Temperature Change of the Ripple Structure in Fully Hydrated Dimyristoylphosphatidylcholine/Cholesterol Multibilayers

Sinzi Matuoka,* Satoru Kato,‡ and Ichiro Hatta§

*Department of Physics, School of Medicine, Sapporo Medical University, Sapporo 060; ‡Department of Physics, Kwansei Gakuin University, Nishinomiya 662; and §Department of Applied Physics, School of Engineering, Nagoya University, Nagoya 464–01, Japan

ABSTRACT The ripple structure was studied as a function of temperature in fully hydrated dimyristoylphosphatidylcholine (DMPC)/cholesterol multibilayers using synchrotron x-ray small-angle diffraction and freeze-fracture electron microscopy. In the presence of cholesterol, the ripple structure appears below the pretransition temperature of pure DMPC multibilayers. In this temperature range the ripple periodicity is relatively large (25–30 nm) and rapidly decreases with increasing temperature. In this region, defined as region I, we observed coexistence of the $P_{\beta'}$ phase and the $L_{\beta'}$ phase. The large ripple periodicity is caused by the formation of the $P_{\beta'}$ phase region in which cholesterol is concentrated and the $L_{\beta'}$ phase region from which cholesterol is excluded. An increase in ripple periodicity also takes place in the narrow temperature range just below the main transition temperature. We define this temperature region as region III, where the ripple periodicity increases dramatically toward the main transition temperature. In region II, between regions I and III, the ripple periodicity decreases gradually with temperature. This behavior is quite similar to that of pure DMPC. Temperature-versus-ripple periodicity curves are parallel among pure DMPC and DMPCs with various cholesterol contents. We explain this behavior in terms of a model proposed by other workers.

INTRODUCTION

The ripple structure appearing in the $P_{\beta'}$ phase of phosphatidylcholine (PC) multibilayer vesicle is characterized by periodic undulated bilayer structure. The periodicity of the ripple structure lies within the range of 12–16 nm in pure PC as reported by x-ray diffraction (Tardieu et al., 1973; Janiak et al., 1976, 1979; Stamatoff et al., 1982; Wack and Webb, 1989), neutron diffraction (Mortensen et al., 1988), freeze-fracture electron microscopy (Luna and McConnell, 1977; Copeland and McConnell, 1980; Hicks et al., 1987; Gebhardt et al., 1977; Rüppel and Sackmann, 1983; Zasadzinski and Schneider, 1987) scanning tunneling microscopy (Zasadzinski et al., 1988). Many theoretical models have been proposed for explaining this undulation in the $P_{\beta'}$ phase (Falkovitz et al., 1982; Marder et al., 1984; Doniach, 1979;Pearce and Scott, 1982; Hawton and Keeler, 1986; McCullough and Scott, 1990). We recently found, in a small-angle x-ray diffraction study (Matuoka et al., 1990), that the ripple periodicity changes as a function of temperature in fully hydrated DMPC multibilayer vesicles. In that report we suggested that the spatial modulation of order parameter in the ripple structure is essential to the temperature change of the ripple periodicity by the Landau/de Gennes theory (de Gennes, 1974). The periodic distribution of the order parameter has been reported by nuclear magnetic resonance and electron spin resonance studies, which give clear evidence of the existence of fluid- and gel-like domains in the $P_{\beta'}$ phase (Wittebort et al., 1981; Schneider et al., 1983; Tsuchida and Hatta, 1988).

It is known that the ripple periodicity expands with increasing cholesterol content, and the ripple structure disappears at ~20 mol % cholesterol (Mortensen et al., 1988; Luna and McConnell, 1977; Copeland and McConnell, 1980). To explain this fact, Copeland and McConnell (1980) have proposed a model in which a ripple ridge consisting of pure DMPC lies between two flat strips containing 20 mol % cholesterol; this results in a periodic structure where two regions of the ripple strip and the flat strip appear alternatively. Mortensen et al. (1988) have reported the temperature dependence of the ripple periodicity in dehydrated and predeuterated DMPC. Based on the cholesterol dependence on the ripple periodicity at a lower temperature region where the so-called $P_{\beta''}$ phase appears, they have proposed a model in which the stripes of cholesterol-rich domains enter regularly. These models imply that cholesterol molecules distribute heterogeneously in the ripple structure. However, it is unknown how these domain structures change with temperature.

It has been reported in differential scanning calorimetry (DSC) studies that the excess heat capacity pretransition is suppressed by adding more than 3.6 mol % (Estep et al., 1978) or 6 mol % (Mabrey et al., 1978; Vist and Davis, 1990; McMullen et al., 1993) cholesterol to PC. However, the ripple structure remains even at 16 mol % (Copeland and McConnell, 1980), or 15 mol % (Hicks et al., 1987) cholesterol content from the experiments of freeze-fracture and at 14 mol % by neutron diffraction (Mortensen et al., 1988). However, the relationship between the appearance of a pretransition in DSC thermograms and ripple formation is still unknown. A study of the ripple periodicity near the pretransition temperature may answer this question.

Received for publication 23 November 1993 and in final form 21 April 1994.

Address reprint requests to Sinzi Matuoka, Department of Physics, Sapporo Medical University, S.1, W.17, Chuo-ku, Sapporo 060, Japan. Tel.:81 11 611 211 (ext. 2211); Fax: 81 11 612 5861; E-mail: matuoka@sapmed.ac.jp.

Abbreviations used in this article: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSC, differential scanning calorimetry.

0006-3495/94/08/728/09 $2.00

In this paper, we make clear how the ripple structure in fully hydrated DMPC/cholesterol changes as a function of temperature; we use synchrotron x-ray small-angle diffraction and freeze-fracture electron microscopy to do this. From the characteristic of the temperature dependence of ripple periodicity, we define three temperature regions in fully hydrated DMPC/cholesterol multibilayers as drawn schematically in Fig. 1.

MATERIALS AND METHODS

DMPC (1,2-dimyristoyl-L-phosphatidylcholine) was purchased from Sigma Chemical Co. (St. Louis, MO) and also from Avanti Polar Lipids, Inc. (Birmingham, AL). These chemicals were used without further purification because there is no other spot except for phosphatidylcholine in thin-layer chromatography. Cholesterol was purchased from Sigma Chemical Co. Multilamellar vesicles were prepared as previously reported (Matuoka et al., 1990). To prepare samples, an appropriate amount of cholesterol dissolved in spectroscopic-grade chloroform (Katayama Chemical Co., Osaka, Japan) was added to chloroform solution of DMPC. After evaporation under a stream of dry nitrogen gas, these DMPC/cholesterol mixtures were put under vacuum for 12–14 h to remove any traces of chloroform and then hydrated with distilled water at ~40°C for 3 h. For x-ray diffraction measurements these samples were concentrated by centrifugation at 9000 *g* for 30 min to a lipid content of 45–52 wt %. The lipid contents (%) were determined by the following equation: $100 \times W_L/W_T$, where W_L represents lipid weight that was measured before the addition of water to dried lipid, and W_T represents total sample weight (lipid + water), which was measured after hydration and centrifugation. The lipid contents that we used were under the excess water condition (Tardieu et al., 1973; Inoko and Mitsui, 1978; Ruocco and Shipley, 1982).

