DYNAMIC PROPERTIES OF THE HAPTENIC SITE OF LIPID HAPTENS IN PHOSPHATIDYLCHOLINE MEMBRANES

Their Relation to the Phase Transition of the Host Lattice

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ABSTRACT The relation between the dynamic properties of the haptenic site of lipid haptens and the phase transition of the host lattice was investigated using head group spin-labeled phosphatidylethanolamines, that is, spin-label lipid haptens (Brûlet, P., and H. M. McConnell, 1976, *Proc. Natl. Acad. Sci. USA.*, 73:2977–2981; Brûlet, P., and H. M. McConnell, 1977, *Biochemistry*, 16:1209–1217). The electron spin resonance (ESR) spectra of the lipid haptens in liposomal membranes showed three narrow resonance lines, whose widths and hyperfine splitting values suggested that the haptenic site, i.e., the spin-label moiety, should be exposed in the water phase. The line width of each peak depended on the host lipid species and on the incubation temperature. A temperature study using dipalmitoylphosphatidylcholine (DPPC) liposomes showed that the dynamic properties of the haptenic site were related to the main phase transition and the subphase transition of the host lattice but not to the prephase transition. The angular amplitudes of the tumbling motion of the haptenic site were estimated using oriented multibilayer systems. The angular amplitude of dipalmitoylphosphatidyl-*N*-[[*N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-carbamoyl]-methyl]-ethanolamine in DPPC membranes was 63° at 2°C, and it increased slightly with an increase in temperature regardless of the phase transition of the host lattice. The value for egg phosphatidylcholine (PC) at 25°C was the same as for DPPC above its main phase transition temperature. Rotational correlation time analysis showed that the axial rotation of the haptenic site was preferable to the tumbling motion of the rotational axis, and the predominance depended on the phase transition, Lc ↔ Lβ' and Pβ' ↔ Lα. Elongation of the spacer arm between the haptenic site and phosphate increased the angular amplitude of the tumbling motion but reduced the effect of the host lattice. Spin-label lipid haptens with unsaturated fatty acyl chains were distributed heterogeneously in DPPC membranes, whereas those with the same fatty acyl chain as the host lattice were distributed randomly. The ESR spectrum of a lipid hapten under its prephase transition temperature showed two components, broad and narrow. This suggests that at least two different domains, a hapten-rich domain and a hapten-poor one, may coexist in membranes. ESR measurements at various temperatures suggested that the haptenic site fraction in the hapten-rich domain decreased in part during the phase transition from Lβ' to Pβ', and disappeared completely in the Lα phase. The spatial mobility and lateral diffusion of lipid haptens will be discussed in greater detail.

INTRODUCTION

The dynamic properties of cell membranes are known to play important roles in various physiological phenomena in biomembranes (Curtain, 1984). To elucidate the dynamic properties of membranes and their effects on biological activities, reconstituted membrane systems have been widely used. Liposomes containing a dinitrophenylated phospholipid or glycolipid as a hapten are susceptible to antibody-activated complement, and the interaction of the hapten with the antibody is affected by the composition of the liposomes and the incubation temperature (Kinsky, 1972). Concerning the effects of the lipid composition, there have been various reports (Brûlet and McConnell, 1976, 1977; Alving et al., 1980; Suzuki et al., 1981; Balakrishnan et al., 1982; Utsumi et al., 1984; Petrossian and Owicki, 1984; Stanton et al., 1984). Brûlet and McConnell (1976, 1977), Alving et al. (1980), Balakrishnan et al. (1982), Petrossian and Owicki (1984), and Stanton et al. (1984) suggested that the membrane composition may affect the extent of exposure and the mobility of the haptenic site, through which the haptenic activity should be regulated. In addition to the fluidity, the lateral distribution of a hapten in membranes seems to affect the antibody–antigen interaction. Some of us (Suzuki et al., 1981; Utsumi et al., 1984) and others (Delmelle et al., 1980; Tillack et al., 1982) found that some of a glycolipid

may form a cluster phase in phosphatidylcholine liposomes below its main phase transition temperature (Tc). The random distribution of a hapten may be favorable for its interaction with an antibody (Suzuki et al., 1981; Utsumi et al., 1984). These facts indicate the diversity of the effects of the bulk lipid on the recognition process in biomembranes.

Recently, the polymorphism of hydrated phospholipids has become much clearer. In the case of dipalmitoylphosphatidyl choline (DPPC) liposomes, three phase transitions, sub-, pre-, and main phase transitions, have been observed at 18°, 35°, and 41°C, respectively (Chapman, 1973; Chen et al., 1980). These conversions have been confirmed to correspond to the transformation of Lc ↔ Lβ' ↔ Pβ' ↔ Lα by x-ray analysis (Ruocco and Shipley, 1982*a*,*b*; Füldner, 1981). The presence of a hexagonal phase and lipidic particles in liposomes composed of other phospholipids was suggested (Cullis and de Kruijff, 1979; Verkleij, 1984). These mesomorphic forms also seem to coexist in biomembranes and to regulate the bioactivities of the membranes (Cullis and de Kruijff, 1979). However, little is known about the influence of membrane polymorphism on the spatial motion of a haptenic site or on the lateral distribution of a lipid hapten in liposomes.

The spin-label technique is very sensitive and useful for determination of the motion in the time-range of 10^{-11}–10^{-7} s (Hyde and Dalton, 1979). McConnell and his colleagues (Brûlet and McConnell, 1976, 1977; Brûlet et al., 1977) developed excellent spin-label lipid haptens, which have 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) at the polar head group of phosphatidylethanolamine, and they found that TEMPO itself can function as a hapten like a dinitrophenyl or a trinitrophenyl group. It is reported that the immunological responses of liposomes containing the spin-label lipid hapten are quite similar to those of liposomes containing glycolipids or other haptens. Therefore these spin-label lipid haptens should be suitable for detail studies on the effects of the bulk lipid on the physical properties of lipid haptens.

In the present work, using both liposomal systems and oriented multibilayer systems, we investigated in great detail the dynamic properties of the spin-label lipid haptens related to the polymorphism of phospholipid membranes. To elucidate the relation of the lateral distribution of the haptens in lipid membranes with their polymorphism, spin-label lipid haptens with unsaturated fatty acyl chains were newly synthesized and the cluster formation of spin-label lipid haptens was also investigated.

