

# Thermotropic and barotropic phase transitions in bilayer membranes of ether-linked phospholipids with varying alkyl chain lengths

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## Abstract

The bilayer phase transitions of a series of ether-linked phospholipids, 1,2-dialkylphosphatidylcholines containing linear saturated alkyl chain ( $C_n=12, 14, 16$  and  $18$ ), were observed by differential scanning calorimetry (DSC) under ambient pressure and light-transmittance measurements under high pressure. The thermodynamic quantities of the pre- and main-transitions for the ether-linked PC bilayer membranes were calculated and compared with those of a series of ester-linked PCs, 1,2-diacylphosphatidylcholines. The thermodynamic quantities of the main transition for the ether-linked PC bilayers showed distinct dependence on alkyl-chain length and were slightly different from those of the ester-linked PC bilayers. From the comparison of thermodynamic quantities for the main transition between both PC bilayers, we revealed that the attractive interaction in the gel phase for the ether-linked PC bilayers is weaker than that for the ester-linked PC bilayers. Regarding the pretransition, although changes in enthalpy and entropy for both PC bilayers were comparable to each other, the volume changes of the ether-linked PC bilayers roughly doubled those of the ester-linked PC bilayers. The larger volume change results from the smallest partial molar volume of the ether-linked PC molecule in the interdigitated gel phase. Further, we constructed the temperature–pressure phase diagrams for the ether-linked PC bilayers by using the phase-transition data. The region of the interdigitated gel phase in the phase diagrams was extended by applying pressure and by increasing the alkyl-chain length of the molecule. Comparing the phase diagrams with those for the ester-linked PC bilayers, it was proved that the phase behavior of the ester-linked PC bilayers under high temperature and pressure is almost equivalent to that of the ether-linked PC bilayers in the vicinity of ambient pressure.

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## 1. Introduction

The fundamental structure of cell membranes is bilayers composed of phospholipids and many functional proteins buried in the membrane. Cell membranes have high fluidity under physiological conditions, which enables permeation of various substances across the membranes. The membrane fluidity is greatly dependent on kinds of phospholipids and membrane compositions. Most phospholipids in cell membranes of living organisms are ester-linked phospholipids, of which the hydrophobic acyl chains bind to the glycerol back-

bone by an ester linkage. The ester-linked phospholipids such as diacylphosphatidylcholine and diacylphosphatidylethanolamine are extensively used for research concerning model membranes. The properties of bilayer membranes for ester-linked phosphatidylcholine (ester-linked PC) containing linear saturated acyl chains are among the most thoroughly investigated using several physico-chemical methods [1]. We have also studied the thermotropic and barotropic phase transitions of a series of ester-linked PC bilayers, and characterized the thermodynamic quantities of the phase transitions and the temperature–pressure phase diagrams [2–6].

On the other hand, there exist ether-linked phospholipids of which the alkyl chains bind to the glycerol backbone by an ether linkage in the cell membranes of some organisms. It is well known that cell membranes of archaebacteria and deep-sea organisms are occupied by many ether-linked phospholipids

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with branched hydrophobic chains. Ether-linked phospholipids such as 1-alkyl-2-acyl-phosphatidylcholine and dialkylphosphatidylcholine are also found in the plasma and organelle membranes of mammalian species, for example, platelet activating factor (1-alkyl-2-acetyl-phosphatidylcholine) is representative of one. However, only a few studies have been conducted on the physical and chemical properties of the ether-linked phospholipid bilayers, in contrast to the many studies which have been done on ester-linked phospholipids. Some comparative studies of ether-linked phosphatidylcholine (ether-linked PC) bilayers with ester-linked PC bilayers [7–15] and studies of their mixtures [16–21] have been reported. In most studies, phospholipids are confined to PCs containing the same hydrophobic chains of C16 (hexadecyl-, palmitoyl-, or both chains). With the exception of Siminovitch et al. [22] and our previous studies [5,23,24], there have been no reports made concerning pressure studies of ether-linked phospholipid bilayer membranes.

In the present study, in order to examine the thermotropic and barotropic phase transitions of the ether-linked phospholipid bilayer membranes, we chose four kinds of ether-linked PCs containing linear saturated alkyl chain, didodecylphosphatidylcholine (*O*-12:0-PC), ditetradecylphosphatidylcholine (*O*-14:0-PC), dihexadecylphosphatidylcholine (*O*-16:0-PC) and dioctadecylphosphatidylcholine (*O*-18:0-PC). We performed differential scanning calorimetry (DSC) and high-pressure light transmittance measurement on these ether-linked PC bilayer membranes. By use of the thermal and pressure data, the thermodynamic quantities for the bilayer phase transitions were calculated and the temperature–pressure phase diagrams were constructed. The effect of alkyl-chain length on the thermodynamic quantities and the phase behavior is considered systematically. Further, the substitution effect of an ether linkage for an ester linkage in the PC molecule on these properties, especially focusing on the bilayer interdigitation, is discussed by comparison with the corresponding results for the homologous series of ester-linked PC bilayers [2,3].

## 2. Materials and methods

Four kinds of ether-linked phospholipids, *O*-12:0-PC (1,2-di-*O*-dodecyl-*sn*-glycero-3-phosphocholine), *O*-14:0-PC (1,2-di-*O*-tetradecyl-*sn*-glycero-3-phosphocholine), *O*-16:0-PC (1,2-di-*O*-hexadecyl-*sn*-glycero-3-phosphocholine) and *O*-18:0-PC (1,2-di-*O*-octadecyl-*sn*-glycero-3-phosphocholine), were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). *O*-16:0-PC was also purchased from Sigma-Aldrich Co. (St. Louis, MO). They were directly used without further purification. Ethylene glycol (guaranteed reagent >99.5%) was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). Water was distilled twice after deionization, where the second step was done from dilute alkaline permanganate solution. The multilamellar vesicles of the ether-linked PC were prepared by suspending each PC in water using a vortex mixer at concentrations of 1.0–7.0 mmol kg<sup>-1</sup> for DSC measurements and at 1.0 or 2.0 mmol kg<sup>-1</sup> for light-transmittance measurements, respectively. The suspensions were sonicated for a few minutes by using a Branson model 185 sonifier at a temperature several degrees above the main-transition temperature of each lipid. Then, the sample solutions were allowed to stand for 24 h at approximately –20 °C (DSC) or 5 °C (DSC and light-transmittance).

The phase transitions of PC bilayer membranes under ambient pressure were observed by use of a Microcal MCS high-sensitivity differential scanning calorimeter (Northampton, MA). After a degas treatment of 10–15 min for each

sample and reference solutions, the measurements started with a heating rate of 0.5 K min<sup>-1</sup>. The endothermic peaks in the DSC thermograms were analyzed by the software ORIGIN from MicroCal. DSC measurements in the presence of ethylene glycol were also performed by using a SSC 5200-DSC 120 calorimeter (SII Nanotechnology Co. Ltd., Chiba, Japan). The prepared sample and reference solutions were sealed up to the amount of 60 µl in DSC silver cells. After thermal equilibrium, the measurements were carried out under a heating rate of 0.5 K min<sup>-1</sup>. The peaks were analyzed by attached software for the apparatus.

The phase transitions under high pressure were observed by two kinds of light-transmittance measurements. One being an isothermal barotropic observation by scanning pressure at a constant temperature, and the other is an isobaric thermotropic observation by scanning temperature at a constant pressure. The high-pressure transmittance apparatus and the detailed procedures of two methods are described elsewhere [23,25].