Synchrotron x-ray small-angle diffraction measurements were performed at Station BL-15A at Photon Factory, National Laboratory for High Energy Physics (Tsukuba, Japan). The design of this small-angle x-ray diffractometer has been described elsewhere in detail (Amemiya et al., 1983). The x-ray beam was horizontally focused and monochromatized (λ = 0.1504 nm). Diffraction profiles were detected with a one-dimensional position-sensitive proportional counter (Rigaku, Tokyo, Japan). The sample-to-detector distance was in the range 1000–1300 mm, where the resolution for scattering angle ($\Delta 2\theta$) was 0.016–0.021°.

Samples were mounted in a hole in an aluminum plate of 1.5 or 2 mm thickness and sealed by Mylar or Kapton films (thickness: 12.5 μm) as described in detail elsewhere (Tenchov et al., 1989). The plate was fixed in a brass holder and immersed directly in water supplied from a temperature-constant water bath (RCS-20D, Messgeräte-Werk Lauda, FRG). The temperature was monitored with a chromel-alumel thermocouple put just immediately adjacent to the sample.

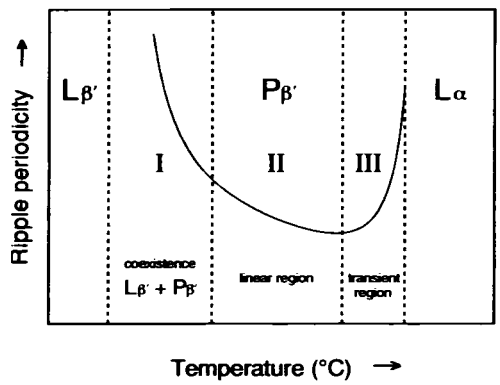

FIGURE 1 Schematic drawing for temperature dependence of ripple periodicity in fully hydrated DMPC in the presence of cholesterol. Regions I, II, and III are classified by the characteristics of temperature dependence of ripple periodicity (see text).

The temperature of the sample was linearly scanned at a rate of 0.25° C/min (Fig. 2) or changed stepwise with an average scanning rate of 0.2–0.3°C/min (Figs. 3 and 5). At every scanning experiment the sample was replaced by a fresh sample to avoid the effects of radiation damage. The maximum cumulative radiation dose was 2 Mrad, so that defects produced by the x rays would be lower than 2%; radiation at a higher level than 10 Mrad yields the breakdown of 10% DPPC according to Caffrey (1984).

The photographs of ripple structures at various temperatures were taken as follows. In the cooling mode, the temperatures of samples were decreased to the objective temperatures after incubation at 50°C for 4–5 h, whereas in heating these were increased from 2.5°C. After storage at these temperatures for 2–3 h, the samples were transferred on copper stages (thickness, 1 mm; diameter, 3 mm). Then the samples on the stages were quenched by plunging them into liquid Freon 22 at −160°C, just above its freezing point. Although this operation was carried out within 1–2 min., we paid attention to the evaporation of water, which could violate the excess water condition of the sample. For this reason, we used a low-lipid concentration of 3 wt % by taking into account water evaporation. The above operation could guarantee the excess water condition, because there was no difference in the results when we used the initial lipid concentration up to 15 wt %. Fracture was carried out at −180°C by a freeze-fracture and etching system (FD-3, Eiko Engineering, Mito, Ibaragi, Japan). Then platinum/carbon shadowing was done from the angle of 30–40°, and vacuum evaporation of carbon from the angle of 90° was performed. The image of these replicas was taken using an electron microscope (JEOL 100C, Nihon Denshi, Tokyo, Japan). Its magnification, calibrated by a carbon grating with 2160 lines/1 mm, was 20000–30000×. The ripple periodicity obtained with the freeze-fracture experiment agreed with the (01) spacing, which ensured that the measurements were made without tilting the replicas.

RESULTS

X-ray small-angle diffraction profiles of fully hydrated DMPC with 1.7 mol %, 3.0 mol %, and 4.0 mol % cholesterol in the cooling run are drawn in Figs. 2 *a*, *b*, and *c*, respectively. These profiles were recorded during cooling from 25°C to 15–18°C at a rate of 0.25°C/min. The peak located from ~0.05 nm^{-1} to ~0.08 nm^{-1} is the (01) peak corresponding to the ripple periodicity from ~13 to ~20 nm. In cooling from 25°C the onsets of the (01) peaks at 0 mol % (pure DMPC), 1.7 mol %, 3.0 mol %, and 4.0 mol % cholesterol began at 23.8°C, 23.7°C, 23.5°C, and 23.3°C, respectively. The main transition temperatures lay within 0.125°C above these temperatures because the profiles were recorded every 0.125°C. Just below the main transition temperature, the (01) peak corresponding to the ripple periodicity appears at ~0.05 nm^{-1} (20 nm), and its intensity is strong. At slightly lower temperatures than the above, the (01) peak rapidly shifts to the higher angle region. The shift of the (01) peak gradually continues down to 22.3°C, 21.9°C, and 20.7°C in the samples with 1.7 mol %, 3.0 mol %, and 4.0 mol % cholesterol, respectively. With further cooling the (01) peak moderately moves toward the smaller angle again, and its intensity becomes smaller in any cholesterol contents.

Because the (01) intensity became weak at low temperatures as presented in Fig. 2, we cooled the samples stepwise and measured the x-ray diffraction profiles at fixed temperatures to improve the signal-to-noise ratio. The results in DMPC with 2.0 mol % cholesterol appear in Fig. 3 *a*. The

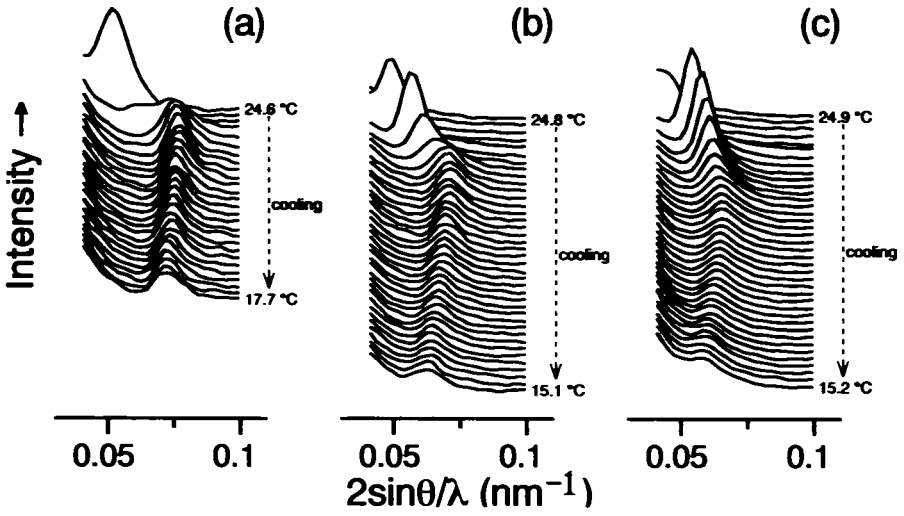

FIGURE 2 The (01) peak profiles displayed with the interval of ~0.25°C. The results are for fully hydrated DMPC with (*a*) 1.7 mol %, (*b*) 3.0 mol % and (*c*) 4.0 mol % cholesterol taken by slow temperature cooling experiments of 0.25°C/min from 25°C to 15°C or to 18°C.