MATERIALS AND METHODS

Lipids

Egg phosphatidylcholine (egg PC) and egg phosphatidylethanolamine (egg PE) were isolated from egg yolk by chromatography on aluminum oxide (Woelm, W-200 neutral) and silicic acid (Mallinckrodt, Inc., Paris, KY) in our laboratory. DPPC, dipalmitoylphosphatidylethanolamine (DPPE), cholesterol, and dicetylphosphate (DCP) were purchased from Sigma Chemical Co. (St. Louis, MO). The purity of lipids was checked by thin-layer chromatography (Merck Kieselgel 60, Darmstadt, West Germany; 0.25-mm thick, $CH_3Cl/CH_3OH/H_2O$ [65:25:4, vol/vol/vol]). All lipids were separately stored as a chloroform solution at −20°C. The concentrations of phospholipids were determined as inorganic phosphate by the method of Gerlach and Deuticke (1963).

Spin-Label Lipid Haptens

Four different spin-label lipid haptens, whose structures and abbreviations are shown in Fig. 1, were prepared by the method of Brûlet and McConnell (1976, 1977) with a slight modification. Dipalmitoylphosphatidyl-*N*-[[*N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-carbamoyl]-methyl]-ethanolamine (SL6-DPPA) and dipalmitoylphosphatidyl-*N*-[4-[*N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-carbamoyl]-butyryl]-ethanolamine (SL9-DPPA) were synthesized through reaction of DPPE with iodoacetamide spin-label and glutaramide spin-label, respectively, at 50°C. Egg yolk phosphatidyl-*N*-[[*N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-carbamoyl]-methyl]-ethanolamine (SL6-eggPA) and egg yolk phosphatidyl-*N*-[4-[*N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-carbamoyl]-butyryl]-ethanolamine (SL9-eggPA) were prepared through reaction with egg PE at room temperature. Purifications were performed by column chromatography (Bio-Sil A, 200–400 mesh; Bio-Rad Laboratories, Richmond, CA) followed by a preparative thin-layer chromatography (Merck Kieselgel 60, 0.25-mm thick). All the spin-label lipid haptens gave a single spot on thin-layer chromatography. The phosphate contents of SL6-DPPA and SL9-DPPA were 2.922 (calc.) and 2.470 (obsd.), and 2.736 (calc.) and 2.016 (obsd.), respectively. That the observed values are lower than the calculated ones might be due to contamination by silicic acid during the preparative thin-layer chromatography. All the spin-label lipid haptens thus obtained were dissolved in chloroform to the same concentration (0.4 mM as phosphorus) and stocked at −20°C. The stock solution of each spin-label lipid hapten showed the same signal intensity in the electron spin resonance (ESR) spectrum, suggesting that there were no appreciable differences in spin density among the four spin-label lipid hapten preparations.

Preparation of Liposomes and Oriented Multibilayers

Multilamellar liposomes composed of PC (1 μmol), DCP (0.1 μmol), and various amounts of a spin-label lipid hapten were prepared in 100 μl of phosphate-buffered saline (PBS; 5 mM sodium phosphate and 0.85 % NaCl; pH 7.4) as described previously (Utsumi et al., 1976).

Oriented multibilayers were prepared as described previously (Naka-

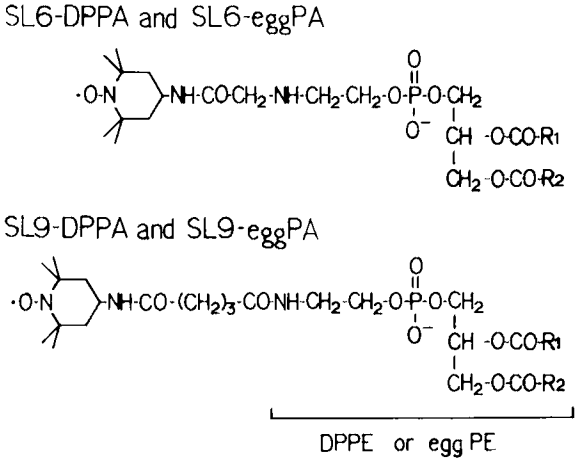

FIGURE 1 Structures of spin-label lipid haptens. The lipid regions of SL6-DPPA and SL9-DPPA were derived from DPPE, whereas those of SL6-eggPA and SL9-eggPA were from egg PE.

gawa et al., 1976). PC, DCP, and a spin-label lipid hapten were mixed at the molar ratio of 1:0.1:0.016 in chloroform, and then the solution was transferred onto a glass plate (20 × 3 mm). After evaporation of the solvent, a very small amount of PBS was dropped onto the lipid film. The preparation was covered with another glass plate to prevent water loss during the measurement.

ESR Measurement

20 μl of the liposomal suspensions was taken up into a disposable micropipette (Drummond Scientific Co., Broomall, PA). The pipette was sealed at one end with a Hemat Sealer, and then kept at a constant position in the ESR cavity. On the other hand, a pair of glass plates sandwiching an oriented multibilayers was placed in a ESR sample tube, which was then inserted into the ESR cavity. The plates were oriented so that the magnetic field was perpendicular or parallel to them. ESR spectra were recorded with a JEOL JES-PE-lX spectrometer (X-band, 100-kHz field modulation, 2.0-gauss modulation width) equipped with a temperature controller.

Schreier et al. (1978) proposed that the line width (ΔHms1) of an individual (hyperfine) line is given by following equation:

$$\Delta\mathrm{Hms1}(m) = \mathrm{A} + \mathrm{B}m = \mathrm{C}m^2,$$

where m is the z component of the nitrogen nuclear spin quantum number (for ^{14}N, with a nuclear spin $I = 1$, the low field, central, and high field lines correspond to $m = +1, 0$, and -1, respectively). Term A includes contributions other than motional. Terms B and C can be defined as functions of the peak-to-peak line width of the central line, ΔHms1(0), and the amplitudes of the mth lines, h(m). From terms B and C, the rotational correlation times, τ_B and τ_C, were calculated using the following equations reported by Schreier et al. (1978):

$$\tau_B = 0.635\Delta\mathrm{Hms1}(0)\,[\mathrm{h}(0)/\mathrm{h}(-1)]^{1/2} - [\mathrm{h}(0)/\mathrm{h}(+1)]^{1/2}\,(\mathrm{ns})$$

$$\tau_C = 0.595\Delta\mathrm{Hms1}(0)\,[\mathrm{h}(0)/\mathrm{h}(+1)]^{1/2} + [\mathrm{h}(0)/\mathrm{h}(-1)]^{1/2} - 2\,(\mathrm{ns})$$