## 3. Results

### 3.1. Thermal behavior of ether-linked PC bilayer membranes at ambient pressure

The DSC thermograms of *O*-14:0-PC, *O*-16:0-PC and *O*-18:0-PC bilayer membranes in a heating scan are presented in Fig. 1. We observed small and large endothermic peaks in the thermograms of the *O*-16:0-PC and *O*-18:0-PC bilayers: a small peak at a low temperature corresponds to the pretransition and a large peak at a high temperature corresponds to the main transition, respectively. In the *O*-14:0-PC bilayer, we observed only a main transition by the DSC measurements. The pretransition could not be obtained with good reproducibility by the DSC method, however, the pretransition temperature of the *O*-14:0-PC bilayer was determined by light transmittance measurement under ambient pressure. The thermal behavior of the ether-linked PC bilayers is quite similar to that of the ester-linked bilayers such as dimyristoylphosphatidylcholine (14:0-PC, so called DMPC), dipalmitoylphosphatidylcholine (16:0-PC, DPPC) and distearoylphosphatidylcholine (18:0-PC, DSPC) [3,26], however, the pretransition is different from that of the latter. It is well known that bilayer membranes of ester-

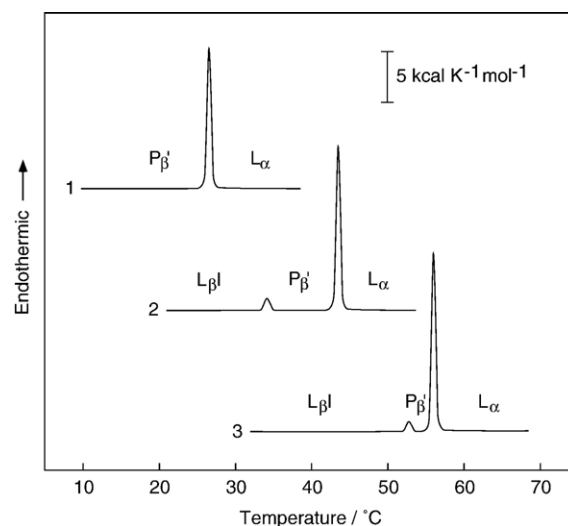


Fig. 1. DSC heating thermograms for solutions of ether-linked PC bilayer membranes: (1) *O*-14:0-PC, (2) *O*-16:0-PC, (3) *O*-18:0-PC.

linked PCs undergo the pretransition from the lamellar gel ( $L_{\beta}'$ ) phase to the ripple gel ( $P_{\beta}'$ ) phase and sequentially the main transition from the  $P_{\beta}'$  phase to the liquid crystal ( $L_{\alpha}$ ) phase. On the other hand, since the ether-linked PC bilayers, at a lower temperature than the pretransition temperature, exist as a phase called the interdigitated gel ( $L_{\beta}I$ ) phase, the pretransition corresponds to the phase transition from the  $L_{\beta}I$  phase to the  $P_{\beta}'$  phase [7–10]. It is a notable characteristic of ether-linked PC bilayers that they spontaneously form the  $L_{\beta}I$  phase solely by hydration under ambient pressure, although formation of the  $L_{\beta}I$  phase in ester-linked PC bilayers requires other driving forces such as addition of amphiphiles [27–29], substitution of solvent [30,31], or the application of high pressure [3–5,32,33]. The phase-transition temperatures and enthalpies ( $\Delta H$ ) of the ether-linked PC bilayer membranes obtained by DSC measurements are listed together with the standard deviations in Table 1. The pre- and main-transition temperatures and the  $\Delta H$  value of the main transition increased with an increase of alkyl-chain length, whereas the pretransition had a similar  $\Delta H$  value to each other. The main-transition temperatures were comparable to those reported by McKeone et al. [16] but their enthalpies were higher than those recorded in the present data.

In the case of *O*-12:0-PC bilayer membrane, a large endothermic peak of ice-melting prevented us from the observation of bilayer phase transition by the DSC method. From the dependence of transition temperatures on alkyl-chain lengths, the pre- and main transitions of the *O*-12:0-PC bilayer are expected to exist at a lower temperature than 0 °C and in the vicinity of 0 °C, respectively. We attempted to determine the phase transitions under the condition of no ice-melting endothermic peak by stepwise solvent substitution from water to ethylene glycol [25,34]. The effect of ethylene glycol on the main transition of the *O*-12:0-PC bilayer together with that of the *O*-14:0-PC bilayer is shown in Fig. 2. The main-transition temperatures and enthalpies of the *O*-12:0-PC and *O*-14:0-PC bilayers increased with increasing ethylene glycol concentration and the dependence of their transition temperatures and enthalpies on ethylene glycol concentration resembled each other. The pretransition of the *O*-12:0-PC bilayer membrane was not detected even if the ethylene glycol solutions were used due to the existence of the transition in the much lower temperature region. Because the extrapolated values of the main-transition temperature and enthalpy to 0 wt.% ethylene glycol concentration in the *O*-14:0-PC bilayer membrane were in good agreement with those obtained from the DSC

Table 1

Temperatures and enthalpies for the phase transitions of ether-linked PC bilayer membranes obtained by DSC

Lipid	Transition	$T$ (°C)	$\Delta H$ (kcal mol <sup>-1</sup> )	$\Delta H$ (kJ mol <sup>-1</sup> )	No. of measurements
<i>O</i> -14:0-PC	$P_{\beta}'/L_{\alpha}$	26.8±0.02	5.4±0.14	22.7±0.57	9
<i>O</i> -16:0-PC	$L_{\beta}I/P_{\beta}'$	34.2±0.39	1.3±0.16	5.3±0.67	11
	$P_{\beta}'/L_{\alpha}$	43.6±0.05	8.1±0.16	33.7±0.66	15
<i>O</i> -18:0-PC	$L_{\beta}I/P_{\beta}'$	53.0±0.29	1.1±0.09	4.6±0.38	16
	$P_{\beta}'/L_{\alpha}$	55.9±0.05	10.8±0.46	45.2±1.92	16

