Biochimica et Biophysica Acta, 1028 (1990) 215–222
Elsevier

BBAMEM 74986

Ethanol induces interdigitated gel phase ($L_{\beta I}$) between lamellar gel phase ($L_{\beta'}$) and ripple phase ($P_{\beta'}$) in phosphatidylcholine membranes: a scanning density meter study

Kazuo Ohki, Kazuo Tamura and Ichiro Hatta

Department of Applied Physics, School of Engineering, Nagoya University, Nagoya (Japan)

(Received 21 March 1990)

Key words: Interdigitated gel phase; Ethanol; Specific volume; Phase diagram; Dipalmitoylphosphatidylcholine; DSC

Effects of ethanol on dipalmitoylphosphatidylcholine (DPPC) and distearoylphosphatidylcholine (DSPC) dispersions were investigated with an automated scanning density meter and a differential scanning calorimeter (DSC). The temperature-dependent profile of specific volume measured by the density meter clearly exhibited phase transitions of the DPPC and the DSPC dispersions as drastic changes in the thermal expansion coefficients. On increasing the ethanol concentration in the DPPC dispersions, the pretransition temperature was reduced faster than the main transition temperature was. An interdigitated gel phase ($L_{\beta I}$) appeared as a region of lower specific volume at the pretransition temperature when the ethanol concentration reached 40 mg/ml. The $L_{\beta I}$ phase spread both its ends in an ethanol-dependent fashion, and the high-temperature end merged to the main transition at 50 mg/ml of ethanol. The temperature-ethanol phase diagram has been determined for DPPC. The transitions from $L_{\beta'}$ to $L_{\beta I}$ and from $L_{\beta I}$ to $P_{\beta'}$ were also observed on the thermograms of DSC measurements. In the DSPC dispersions, the $L_{\beta I}$ phase was induced between the $L_{\beta'}$ and the $P_{\beta'}$ phases by a lower ethanol concentration (about 20 mg/ml).

Introduction

Phospholipid molecules, the major lipid components of biomembranes, assemble spontaneously in aqueous environments and form a bilayer structure which represents a minimum energy configuration resulting preimarily from the exclusion of the hydrocarbon chains by water molecules [1]. In the lipid bilayer model for biomembranes, the acyl chains from one monolayer do not penetrate the apposing monolayer. Although the penetration may occur in fluid phase, the chains are only slightly interpenetrated. On the other hand, studying artificial lipid membranes, it has been well documented in the literature that the hydrated lipids are able to adopt a variety of phases in addition to the bilayer structure described above. These phases have been recognized by Luzzatti et al. using the X-ray diffraction method [2], and the hexagonal II pahse for phosphatidylethanolamine has been more closely examined by Cullis and De Kruijff employing ^{31}P-NMR techniques [3]. Phosphatidic acid, which is produced in the metabolism of inositol phospholipid in a stimulated cell, plays the role of a Ca^{2+}-translocator in membranes [4], and the formation of the hexagonal II phase is proposed for the mechanism of the transmembrane shuttle [5]. X-ray diffraction measurements have revealed that an interdigitated phase appears in a gel state of dipalmitoylphosphatidylglycerol [6]. This finding has given a new idea to the models of lipid membranes: acyl chains are able to penetrate the apposing monolayer under certain conditions. Now it is known that the interdigitated structures are also formed in phosphatidylcholine dispersions in the presence of chlorpromazine [7], glycerol [8] or ethanol [9–11], in Tris buffer [12] and under high pressure [13,14]. And dihexadecylphosphatidylcholine (DHPC), ether-linked phosphatidylcholine, exhibits the interdigitated gel phase being hydration dependent [15,16]. Compared with these phospholipids, having a pair of the same acyl chains, the interdigitate structure is supposed to be an essential alignment for mixed-chain phosphatidylcholines [17–20], a lysophosphatidylcholine [21] and cerebroside sulfates with a long acyl chain [22], especially at low temperatures. These studies of interdigitated membrane structures are reviewed by Slater and Huang [23].

Abbreviations: DSC, differential scanning calorimetry, DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DHPC, dihexadecylphosphatidylcholine.

Correspondence: K. Ohki, Department of Applied Physics, School of Engineering, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan.

The X-ray diffraction is the most powerful method to solve in detail the structural characteristics of membranes. Indeed, in order to study the interdigitated structures, the X-ray diffraction technique (including the neutron diffraction [13]) which gives information about bilayer thickness and chain packing has been used in most studies except a few using infrared spectroscopy [14,24], fluorescent probe (DPH) [25] and spin labeling [26,27]. The X-ray diffraction study of Simon and McIntosh has revealed that the chain packing of DPPC in the ethanol-induced $L_{\beta I}$ phase is closer than that of $L_{\beta'}$ phase [28]. In general, molecular packing is directly measured by a density meter. Wilkinson et al. have shown that the specific volume of the interdigitated gel phase ($L_{\beta I}$) is less than that of the lamellar gel phase ($L_{\beta'}$) in dipalmitoylphosphatidylglycerol bilayers [12]. Recently a high-sensitive dilatometer (density meter) is available for monitoring phase changes in lipid dispersions by measuring specific volumes [29,30]. And compared with a differential scanning calorimeter, a scanning type of the dilatometer has the advantage that it is relatively easy to follow very slow process of a phase change with a long relaxation time [31].