The apparent rotational correlation time, τ_R, was also calculated using the following equation (Keith et al., 1970; Utsumi et al., 1985):

$$\tau_R = 0.65\,\Delta\mathrm{Hms1}(0)\,[\mathrm{h}(0)/\mathrm{h}(-1)]^{1/2} - 1\,(\mathrm{ns}).$$

The order parameter was calculated with the following equation (Hubbell and McConnell, 1971):

$$S = \frac{\mathrm{A}_\parallel - \mathrm{A}_\perp}{(\mathrm{A}yy + \mathrm{A}zz)/2 - \mathrm{A}xx} \cdot \frac{ao'}{ao},$$

where $ao' = (\mathrm{A}xx + \mathrm{A}yy + \mathrm{A}zz)/3$ and $ao = (\mathrm{A}_\perp + 2\mathrm{A}_\parallel)/3$, and $\mathrm{A}xx$, $\mathrm{A}yy$, and $\mathrm{A}zz$ are 5.2, 5.2, and 31 gauss, respectively, which are the principal hyperfine splittings of 1-oxyl-2,2,6,6-tetramethyl-4-piperidone (Griffith et al., 1965).

RESULTS

ESR Spectra of Spin-Label Lipid Haptens with Saturated Fatty Acyl Chains in Lecithin Liposomes

The spin-label lipid haptens with saturated fatty acyl chains, SL6-DPPA and SL9-DPPA, were incorporated into egg PC- or DPPC-liposomes, and then the ESR spectra of the four preparations were measured at 25°C (Fig. 2). All the spectra consisted of three narrow lines, suggesting that the spin-label moiety in the molecules

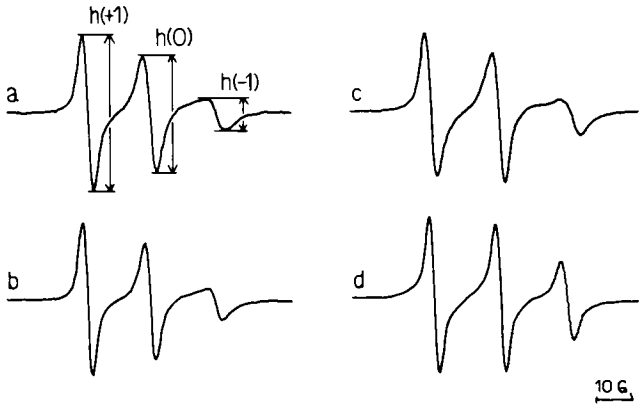

FIGURE 2 Typical ESR spectra of SL6- and SL9-DPPA in egg PC or DPPC liposomes at 25°C. 1.43 mol% of a spin-label lipid hapten was incorporated into liposomes composed of phosphatidylcholine and DCP (molar ratio 1:0.1). The combinations of phosphatidylcholine and spin-label lipid haptens were (*a*) egg PC–SL6-DPPA, (*b*) egg PC–SL9-DPPA, (*c*) DPPC–SL6-DPPA, and (*d*) DPPC–SL9-DPPA. The ESR spectra were measured at 9.25 GHz and 2.0 gauss modulation width.

exhibits a rapid tumbling motion in the liposomal membranes. The peak height ratio among the three resonance lines depended on the bulk lipid species. For egg PC liposomes, the peak height decreased gradually with an increase in magnetic field intensity, while for DPPC liposomes no appreciable difference was observed between the low field peak height (h(+1)) and the central one (h(0)). In addition, SL6-DPPA gave asymmetric resonance lines with respect to the baseline in DPPC liposomes.

Egg PC and DPPC liposomes are known to be in the liquid crystalline state (Lα) and the gel state (Lβ′) at 25°C, respectively. To determine whether or not the state of the host lattice is responsible for the difference in the spectra between egg PC and DPPC liposomes, ESR spectra of SL6-DPPA in DPPC liposomes were recorded at various temperatures (Fig. 3). The ESR spectrum of SL6-DPPA at 45°C (Fig. 3 *a*) was almost the same as that in the case of egg PC liposomes at 25°C (Fig. 2 *a*), suggesting that the dynamic properties of the bulk lipid should influence the peak height ratio. Very interestingly, the spectrum of SL6-DPPA at 2°C was quite different from the other spectra (Fig. 3 *c*). The spectral change showed reversibility with incubation temperature. The central peak in the spectrum at 2°C was the highest, and each resonance line at 2°C was broader than the corresponding line at 25° or 45°C. This suggested that the spin-label moiety in SL6-DPPA should be relatively restricted in motion at 2°C.

The peak height ratio (h(+1)/h(0)) of SL6-DPPA was plotted against the incubation temperature (Fig. 4). The ratio increased with an increase in temperature up to 43°C, and then decreased. A discontinuous point around 20°C and a steep increase between 40° and 43°C were observed. These temperatures are close to the subphase transition temperature (Tsub) and Tc of DPPC membranes (Chap-

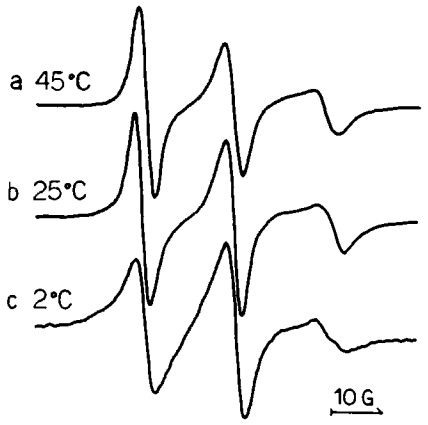

FIGURE 3 ESR spectra of SL6-DPPA in DPPC liposomes at various temperatures. The method of preparation of liposomes and the ESR measurement conditions are given in the legend to Fig. 2. The temperatures of the ESR measurements are indicated on the left sides of the spectra.

man, 1973; Chen et al., 1980). Such a change in h(+1)/h(0) around Tc was also observed for dimyristoylphosphatidylcholine (DMPC) liposomes, in accord with the results of Humphries (1980), but the decrease above Tc was not so abrupt as that in DPPC liposomes (data not shown). A similar temperature dependence was also observed in the ESR spectra of SL9-DPPA, but the thermotropic change was much smaller than that in the case of SL6-DPPA (Fig. 4). This small change may suggest that the longer spacer length may lessen the influence of the host lattice. Despite the great distance between the spin-label moiety and the membrane surface, these results imply that the physical properties of the host lattice should affect the mode and the rate of motion of the spin-label moiety.

