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# ULTRASONIC MEASUREMENTS OF TWO-COMPONENT LIPID BILAYER SUSPENSIONS

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Two-component lipid bilayers of dipalmitoylphosphatidylcholine and dimyristoylphosphatidylcholine were studied by measuring ultrasonic velocity and absorption at 3 MHz. The phase diagram of the two-component lipid bilayers is discussed based upon the transition anomalies of the ultrasonic velocity as well as absorption, and it is suggested that this binary system has two critical points. The bulk modulus of lipid bilayers was determined from the ultrasonic velocity to be $(2.2–3.0)\times10^{10}$ dyne/cm$^2$, whereas the bulk viscosity calculated from the absorption was 10–20 P except for the transition regions.

## 1. Introduction

Biological membranes are essentially multicomponent systems, which are composed of lipids, proteins and other components. Lipid bilayers in biological membranes also consist of many kinds of lipids whose hydrocarbon chains and polar head groups are different [1]. Therefore, the study of multicomponent lipid bilayers is very important for the understanding of static as well as dynamic structures of biological membranes. For this purpose, a number of techniques have been applied to simple two-component systems: binary mixtures of synthetic phosphatidylcholines [2–4] or of phosphatidylcholine and cholesterol [5–9].

A common feature of two-component lipid bilayers is the broadening of the gel-to-liquid crystal transition. Single-component lipid bilayers of synthetic phosphatidylcholine show a very sharp endothermic peak due to the gel-to-liquid crystal transition in which the orientational as well as positional order of hydrocarbon chains disappears [10]. However, the transition becomes broader when a second component such as another phosphatidylcholine, cholesterol or protein is incorporated into the lipid bilayer [2–9,11]. Lateral phase separation of lipids was proposed to be the mechanism of this broadening effect of binary lipid mixtures [3–5]. However, phase separation in the transition regions is only one of the possible phase diagrams of binary mixtures [12], and it seems that other mechanisms of broadening have been given little consideration in the case of two-component lipid bilayers. Therefore, we suppose that all typical phase diagrams of binary mixtures should be examined before elucidating the phase diagram of two-component lipid bilayers.

Ultrasonic measurement has several characteristics among the many techniques of membrane research:

(1) it is very sensitive to cooperative phenomena [13,14],

(2) the ultrasonic velocity and absorption are directly related to the bulk modulus and viscosity, respectively [14,15], and

(3) dilute and turbid samples may be used.

Therefore, ultrasonic measurement is particularly suitable for the investigation of the phase transition of lipid bilayer suspensions, which are turbid and undergo a highly cooperative transition.

In the present work, we have measured the ultrasonic velocity as well the absorption of binary mixtures of dipalmitoyl- and dimyristoylphospha-

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tidylcholines by a differential apparatus [16] in order to study the phase behavior as well as the mechanical properties of two-component lipid bilayers. The results indicate that the details of the transition anomalies are best explained if we assume the existence of critical points. The bulk modulus as well as the viscosity are also estimated from the ultrasonic velocity and absorption of lipid bilayer suspensions.

## 2. Materials and methods

### 2.1. Preparation of liposomes

Dipalmitoyl-L-α-phosphatidylcholine and dimyristoyl-L-α-phosphatidylcholine (from Sigma Chemical Co.) were obtained chromatographically pure and used without further purification. Salts and solvents of reagent grade and twice-distilled water were used for preparation.

Multilamellar large liposomes were prepared as described previously [14]. Lipids were dissolved in chloroform in order to confirm the mixing of two kinds of lipids. After evaporating the chloroform in a rotary evaporator, an aqueous solvent of 150 mM NaCl of pH 7 was added and agitated by a vortex mixer at a temperature of $\approx 50°C$. The size of the multilamellar liposomes thus prepared ranged from less than a micrometer to tens of micrometers, and there was no difference among samples of various compositions.

We prepared seven samples with different mole fractions of dipalmitoylphosphatidylcholine and dimyristoylphosphatidylcholine, as listed in table 1. The mole fractions of dipalmitoylphosphatidylcholine were 0, 13.4, 31.6, 48.0, 64.9, 86.6 and 100% and the weight concentration of these samples was in the range from 2.6 to 2.8 mg/ml. All the samples were prepared under the same conditions.