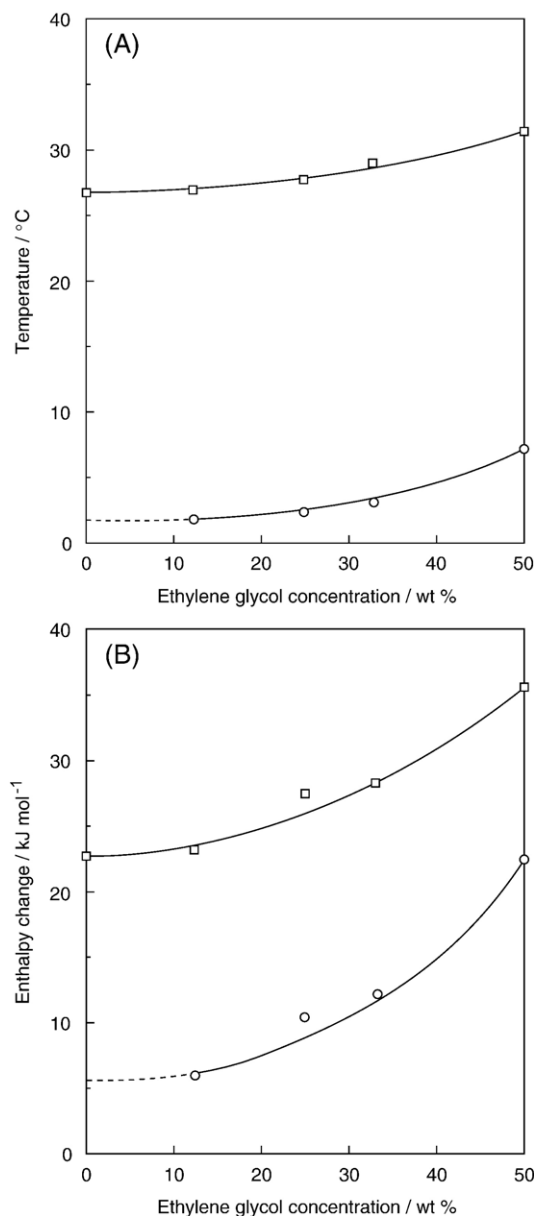


Fig. 2. Main transition of ether-linked PC bilayer membranes observed in ethylene glycol solutions. (A) transition temperature, (B) transition enthalpy; (○) *O*-12:0-PC, (□) *O*-14:0-PC.

measurements by use of Microcal's calorimeter, the corresponding values of the *O*-12:0-PC bilayer membrane were determined by the same extrapolation procedure, which are also given in Table 2.

### 3.2. Phase transitions of ether-linked PC bilayer membranes under high pressure

The phase transition of lipid bilayers induces abrupt changes in membrane properties. There are several methods for detecting the changes. One of the methods utilizes the change of the turbidity of the phospholipid vesicle suspension, for example, translucent in the liquid crystalline state and less translucent in the gel state, which can be monitored by light

Table 2  
Thermodynamic properties for the phase transitions of ether-linked PC bilayer membranes

Lipid	Transition	$T$ (K)	$T$ (°C)	$dT/dp$ (K MPa <sup>-1</sup> )	$\Delta H$ (kJ mol <sup>-1</sup> )	$\Delta S$ (J K <sup>-1</sup> mol <sup>-1</sup> )	$\Delta V$ (cm <sup>3</sup> mol <sup>-1</sup> )
O-12:0-PC	$L_{\beta}I/P_{\beta}'$	223.2	-50.0 <sup>a</sup>	0.280	–	–	–
	$P_{\beta}'/L_{\alpha}$	274.9	1.7	0.197	5.5	20	3.9
O-14:0-PC	$L_{\beta}I/P_{\beta}'$	281.5	8.3	0.294	–	–	–
	$P_{\beta}'/L_{\alpha}$	300.0	26.8	0.229	22.7	76	17.3
O-16:0-PC	$L_{\beta}I/P_{\beta}'$	307.4	34.2	0.308	5.3	17	5.3
	$P_{\beta}'/L_{\alpha}$	316.8	43.6	0.238	33.7	106	25.3
O-18:0-PC	$L_{\beta}I/P_{\beta}'$	327.2	53.0	0.314	4.6	14	4.4
	$P_{\beta}'/L_{\alpha}$	330.1	55.9	0.245	45.2	137	33.5

<sup>a</sup> The value was determined from the extrapolation of  $T$ - $p$  curve to ambient pressure.

absorbance (transmittance) or scattering. Turbidity change mainly results from the change in optical properties of the membrane such as a refractive index, and it is attributable to multiple factors such as changes in vesicle size and in fluctuation of movement between hydrocarbon chains of lipid molecules, etc [35,36]. We adopted the above light-transmittance measurement of vesicle suspension to detect phase transition under high pressure.

Fig. 3 demonstrates one example of phase-transition detection by the light-transmittance method under high pressure, namely, the isobaric thermotropic observations of O-16:0-PC bilayer phase transition in the heating process at various pressures. We observed two abrupt changes in the transmittance vs. temperature curve under ambient pressure. The temperatures of both changes coincided with the pre- and main-transition temperatures determined by the DSC measurements. We cannot distinguish the difference in gel phases between ether- and ester-linked PC bilayer membranes on DSC thermograms, but can distinguish between the gel phases in the transmittance measurements because the pretransition of the former was accompanied by a decrease

in transmittance, while that of the latter was accompanied by an increase in transmittance (curve 4), as shown in the figure. Rowe et al. [37,38] have shown similar behavior regarding transmittance curves in alcohol-induced interdigitation of ester-linked PC and ether-linked PC bilayers. The pre- and main-transition temperatures were elevated by applying pressure. Regarding the reversibility of phase transitions, the main transition ( $P_{\beta}'/L_{\alpha}$  transition) is reversible, whereas there exists thermal hysteresis in the pretransition ( $L_{\beta}I/P_{\beta}'$  transition) because the  $L_{\beta}I$  phase formation is a slow process [23,32,39]. Both transition temperatures of the O-12:0-PC and O-14:0-PC bilayer membranes, which could be determined with difficulty or not by the DSC measurements, were easily observed in the high-pressure region because of the elevation of transition temperatures by the application of pressure. Similarly, the phase-transition pressures of ether-linked PC bilayer membranes were determined as a function of temperature from transmittance vs. pressure curves in the isothermal barotropic observations [23].

### 3.3. Phase diagrams of ether-linked PC bilayer membranes

The temperature ( $T$ )-pressure ( $p$ ) phase diagrams of four ether-linked PC bilayer membranes are respectively illustrated in Fig. 4. We immediately notice that all the PC bilayer membranes show similar phase behavior. The pre- and main-transition temperatures increased by applying pressure. The  $T$ - $p$  curve for the pretransition is almost linear while that for the main-transition has a slightly upward convex. Since the pressure dependence of transition temperature ( $dT/dp$ ) was greater in pretransition than that in main transition, both transition curves intersected with each other in the high pressure region. The intersection point formed a triple point on the phase diagram among the  $P_{\beta}'$ ,  $L_{\beta}I$  and  $L_{\alpha}$  phases. The pressures and temperatures of the triple point were 225 MPa and 73 °C for O-14:0-PC, 110 MPa and 70 °C for O-16:0-PC and 50 MPa and 68 °C for O-18:0-PC, respectively. The point of the O-12:0-PC bilayer membrane was approximately obtained as 420 MPa and 83 °C from the intersection between the extrapolated pre- and main-transition curves to high pressure. At higher pressures than the triple point, the  $P_{\beta}'$  phase disappeared and we observed only the main transition from the  $L_{\beta}I$  phase to the  $L_{\alpha}$  phase. The new pressure-induced phase as seen in ester-linked PC bilayer

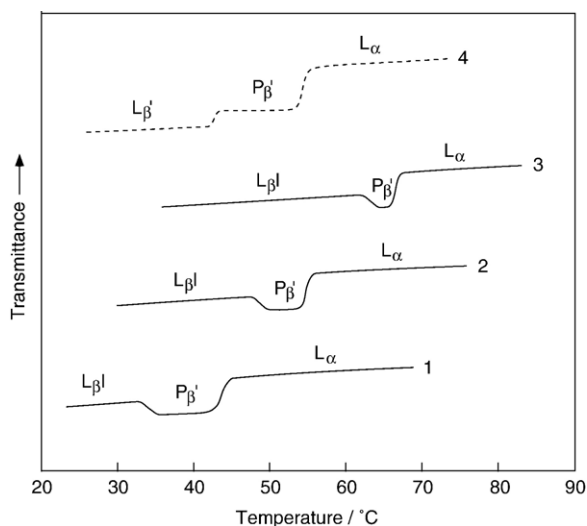


Fig. 3. Typical curves for isobaric thermotropic phase transitions of O-16:0-PC bilayer membrane in the heating process at various pressures: (1) 0.1 MPa, (2) 45 MPa, (3) 91 MPa, (4) 16:0-PC bilayer membrane at 73 MPa.