The unexpected spectra shown in Fig. 2 imply that a dominant rotational axis should exist. To elucidate the motional mode of the spin-label moiety, evaluation of two different rotational correlation times, τ_B and τ_C, as proposed by Schreier et al. (1978), is very useful. For a rapid isotropic motion, τ_B should equal τ_C. However, anisotropic rotation about the x-axis or the other axes should make τ_B larger or smaller than τ_C, respectively. The ratio of the two (τ_B/τ_C) might be a suitable parameter for the degree of anisotropy of the motion and the direction of the preferred axis. Thus τ_B and τ_C of SL6-DPPA in liposomes were calculated from the three peak heights, h(+1), h(0), and h(−1), and the line width of the central peak, ΔHms1(0). In Fig. 5, τ_B/τ_C ratios are plotted against temperature. At 2°C, τ_B/τ_C was slightly smaller than 1.0. But with an increase in temperature, τ_B/τ_C increased, and above 15°C, it became larger than 1.0, indicating that the spin-label moiety of SL6-DPPA rotates predominantly about the x-axis above 15°C. A discontinuous point around 20°C and a steep increase between 40° and 43°C were observed, coinciding with the behavior of the peak height ratio, h(+1)/h(0), although τ_B/τ_C was almost constant above 43°C (refer to Fig. 4). The apparent rotational correlation time (τ_R) of the spin-label moiety was also plotted against temperature (Fig. 5). τ_R decreased with an increase in temperature. It is noteworthy that above 43°C, τ_R

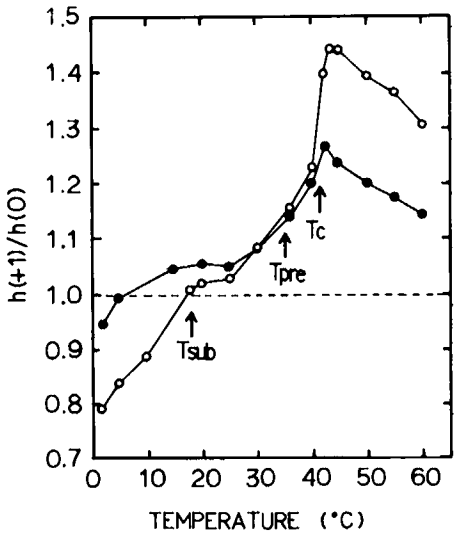

FIGURE 4 Temperature dependency of the peak height ratio (h(+1)/h(0)) of the ESR spectra for SL6- and SL9-DPPA in DPPC liposomes. 1.43 mol% of SL6-DPPA or SL9-DPPA was incorporated into liposomes composed of DPPC and DCP (molar ratio 1:0.1), and then ESR spectra were measured at various temperatures. The peak heights (h(+1) and h(0)) are as indicated in Fig. 2. The peak height ratios, h(+1)/h(0), of SL6-DPPA (○) and SL9-DPPA (●) were plotted against temperature. The three arrows in the figure indicate the main (Tc), pre- (Tpre), and subphase transition temperatures (Tsub) of DPPC membranes, respectively.

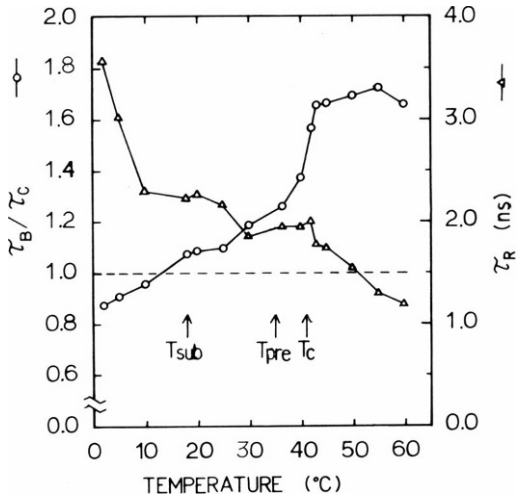

FIGURE 5 Temperature dependency of the rotational correlation time ratio (τ_B/τ_C) and the apparent rotational correlation time (τ_R) of SL6-DPPA incorporated into DPPC liposomes. Two rotational correlation times, τ_B and τ_C, were calculated from the ESR spectra for SL6-DPPA shown in Fig. 4, as described under Materials and Methods, and the ratio of the two, τ_B/τ_C, was plotted against temperature (○). The three arrows in the figure are explained in the legend to Fig. 4. The apparent rotational correlation time, τ_R (Δ), was calculated as described under Materials and Methods.

decreased remarkably despite the constant τ_B/τ_C. τ_R should be correlated to the rate of the tumbling motion of the spin-label moiety when the molecular motion is isotropic. The remarkable decrease in τ_R indicates an increase in the tumbling motion of the spin-label moiety above 43°C.

ESR Spectra of Spin-Label Lipid Haptens in Oriented Multibilayers

The spin-label moiety of a spin-label lipid hapten should show two motions, axial rotation and rapid tumbling. The peak height ratio among the three resonance lines should be affected by the angular amplitude of the tumbling motion in addition to the rate of the axial rotation and the tumbling motion. To determine the angular amplitude, ESR spectra of oriented multibilayer systems were recorded with the external magnetic field parallel and perpendicular to the plane of the membrane surface. Typical ESR spectra of SL6-DPPA at 45° and 2°C are shown in Fig. 6. The spectra for the perpendicular orientation seem to be contaminated by the component for the parallel orientation, causing the appearance of two signals in the higher magnetic field. This contamination was often observed in the oriented multibilayer systems (Smith and Butler, 1976), possibly due to an amount of spin-label molecules, on the edge of the membranes, that is not negligible. Thus an apparent hyperfine splitting was defined with the half distance in gauss measured between the low field line and the high field line from the point where each intersects the baseline. $A_\perp$ and $A_\parallel$ denote the splittings observed for the two principal orientations of the bilayers to the magnetic field. Using $A_\perp$ and $A_\parallel$, the order