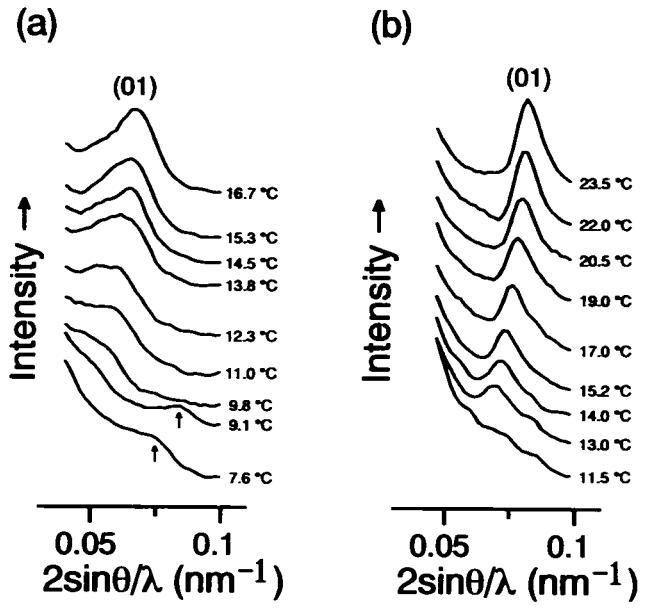

FIGURE 3 X-ray diffraction profiles of fully hydrated DMPC (*a*) with 2.0 mol % cholesterol and (*b*) without cholesterol. The samples were cooled from 30°C to 7.6°C in (a) and from 27°C to 11.5°C in (b) stepwise. Average cooling rates in (*a*) and (*b*) were 0.14°C/min and 0.23°C/min, respectively. The arrows in (*a*) represent the (02) peak appearing at low temperatures.

(01) peak was still observed at 9.8°C, which is below the pretransition temperature of pure DMPC, although the (01) peak is hardly detectable at 11.5°C in pure DMPC as shown in Fig. 3 *b*. In DMPC with 2.0 mol % cholesterol at 9.1 and 7.6°C a diffraction peak appears at 0.072 nm^{-1} (13.9 nm), and 0.084 nm^{-1} (11.9 nm), respectively (see arrows in Fig. 3 *a*). Also in DMPC with 3.0 mol % cholesterol, a peak located at 13.7 nm was observed at 7.5°C (data not shown). Near this temperature (at 7.2°C) the ripple periodicity obtained with freeze-fracture electron microscopy was 24.6 ± 1.0 nm. Taking the difference between electron microscopy and x-ray diffraction into consideration, the above value of ripple periodicity is approximately twice the spacings obtained from x-ray diffraction. These facts indicate that the peaks observed near 11–14 nm are the (02) peaks, although the (01) peaks, of which spacing should be twice that of the above peaks, were not observable.

In an overall temperature range, we estimated the (01) spacings from the peak position of maximum intensity of the (01) or (02) peak. These spacings were nearly equal to the ripple periodicity observed with freeze-fracture electron microscopy. In Fig. 4, the (01) spacings of DMPC with and without cholesterol in cooling are plotted as a function of temperature. Based on the temperature dependence of the ripple periodicity, we classified temperature regions I, II, and III from low to high temperatures (see Figs. 1 and 4). Region I ranges below the pretransition temperature (10.9°C, Parente and Lentz, 1984) of pure DMPC multibilayers. In

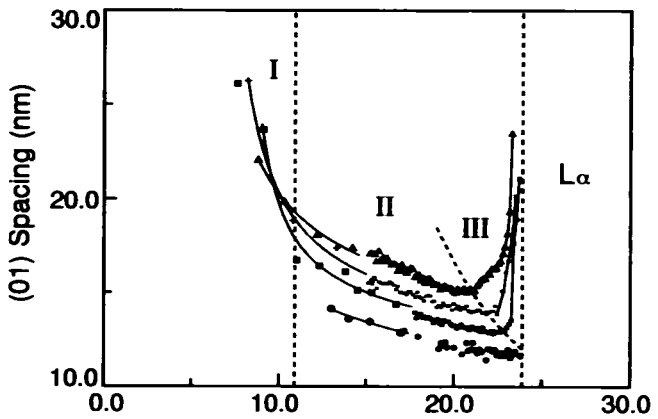

FIGURE 4 Temperature dependence of the (01) spacing of DMPC multibilayer. The measurements were performed in the two temperature regions. In the lower temperature region for 0 mol % (●), 2.0 mol % (■), 3.0 mol % (+), and 4.6 mol % (▲) cholesterol and in the higher temperature region 0 mol % (○), 1.7 mol % (□), 3.0 mol % (×) and 4.0 mol % (△) cholesterol in higher temperature region. The former series are obtained in the stepwise cooling experiment, and the latter is taken by slow temperature scanning experiments of 0.25°C/min. The dashed straight lines represent the pretransition temperature of 10.9°C and the main transition temperature of 23.9°C in DMPC without cholesterol as described by Parente and Lentz (1984). The dashed curved line was drawn for viewing the boundary between regions II and III.

this region the ripple periodicity in DMPC with cholesterol decreases markedly with increasing temperature. The boundary between regions I and II lies at the temperature where the slope of the (01) spacing-versus-temperature curve changed from steep to gentle with increasing temperature. As seen in Fig. 4, the boundary is not sharp and, furthermore, depends on cholesterol content. Therefore, we use region I to indicate the distinct region where the temperature dependence of the (01) spacing is different from that in region II. Region II lies in the middle temperature region, where the curve of temperature-versus-ripple periodicity runs parallel with that of the pure DMPC (see Fig. 4). Region III ranges close to the main transition temperature (23.9°C, Parente and Lentz, 1984), where the ripple periodicity increases rapidly toward the main transition. Region III is also used for distinguishing the particular region where the temperature dependence of the (01) spacing is different from that in region II. Such behavior of the temperature dependence of the (01) spacing has not been pointed out so far. In this paper, we adopt on trial the above three regions for convenience of consideration.