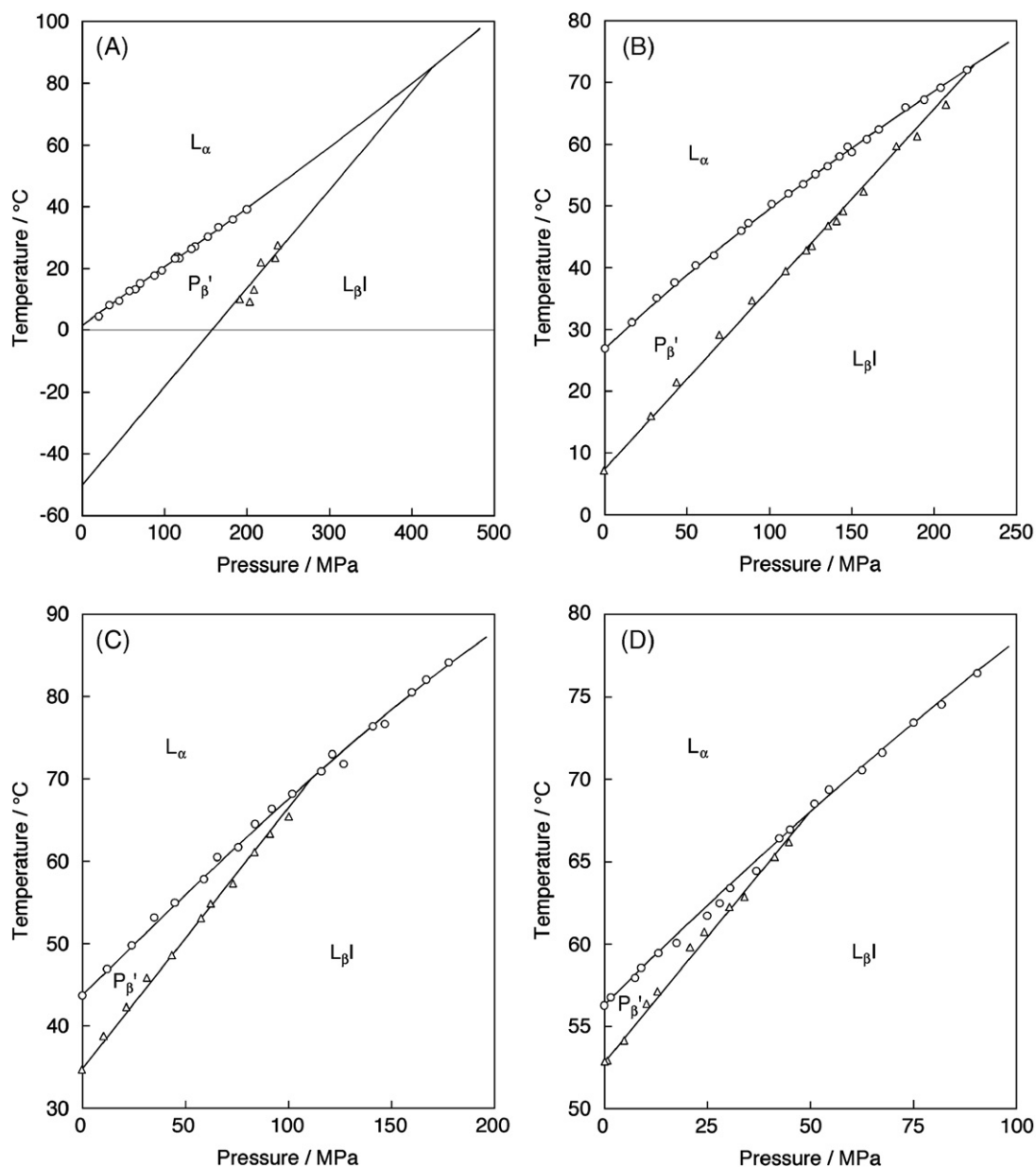


Fig. 4. Temperature–pressure phase diagrams of ether-linked PC bilayer membranes: (A) *O*-12:0-PC, (B) *O*-14:0-PC, (C) *O*-16:0-PC, (D) *O*-18:0-PC. Phase transitions: (○) main transition;  $P_{\beta'}/L_{\alpha}$  or  $L_{\beta I}/L_{\alpha}$ , ( $\Delta$ ) pretransition;  $L_{\beta I}/P_{\beta'}$ .

membranes [3–5] was not observed in any bilayer membranes. The pretransition temperature of the *O*-12:0-PC bilayer membrane under ambient pressure, which could not be obtained from either the DSC or light-transmittance measurements, was approximately determined as  $-50$  °C from the extrapolation of the  $T$ – $p$  curve to 0.1 MPa.

#### 4. Discussion

##### 4.1. Thermodynamic properties for phase transitions of ether-linked PC bilayer membranes

Other thermodynamic quantities associated with the phase transitions under ambient pressure, entropy ( $\Delta S$ ) and volume

( $\Delta V$ ) changes, were calculated from the equations held at phase equilibrium:

$$\Delta S = \Delta H/T \quad (1)$$

and

$$\Delta V = \Delta H(dT/dp)/T, \quad (2)$$

where the  $T$  and  $\Delta H$  values were taken from Table 1 and the  $dT/dp$  values obtained from Fig. 4 were used. In Table 2 the resulting quantities of the ether-linked PC bilayers are summarized. The corresponding results for the homologous series of ester-linked PC bilayers, which were obtained in the previous study [2,3], are also given in Table 3 for comparison.

Table 3  
Thermodynamic properties for the phase transitions of ester-linked PC bilayer membranes [2,3]

Lipid	Transition	$T$ (K)	$T$ (°C)	$dT/dp$ (K MPa <sup>-1</sup> )	$\Delta H$ (kJ mol <sup>-1</sup> )	$\Delta S$ (J K <sup>-1</sup> mol <sup>-1</sup> )	$\Delta V$ (cm <sup>3</sup> mol <sup>-1</sup> )
12:0-PC	P' <sub>β</sub> /L <sub>α</sub>	270.9	-2.3	0.199	7.5	28	5.5
13:0-PC	L' <sub>β</sub> /P' <sub>β</sub>	272.4	-0.8 <sup>a</sup>	0.140	2.1 <sup>a</sup>	8	1.1
	P' <sub>β</sub> /L <sub>α</sub>	286.8	13.6	0.210	16.0	56	11.7
14:0-PC	L' <sub>β</sub> /P' <sub>β</sub>	287.1	13.9	0.130	4.0	14	1.8
	P' <sub>β</sub> /L <sub>α</sub>	297.1	23.9	0.212	24.7	83	17.6
15:0-PC	L' <sub>β</sub> /P' <sub>β</sub>	297.8	24.6	0.120	4.2	14	1.7
	P' <sub>β</sub> /L <sub>α</sub>	307.0	33.8	0.215	30.3	99	21.2
16:0-PC	L' <sub>β</sub> /P' <sub>β</sub>	307.5	34.3	0.130	4.6	15	1.9
	P' <sub>β</sub> /L <sub>α</sub>	315.2	42.0	0.220	36.4	115	25.4
17:0-PC	L' <sub>β</sub> /P' <sub>β</sub>	316.1	42.9	0.130	4.6	15	1.9
	P' <sub>β</sub> /L <sub>α</sub>	322.1	49.0	0.224	41.4	129	28.8
18:0-PC	L' <sub>β</sub> /P' <sub>β</sub>	324.1	50.9	0.140	5.0	15	2.2
	P' <sub>β</sub> /L <sub>α</sub>	328.8	55.6	0.230	45.2	137	31.6

<sup>a</sup> From data of Lewis et al. [26].