Approximate phase diagrams including the $L_{\beta I}$ phase have been proposed for DPPC/glycerol and DPPC/ethanol systems by McDaniel et al. [8] and Nambi et al. [25], respectively. In the DPPC/glycerol phase diagram, the lamellar gel phase ($L_{\beta'}$) and the ripple phase ($P_{\beta'}$) in pure water are replaced by the interdigitated gel phase ($L_{\beta I}$) in the presence of glycerol. And, in the DPPC/ethanol phase diagram, the $P_{\beta'}$ phase changes into the $L_{\beta I}$ phase above a threshold concentration of ethanol. On the contrary, it has been shown in the phase diagram of the DHPC/water system that the phase proceeds from the $L_{\beta I}$ phase to the $P_{\beta'}$ and then to the fluid phase (L_α) as the temperature increases [15]. Therefore, the question is whether the $P_{\beta'}$ phase does exist between the $L_{\beta I}$ phase and the L_α phase or is replaced with the $L_{\beta I}$ phase in the temperature-composition phase diagram of the DPPC/aqueous ethanol system.

In the present report, the ethanol-induced interdigitated gel phase of DPPC was examined by a high-sensitive density meter and DSC. We have shown that the scanning density meter is a powerful method to study phase behavior of lipid membranes. By measuring the temperature dependence of the specific volume at various concentrations of ethanol, we have proposed a refined phase diagram which has the ripple phase ($P_{\beta'}$) between the interdigitated gel phase ($L_{\beta I}$) and the fluid phase (L_α) in the narrow range of ethanol concentration. These results have been confirmed in the DSPC/aqueous ethanol system.

Materials and Methods

Synthetic 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-stearoyl-*sn*-glycero-3-phosphocholine (DSPC) were purchased from Sigma Chemical Co. (St. Louis, MO). Ethanol (ethyl alcohol, analytical grade) was purchased from Katayama Chemicals (Osaka, Japan). Multilamellar vesicles were prepared by taking the lipid (60 μmol) from the chloroform solution into a small test tube and evaporating the solvent first by a nitrogen stream then under reduced pressure. Distilled water (3 ml; specific resistance = 17.8 M$\Omega \cdot$ cm) was poured into the tube, and then the mixture was incubated in a water-bath at 52° C for 4 h with occasional vortexing to make homogeneous dispersions. In case of investigating ethanol effects, a desired amount of ethanol was added into the lipid dispersion. The dispersions were degassed in Shodex DEGAS KT15 (Showa Denko, Tokyo) for 2 h in order to prevent bubble formation, and injected into an external measuring cell DMA602HT. Pure water was injected into another external measuring cell DMA602HT as a reference in a density meter DMA60 (Anton Paar, Austria). Density of the dispersions was measured at every 25 s with the density meter equipped with a compact thermostat Lauda RCS20D (Lauda Dr. R. Wobser GmbH & Co.) at a scanning rate of 0.03 K/min. The temperature control and data acquisition were processed by a personal computer PC9801 (NEC, Tokyo). The measured values of density have been converted to the specific volumes by the equation:

$$v = (1/d')[1 - (d - d')/c]$$

where v is specific volume of DPPC (cm^3/g), d' is density of solvent (g/cm^3), d is the measured density of the dispersions and c is concentration of DPPC (g/cm^3). In order to calibrate the specific volume of DPPC obtained with the density meter, the specific volume of the DPPC dispersions in pure water was measured by sedimentation method in D_2O/H_2O mixture [32]. At 20° C, the density meter and the sedimentation method gave 0.9470442 ml/g and 0.9368 ml/g, respectively. The relative error was 1%.

For DSC measurements, DPPC dispersions (340 mM) including a desired concentration of ethanol were prepared by a procedure similar to that described above. 15 μl of the dispersions were taken into a pan (Du Pont 900796.901), and then the pan and a cover (Du Pont 900790.901) were sealed hermetically. Calorimetric scans were performed with a SEIKO DC10 and SSC580 differential scanning calorimeter (SEIKO I&E, Tokyo) at a heating scan rate 1.0 K/min as described previously [33].

Results

It is well known that multilamellar liposomes of DPPC in excess water exert lamellar crystalline (L_c), lamellar gel ($L_{\beta'}$), ripple ($P_{\beta'}$) and lamellar fluid (L_α) phases as the temperature increases. The phase behavior is an important feature in studying physical properties of lipid membranes since the phase transitions are accompanied with changes in various physical properties of the membranes. For instance, arrangement or packing of lipid molecules differs from phase to phase. Therefore, by measuring density of lipid membranes, we can monitor the phase behavior. As shown in Fig. 1, a high-sensitive density meter demonstrated how the specific volume of DPPC membranes changed as the temperature increased and how ethanol affected the profile of the phase transitions. In these cases, the multilamellar liposomes of 20 mM DPPC were prepared at 52° C and the temperature scan started from 10° C just after the dispersions were injected into the cell of the density meter. Hence, no subtransition (L_c phase to $L_{\beta'}$ phase) was observed because the DPPC system had not been relaxed into the L_c phase. In the absence of ethanol, the specific volume increased at a constant expansion coefficient ($75 \cdot 10^{-5}$ ml/g per K) as the temperature was raised, and pretransition and main transition were observed at 34.8° C and 41.6° C, respectively (Fig. 1a). The $L_{\beta'}$, $P_{\beta'}$ and L_α phases are clearly separated by drastic jumps of specific volume, i.e., large thermal expansion coefficients. And these transition temperatures agree with the reported values. Addition of ethanol into the dispersion altered the profile of specific volume vs. temperature. The pretransition temperature was lowered remarkably by ethanol, or the $P_{\beta'}$ phase was extended to a lower temperature. 20 mg/ml of ethanol reduced the temperatures of the pretransition and the main transition to 32.5° C and 40.6° C, respectively (Fig. 1b). In the presence of 40 mg/ml of ethanol, the thermal expansion coefficient became zero around 25° C where the pretransition located just above, and the main transition temperature was lowered to 39.7° C (Fig. 1c). When the concentration of ethanol was raised to 42 mg/ml, interdigitated gel phase ($L_{\beta I}$) which ranged from 25.7° C to 33.7° C broke out as a region of lower specific volume (Fig. 1d). This observation agrees with the report that the specific volume of the $L_{\beta I}$ phase is less than that of the $L_{\beta'}$ phase in bilayer [12]. The $L_{\beta I}$ phase became clearer in the presence of 45 mg/ml of ethanol (Fig. 1e). As the ethanol concentration increased, the $L_{\beta I}$ phase further expanded and the high-temperature end merged to the main transition in the presence of 50 mg/ml of ethanol (Fig. 1f). In a narrow range of ethanol concentration (42–48 mg/ml), the $P_{\beta'}$ phase existed between the $L_{\beta I}$ and the L_α phases in the DPPC dispersions, which was first observed by the present experiment.