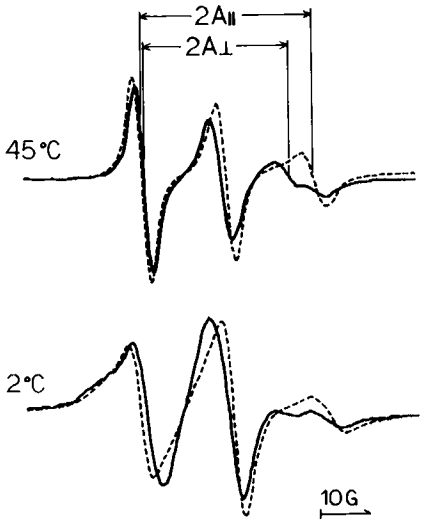

FIGURE 6 Typical ESR spectra for SL6-DPPA in oriented multibilayers. 1.43 mol% of SL6-DPPA was incorporated into oriented multibilayers composed of DPPC and DCP (molar ratio 1:0.1). Spectra were measured with the external magnetic field perpendicular (—) and parallel (---) to the surface of the multibilayers. $A_\perp$ and $A_\parallel$ denote the perpendicular and parallel components of the hyperfine splitting, respectively. The temperatures of ESR measurements are indicated on the left sides of the spectra.

parameters and angular amplitudes were calculated assuming a restricted random walk model for the spin-label moiety (Jost et al., 1971) (Table I). The angular amplitude of SL6-DPPA in DPPC bilayers increased with an increase in temperature from 63° at 2°C to 75° at 45°C. The value at 45°C is the same within experimental error as that for egg PC at 25°C. The angular amplitudes of SL9-DPPA were, however, close to "90°" despite membrane composition and temperature. These results indicate that for spin-label lipid haptens with shorter spacer arms, membrane fluidity influences the angular amplitude of the spin-label moiety, but that a spacer of sufficient length can cancel out the effect of the fluidity.

The peak height ratios, $h(+1)/h(0)$, of the oriented multibilayers are also shown in Table I. For both spin-label lipid haptens, the ratio was highest in DPPC bilayers at 45°C, followed by in egg PC ones at 25°C, DPPC ones at 25°C, and DPPC ones at 2°C, but the difference was smaller for SL9-DPPA than SL6-DPPA. For SL6-DPPA the ratio for the perpendicular spectrum was higher than that for the parallel one, while no appreciable difference was observed between the perpendicular and parallel spectra for SL9-DPPA. This distinction between SL6-DPPA and SL9-DPPA may be due to the difference in the angular amplitude of the tumbling motion. The peak height ratios for the oriented multibilayer systems were in agreement with those for liposomal systems. No relation was observed between the angular amplitude and the peak height ratio. Therefore, it may be concluded that the variation in the peak height ratio in Fig. 4 is due to a change in the rate of the axial rotation and/or the tumbling motion of the spin-label moiety, and not to the angular amplitude.

ESR Spectra of Spin-Label Lipid Haptens with Unsaturated Fatty Acyl Chains in Lecithin Liposomes

The spin-label lipid haptens with unsaturated fatty acyl chains, SL6-eggPA and SL9-eggPA, were incorporated into egg PC- or DPPC-liposomes, and then the ESR spectra of the resulting four preparations were measured at 25°C (Fig. 7). Fig. 7, *a* and *b* shows the spectra of SL6-eggPA and SL9-eggPA in egg PC liposomes, respectively. These spectra were almost the same as those of the corresponding analogues with saturated fatty acyl chains (refer to Fig. 2). In contrast, the ESR spectra in DPPC liposomes (Fig. 7, *c* and *d*) exhibited an interesting feature. On comparison of the spectra for DPPC liposomes with those for egg PC liposomes, a broader component was discernible as a shoulder on the side of each of the three narrow lines. The presence of the broader components was seen more clearly in the ESR spectra for DPPC liposomes containing various amounts of SL9-eggPA (Fig. 8). The relative intensity of the broader resonance lines ($\Delta Hms1(0)$ = 7.4 gauss) increased with an increase in SL9-eggPA concentration with respect to DPPC, while the

TABLE I
ESR PARAMETERS OF SPIN-LABEL LIPID HAPTENS IN ORIENTED MULTIBILAYERS

Spin-label lipid haptens	Host lipid	Temp.	Order parameter*	Angular amplitude‡	h(+1)/h(0)		
					Oriented multibilayers		Liposomes§
		°C		*degrees*	⊥	∥	
SL6-DPPA	DPPC	2	0.33	63.0	0.79	0.67	0.76
	DPPC	25	0.31	64.4	1.07	1.02	1.03
	DPPC	45	0.16	75.2	1.52	1.35	1.45
	Egg PC	25	0.15	76.0	1.29	1.26	1.30
SL9-DPPA	DPPC	2	0.02	87.8	0.86	0.94	0.97
	DPPC	25	0.03	86.7	1.11	1.10	1.06
	DPPC	45	0.03	86.7	1.30	1.33	1.24
	Egg PC	25	0.03	86.7	1.20	1.24	1.22

*The order parameter was calculated using the hyperfine splitting values for oriented multibilayers, as defined in Fig. 5, as described under Materials and Methods.
‡The angular amplitude of the haptenic site was calculated as described in the text.
§The peak height ratio obtained for the corresponding liposomes is given in the right column for reference.

relative intensity of the narrow ones ($\Delta Hms1(0) = 3.7$ gauss) remained almost constant. This dose-responsiveness and symmetric shape of the broader lines indicate that the broader lines in the case of SL9-eggPA were caused by spin-exchange broadening due to the formation of the spin-label rich domain (Humphries, 1980) and not by the immobilization of the spin-label moiety. Therefore, it may be concluded that a spin-label lipid hapten with unsaturated fatty acyl chains forms a hapten-rich domain in DPPC membranes at 25°C.

Temperature Dependence of Hapten-rich Domain Formation

To determine the amount of a spin-label lipid hapten in the hapten-rich domain, the ESR spectra of SL9-eggPA in DPPC liposomes were compared with those in the case of SL9-DPPA, because SL9-DPPA has been suggested by Humphries (1980) to be distributed homogeneously in DPPC liposomal membranes (Fig. 9). At 25°C, the broad component at the side of each signal of SL9-DPPA was clearly confirmed, and the signal intensity of SL9-eggPA was about one-third that of SL9-DPPA. The contribution of the broad component to the signal intensity was much smaller than that of the narrow component, since the height of a peak is inversely proportional to the square of the line width of the ESR signal. Therefore, in a spectrum composed of narrow and broad components, the total signal intensity should decrease with an increase in the ratio of the broad component to the narrow one. Comparison of the signal intensity of SL9-eggPA to that of SL9-DPPA showed that about two-thirds of SL9-eggPA may be present as a hapten-rich domain at 25°C in DPPC membranes. At 45°C the spectrum of SL9-eggPA coincided completely with that of SL9-DPPA, suggesting that the hapten-rich domain of SL9-eggPA disappears at 45°C and that SL9-eggPA is dispersed homogeneously in DPPC membranes at 45°C.