In all three regions, the temperature dependence of the (01) spacing in heating was basically similar to that in cooling (data not shown). However, we observed hysteresis at pretransition in pure DMPC. The ripple structure in pure DMPC appears at a temperature of 13.3–15.8°C in heating (Matuoka et al., 1990), which is somewhat higher than that in cooling (11.5–13.0°C) as seen in Fig. 3 *b*. At the main transition temperature of pure DMPC, we observed weak temperature hysteresis, i.e., the main transition temperature in heating was slightly higher than that in cooling. The extent of this temperature hysteresis is 0.1–0.2°C both in the absence and the presence of cholesterol.

Region I

Below 11°C, even lower than the pretransition temperature of pure DMPC, the (01) peak remains in the presence of cholesterol indicating the existence of the ripple structure. However, in this region the coexistence of two lamellar structures is observed. Figs. 5 *a*, *b*, and *c* present x-ray small-angle diffraction profiles of fully hydrated DMPC multibilayer vesicles containing 2.0, 3.0, and 4.6 mol % cholesterol in cooling from 28°C to 2°C, respectively. It is clear that the lamellar (10) and (20) peaks of DMPC with 4.6 mol % cholesterol split into two peaks below 10°C (see Fig. 5 *c*). Although not so obvious as in the case of 4.6 mol %, it is clear that the (10) and the (20) peaks in DMPC with 2.0 and 3.0 mol % cholesterol are composed of two diffraction peaks. Thus, we can deconvolute the x-ray diffraction profiles in 0.1–0.2 nm^{-1} into two Lorentzians by the Simplex method (Okumura, 1986). We denote these two peaks located near 0.13 nm^{-1} and 0.15 nm^{-1} denoted by peaks 1 and 2, respectively. In Figs. 6 *a*, *c*, and *e*, the (10) spacings of peaks 1 and 2 in region I together with the other temperature region are displayed for 2.0, 3.0, and 4.6 mol % cholesterol, respectively. The normalized proportion of peak 2 ($I_2/(I_1+I_2)$) in region I is plotted in Figs. 6 *b*, *d*, and *f*, where I_1 and I_2

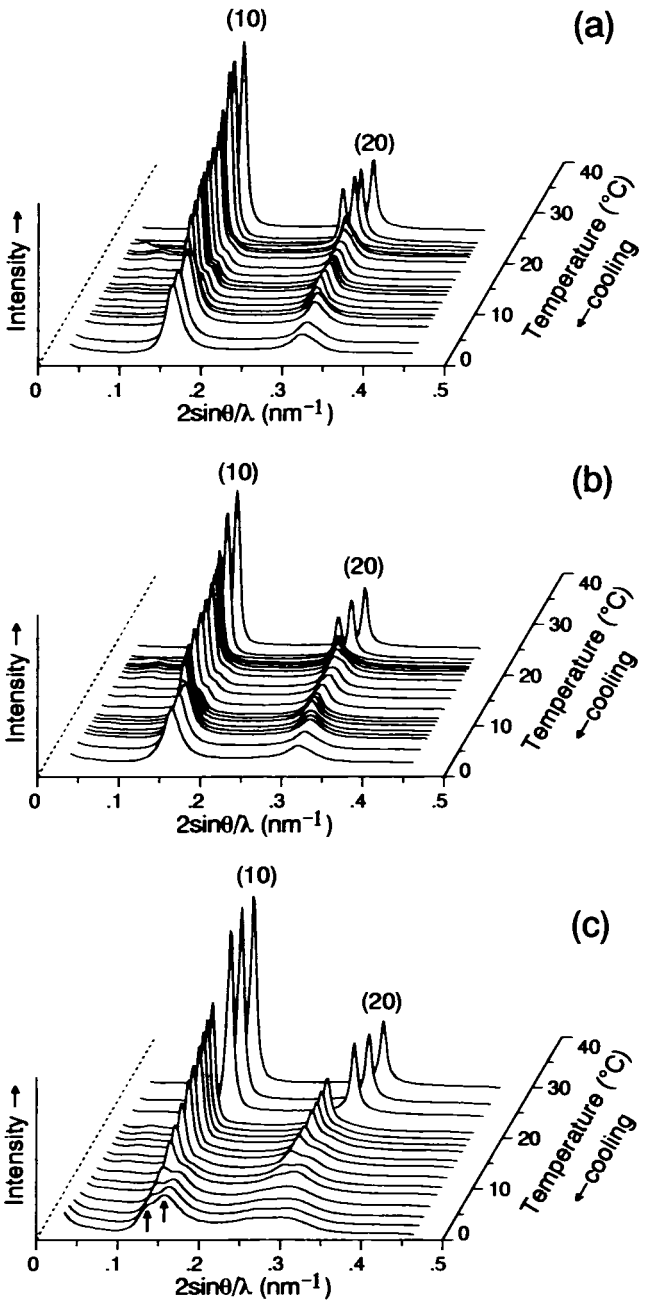

FIGURE 5 Small-angle x-ray diffraction profiles of fully hydrated DMPC multibilayer with cholesterol of (*a*) 2.0 mol %, (*b*) 3.0 mol %, and (*c*) 4.6 mol %. Temperatures of the samples were cooled from 30°C to 2°C stepwise giving an average cooling rate of 0.2–0.3°C/min. The (10) and (20) peaks caused by the lamellar periodicity are observed for the $L_{\beta'}$, $P_{\beta'}$ and L_α phases. The arrows in (*c*) represent the two (10) peaks.

represent the integrated intensity of peaks 1 and 2, respectively. This exhibits that the normalized proportion of peak 2 increases with decreasing temperature (see Figs. 6 *b*, *d*, and *f*), consistent with the fact that the origin of peak 2 is the $L_{\beta'}$ phase. Thus, we conclude that the coexistence of the $L_{\beta'}$ phase and the $P_{\beta'}$ phase occurs in region I.

Rand et al. (1980) have reported a similar coexistence of the two lamellar (10) spacings in DPPC with cholesterol. They have concluded that one of the (10) peaks located at the

