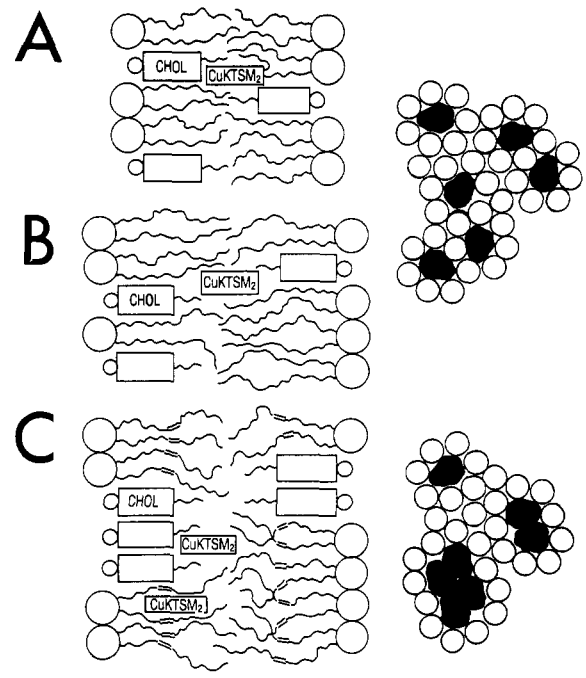

FIGURE 8: Schematic snapshot drawings of DMPC–cholesterol (A), DSPC–cholesterol (B), and DOPC–cholesterol (C) membranes with $CuKTSM_2$. The drawings on the right side show the top-view models for saturated PC–cholesterol (top) and for unsaturated PC–cholesterol (bottom) membranes. The open circles represent the alkyl chains, and the solid structures [after Martin and Yeagle (1978)] indicate cholesterol molecules.

Saxton (1982) theoretically showed that lateral diffusion of molecules in lipid bilayer membranes can be hindered by the presence of less-fluid lipid domains. The presence of large and stable cholesterol-rich domains would then decrease the lateral diffusion coefficient of PC in unsaturated PC–cholesterol membranes. However, in DOPC membranes, inclusion of 30 mol % cholesterol does not affect lateral diffusion of PC with fluorescence-labeled PC, while it decreases the lateral diffusion coefficient by a factor of 4 in the liquid-crystalline DMPC membranes (Kusumi et al., 1986). These results are, therefore, consistent with the concluson above that cholesterol-rich (oligomeric) domains are small (several lipids) and/or of short lifetime.

An alternative explanation may be that W is the same both in cholesterol-rich domains and in DOPC-rich domains. Since W in the central part of the bilayer is large (also see the next section), this idea may be good for the results with ASL. However, we do not favor this explanation because (1) CSL, as well as ASL, did not detect the effect of cholesterol on W and (2) W in the cholesterol-rich domains is very likely to be smaller because our previous observation showed that the cone angle and the wobbling rotational diffusion coefficient of CSL and ASL are much smaller in the cholesterol-rich domains than in DOPC membranes.

Since DEPC membranes are strongly affected by incorporation of cholesterol, it is concluded that the nonconformability of cis double bonds with cholesterol is the key factor for microimmiscibility of cis-unsaturated PC and cholesterol.

Deuterium NMR of deuterated lipids can provide information on events at the time scale of 10^{-5} s. A phospholipid

molecule can cover an area of more than 1000 lipids during this time by lateral diffusion in the membrane. Stockton and Smith (1976) observed an ordering effect of cholesterol on perdeuterated stearic acids in EYPC membranes using this method. Their result is in accord with our model in which the cholesterol-rich domain is forming and dispersing continually at time scales shorter than 2×10^{-6} s.

It would be of interest to relate the present results to the phase diagram of unsaturated PC–cholesterol membranes. However, such a phase diagram has not been available. The phase diagrams for DMPC–cholesterol (Recktenwald & McConnell, 1980) and DPPC–cholesterol (Ipsen et al., 1987; Vist & Davis, 1990) membranes suggest a possibility that the two-phase region extends to higher cholesterol concentrations for the unsaturated PC–cholesterol membranes.