To elucidate the relation of the phase transition of the

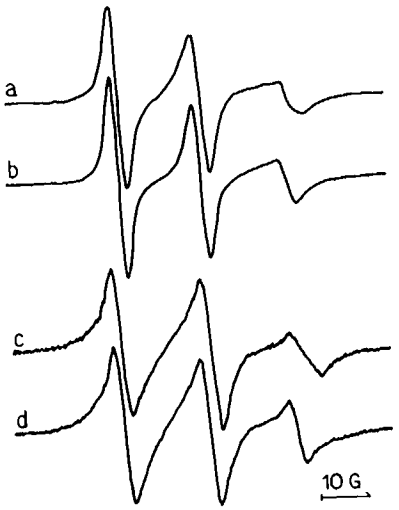

FIGURE 7 Typical ESR spectra for SL6- and SL9-eggPA in egg PC or DPPC liposomes at 25°C. The method of preparation of liposomes and the ESR measurement conditions are given in the legend to Fig. 2. The combinations of phosphatidylcholine and spin-label lipid haptens were (*a*) egg PC–SL6-eggPA, (*b*) egg PC–SL9-eggPA, (*c*) DPPC–SL6-eggPA, and (*d*) DPPC–SL9-eggPA.

FIGURE 8 ESR spectra for DPPC liposomes containing various amounts of SL9-eggPA. SL9-eggPA was incorporated into liposomes composed of DPPC and DCP (molar ratio 1:0.1) in the amounts of 5.5 mol% (—), 18.9 mol% (-·-), and 48.2 mol% (---). The concentration of DPPC was 10 mM. The relative amplifications for the spectra were 5, 2, and 1 for 5.5, 18.9, and 48.2 mol%, respectively. The spectra were measured at 25°C as described in the legend to Fig. 2.

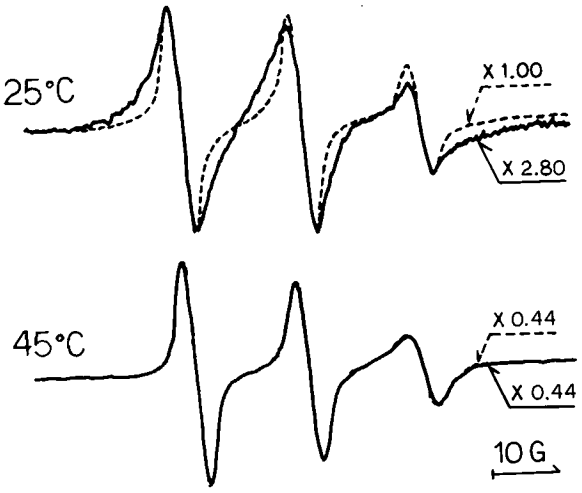

FIGURE 9 Comparison of the ESR spectra for SL9-eggPA and SL9-DPPA in DPPC liposomes at different temperatures. ESR spectra of DPPC liposomes containing 1.43 mol% of SL9-eggPA (—) were recorded at 25° and 45°C, and then compared with the spectra with the same concentration of SL9-DPPA (---). The method of preparation of liposomes and the ESR measurement conditions are given in the legend to Fig. 2. The relative amplifications are indicated on the right sides of the spectra.

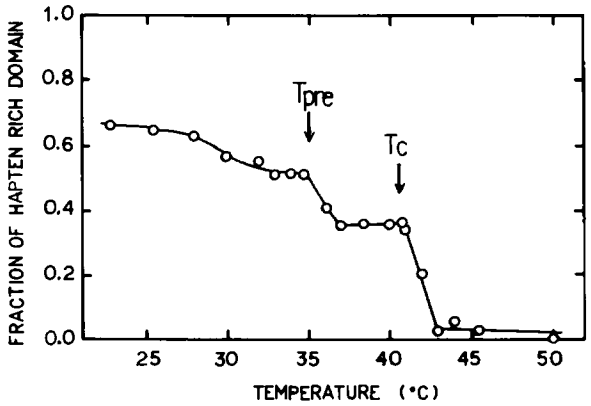

FIGURE 10 Temperature dependency of the SL9-eggPA fraction in the hapten-rich domain. DPPC liposomes containing either SL9-DPPA or SL9-eggPA were prepared as described in the legend to Fig. 2, and ESR spectra were recorded at various temperatures. The SL9-eggPA fraction in the hapten-rich domain was calculated using the equation given in the text. The two arrows in the figure indicate the main (Tc) and prephase transition temperatures (Tpre) of DPPC membranes, respectively.

bulk lipid to the formation of a hapten-rich domain, the spectra for DPPC liposomes containing 1.4 mol% of either SL9-eggPA or SL9-DPPA were measured at various temperatures. As an apparent indication of the amount of SL9-eggPA in the hapten-rich domain, the following parameter was evaluated:

$$\left[\begin{array}{l}\text{Apparent}\\ \text{fraction}\\ \text{of SL9-eggPa}\\ \text{in the hapten-}\\ \text{rich domain}\end{array}\right]_{t°C} = \frac{[h(0)_{\text{SL9-DPPA}}]_{t°C} - [h(0)_{\text{SL9-eggPA}}]_{t°C}}{[h(0)_{\text{SL9-DPPA}}]_{t°C}}.$$

Here h(0) is the normalized central peak height with respect to the peak height of the internal standard (Mn^{2+}). Fig. 10 shows the apparent fraction of SL9-eggPA in the hapten-rich domain as a function of the incubation temperature. As the incubation temperature increased, two steep decreases were observed around 35° and 41°C. The hapten-rich domain decreased to two-thirds at 35°C and disappeared completely above 43°C. The prephase transition temperature (Tpre) and Tc of DPPC liposomes have been reported to be 35° and 41°C, respectively (Chapman, 1973), indicating that the phase transition of DPPC membranes from Lβ' to Pβ' may in part cause lateral diffusion of the spin-label lipid hapten besides the main phase transition from Pβ' to Lα. However, we should not neglect the possibility that a rippled structure in the Pβ' phase may contribute to the reduction of the apparent hapten-rich domain fraction.