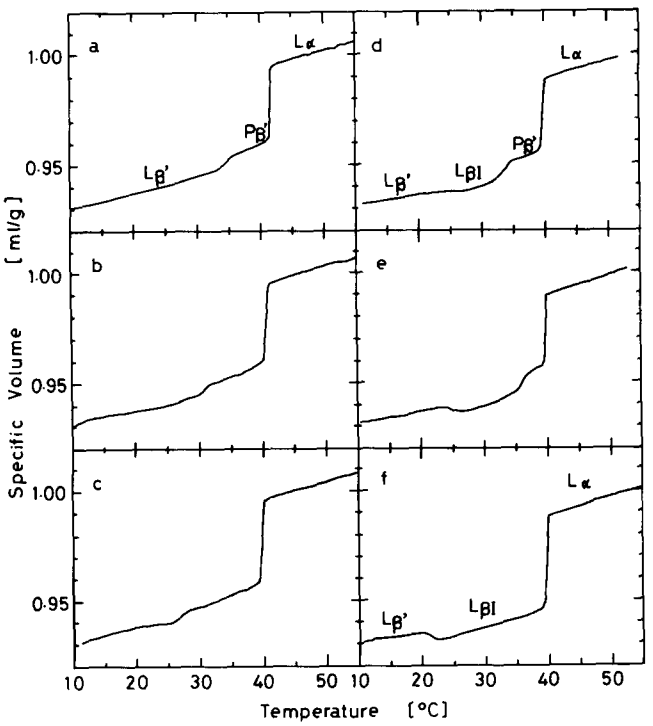

Fig. 1. Specific volume vs. temperature of DPPC dispersions in the presence of (a) no ethanol, (b) 20 mg/ml ethanol, (c) 40 mg/ml ethanol (d) 42 mg/ml ethanol (e) 45 mg/ml ethanol and (f) 50 mg/ml ethanol. The concentration of DPPC in the dispersions was 20 mM. Temperature was scanned at rates of 0.03 K/min. $L_{\beta'}$, $L_{\beta I}$, $P_{\beta'}$ and L_α indicate lamellar gel, interdigitated gel, ripple and lamellar fluid phases, respectively.

Then, by using DSC, we studied the thermal properties of DPPC dispersions and ethanol effects on them. For DSC measurements, a higher concentration of DPPC dispersions (340 mM) were prepared in various concentrations of ethanol. In the absence of ethanol, the thermogram of the DPPC dispersions showed pretransition at 34.9° C and main transition at 40.6° C (Fig. 2a). The transition temperatures of the pre- and main transitions were lowered to 26.5° C and 39.4° C, respectively, by the addition of 45 mg/ml of ethanol (Fig. 2b). In the presence of 47.5 mg/ml ethanol, an endothermic trough appeared around 36° C which could be attributed to the transition from the $L_{\beta I}$ phase to the $P_{\beta'}$ phase (Fig. 2c). And an endothermic trough around 24° C indicated the transition from the $L_{\beta'}$ phase to the $L_{\beta I}$ phase. The three endothermic troughs still existed in the presence of 50 mg/ml of ethanol (data not shown), and the middle trough disappeared from the thermogram in the presence of 55 mg/ml ethanol (Fig. 2d). The present DSC study has proven that the appearance of the interdigitated gel phase is manifested on the thermogram. The ethanol concentrations for the appearance and disappearance of the interdigitated gel phase were a little bit higher, compared with those of the density meter experiments (Fig. 1). This might be due to the

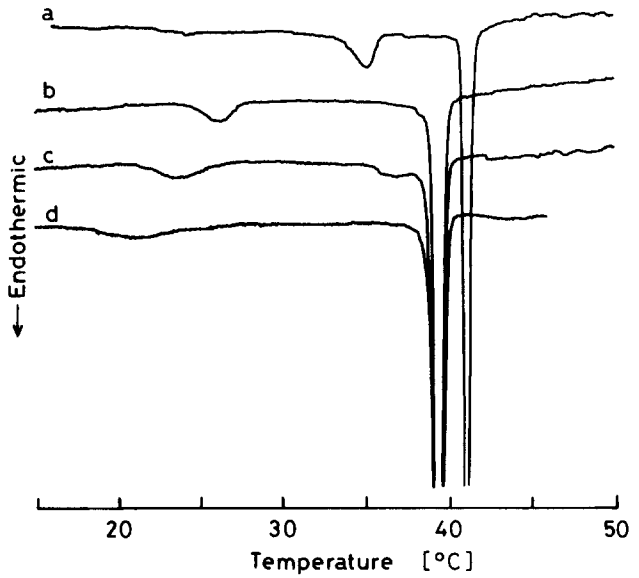

Fig. 2. DSC scans of DPPC dispersions in the presence of (a) no ethanol, (b) 45 mg/ml ethanol, (c) 47.5 mg/ml ethanol and (d) 55 mg/ml ethanol. The concentration of DPPC in the dispersions was 340 mM. The rate of heating scan was 1 K/min.

different concentrations of the DPPC dispersions; 340 mM for DSC and 20 mM for the density meter.