All thermodynamic quantities of the main transition for the ether-linked PC bilayers increased with an increase in alkyl-chain length, while those of the pretransition were comparable to each other except for the transition temperature. The chain-length dependence of  $\Delta H$  and  $\Delta V$  values of the main transition for the ether- and ester-linked PC bilayers is compared in Fig. 5. The  $\Delta H$  and  $\Delta V$  values for the bilayer membranes of the ether-linked PCs were close to those of the ester-linked PCs with the same hydrophobic chain lengths, and the chain-length dependence of  $\Delta H$  and  $\Delta V$  values for both PCs was almost the same and is indicated by a smooth curve, not a linear function. Both PC bilayer membranes undergo the same kind of main transition; the P'<sub>β</sub>/L<sub>α</sub> transition. The main transition is caused by a chain order-to-disorder change, namely the chain melting, due to the *trans-gauche* conformational change of the hydrophobic chain. Therefore, we can say that the thermodynamic quantities of the main transition are almost the same magnitude for the bilayer membranes of both PCs with the same chain lengths irrespective of the difference in linkage of the glycerol backbone and the magnitude is proportional to the chain length. Similar thermodynamic quantities between the ether- and ester-linked PC bilayers may be attributable to the same number of the *trans-gauche* conformational changes of the hydrophobic chain, that is, the same number of methylene groups between the terminal methyl group and ether-linked carbon in the ether-linked PC molecule and those between the terminal methyl group and carbonyl carbon in the ester-linked PC molecule.

#### 4.2. Difference in thermodynamic properties between ether- and ester-linked PC bilayer membranes

In Table 4, the difference in the thermodynamic properties of the main transition between the bilayer membranes of the ether- and ester-linked PCs with the same chain lengths are listed. Here the values of the difference were obtained by subtraction of the properties for the ether-linked PC bilayers from those for the ester-linked PC bilayers such as:

$$\Delta Y(1) = Y(n:0\text{-PC}) - Y(O\text{-}n:0\text{-PC}),$$

$$Y = T, \Delta H, \Delta S \text{ and } \Delta V, \quad (3)$$

where  $n$  is the carbon number of the hydrophobic chain. The  $\Delta H$ ,  $\Delta S$  and  $\Delta V$  values of the ether-linked PC bilayers, except the *O*-18:0-PC bilayer, had slightly smaller values than those of the ester-linked PC bilayers. However, it is noted that all the main-transition temperatures of the ether-linked PC bilayers are slightly higher than those of the ester-linked PC bilayers. The phase-transition temperatures of phospholipid bilayers are known to be greatly dependent on the hydrophilic part of the lipid molecule. We have shown in the previous study [4,6] that the phase-transition temperatures of *N*-methylated phosphatidylethanolamines increase with a decrease in the size of the polar head group, and are proportional to the ability of hydrogen-bond formation between the head groups. The ether bond has a smaller size and more reduced polarity than the ester bond. Hence, the degree of hydration in the head group of the ether-linked PC molecules is expected to be lower than that of the ester-linked PC bilayers. Channareddy and Janes [13] have investigated the amount of bound water in the *O*-16:0-PC and 16:0-PC bilayers by a dual radiolabel centrifugal technique and suggested that 8.1 water molecules are bound in the P'<sub>β</sub> phase of the 16:0-PC bilayer, whereas the number drops to 5.4 in the P'<sub>β</sub> phase of the *O*-16:0-PC bilayer. Because the lesser hydration of the ether bond than the ester bond favors tighter packing of the bilayer, this seemingly explains the elevation in the main-transition temperatures of ether-linked PC bilayers as compared with those of ester-linked PC bilayers.

However, the nature of the ether bond mentioned above is incompatible with the thermodynamic quantities of the main transition and with the fact that the ether-linked PC bilayers undergo the chain interdigitation at temperatures below the pretransition temperatures. Furthermore, Lewis et al. have reported in their comparative study of *O*-16:0-PC and 16:0-PC bilayers by Fourier transform infrared spectroscopy [12,40], that the *O*-16:0-PC bilayer facilitates greater hydration of the head group and polar/apolar interfacial regions of the bilayer than the 16:0-PC bilayer. They suggested the incompatibility between the nature of linkages and the bilayer hydration and also pointed out that there is a change in the preferred conformation and/or orientation of the glycerol backbone. Most studies of ether-linked PC bilayer membranes have been

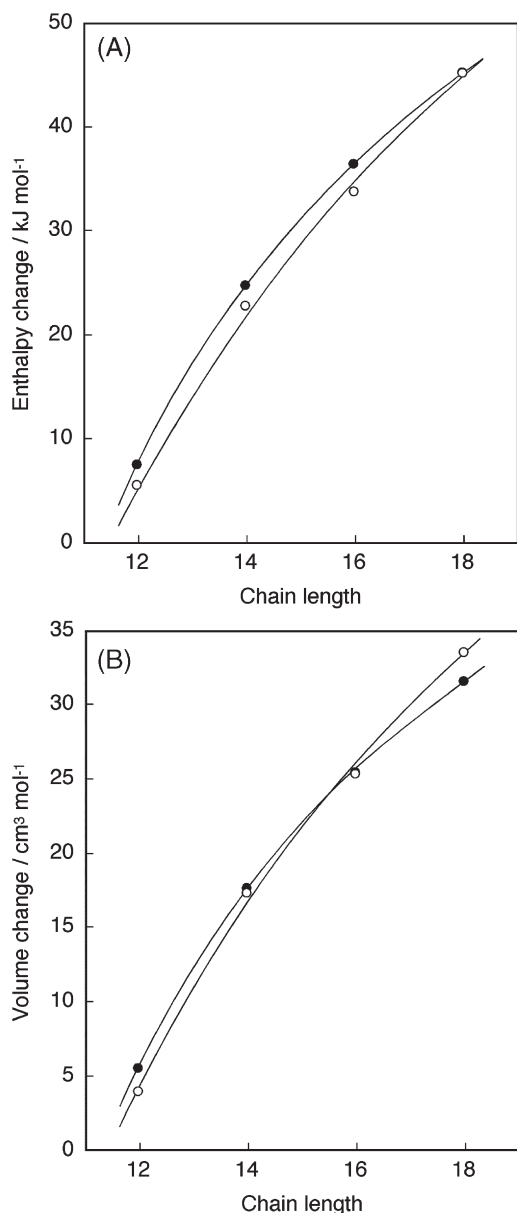


Fig. 5. Effect of hydrophobic chain length on main-transition temperature and enthalpy of ether- and ester-linked PC bilayer membranes: (A) transition enthalpy, (B) transition volume; (○) ether-linked PC, (●) ester-linked PC.

performed on the *O*-16:0-PC bilayer and the results have been compared with those of 16:0-PC bilayer without exception. In these studies, the part of hydrophobic chain for the ether- and ester-linked PC molecules is implicitly regarded as equivalent. As is seen from Tables 2 and 3, thermodynamic properties of phospholipid bilayer membranes are dependent on the hydrophobic chain length of the lipid molecule as well as the hydrophilic part. We speculate that the incompatibility arises from the notion favoring the equivalency of the hydrophobic part of both ether- and ester-linked PC molecules with the same hydrophobic chain lengths.