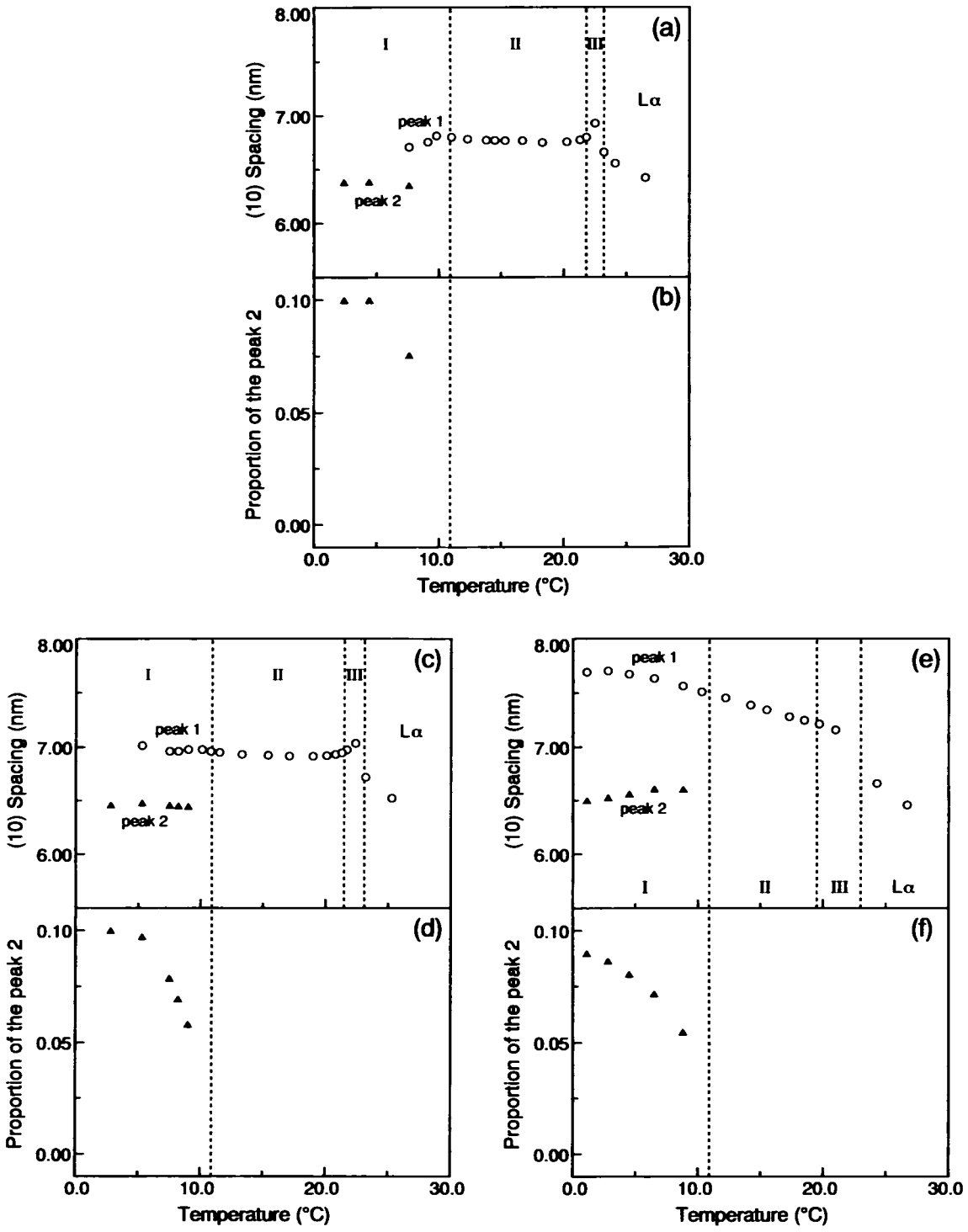

FIGURE 6 Temperature dependence of the (10) spacing of DMPC multibilayer with cholesterol of (*a*) 2.0 mol %, (*c*) 3.0 mol %, and (*e*) 4.6 mol %. The smaller angle spacing (peak 1) and the wider angle spacing (peak 2) are denoted as ○ and ▲, respectively, where peak profiles were separated into two peaks of Lorenzian by the Simplex method. The proportion of integrated intensity of peak 2 ($= I_2/(I_1 + I_2)$) in the presence of cholesterol of 2.0 mol %, 3.0 mol %, and 4.6 mol % are plotted in (*b*), (*d*), and (*f*), respectively, where I_1 and I_2 denote the integrated intensity of peaks 1 and 2, respectively.

higher angle region (shorter spacing) is caused by to the pure DPPC domains, because its spacing is just the same as that of pure DPPC in the $L_{\beta'}$ phase. This higher angle (10) peak in DPPC-cholesterol system corresponds to peak 2 in the present results of DMPC-cholesterol. Thus, it is possible that peak 2 may come from the domains of pure DMPC in the $L_{\beta'}$ phase on the analogy of the DPPC-cholesterol system. However, the spacings of peak 2 at 2.0 mol %, 3.0 mol %, and 4.6 mol % cholesterol content are 6.36 nm, 6.42 nm and 6.42 nm, respectively, which are slightly longer than that of pure

DMPC in the $L_{\beta'}$ phase (6.02 nm). Thus, the domain of peak 2 is modified by incorporating a small amount of cholesterol, but it is close to the pure DMPC domain. If this is the case, the domain of peak 1 includes a large amount of cholesterol. The curve of temperature versus spacing of peak 1 in region I is smoothly connected from that of region II as seen in Figs. 6 *a*, *c*, and *e*, suggesting that the domain of peak 1 has a characteristic of the $P_{\beta'}$ phase. Thus, the modified $L_{\beta'}$ phase domain including a small amount of cholesterol and the modified $P_{\beta'}$ domain including a large amount of cholesterol coexist in region I.

In DMPC with 2.0 mol % cholesterol, the coexistence of the $L_{\beta'}$ phase and the $P_{\beta'}$ phase was observed only at a data point of 7.6°C (see Figs. 6 *a* and *b*), whereas in the higher concentration of 3.0 mol % (Figs. 6 *c* and *d*) and 4.6 mol % (Figs. 6 *e* and *f*), the coexistence occurs between 5 and 9°C and 0 and 9°C, respectively. This indicates that the temperature range where the $L_{\beta'}$ phase and the $P_{\beta'}$ phase coexist is wider at higher cholesterol contents. This is consistent with the fact that the pretransition peak of DSC broadens as increasing cholesterol contents (McMullen et al., 1993; Koynova et al., 1985).

Below 9–10°C the (01) peak intensity becomes weak, its spacing shifts to a much smaller angle region, and the peak smears out. Such behavior of the ripple periodicity was measured also from freeze-fracture electron micrographs. Typical electron micrographs of fully hydrated DMPC with 3.0 mol % cholesterol are displayed in Fig. 7. The ripple periodicity at 5.7°C and 9.3°C reaches 31.1 nm (Fig. 7 *c*) and 20.6 nm (Fig. 7 *b*), respectively, showing the formation of ripple structure with large periodicity. At 22.0°C, the ripple periodicity at Fig. 7 *a* is 14.3 nm, which is in good agreement with the results reported previously (Copeland and McConnell, 1980). The ripple periodicities obtained with freeze-fracture electron microscopy in fully hydrated DMPC with 3.0 mol % and 4.8 mol % cholesterol are plotted as a function of temperature with filled circle in Figs. 8 *a* and *b*, respectively. The results of 3.0 mol % and 4.6 mol % cholesterol obtained with x-ray diffraction are plotted in the same figures with open circles. The (01) spacings are in good agreement with the ripple periodicity obtained with freeze-fracture experiments. This suggests that the lattice parameter γ (the interior angle of the two-dimensional monoclinic unit cell) lies near 90°, because the (01) spacing is represented by $b \cdot \sin\gamma$, where b is the longer side of the unit cell and equals the ripple periodicity (Tardieu et al., 1973; Janiak et al., 1976).