DISCUSSION

In this study, we investigated the spatial mobility of the haptenic site and the topographic distribution of lipid haptens in connection with the thermotropic phase transition of liposomal membranes, using a spin-label technique. The four lipid haptens used in the present study carry a nitroxide group as the haptenic site in the polar head of a phospholipid. The physical properties of liposomal membranes were found to affect both the motional state and the distribution of a spin-label lipid hapten, but the modes of the effects were quite different from each other.

Some reports have suggested that haptenic sites of some artificial lipid haptens like a dinitrophenyl group or a fluorescein group may be buried in the hydrophobic region of membranes or associated with the membrane head group region if the membranes are in a fluid state (Balakrishnan et al., 1982; Staton et al., 1984). However, in our case, we can neglect the possibility that a nitroxide moiety of the spin-label hapten might be embedded into the hydrocarbon core of membranes, since the hyperfine splitting values of SL9-DPPA in liposomes (17.15 G for egg PC-liposomes and 17.25 G for DPPC-liposomes) were very close to that of glutaramide spin-label in water (17.29 G) which is a haptenic site of SL9-DPPA with a spacer region, and much larger than those of SL9-DPPA and glutaramide spin-label in chloroform (16.10 G and 16.08 G, respectively).

The spectra of spin-label lipid haptens in membranes were rather anisotropic, and the low field peak was the narrowest of the three sharp lines. This characteristic coincided with the results obtained by Smith's group (Hsia et al., 1970; Schreier-Muccillo et al., 1973) using the stearamide spin-label and by McConnell's group (Brûlet et al., 1977; Brûlet and McConnell, 1977; Humphries and McConnell, 1977; Humphries, 1980) using the same spin-label as us. According to Griffith and Jost (1976), axial rotation about the x-axis should result in the spectrum with the narrowest low field peak, while rotation about the other

axes lead to the central peak being the narrowest. Therefore the observation of a characteristic spectrum in which the low field peak is the narrowest (Figs. 2 and 3, *a* and *b*) indicates that in the present case the spin-label moiety of each spin-label hapten predominantly undergoes axial rotation about the *x*-axis in membranes.

The ESR spectrum of a spin-label lipid hapten in DPPC membranes slightly changed with the incubation temperature, and the ratio of the low field peak height (h(+1)) to the central peak height (h(0)) showed a discontinuous point around 20°C and a remarkable change between 40° and 43°C (Fig. 4). The peak height ratio among the three resonance lines should be influenced by (*a*) the rate of axial rotation of the spin-label moiety, (*b*) the rate of tumbling motion of the rotational axis, and (*c*) the angular amplitude of the tumbling motion. ESR spectra of the oriented multibilayers (Fig. 6 and Table I) revealed that there was a little ordering of the rotational axis in SL6-DPPA but not in SL9-DPPA. There was no relation between the peak height ratio and the angular amplitude, suggesting that in the case of a spin-label lipid hapten, the angular amplitude of the rotational axis should not affect the peak height ratio. Therefore, the change in the peak height ratio indicates a change in the rate of axial rotation of the spin-label moiety and/or of the tumbling motion of the rotational axis.

Calculation of the rotational correlation time ratio (τ_B/τ_C) should be used for estimating the contributions of the axial rotation and the tumbling motion. τ_B/τ_C of SL6-DPPA was >1.0 above 15°C (Fig. 5), indicating that the rate of *x*-axis rotation of the spin-label moiety is more predominant than that of the tumbling motion of the rotational axis, in agreement with the above discussion. τ_B/τ_C also showed a discontinuous point around 20°C and a remarkable change between 40° and 43°C (Fig. 5). These findings imply that the anisotropy in the motion of haptenic site changed markedly around 20°C and at 40°–43°C.

Recent studies (Chen et al., 1980; Füldner, 1981; Ruocco and Shipley, 1982*a*,*b*) demonstrated that DPPC liposomes undergo the following three transitions:

$$\mathrm{Lc} \overset{\text{Tsub (18°–20°C)}}{\rightleftharpoons} \mathrm{L}_{\beta'} \overset{\text{Tpre (35°C)}}{\rightleftharpoons} \mathrm{P}_{\beta'} \overset{\text{Tc (41°C)}}{\rightleftharpoons} \mathrm{L}_{\alpha'}.$$

The temperatures at which a discontinuous point and a remarkable change in the peak height ratio and the rotational correlation time ratio were (Figs. 4 and 5) close to Tsub and Tc, respectively.

The Lβ' phase was characterized by ordered hydrocarbon chains of the lipid and by a surface area per hydrocarbon chain of 19.5 Å^2, whereas in the Lc phase there was a more order, dehydrated and probably three-dimensional lattice, in which the chains were more closely packed (19.0 Å^2/chain), and neighboring lipids might be dispersed zigzag with one another (Ruocco and Shipley, 1982*b*). SL6-DPPA gave a broad isotropic ESR spectrum below 20°C, and the apparent rotational correlation time, τ_R, was extremely great below 10°C, indicating immobilization of the spin-label moiety. The rigid packed structure in the Lc phase could cause immobilization of the spin-label moiety of a lipid hapten. It should, however, be noted that the occurrence of the Lc phase required the cooling of the liposomes at low temperature (0°C) for several days (Chen et al., 1980; Ruocco and Shipley, 1982*a*,*b*; Füldner, 1981). Here a sample was cooled for only a few minutes in the temperature-controlled cavity of an ESR spectrometer. Thus the Lc phase might not have been formed completely in our case. Indeed, ^{31}P-NMR observation revealed the requirement of cooling at 5°C for a long period for the production of a "slow motion" signal (Füldner, 1981). But a rapid modification of the hydrocarbon chain packing mode has been reported in an x-ray diffraction study from 0 to 1.5 h at −2°C (Ruocco and Shipley, 1982*b*). In addition, using a saturation-transfer ESR technique, Marsh (1980) also observed a rapid decrease in the rotational motion of phosphatidylcholine spin-labeled at the fatty acyl chain in DPPC membranes on cooling below 25°C. These facts suggest that some partial rearrangement of lipid molecules may occur rapidly below 20°C, leading to a change from the Lβ' to the Lc phase in DPPC membranes, and such a change could cause the discontinuous point around 20°C observed in Figs. 4 and 5. The steep increase in τ_B/τ_C for SL6-DPPA at 41°–43°C indicates that the preference for the *x*-axis rotation of the spin-label moiety to the tumbling motion of the rotational axis increased significantly around this temperature. As described above, 41°C is Tc of DPPC membranes and the main phase transition is attributed to the conversion between Pβ' and Lα. The Pβ' phase was a rippled gel phase, while the Lα phase was a liquid-crystalline phase (Ververgaert et al., 1973; Janiak et al., 1976; Luna and McConnell, 1977). The rotational diffusion of lipid molecules in the Lα phase was reported to be significantly faster than that in the Pβ' phase (Marsh, 1980). Therefore the Pβ' ↔ Lα conversion may contribute to the remarkable change in h(+1)/h(0) seen in our work.