The most noticeable difference between ether- and ester-linked PC molecules is the absence and the presence of carbonyl carbon. The ester-linked PC with almost the same hydrophobic part as *O*-16:0-PC is 17:0-PC, not 16:0-PC. It is useful to

examine the effect of carbonyl carbon on the thermodynamic properties of main transition. For this purpose, we evaluated the difference between the properties for the ether-linked PC bilayers and those for the ester-linked PC bilayers by the following relation:

$$\Delta Y(2) = Y((n+1):0\text{-PC}) - Y(O\text{-}n:0\text{-PC}),$$

$$Y = T, \Delta H, \Delta S \text{ and } \Delta V. \quad (4)$$

The above values correspond to the change of thermodynamic quantities of PC bilayer by the introduction of two carbonyl groups into the linkage between glycerol backbone and alkyl chains. The values of the difference calculated by using the values in Tables 2 and 3 are given in Table 4. The  $\Delta Y(2)$  values became all positive and decreased in the bilayer membranes of PC with longer hydrophobic chains. That is, the introduction of two carbonyl groups increased the thermodynamic properties and the effect was weakened with an elongation of hydrophobic chains. Since the introduction of two carbonyl groups brings about an increase in the probability of one more *trans-gauche* conformational change of the hydrophobic chains simultaneously, we also calculated the change of thermodynamic properties by the introduction of two methylene groups instead of the carbonyl carbons and compared both the effects on the PC bilayers. It should be noted that the effect of the introduction of two methylene groups on the thermodynamic properties of the main transition can be directly evaluated from Eqs. (3) and (4),

$$\Delta Y(3) = \Delta Y(2) - \Delta Y(1) = Y((n+1):0\text{-PC})$$

$$- Y(n:0\text{-PC}), Y = T, \Delta H, \Delta S \text{ and } \Delta V. \quad (5)$$

The resulting  $\Delta Y(3)$  values are also given in Table 4. The introduction of two methylene groups also increased all thermodynamic properties and decreased the thermodynamic properties with an elongation of chains in the same manner as the introduction of two carbonyl groups. Upon careful comparison of the introduction of carbonyl and methylene groups, the carbonyl group is less effective for the elevation of transition temperature, while the thermodynamic quantities (such as  $\Delta H$ ,  $\Delta S$  and  $\Delta V$ ) are elevated more effectively by the carbonyl group than by the methylene group. The positive values of  $\Delta Y(2)$  and  $\Delta Y(3)$  clearly indicate that the introduction of carbonyl carbons and methylene groups stabilizes the gel phase of the PC bilayer membranes, so that the main-transition temperature elevates. This stabilization of the gel phase may be attributable to the increase in lateral interaction between the phospholipid molecules in the bilayer; the greater hydrophilic interaction by hydration around carbonyl groups and the enhanced van der Waals interaction between hydrophobic chains. Taking into account that  $\Delta Y(1) = \Delta Y(2) - \Delta Y(3)$ , it can be said from the  $\Delta H$ ,  $\Delta S$  and  $\Delta V$  values that the substitution of ester carbonyl for the ether-linked methylene group also provides the stabilization of the gel phase, and the stabilization effect is canceled out in the bilayer membranes of PC with longer chains such as carbon number 18. In the main-transition temperatures, both the introduction of carbonyl groups and methylene groups increases

Table 4  
Difference in thermodynamic properties for the main transitions between two kinds of PC bilayer membranes

Quantity	Lipid combination	$\Delta T$ (°C)	$\Delta(\Delta H)$ (kJ mol <sup>-1</sup> )	$\Delta(\Delta S)$ (J K <sup>-1</sup> mol <sup>-1</sup> )	$\Delta(\Delta V)$ (cm <sup>3</sup> mol <sup>-1</sup> )
$\Delta Y(1)$	12:0-PC– <i>O</i> -12:0-PC	–4.0	2.0	8	1.6
	14:0-PC– <i>O</i> -14:0-PC	–2.9	2.0	7	0.3
	16:0-PC– <i>O</i> -16:0-PC	–1.6	2.7	9	0.1
	18:0-PC– <i>O</i> -18:0-PC	–0.3	0	0	–1.9
$\Delta Y(2)$	13:0-PC– <i>O</i> -12:0-PC	11.9	10.5	36	7.7
	15:0-PC– <i>O</i> -14:0-PC	7.0	7.6	23	3.9
	17:0-PC– <i>O</i> -16:0-PC	5.0	7.7	23	3.5
$\Delta Y(3)$	13:0-PC–12:0-PC	15.9	8.5	28	6.1
	15:0-PC–14:0-PC	9.9	5.6	16	3.6
	17:0-PC–16:0-PC	6.6	5.0	14	3.4

The differences were obtained by subtraction of the bilayer properties for the right side PC from those for the left side PC.

the transition temperatures, which leads to the stabilization of the gel phase. However, since the transition-temperature elevation brought upon by the introduction of the carbonyl group is smaller than that by the methylene group, the  $\Delta Y(1)$  values become apparently negative in spite of the stabilization of the gel phase. The above consideration among the  $\Delta Y(1)$ ,  $\Delta Y(2)$  and  $\Delta Y(3)$  values leads us to the conclusion that the interaction in the ether-linked PC bilayer membranes is weaker than that in the ester-linked PC bilayer membranes, although the main-transition temperatures of the former are apparently higher than those of the latter. The reduced interaction between the head group of the *O*-16:0-PC bilayer membranes has been reported [41–43].

#### 4.3. Volume behavior of gel phases of ether-linked PC bilayer membranes

The bilayer membranes of the ether- and ester-linked PCs undergo different kinds of pretransitions: the  $L_{\beta}I/P_{\beta}'$  transition for the ether-linked PC bilayers and the  $L_{\beta}'/P_{\beta}'$  transition for the ester-linked PC bilayers. We expected great differences in the thermodynamic quantities of the pretransitions between both PC bilayers, but the detectable difference was not observed in the  $\Delta H$  and  $\Delta S$  values for both PC bilayers, as seen in the comparable values of the *O*-16:0-PC and *O*-18:0-PC bilayers with the 16:0-PC and 18:0-PC bilayers. However, the  $dT/dp$  values of the *O*-16:0-PC and *O*-18:0-PC bilayers were about twice as large as those of the 16:0-PC and 18:0-PC bilayers, hence, the  $\Delta V$  values of the former roughly doubled those of the latter. The pretransition corresponds to the reconstruction of bilayer packing and the thermodynamic quantities are known to be much smaller than those of the main transition [3,12,26]. The pretransition of ether-linked PC bilayers is the transition between bilayer and non-bilayer, not between bilayers like ester-linked PC bilayers, although both the pretransitions are the transitions between gel phases. Regarding the ester-linked PC bilayers, it was found from the  $\Delta V$  values that the difference in partial molar volume of the PC molecule between the  $L_{\beta}'$  and  $P_{\beta}'$  phases was small (ca. 2 cm<sup>3</sup> mol<sup>-1</sup>). The hydrophobic chains in the  $L_{\beta}'$  phase are tilted with respect to the bilayer normal and orderly packed in a regular hexagonal lattice in the lamellae while in the  $P_{\beta}'$  phases the bilayer membranes are solely distorted by a periodic ripple in the plane of the lamellae with no alterations in the structure of the chains. So, the partial molar