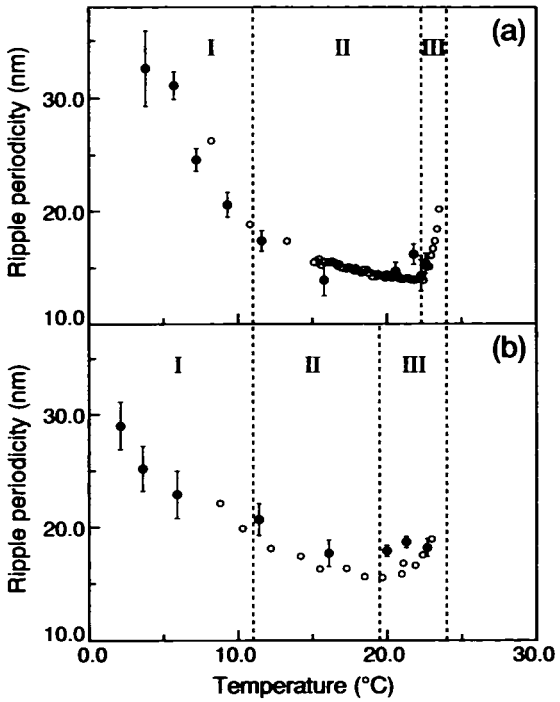

FIGURE 8 Temperature dependence of the ripple periodicity (●) obtained from freeze-fracture electron microscopy and the (01) spacing (○) with x-ray diffraction in fully hydrated DMPC with (*a*) 3.0 mol % and (*b*) 4.8 mol % (freeze-fracture) and 4.6 mol % (x-ray diffraction) cholesterol.

In region I, the slope of temperature-versus-ripple periodicity curve becomes more gentle with increasing cholesterol contents as shown in the results of 3.0 mol % and 4.8 mol % cholesterol of Fig. 8. As a result, at ~5°C, the ripple periodicity decreases with higher cholesterol content; ripple periodicity is 30 nm for 3 mol % and 23 nm for 4.9 mol %.

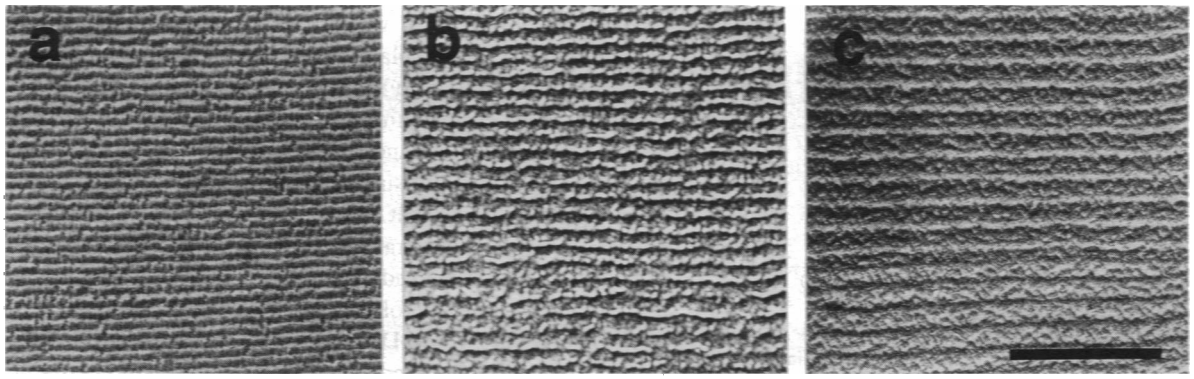

FIGURE 7 Freeze-fracture electron micrographs of multilamellar vesicles of fully hydrated DMPC with 3.0 mol % cholesterol quenched from (*a*) 22.0°C, (*b*) 9.3°C and (*c*) 5.7°C. The bar in (c) is 200 nm long.

This tendency is opposite that in region II, where ripple periodicity increases at higher cholesterol content.

Region II

In region II the (01) spacing decreases with increasing temperature rather moderately compared with region I as in Fig. 4. The upper temperature boundary of region II lies at 21.9°C and 20.7°C for 3.0 mol % and 4.6 mol % cholesterol, respectively. The upper boundary of region II shifts toward the lower temperatures with increasing cholesterol content.

Fig. 9 presents cholesterol dependence of (01) spacing at 15.2°C, 17.8°C, and 20.0°C in region II. Below 15.2°C, we did not have enough data to elucidate the cholesterol dependence of the (01) spacing. The regression lines obtained with the least-squares method exhibit that the (01) spacings linearly increase with cholesterol content.

Region III

Just below the main transition temperature, the ripple periodicity reached 20–25 nm in the presence of cholesterol (see Fig. 4). In cooling in region III the ripple periodicity decreased rapidly and achieved a minimum value at 22.5°C, 22.0°C, and 21.0°C in 1.7 mol %, 3.0 mol %, and 4.0 mol % cholesterol content, respectively. We define the temperature giving the minimum ripple periodicity as the lower end of region III. Thus, region III moved to the lower temperature with increasing cholesterol contents. The ripple periodicity in pure DMPC does not increase just below the main transition, indicating absence of region III. However, even in 1.0 mol % cholesterol, region III appears in the narrow temperature range (data not shown).

DISCUSSION

The characteristic of region II is that the temperature-versus-ripple periodicity in the presence of cholesterol run nearly parallel with that in pure DMPC, where ripple periodicity decreases with increasing temperature as reported previously (Matuoka et al., 1990). We explain this observation by using the model proposed by Copeland and McConnell (1980) where a ripple strip of pure DMPC microdomain corresponding to the width of a single ripple ridge lies between two flat strips of cholesterol-rich region (20 mol % cholesterol). They have proposed this model based on the fact that the ripple periodicity linearly increases with cholesterol content (Copeland and McConnell, 1980). Suppose that the strip of pure DMPC microdomain has the same temperature dependence as pure DMPC, and the width of flat strip of cholesterol-rich region has little temperature dependence. The temperature dependence of the total ripple periodicity may be mainly caused by that of the strips of pure DMPC microdomains. If this is the case, the difference in ripple periodicity among DMPCs with various cholesterol contents results from the width of the flat strip of cholesterol-rich domain. As a result, this difference is constant at any temperature in region II, leading to the parallel temperature-versus-ripple periodicity curves as in Fig. 4. It is not clear why cholesterol in the ripple structure does not distribute homogeneously but prefers to form in the cholesterol-rich domains. However, this may be related to the spatial modulation of order of hydrocarbon chain (Wittebort et al., 1981; Schneider et al., 1983; Tsuchida and Hatta, 1988).

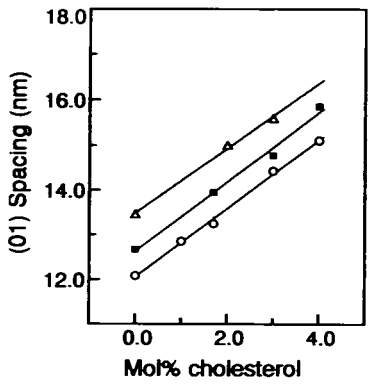

FIGURE 9 Cholesterol dependence of (01) spacing at 15.2°C (△), 17.8°C (■) and 20°C (○). These values were obtained from the data of DMPC-cholesterol in cooling as shown in Fig. 4. The lines represent the regression lines obtained with the least-squares method.