The main transition temperature of DPPC dispersions was affected by ethanol in a biphasic manner. When the ethanol concentration exceeded 50 mg/ml, the main transition temperature began to rise. Fig. 3 shows the main transition temperature vs. ethanol concentration obtained by the density meter and DSC experiments. Below 35 mg/ml of ethanol, the main transition temperature dropped linearly as the ethanol concentration increased. And the main transition temperature rose asymptotically up to about 42° C after the ethanol concentration passed 55 mg/ml. Although the ethanol concentration required for raising the main transition temperature was slightly higher in the DSC experiment, the profiles of ethanol dependency by the density meter and the DSC agreed very well. The present result is also identical to that reported by Rowe [9] with a spectrometer monitoring the absorbance at 400 nm.

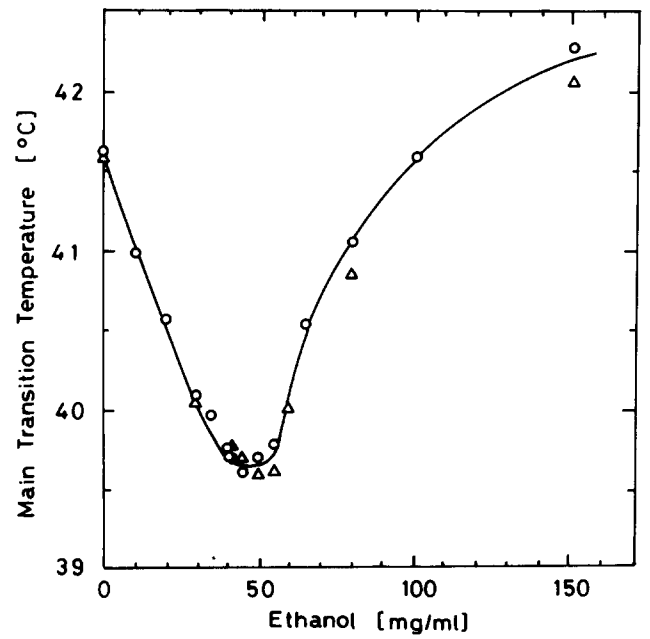

Fig. 3. Main transition temperature vs. ethanol concentration in DPPC dispersions. The transition temperatures were obtained by (○) the density meter and (△) DSC.

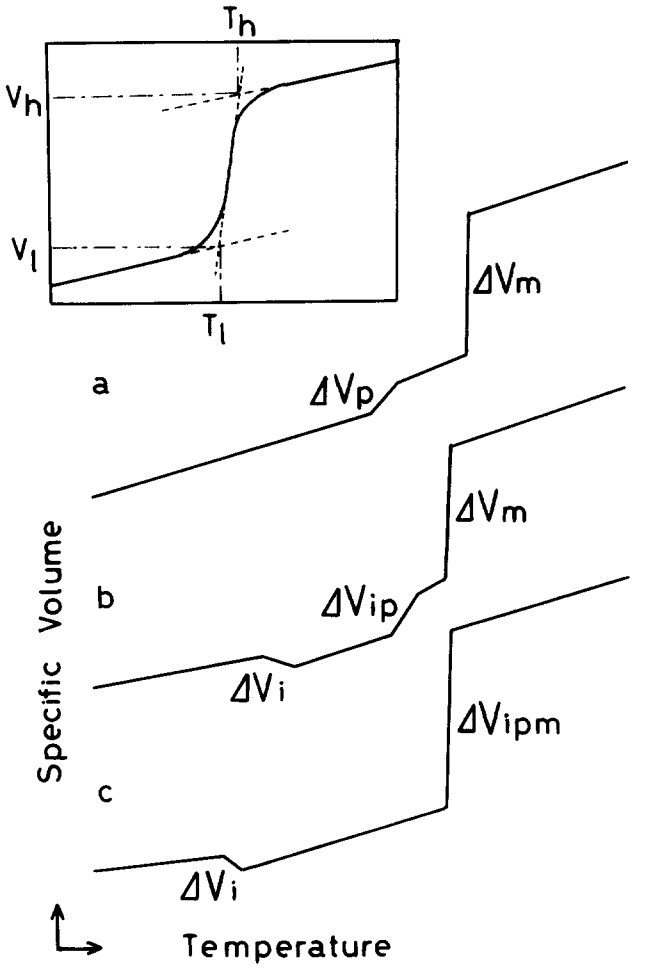

Fig. 4. Schematic profiles of specific volume vs. temperature of DPPC dispersions in the presence of (a) no ethanol, (b) 40–50 mg/ml ethanol and (c) more than 50 mg/ml ethanol. ΔV, ΔV_p, ΔV_{ip}, ΔV_{ipm} and ΔV_m are changes in specific volumes at the transitions of $L_{\beta'}$ to $L_{\beta I}$, $L_{\beta'}$ to $P_{\beta'}$, $L_{\beta I}$ to $P_{\beta'}$, $L_{\beta I}$ to L_α, and $P_{\beta'}$ to L_α, respectively. How to estimate the change in specific volume at a transition is shown in the inset. Change in specific volume and phase transition temperature are obtained as $\Delta V = V_h - V_l$ and $T = (T_h + T_l)/2$, respectively.