volumes of the PC molecule in the  $L_{\beta}'$  and  $P_{\beta}'$  phases may have values similar to each other. In the case of the ether-linked PC bilayers, the hydrophobic chains in the  $L_{\beta}I$  phase are fully interdigitated and exist in a more condensed state than any other gel phase. The  $L_{\beta}I/P_{\beta}'$  transition accompanies a larger packing change in the bilayer than the  $L_{\beta}'/P_{\beta}'$  transition. Therefore, we may say that the large  $\Delta V$  values for the ether-linked PC bilayers (ca. 5 cm<sup>3</sup> mol<sup>-1</sup>) result from the smallest partial molar volume of the PC molecule in the  $L_{\beta}I$  phase. Ohki et al. [44] have proved by scanning densitometry, for the system of ester-linked PC bilayers containing ethanol, that the partial molar volumes of ester-linked PC bilayers in the gel phase increase in the order of the  $L_{\beta}I$ ,  $L_{\beta}'$  and  $P_{\beta}'$  phases. The present results are entirely consistent with their data and suggest the denser packing of the  $L_{\beta}I$  phase.

#### 4.4. Effect of ethylene glycol on thermal behavior of ether-linked PC bilayer membranes

We observed that the main-transition temperatures and enthalpies of the *O*-12:0-PC and *O*-14:0-PC bilayer membranes varied depending on ethylene glycol concentrations as is seen in Fig. 2. Although the effect of ethylene glycol on the transitions at low concentrations is marginal, the addition of a large amount of ethylene glycol significantly affects the transitions. Ethylene glycol is often used as an antifreeze because of its dehydration ability from its strong hydrophilic nature. At high concentrations ethylene glycol molecules displace water molecules in the interfacial region of the bilayer membranes. The displacement may bring about new interaction between the polar head groups of the PC molecules and the ethylene glycol molecules. Judging from the elevation of the main-transition temperatures and enthalpies, such interactions containing dipole–dipole or ion–dipole between them, enhance the stability of the gel phase. Furthermore, it is necessary to take into account another factor regarding changes in thermodynamic quantities of the main-transition. The main transition from the  $P_{\beta}'$  phase to the  $L_{\alpha}$  phase probably converts into the transition from the  $L_{\beta}I$  phase to the  $L_{\alpha}$  phase in regions of high ethylene glycol concentration. In bilayer membranes of the ester-linked PCs such as 16:0-PC and 18:0-PC, it was confirmed that the  $L_{\beta}I$  phase instead of the  $P_{\beta}'$  phase is induced at a certain concentration of ethylene glycol or glycerol with the disappearance of the pretransition



[31,45–48]. We speculate similar induction of the  $L_{\beta}I$  phase in the  $O$ -12:0-PC and  $O$ -14:0-PC bilayer membranes. However, since the pretransition in both bilayers in the presence of ethylene glycol was not observed due to their low transition temperatures and small enthalpies, we could not prove the  $L_{\beta}I/L_{\alpha}$  transition at high ethylene glycol concentrations in the present study.

#### 4.5. Effect of alkyl-chain length on phase diagrams of ether-linked PC bilayer membranes

As is shown in the  $T$ - $p$  phase diagrams of four ether-linked PC bilayer membranes in Fig. 4, the pretransition temperature approached the main-transition temperature and the triple point among the  $P'_{\beta}$ ,  $L_{\beta}I$  and  $L_{\alpha}$  phases shifted to the lower temperature and pressure region with an increase in alkyl-chain length. Therefore, the region of the  $L_{\beta}I$  phase was extended while that of the  $P'_{\beta}$  phase was markedly reduced by increasing the hydrophobicity of the PC molecule. Pressure reduces the volume of a system and accelerates to shift toward the state having a smaller partial molar volume. The  $\Delta V$  values of the pre- and main-transition suggest that the partial molar volumes of ether-linked PC bilayers in the  $L_{\beta}I$  phase is the smallest of all phases in this study. In the high-pressure region, the  $L_{\beta}I$  phase is more stable than the  $P'_{\beta}$  phase and extends its phase region up into the high temperature range. Furthermore, the formation of the  $L_{\beta}I$  phase is accelerated by the elongation of saturated hydrocarbon chains, namely, by the enhancement of the van der Waals interaction between hydrophobic chains of PC molecules. We showed in the

previous study [3] that the pressure-induced interdigitation of the ester-linked PC bilayer membranes is easier for the PC with a longer acyl-chain length. The increase in the van der Waals interaction synergizes the effect of pressure, which brings about the further extension of the  $L_{\beta}I$  phase (in other words, shrinkage of the  $P'_{\beta}$  phase) in the ether-linked PC bilayer membranes.

#### 4.6. Difference in phase diagram between ether- and ester-linked PC bilayer membranes

Finally, let us consider the relation of the barotropic phase behavior between the ether- and ester-linked PC bilayer membranes. In Fig. 6 the  $T$ - $p$  phase diagrams of the  $O$ -16:0-PC and 16:0-PC bilayer membranes and those of the  $O$ -18:0-PC and 18:0-PC bilayer membranes are compared, respectively. The phase diagrams of the ether-linked PC bilayers are much simpler than the corresponding ester-linked PC bilayers [3–5] because of the lack of a pressure-induced phase. We immediately notice that the phase diagram of the ether-linked PC bilayer overlaps that of the ester-linked PC bilayer in the high temperature and pressure region. The substitution of an ether linkage for an ester linkage brings about the appearance of the  $L_{\beta}I$  phase under ambient pressure. Accordingly, it can be said that the phase behavior of an ester-linked PC bilayer under high temperature and pressure is almost equivalent to that of an ether-linked PC bilayer in the vicinity of ambient pressure. Furthermore, the overlap of the phase diagrams between the ether- and ester-linked PC bilayers extended to the lower pressure region with an increase in hydrophobic chain-length.