In region I the ripple structure was observed in the presence of cholesterol even below the pretransition temperature of pure DMPC. This is consistent with the results reported by Rock et al. (1989), where the ripple structure of DPPC with 7 mol % cholesterol persists even after 2 weeks at 30°C, lower than the pretransition temperature of pure DPPC.

From the observation of the splitting of the (10) peak profiles, it has been shown that the modified $L_{\beta'}$ phase domain including a small amount of cholesterol and the modified $P_{\beta'}$ phase domain including a large amount of cholesterol coexist in region I. Based on this fact, we consider the mechanism for forming large ripple periodicity. On lowering temperature from region II to region I, the domain of the $L_{\beta'}$ phase is squeezed from the domain of the $P_{\beta'}$ phase with cholesterol-rich DMPC and, on the other hand, cholesterol molecules are further incorporated in the domain of the $P_{\beta'}$ phase with cholesterol-rich DMPC. As a result, in the domain of the $P_{\beta'}$ phase in region I the flat strip of cholesterol-rich region becomes wider, and then the ripple periodicity is larger. This explains why the temperature dependence of ripple periodicity in region I becomes steep.

The temperature range where the $L_{\beta'}$ and $P_{\beta'}$ phases coexist becomes wider and the pretransition takes place more gradually with increasing cholesterol contents (see Fig. 6). This fact is consistent with DSC studies, which show that the width of the pretransition increases by adding a small amount of cholesterol, and the pretransition is not seen above 3.6–6.0 mol % cholesterol content (Estep et al., 1978; Mabrey et al., 1978; Vist and Davis, 1990; McMullen et al., 1993). We think that the suppression of the pretransition does not mean disappearance of the $P_{\beta'}$ phase but broadening of the thermal peak of pretransition. In fact, the ripple (01) diffraction peak

is present at 10 mol % cholesterol content (data not shown), which is consistent with the results of freeze-fracture electron microscopy (Copeland and McConnell, 1980; Hicks et al., 1987) and neutron diffraction (Mortensen et al., 1988). This observation agrees with the results obtained by Koynova et al. (1985) in which pretransition peak in DSC is observed with up to 11 mol % cholesterol content.

With increasing cholesterol content, the slope of the temperature-versus-ripple periodicity curve in region I becomes more gentle, as shown in the plots for 3.0 mol % and 4.8 mol % cholesterol of Fig. 8. This is interpreted as follows. At higher cholesterol content the broadening of the pretransition was observed in DSC and then the modified $L_{\beta'}$ phase domain was formed over a wide temperature range. Thus, the incorporation of cholesterol into the modified $P_{\beta'}$ domain in cooling takes place more gradually at higher cholesterol contents. This causes the more gradual increase of ripple periodicity at higher cholesterol contents in cooling. Whether this process is a kinetic or an equilibrium phenomenon remains uncertain. At present we think that this is behavior in an equilibrium state, because we observed the same behavior in the samples that were stored at the objective temperatures for 2–3 h in freeze-fracture experiments.

In region III, the ripple periodicity increased toward the main transition temperature. As in region I it seems possible to explain this long ripple periodicity in terms of the phase separation in the main transition region. However, there is no evidence for the existence of the large-scale cholesterol-rich ripple structure because the lamellar (10) peak exhibits a single peak (see Fig. 5) indicating the absence of large-scale phase separation. Thus, the overall changes of the ripple structure may happen in region III.

Mortensen et al. (1988) have published the results with the ripple structure of perdeuterated DMPC (DMPC-d_{54}) multibilayer in the presence and absence of cholesterol by small-angle neutron scattering. They have observed that the temperature-versus-ripple periodicity curve is steeper at 10–12°C than at 12–20°C in 2 mol % cholesterol (Fig. 10, Mortensen et al., 1980), and the ripple periodicity increases at high temperature bounds of the $P_{\beta'}$ phase (Figs. 6 *b*, 8 *b*, and 9 *b*, Mortensen et al., 1980). These features are similar to those of regions I and III. Thus their results are consistent with ours, although they measured samples with only 17% hydration.

In DMPC with 2 mol % cholesterol, Mortensen et al. (1988) have observed another ripple phase referred to as $P_{\beta''}$ phase with long ripple periodicity. This structure of $P_{\beta''}$ phase becomes dominant below 11°C instead of the normal ripple structure of the $P_{\beta'}$ phase. The long ripple periodicity in region I observed in this study might be related to this $P_{\beta''}$ phase. However, the neutron scattering intensity associated with the $P_{\beta''}$ phase by Mortensen et al. increases at lower temperatures, whereas the (01) intensity of x-ray diffraction decreases in our study. In addition, they did not observe the coexistence of the $L_{\beta'}$ and $P_{\beta'}$ phases. This discrepancy might be caused by dehydration or deuteration of DMPC in the sample.

We are grateful to Mr. Taizo Ishihama for help in the freeze-fracture electron microscopy experiments. We also thank Professors M. Akiyama, K. Ohki, and P. Quinn for useful discussions, and Dr. Y. Amemiya for help in the x-ray measurements.

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan.

REFERENCES

Amemiya, A., K. Wakabayashi, T. Hamanaka, T. Wakabayashi, T. Matsushita, and H. Hashizume. 1983. Design of a small-angle X-ray diffractometer using synchrotron radiation at the photon factory. *Nucl. Instrum. Methods.* 208:471–477.

Caffrey, M. 1984. X-radiation damage of hydrated lecithin membranes detected by real-time X-ray diffraction using wiggler-enhanced synchrotron radiation as the ionizing radiation source. *Nucl. Instrum. Methods.* 222: 329–338.

Copeland, B. R., and H. M. McConnell. 1980. The rippled structure in bilayer membranes of phosphatidylcholine and binary mixtures of phosphatidylcholine and cholesterol. *Biochim. Biophys. Acta.* 599:95–109.

de Gennes, P. G. 1974. The Physics of Liquid Crystals Oxford University Press, London.

Doniach, S. 1979. A thermodynamic model for the monoclinic (ripple) phase of hydrated phospholipid bilayers. *J. Chem. Phys.* 70: 4587–4596.

Estep, T. N., D. B. Mountcastle, R. L. Biltonen, and T. E. Thompson. 1978. Studies on the anomalous thermotropic behavior of aqueous dispersions of dipalmitoylphosphatidylcholine-cholesterol mixtures. *Biochemistry.* 17:1984–1989.

Falkovitz, M. S., M. Seul, H. L. Frisch, and H. M. McConnell. 1982. Theory of periodic structures in lipid bilayer membranes. *Proc. Natl. Acad. Sci. USA.* 79:3918–3921.