Above 43°C, h(+1)/h(0), τ_B/τ_C and τ_R showed different temperature dependencies. h(+1)/h(0) and τ_R decreased with an increase in temperature, while τ_B/τ_C remained almost constant. Here we cannot evaluate the molecular motion exactly on the basis of τ_R, since the molecular motion of the haptenic site of spin-label lipid haptens was somewhat anisotropic. However, if the term "apparent free tumbling" is permitted, the decrease of τ_R indicates that the rate of apparent free tumbling may increase above 43°C. The almost constant value of τ_B/τ_C suggests that the degree of anisotropy may not increase above 43°C. But at this moment we should not neglect the possibility that τ_B/τ_C might be the upper limit value under these experimental conditions.

Rubenstein et al. (1980) reported the following observation, when the same spin-probe as SL6-DPPA was incorpo-

rated into DMPC liposomes. As the temperature increased, the normalized low field peak height increased above 14°C with a steep change around 24°C, which corresponds to Tc of DMPC membranes. They interpreted the increase as reflecting an increase in the head group motional freedom involving lateral diffusion. Here, a rise in the lateral diffusion should broaden all three resonance lines equally (Scandella et al., 1972; Humphries, 1980). In the present study, however, the rate of the increase in h(+1) was not parallel with that of the increase in h(0). Therefore, the contribution of the lateral diffusion to the peak height ratio should be negligible in our system. We analyzed the head group motional freedom into the following three modes: the rate of axial rotation, the rate of the tumbling motion, and the angular amplitude of the tumbling motion. The temperature dependence of the normalized low field peak height above Tc was the inverse of that of h(+1)/h(0), while below Tc the two were well correlated with each other. This discrepancy should be due to the remarkable increase in h(0) above Tc. The increase in the normalized low field peak height seems to be rather close to the decrease in τ_R. Considering the τ_B/τ_C above Tc, the discrepancy between the normalized low field peak height and h(+1)/h(0) might be due to some difference in the contributions of the tumbling motion to the two parameters.

Using ganglioside and globoside probes that were spin-labeled at their carbohydrate regions, Grant and his colleagues (Lee et al., 1980; Peters et al., 1982) observed remarkable breaks in Arrhenius plots of the apparent correlation times around Tpre of the host lattice, but not at Tc. This discrepancy between their data and ours may be due to a difference in the chemical structures of the spin-probes used and/or in the motional modes of the probes, since the spin-label moieties of their glycolipid probes may undergo pseudo-isotropic rotation (Sharom and Grant, 1978).

The presence of an unsaturated fatty acyl chain in a spin-label lipid hapten caused a phase separation in the DPPC membranes containing the spin-label lipid hapten. The occurrence of a phase separation in liposomal membranes has been reported for various systems. In 1973, Shimshick and McConnell suggested for the first time the occurrence of a phase separation in DPPC-DMPC and DPPC-DPPE mixed liposomes, on the basis of the results of TEMPO spin-label partition. This phase separation has been confirmed in differential scanning calorimetric studies (Mabrey and Sturtevant, 1976). These observations suggested that liposomes composed of phospholipids with different acyl chains separate into different phases. Another type of phase separation has been observed in lecithin liposomes containing glycolipids, on the use of various techniques such as differential scanning calorimetry, ESR, and freeze-fracture electron microscopy (Sillerud et al., 1979; Suzuki et al., 1981; Utsumi et al., 1984; Delmelle et al., 1980; Tillack et al., 1982). In this case, the difference in the polar head group may contribute to the formation of a glycolipid-rich domain in the liposomal membranes. In the present study, spin-label lipid haptens with the same acyl chain as the host lattice did not show any phase separation. These results indicate that attachment of the TEMPO moiety should not be the driving force for the phase separation.

Aggregates or clusters of spin-probes with a spin-label moiety at the fatty acyl chain gave a broad singlet line in the phase-separated state (Utsumi et al., 1978; Suzuki et al., 1981; Kanda et al., 1982; Utsumi et al., 1984; Manabe et al., 1986). In the present study, spin-label lipid haptens with unsaturated fatty acyl chains did not give a broad singlet resonance line but triplet lines. As reported by Delmelle et al. (1980), a nitroxide radical attached to a polar head group of a lipid may tend to give triplet resonance lines in the cluster phase.

The apparent hapten-rich domain fraction depended on the incubation temperature. The cluster phase has been reported to occur in the gel phase and to disappear above Tc of the host lattice (Delmelle et al., 1980; Tillack et al., 1982; Utsumi et al., 1984). In the present study, spin-label lipid haptens were also dispersed completely above 41°C, which is Tc of DPPC membranes. But it is noteworthy that a distinguishable decrease was observed around 35°C in the hapten-rich domain fraction (Fig. 10). This temperature is close to Tpre of DPPC liposomes (Chapman, 1973). Recently, McConnell and his colleague have extensively studied the relation between heterogeneous distribution of membrane components and a phase transition of host lattice using fluorescently labeled phospholipid (McConnell et al., 1984; Seul et al., 1985; Tamm and McConnell, 1985). They succeeded in a direct observation of the fluorescent cluster with epi-fluorescence microscopy using planar DPPC layers on alkylated-solid supports and suggested that both Tpre and Tc of the host lattice may affect the lateral heterogeneity in membranes. However, in temperature dependence of arrangement of labeled lipids, there seems to be some difference between their observation and ours. This discrepancy may arise from the difference in host lattice. In the present study, of course, we should not neglect the possibility that only the spin-exchange interaction becomes weak without any increase in lateral diffusion, because the collision frequency of the nitroxide moiety might be less at the top of the ripple structure.

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