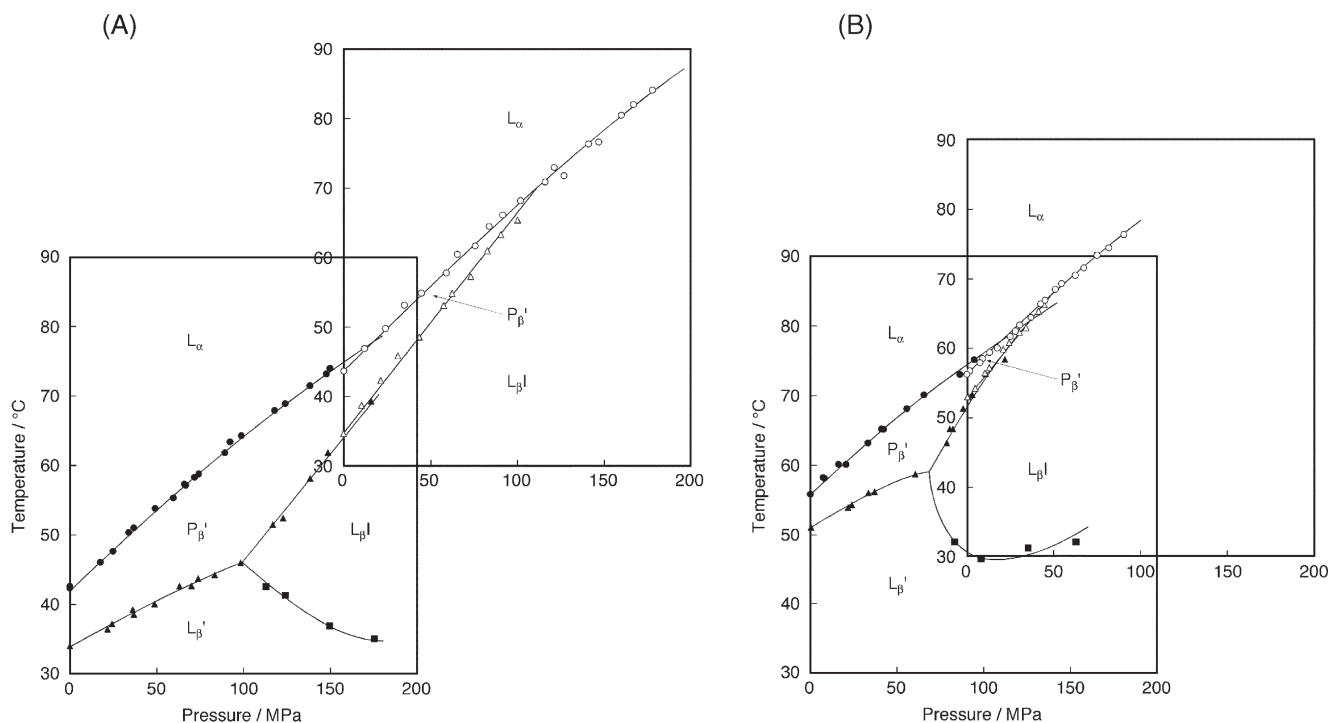


Fig. 6. Comparison of temperature–pressure phase diagram of an ether-linked PC bilayer membrane with that of an ester-linked PC bilayer membrane: (A)  $O$ -16:0-PC and 16:0-PC, (B)  $O$ -18:0-PC and 18:0-PC. Phase transitions: ( $\circ$  and  $\bullet$ ) main transition;  $P'_{\beta}/L_{\alpha}$ , ( $\Delta$  and  $\blacktriangle$ ) pretransition;  $L_{\beta}I/P'_{\beta}$  or  $L_{\beta}I/P_{\beta}$ , ( $\blacksquare$ )  $L_{\beta}I/L_{\beta}I$ . Open and closed symbols indicate the results for ether- and ester-linked PC bilayer membranes, respectively.

This implies that the elongation of hydrophobic chain-length brings the phase behavior of both PC bilayers into closer relationship. The ether-linked PC molecules can form the  $L_{\beta}I$  phase easily by only hydration without requiring special environments under high pressure, whereas the interdigitation of the ester-linked PC bilayer membranes requires high pressure. The difference in the  $L_{\beta}I$  phase formation between both PC bilayers is closely related to the difference in interaction between PC molecules. The bilayer interdigitation occurs as a result of disruption of an interaction balance for bilayer formation. The interdigitation of a phospholipid bilayer is caused by the cohesive forces between hydrophobic chains and the repulsive forces of a hydrophilic part, like the polar head groups. From the above point of view, the effects of hydrophilic solutes and pH on the interdigitation of the *O*-16:0-PC bilayer membrane have been already discussed on the basis of the repulsive interaction between the polar head group [42,43,46]. However, the effects of pressure and hydrophobic chain-lengths on the bilayer interdigitation have not been referred to in previous studies on ether-linked PC bilayers, and we were able to show the above effects systematically in this study. We also consider that the repulsive interaction is a main factor of bilayer interdigitation for the ether-linked PCs. In the ether-linked PC bilayers, the interaction between the polar head groups is weaker (i.e., more repulsive) than that in the ester-linked PC bilayers as mentioned above, enabling it to interpenetrate the alkyl chains into the opposed monolayer. This brings about the interdigitation by only hydration at ambient pressure, and the interdigitation is stabilized by the enhanced van der Waals interactions with applying pressure and with an increase in alkyl-chain length. Regarding the interdigitation of the ester-linked PC bilayers under high pressure and its acyl-chain dependence, since pressure and hydrophobicity both accelerate the van der Waals interactions between acyl chains, we speculate that it may be mainly caused by the strong repulsive interaction between the polar head groups, which is newly induced by pressure so as to produce a smaller bilayer volume, e.g., by the pressure-induced conformational change of the head groups. The induced interaction is also dependent on the acyl chain-length. We have shown [3] that when longer chains interdigitate, larger volume change of the interdigitation is expected. This explains that the interdigitation becomes favorable for longer chains at lower pressures. We can conclude from these considerations that the phase behavior of the ether-linked PC bilayer membranes essentially corresponds to that of the ester-linked PC bilayer membranes in the high temperature and pressure region, and the difference in the phase behavior between both PC bilayers is reduced with an increase in hydrophobic-chain length.

## 5. Conclusions

The phase behavior and the thermodynamic properties of ether-linked PC bilayer membranes were investigated in the present study. The most notable point of the ether-linked PC bilayers is the spontaneous formation of non-bilayer structure, the  $L_{\beta}I$  phase, without other driving forces unlike

ester-linked PC bilayers. The bilayer interdigitation is attributable to the weaker interaction between polar head groups in the ether-linked PCs than that in the ester-linked PCs. Comparing the thermodynamic properties of main transition for the ether-linked PC bilayers with those for the ester-PC bilayers, we revealed weak interaction of the ether-linked PC bilayers in the gel phase. Pressure promotes the interdigitation, and the elongation of alkyl-chain length in the lipid molecule also promotes the interdigitation. Both effects extend the region of the  $L_{\beta}I$  phase on the phase diagram synergistically. Taking into consideration that membrane lipids of archaeobacteria and deep-sea organisms consist of almost all ether-linked phospholipids and that an ether-linkage is chemically more stable than an ester-linkage, it is very interesting that the phase behavior of the ether-linked PC bilayer membranes bears a strong resemblance to that of the ester-linked PC bilayer membranes under such extreme conditions as high temperature and pressure. The fact that archaeobacteria and deep-sea organisms are abundant in ether lipids seems to be counterintuitive because the phase diagrams of ether lipid bilayers at higher temperature and higher pressure are farther away from those of ester lipid at ambient pressure. We speculate that this might be closely related to organic evolution associated with environmental changes. Archaeobacteria living in extreme conditions have chemically stable ether-lipids while bacteria and eukaryotes living in normal conditions have easily decomposable ester-lipids which may play an important role in environmental adaptation. Although the switch from ether-lipids to ester-lipids in the evolutionary process has not yet been made clear, the present phase behavior corresponds favorably with the direction of organic evolution associated with the environmental change from being at high pressure to low pressure. It is expected that further research concerning ether-linked PC bilayers will give us the fundamental information needed regarding the mechanism of environmental adaptation for living organisms.

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