Gebhardt, C., H. Gruler, and E. Sackmann. 1977. On domain structure and local curvature in lipid bilayers and biological membranes. *Z. Naturforsch. Sect. C Biosci.* 32c:581–596.

Hawton, M. H., and W. J. Keeler. 1986. van der Waals energy of lecithins in the ripple phase. *Phys. Rev. A.* 33:3333–3340.

Hicks, A., Dinda, M., and M. A. Singer. 1987. The ripple phase of phosphatidylcholines: effect of chain length and cholesterol. *Biochim. Biophys. Acta.* 903:177–185.

Inoko, Y., and T. Mitsui. 1978. Structural parameters of dipalmitoyl phosphatidylcholine lamellar phases and bilayer phase transitions. *J. Phys. Soc. Jpn.* 44:1918–1924.

Janiak, M. J., D. M. Small, and G. G. Shipley. 1976. Nature of the thermal pretransition of synthetic phospholipids: dimyristoyl- and dipalmitoyllecithin. *Biochemistry.* 15:4575–4580.

Janiak, M. J., D. M. Small, and G. G. Shipley. 1979. Temperature and compositional dependence of the structure of hydrated dimyristoyl lecithin. *J. Biol. Chem.* 254:6068–6078.

Koynova, R. D., A. I. Boyanov, and B. G. Tenchov. 1985. On the phase diagram of an L-dipalmitoylphosphatidylcholine/cholesterol mixture. *FEBS Lett.* 187:65–68.

Luna, E. J., and H. M. McConnell. 1977. The intermediate monoclinic phase of phosphatidylcholines. *Biochim. Biophys. Acta.* 466:381–392.

Mabrey, S., P. L. Mateo, and J. M. Sturtevant. 1978. High-sensitivity scanning calorimetric study of mixtures of cholesterol with dimyristoyl- and dipalmitoyl-phosphatidylcholines. *Biochemistry.* 17:2464–2468.

Marder, M., H. L. Frisch, J. S. Langer, and H. M. McConnell. 1984. Theory of the intermediate rippled phase of phopholipid bilayers. *Proc. Natl. Acad. Sci. USA.* 81:6559–6561.

Matuoka, S., S. Kato, M. Akiyama, Y. Amemiya, and I. Hatta. 1990. Temperature dependence of the ripple structure in dimyristoylphosphatidylcholine studied by synchrotron X-ray small-angle diffraction. *Biochim. Biophys. Acta.* 1028:103–109.

McCullough, W. S., and H. L. Scott. 1990. Statistical-mechanical theory of the ripple phase of lipid bilayers. *Phys. Rev. Lett.* 65:931–934.

McMullen, T. P. W., R. N. A. H. Lewis, and R. N. McElhaney. 1993. Differential scanning calorimetric study of the effect of cholesterol on the thermotropic phase behavior of a homologous series of linear saturated phosphatidylcholines. *Biochemistry.* 32:516–522.

Mortensen, K., W. Pfeiffer, E. Sackmann, and W. Knoll. 1988. Structural properties of a phosphatidylcholine-cholesterol system as studied by small-angle neutron scattering:ripple structure and phase diagram. *Biochim. Biophys. Acta.* 945:221–245.

Okumura, H. 1986. Introduction to Data Analysis by Personal Computer. Gijutsu Hyoron Sha, Tokyo (in Japanese).

Parente, R. A., and B. R. Lentz. 1984. Phase behavior of large unilamellar vesicles composed of synthetic phospholipids. *Biochemistry.* 23: 2353–2362.

Pearce, P. A., and H. L. Scott Jr. 1982. Statistical mechanics of the ripple phase in lipid bilayers. *J. Chem. Phys.* 77:951–958.

Rand, R. P., V. A. Parsegian, J. A. C. Henry, L. J. Lis, and M. McAlister. 1980. The effect of cholesterol on measured interaction and compressibility of dipalmitoylphosphatidylcholine bilayers. *Can. J. Biochem.* 58: 959–968.

Rock, P., T. E. Thompson, and T. W. Tillack. 1989. Persistence at low temperature of the $P_{\beta'}$ ripple in dipalmitoylphosphatidylcholine multilamellar vesicles containing either glycosphingolipids or cholesterol. *Biochim. Biophys. Acta.* 979:347–351.

Ruocco, M., and G. G. Shipley. 1982. Characterization of the subtransition of hydrated dipalmitoylphosphatidylcholine bilayers. *Biochim. Biophys. Acta.* 691:309–320.

Rüppel, D., and E. Sackmann. 1983. On defects in different phases of two-dimensional lipid bilayers. *J. Physique.* 44:1025–1034.

Schneider, M. B., W. K. Chan, and W. W. Webb. 1983. Fast diffusion along defects and corrugations in phospholipid $P_{\beta'}$ liquid crystals. *Biophys. J.* 43:157–165.

Stamatoff, J., B. Feuer, H. J. Guggenheim, G. Tellez, and T. Yamane. 1982. Amplitude of rippling in the $P_{\beta'}$ phase of dipalmitoylphosphatidylcholine bilayers. *Biophys. J.* 38:217–226.

Tardieu, A., V. Luzzati, and F. C. Reman. 1973. Structure and polymorphism of the hydrocarbon chains of lipids: a study of lecithin-water phases. *J. Mol. Biol.* 75:711–733.

Tenchov, B. G., H. Yao, and I. Hatta. 1989. Time-resolved x-ray diffraction and calorimetric studies at low scan rates. I. Fully hydrated dipalmitoylphosphatidylcholine (DPPC) and DPPC/water/ethanol phases. *Biophys. J.* 56:757–768.

Tsuchida, K., and I. Hatta. 1988. ESR studies on the ripple phase in multilamellar phospholipid bilayers. *Biochim. Biophys. Acta.* 945:73–80.

Vist, M. R., and J. H. Davis. 1990. Phase equilibria of cholesterol/dipalmitoyl phosphatidylcholine mixtures: ^2H nuclear magnetic resonance and differential scanning calorimetry. *Biochemistry.* 29:451–464.

Wack, D. C., and W. W. Webb. 1989. Synchrotron x-ray study of the modulated lamellar phase $P_{\beta'}$ in the lecithin-water system. *Phys. Rev. A.* 40: 2712–2730.

Wittebort, R. J., C. F. Schmidt, and R. G. Griffin. 1981. Solid-state carbon-13 nuclear magnetic resonance of the lecithin gel to liquid-crystalline phase transition. *Biochemistry.* 20:4223–4228.

Zasadzinski, J. A. N., and M. B. Schneider. 1987. Ripple wavelength, amplitude, and configuration in lyotropic liquid crystals as a function of effective headgroup size. *J. Physique.* 48:2001–2011.

Zasadzinski, J. A. N., J. Schneir, J. Gurley, V. Elings, and P. K. Hansma. 1988. Scanning tunneling microscopy of freeze-fracture replicas of biomembranes. *Science.* 239:1013–1015.