It is possible to classify the profiles of specific volume vs. temperature in the DPPC/aqueous ethanol system into three types (Fig. 4). In the absence and below 40 mg/ml of ethanol, the profile includes pretransition and main transition, and the changes of specific volumes are represented by ΔV_p and ΔV_m, respectively (Fig. 4a). The

TABLE I

Changes of specific volumes at phase transitions in DPPC dispersions

Changes of specific volumes at the phase transitions in three different patterns, ΔV_i, ΔV_p, ΔV_{ip}, ΔV_{ipm} and ΔV_m, are indicated in Fig. 4. The values represent averages of three experiments.

Ethanol (mg/ml)	ΔV_i (ml/g)	ΔV_p (ml/g)	ΔV_{ip} (ml/g)	ΔV_{ipm} (ml/g)	ΔV_m (ml/g)	$-\Delta V_i + \Delta V_p$ (ml/g)	$-\Delta V_i + \Delta V_p + \Delta V_m$ (ml/g)
0	–	0.0045	–	–	0.0327	–	–
30	–	0.0046	–	–	0.0331	–	–
45	−0.0036	–	0.0085	–	0.0309	0.0082	–
50	−0.0037	–	–	0.0426	–	–	0.0414

second profile has a transition from the $L_{\beta'}$ phase to the $L_{\beta I}$ phase (ΔV_i) and a transition from the $L_{\beta I}$ phase to the $P_{\beta'}$ phase (ΔV_{ip}) in addition to the main transition in the concentration range of ethanol between 42 and 48 mg/ml (Fig. 4b). And the third profile has a transition from the $L_{\beta'}$ phase to the $L_{\beta I}$ phase (ΔV_i) and a transition from the $L_{\beta I}$ phase to the L_α phase (ΔV_{ipm}) in the ethanol concentrations above 50 mg/ml (Fig. 4c). These changes of specific volumes at the transitions are summarized in Table I. The change in specific volume at the phase transition is supposed to reflect the difference of molecular packing between the two phases. And, in the DPPC dispersions, the thermal expansion coefficients of the $L_{\beta'}$, $P_{\beta'}$ and L_α phases are not so different; $75 \cdot 10^{-5}$, $123 \cdot 10^{-5}$ and $104 \cdot 10^{-5}$ ml/g per K, respectively. Therefore, it is suggested that a superposition rule exists among the changes of specific volume at the phase transitions. By substituting ΔV_i and ΔV_p with −0.0036 and 0.0046 ml/g, respectively, $-\Delta V_i + \Delta V_p$ gives 0.0082 ml/g, which is almost equal to 0.0085 ml/g of ΔV_{ip}. This fact suggests that the specific volume reduced by the transition to the $L_{\beta I}$ phase is recovered to the level of the $P_{\beta'}$ phase by the transition of ΔV_{ip}. So the ΔV_{ip} transition can be attributed to the sum of the ΔV_i and ΔV_p transitions. If we choose −0.0037, 0.0046, 0.0331 ml/g for ΔV_i, ΔV_p and ΔV_m, respectively, in calculating the sum, $-\Delta V_i + \Delta V_p + \Delta V_m$ gives 0.0414 ml/g, which agrees well with 0.0426 ml/g of ΔV_{ipm}. This result confirms that the phase transition from $L_{\beta I}$ to L_α is a superposition of three transitions, i.e., $L_{\beta'}$ to $L_{\beta I}$, $L_{\beta I}$ to $P_{\beta'}$ and $L_{\beta I}$ to L_α.

Temperature-dependent profiles of the specific volume were also investigated in DSPC dispersions. In the absence of ethanol, pretransition and main transition were observed at 50.3° C and 54.9° C, respectively (Fig. 5a). No subtransition was observed in this profile since the DSPC dispersions had not been exposed to a low temperature for a time long enough for relaxing into the L_c phase. The interdigitated gel phase ($L_{\beta I}$) existed between the $L_{\beta'}$ and $P_{\beta'}$ phases at an ethanol concentration of 20 mg/ml (Fig. 5b). Then the $L_{\beta I}$ phase expanded and became in contact with the main transition in the presence of 25 mg/ml ethanol (Fig. 5c). Compared with the DPPC dispersions, the appearance of the $L_{\beta I}$ phase and the disappearance of the $P_{\beta'}$ phase were observed at lower concentrations of ethanol. Rowe [9] has reported that the effect of ethanol on the main transition depends on the chain length of the saturated phosphatidylcholines. The dependence of chain length in the present experiment is identical to the reported one.

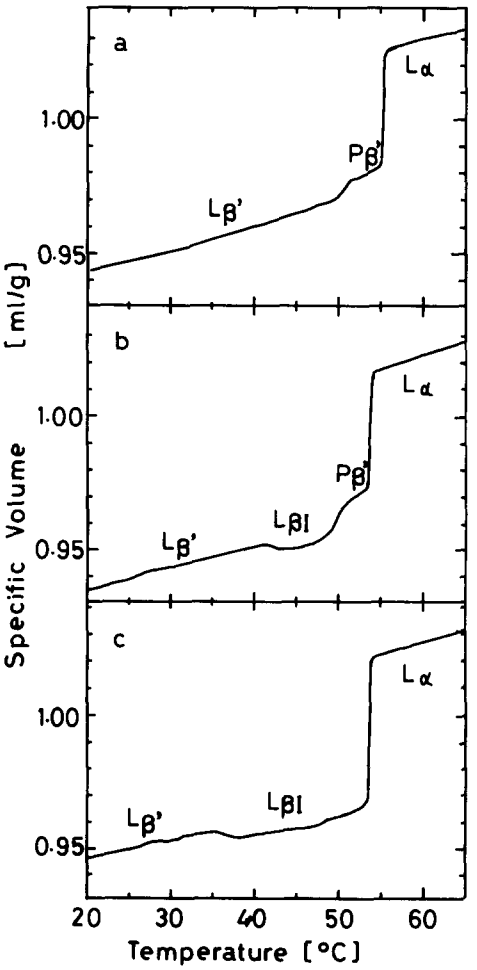

Fig. 5. Specific volume vs. temperature of DSPC dispersions in the presence of (a) no ethanol, (b) 20 mg/ml ethanol and (c) 25 mg/ml ethanol. The concentration of DSPC in the dispersions was 20 mM. Temperature was scanned at rates of 0.03 K/min.