In conclusion, the structural nonconformability between the rigid bend at the cis double bond in the alkyl chain and the rigid tetracyclic ring structure of cholesterol induces *fluid-phase microimmiscibility* in cis-unsaturated PC–cholesterol membranes (Figure 8).

Free Volume Created in the Central Part of the Bilayer Due to the Short Bulky Tetracyclic Ring of Cholesterol. In saturated PC membranes above the phase transition temperature of the host lipids, the presence of cholesterol decreased the $CuKTSM_2$ transport parameter (Figures 3, 4, and 6). The cholesterol effect is larger in DMPC membranes than in DSPC membranes. These results can be explained by the mismatch in the hydrophobic length between cholesterol (the bulky tetracyclic ring, in particular) and PC in DSPC–cholesterol membranes, which create the free volume in the central region of the membrane.

The cholesterol molecule contains three well-distinguished regions: small polar hydroxyl group, rigid platelike steroid ring, and alkyl-chain tail. When cholesterol intercalates into the membrane, its polar hydroxyl group is positioned near the middle of the glycerol backbone region of the PC molecule. It separates the PC head groups and decreases the interaction between them. Water molecules come into the free space between the separated head groups (Kusumi et al., 1986). The rigid steroid ring intercalates between hydrocarbon chains, interacts with them, and promotes the trans conformation in PC alkyl chains (if there is no double bond) from the membrane surface to a depth of about the 7th to 10th carbon (McIntosh, 1978). The rest of the PC alkyl-chain tails stay flexible. The presence of cholesterol increases the free space in the central part of the bilayer because the cross section of the steroid ring is larger than that of its hydrocarbon tail. This "fluid" region created in the center of the bilayer by the presence of cholesterol is much wider in DSPC membranes than in DMPC membranes, thereby accommodating $CuKTSM_2$ molecules more easily in DSPC membranes. These models are displayed schematically in Figure 8. In DSPC–cholesterol membranes, W profiles show a large spatial variation (Figure 8) because cholesterol increases the lipid packing in and near the head-group region while it decreases the packing in the middle of the bilayer.

Concluding Remarks. Several models have been proposed for the structure of the liquid-crystalline phase of PC–cholesterol membranes on the basis of various physicochemical studies (Engleman & Rothman, 1972; Müller-Landau & Cadenhead, 1979b; Rogers et al., 1979; Presti et al., 1982; Pink & MacDonald, 1988). Hui (1988) reported that cholesterol-rich domains show a ribbon-like structure (20–30 nm × several micrometers) as observed by the diffraction-contrast electron microscopy (40 mol % cholesterol in DPPC at 35 °C). In these types of investigations, we think it is important to pay attention to the following three factors in cholesterol-containing membranes: (1) the conformational mismatch between the rigid ring structure of cholesterol and the bend at the cis double bonds in unsaturated alkyl chains, (2) the mismatch in hydrophobic length between cholesterol and PC alkyl chains, and (3) the time–space scales of the lipid domains (cholesterol oligomers). Since the plasma membranes of eukaryotic cells contain various amounts of cholesterol and a variety of phospholipid alkyl chains, the delicate balance among PC–PC, PC–cholesterol, and cholesterol–cholesterol interactions may play important roles in formation of specialized domain structures in the plasma membranes (Bridgman & Nakajima, 1981; Karnovsky et al., 1982; Kusumi & Hyde, 1982; Kusumi et al., 1983; Palade, 1985; Castuma & Brenner, 1986).

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Registry No. DMPC, 18194-24-6; DLPC, 18194-25-7; DPPC, 63-89-8; POPC, 26853-31-6; DSPC, 816-94-4; DOPC, 4235-95-4; DEPC, 56782-46-8; $CuKTSM_2$, 53109-51-6; cholesterol, 57-88-5.

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