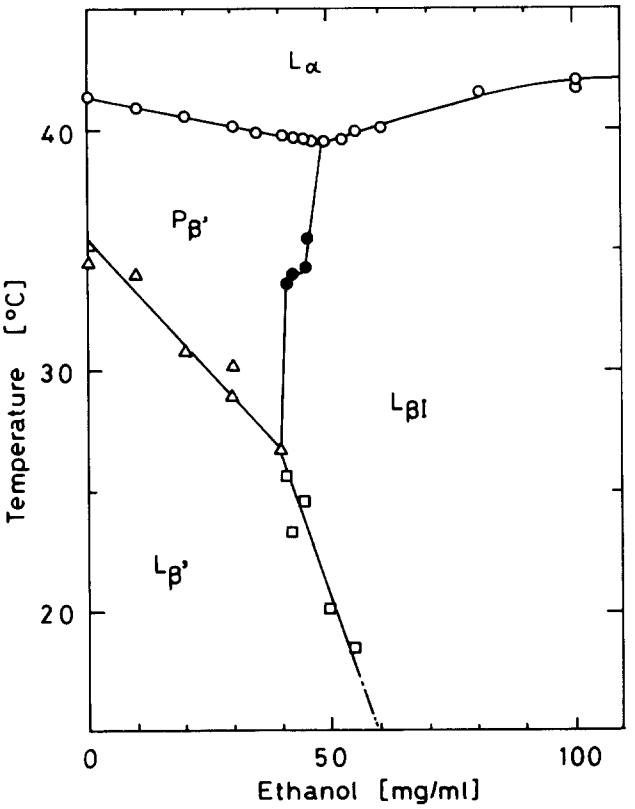

Fig. 6. Phase diagram of DPPC/aqueous ethanol. Each point is determined by the density meter measurement. L_α, $P_{\beta'}$, $L_{\beta I}$ and $L_{\beta'}$ are lamellar fluid, ripple, interdigitated gel and lamellar gel phase, respectively.

Fig. 6 shows the phase transition temperature vs. the ethanol concentration phase diagram for the DPPC dispersions. As the ethanol concentration increases, the ripple phase ($P_{\beta'}$) expands in compensation for the reduction of the lamellar gel phase ($L_{\beta'}$). And the interdigitated gel phase ($L_{\beta I}$) breaks out at the lower triple point which is the break of the $L_{\beta'}$ border line. Then the $L_{\beta I}$ phase expands rapidly over the $P_{\beta'}$ and $L_{\beta'}$ phases. And the $P_{\beta'}$ phase disappears at the upper triple point which is the break of the L_α border line. In the narrow range of ethanol concentration, the DPPC dispersions exhibit four phases; $L_{\beta'}$, $L_{\beta I}$, $P_{\beta'}$ and L_α.

Discussion

This paper describes the phase behavior of the DPPC dispersions in the presence of ethanol by use of a high-sensitive density meter. In general, each phase has its own arrangement of molecules. If a phase transition of lipid membranes is accompanied by a change in molecular packing, it is possible to monitor the transition by measuring the density or the specific volume. Practically, Wiener et al. have demonstrated by accurate measurements that the specific volumes of DPPC and DLPE bilayer dispersions change sharply at phase transition temperatures [34]. They obtained the changes in specific volume of 0.004 ml/g for pretransition (ΔV_p) and 0.038 ml/g for main transition (ΔV_m) in the DPPC dispersions. The difference between these values and ours was about 15%; greater ΔV_p and smaller ΔV_m were obtained in the present experiment. Our absolute specific volumes in lamellar gel phase ($L_{\beta'}$) and ripple phase ($P_{\beta'}$) agreed well with their values, but the value in fluid phase (L_α) is smaller than the reported one. And, in the absence of ethanol, the coefficients of thermal expansion in the present experiment, calculated by $\alpha = -dV/dT$, were $75 \cdot 10^{-5}$, $123 \cdot 10^{-5}$ and $104 \cdot 10^{-5}$ ml/g per K in $L_{\beta'}$, $P_{\beta'}$ and L_α, respectively. These values agree generally with those obtained by Wiener et al. [34].

X-ray diffraction is a powerful method to investigate membrane structures, and it has been successfully applied to studies of interdigitated structures [6,7,28,35]. These studies have demonstrated that the bilayer thickness of chlorpromazine-induced interdigitated phase of DPPC contracts to 30 Å in comparison with 49 Å of non-penetrated bilayers [7] and that the packing arrangement of acyl cahins in DPPC changes from 4.21 Å to 4.09 Å when $L_{\beta'}$ phase goes into ethanol-induced $L_{\beta I}$ phase [28]. The present experiment using a density meter has exhibited such changes in molecular packing of DPPC membranes as a decrease in the specific volume. The specific volume is 0.9464 ml/g at the high-temperature end of $L_{\beta'}$ phase in the presence of 45 mg/ml ethanol (at 23.67 °C). Therefore, −0.0036 ml/g of ΔV_i indicates that the specific volume decreases by 3.8% during the transition from the $L_{\beta'}$ phase to the $L_{\beta I}$ phase. This small decrease of specific volume suggests that the DPPC membranes expands laterally in compensation for the large reduction of thickness by the transition to the $L_{\beta I}$ phase.

A large number of studies have been done on effects of alcohol on lipid membranes since this subject is closely related to anesthetic actions and alcoholism. Rowe has observed a biphasic effect of ethanol concentration on the main phase transition temperature of saturated phosphatidylcholines having acyl chain lengths from 14 to 21 [9]. In this biphasic effect, critical concentrations of ethanol are 50 mg/ml for DPPC and 25 mg/ml for DSPC; ethanol reduces and raises the main transition temperature below and above the critical value, respectively. These reported values correspond to the concentrations where the $L_{\beta I}$ phase drives out the $P_{\beta'}$ phase and becomes in contact with the L_α phase in the present study. Above the critical concentration, further expansion of the $L_{\beta I}$ phase has proved to raise the main transition temperature.

The present density meter study has revealed effect of ethanol concentration on the pretransition of DPPC dispersions in detail. Ethanol reduces the pretransition temperature, which is in good agreement with that shown by spectrophotometry [10] and fluorometry [25].

Our new finding is that ethanol-induced interdigitated gel phase ($L_{\beta I}$) breaks out between $L_{\beta'}$ phase and $P_{\beta'}$ phase in the phosphatidylcholine dispersions. According to the studies done so far, no $P_{\beta'}$ phase between $L_{\beta I}$ and L_α phases has been shown in the phase diagrams of DPPC/aqueous ethanol and DSPC/aqueous ethanol systems [25]. And, in the phase diagram of DPPC/glycerol system, it is shown that $L_{\beta I}$ phase replaces the $L_{\beta'}$ and $P_{\beta'}$ gel phases [8]. From a thermodynamical viewpoint, the most probable phase is obtained by comparing the chemical potentials of the phases under the given condition. On the other hand, the phase transition temperature of phospholipid membranes is theoretically determined by the sum of the attractive interactions, steric constant and *trans-gauche* configurations [36]. The decrease of the phase transition temperature implies that ethanol destabilizes the lipid organization by modifying the intermolecular interactions and that the ethanol effect consequently makes the higher-temperature phase ($P_{\beta'}$) more stable than the lower-temperature one ($L_{\beta'}$) by changing the chemical potentials. The $P_{\beta'}$ phase replaces the $L_{\beta'}$ phase in the DPPC dispersions as the ethanol concentration increases up to 40 mg/ml. At this ethanol concentration, the $L_{\beta I}$ phase appears on the boundary of the $L_{\beta'}$ and $P_{\beta'}$ phases probably because the $L_{\beta I}$ phase is thermodynamically more stable than the other two phases at the temperature. The relations of the $L_{\beta I}$ phase with the $L_{\beta'}$ and $P_{\beta'}$ phases have been reported in the gel phase of the dihexadecylphosphatidylcholine (DHPC)/water system. The stability between the $L_{\beta'}$ phase and the $L_{\beta I}$ phase depends on hydration. At higher hydration, above 32% water, the $L_{\beta I}$ phase is favorable in the gel phase of DHPC [16]. And the temperature-composition phase diagram of the DHPC/water system shows that the $L_{\beta I}$ phase locates below the $P_{\beta'}$ phase in the higher hydration [15]. Veiro et al. have investigated the effect of short chain alcohols on the phase behavior of DHPC [37]. As the alcohol concentration is increased, the $L_{\beta I}$ to $P_{\beta'}$ transition and $P_{\beta'}$ to L_α transition of DHPC merge at the threshold concentration of the biphasic effect. These observations agree well with our observations in the DPPC/aqueous ethanol system. The present study suggests that there generally exist four polymorphic phases in the gel phase of phospholipids; they are L_c, $L_{\beta'}$, $L_{\beta I}$ and $P_{\beta'}$ phases. Appearance of each phase in a phospholipid membrane system might be determined by the balance among intermolecular interactions such as hydrophobic force, electrostatic force and Van der Waals force. The factors influencing the intermolecular interactions are supposed to be molecular structures such as ester-, ether-linkage and polar head, hydration of lipid molecules, alcohols, and so on.

The boundary between the $P_{\beta'}$ phase and the $L_{\beta I}$ phase in the phase diagram of DPPC/aqueous ethanol has not been determined in the fluorometric study by Nambi et al. [25]. By using a density meter, we have determined the boundary. The triple point among the $L_{\beta'}$, $L_{\beta I}$ and $P_{\beta'}$ phases is obtained at 26.7°C at 40 mg/ml of ethanol. This corresponds to the starting point of the suppression of the pretransition that has been reported by Veiro et al.; at 39.1 mg/ml of ethanol (0.85 mol/l) [10]. Another triple point among the $L_{\beta I}$, $P_{\beta'}$ and L_α phases is determined to locate at 39.5°C at 49 mg/ml of ethanol. This is the reflection point of the biphasic ethanol effect on the main transition temperature. As described above, the biphasic effect is apparently the result of the expansion of the $L_{\beta I}$ phase replacing the $P_{\beta'}$ phase and expelling the L_α phase.

By use of freeze-fracture electron microscopy, the $P_{\beta'}$ phase is characterized as a ripple structure of bilayers. And at least two different ripple structures have been recognized; ripples with normal wavelength and with 2-fold wavelength [38,39]. Preliminary experiments using freeze-fracture electron microscopy show that the ripple structure with 2-fold wavelength is less stable than the normal ripple structure and that the ripple structure with 2-fold wavelength appears in the $P_{\beta'}$ phase which exists between the $L_{\beta I}$ and L_α phases in the presence of ethanol.

Calorimetry is widely used in studying the phase behavior of lipid membrane since the phase transition is accompanied by excess heat capacity. By use of DSC, Lohner et al. have observed the phase transition from $L_{\beta I}$ to $P_{\beta'}$ in DHPC [40]. As the density meter was able to observe phase transitions from $L_{\beta'}$ to $L_{\beta I}$ and from $L_{\beta I}$ to $P_{\beta'}$ in phosphatidylcholine dispersions, we have tried to detect these transitions by DSC and succeeded in measuring them. In addition to X-ray differaction, a high-sensitivity density meter and DSC are progressive methods to study the phase behavior of lipid membranes, in particular interdigitated phases.

Acknowledgment

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

References

1 Tanford, C. (1980) The hydrophobic effect, 2nd Edn., John Wiley & Sons, New York.
2 Luzatti, V., Gulik-Krzywicki, T. and Tardieu, A. (1968) Nature 218, 1031–1034.
3 Cullis, P.R. and De Kruijff, B. (1978) Biochim. Biophys. Acta 507, 207–218.
4 Ohki, K., Nagaoka, S., Sogami, M. and Nozawa, Y. (1986) Chem. Phys. Lipids 39, 237–249.
5 Small, E.B., Nicolay, K., Mandersloot, J.G., De Gier, J. and De Kruijff, B. (1987) Biochim. Biophys. Acta 897, 453–466.
6 Ranck, J.L., Keira, T. and Luzzati, V. (1977) Biochim. Biophys. Acta 438, 432–441.

7 McIntosh, T.J., McDaniel, R.V. and Simon, S.A. (1983) Biochim. Biophys. Acta 731, 109–114.
8 McDaniel, R.V., McIntosh, T.J. and Simon, S.A. (1983) Biochim. Biophys. Acta 731, 97–108.
9 Rowe, E.S. (1983) Biochemistry 22, 3299–3305.
10 Veiro, J.A., Nambi, P., Herold, L.L. and Rowe, E.S. (1987) Biochim. Biophys. Acta 900, 230–238.
11 Rowe, E.S. (1985) Biochim. Biophys. Acta 813, 321–330.
12 Wilkinson, D.A., Tirrell, D.A., Turek, A.B. and McIntosh, T.J. (1987) Biochim. Biophys. Acta 905, 447–453.
13 Braganza, L.F. and Worcester, D.L. (1986) Biochemistry 25, 2591–2596.
14 Siminovitch, D.J., Wong, P.T.T. and Mantsch, H.H. (1987) Biochim. Biophys. Acta 900, 163–167.
15 Laggnr, P., Lohner, K., Degovics, G., Muller, K. and Schuster, A. (1987) Chem. Phys. Lipids 44, 31–60.
16 Kim, J.T., Mattai, J. and Shipley, G.G. (1987) Biochemistry 26, 6592–6598.
17 McIntosh, T.J., Simon, S.A., Ellington, J.C., Jr. and Porter, N.A. (1984) Biochemistry 23, 4038–4044.
18 Hui, S.W., Mason, J.T. and Huang, C.-H. (1984) Biochemistry 23, 5570–5577.
19 Xu, H. and Huang, C.-H. (1987) Biochemistry 26, 1036–1043.
20 Huang, C.-H. and Mason, J.T. (1986) Biochim. Biophys. Acta 864, 423–470.
21 Hui, S.W. and Huang, C.-H. (1986) Biochemistry 25, 1330–1335.
22 Stinson, R.H. and Boggs, J.M. (1989) Biochim. Biophys. Acta 986, 234–240.
23 Slater, J.L. and Huang, C.-H. (1988) Prog. Lipid Res. 27, 325–359.
24 Wong, P.T. and Huang, C. (1989) Biochemistry 28, 1259–1263.
25 Nambi, P., Rowe, E.S. and McIntosh, T.J. (1988) Biochemistry 27, 9175–9182.
26 Boggs, J.M., Rangaraj, G. and Watts, A. (1989) Biochim. Biophys. Acta 981, 243–253.
27 Wang, H.-Y., Tummler, B. and Boggs, J.M. (1989) Biochim. Biophys. Acta 985, 182–198.
28 Simon, S.A. and McIntosh, T.J. (1984) Biochim. Biophys. Acta 773, 169–172.
29 Wilkinson, D.A. and Nagle, J.F. (1978) Anal. Biochem. 84, 263–271.
30 Nagle, J.F. and Wilkinson, D.A. (1978) Biophys. J. 23, 159–175.
31 Tristram-Nagle, S., Wiener, M.C., Yang, C.-P. and Nagle, J.F. (1987) Biochemistry 26, 4288–4294.
32 Huang, C. and Charlton, J.P. (1971) J. Biol. Chem. 246, 2555–2560.
33 Ohki, K. (1988) J. Biochem. 104, 14–17.
34 Wiener, M.C., Tristram-Nagle, S., Wilkinson, D.A., Campbell, L.E. and Nagle, J.F. (1988) Biochim. Biophys. Acta 938, 135–142.
35 Tenchov, B.G., Yao, H. and Hatta, I. (1989) Biophys. J. 56, 757–768.
36 Nagle, J.F. (1973) J. Chem. Phys. 58, 252–264.
37 Veiro, J.A., Nambi, P. and Rowe, E.S. (1988) Biochim. Biophys. Acta 943, 108–111.
38 Inoko, Y., Mitsui, T., Ohki, K., Sekiya, Y. and Nozawa, Y. (1980) Phys. Stat. Sol (a) 61, 115–121.
39 Copeland, B.R. and McConnell, H.M. (1980) Biochim. Biophys. Acta 599, 95–109.
40 Lohner, K., Schuster, A., Degovics, G., Muller, K. and Laggner, P. (1987) Chem. Phys. Lipids 44, 61–70.