Table 1
Composition and transition temperature of samples

| Sample number | Mole fraction of DPPC ($\chi$) | Concentration (mg/ml) | $T_{main}$ (°C) | $T_{pre}$ (°C) |
|---|---|---|---|---|
| 1 | 0.0 | 2.6 | 24.4 | 14 |
| 2 | 13.4 | 2.7 | 26.9 | – |
| 3 | 31.6 | 2.7 | 28.5 | – |
| 4 | 48.0 | 2.8 | 33.4 | – |
| 5 | 64.9 | 2.6 | 37.0 | – |
| 6 | 86.6 | 2.7 | 39.6 | 29 |
| 7 | 100.0 | 2.7 | 41.4 | 34 |

### 2.2. Ultrasonic measurements

Ultrasonic velocity and absorption in liposome suspensions were measured by a differential apparatus which is based upon a "sing-around" method [16]. A block diagram of the apparatus is shown in fig. 1. An ultrasonic pulse is excited in one of the cells by a pulse generator, and reflected many times in the cell until it is completely attenuated. The echo signals are converted into rectangular pulses by a triggering circuit, and the pulse generator is retriggered by the $n$th echo of the preceding excitation. Then, the pulse generator, one of the cells and the triggering circuit form a self-excited pulse generator for which the period $T$ of the pulse sequence is expressed as

$$T = T_e + 2nL/V. \quad (1)$$

Where, $T_e$ and $L$ are the electric delay time and the effective cell length, respectively, and $V$ is the ultrasonic velocity of the sample liquid. We have used two cells which have the same cell constants: one contains a liposome suspension and the other the solvent. By taking the difference between the two cells, we can determine the excess ultrasonic velocity $\Delta V$ and absorption $\Delta\alpha$ by the following

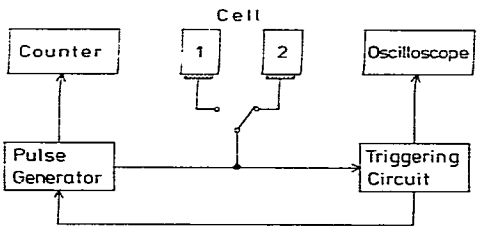


Fig. 1. Block diagram of differential apparatus for the measurements of ultrasonic velocity and absorption at 3 MHz.

equations:

$$\Delta V = -\left[2nL/(T-T_c)^2\right]\Delta T, \quad (2)$$

$$\Delta\alpha = -\ln(E/E_s)/2nL, \quad (3)$$

where $E$ and $E_s$ are the amplitudes of the echo signal in the liposome suspension and the solvent which were monitored by an oscilloscope. The very large background of the ultrasonic velocity in water and the drift of the electric circuits are cancelled by the direct measurement of excess value of the period. This apparatus was originally designed for ultrasonic velocity measurements and the principle and performance have been reported elsewhere [16].

We have improved the apparatus for absorption measurements in this work. Since the effect of viscous drag of the solvent is cancelled by taking the excess absorption, the apparent absorption determined by eq. (3) generally consists of three terms:

$$\Delta\alpha = \Delta\alpha_{abs} + \Delta\alpha_{sca} + \Delta\alpha_{imp}, \quad (4)$$

where, $\Delta\alpha_{abs}$ and $\Delta\alpha_{sca}$ represent the absorption and scattering of sound by suspended membrane vesicles, respectively, and $\Delta\alpha_{imp}$ is the term due to the change in the mechanical impedance matching at the transducer surface. The real absorption $\Delta\alpha_{abs}$ is related to the membrane viscosity as well as to the critical slowing down of the structural relaxation time of the membrane in which we are interested. The scattering term $\Delta\alpha_{sca}$ represents the sound extinction due to the scattering. It can be shown by Okano's theory of sound scattering that $\Delta\alpha_{sca}$ is much smaller than $\Delta\alpha_{abs}$, which is discussed in section 5 [17]. When the mechanical impedances of sample and solvent are different, this causes a variation of the reflection coefficient of sound at the transducer surface and results in an apparent change in the absorption coefficient, $\Delta\alpha_{imp}$. In the present work, the difference in the mechanical impedance between liposome suspensions and solvent is estimated to be less than 0.02%. So, the change in the signal amplitude due to $\Delta\alpha_{imp}$ has to be much smaller than 0.2% which is the relative accuracy of amplitude measurement by an oscilloscope. Therefore, we may assume that the excess absorption determined by eq. (3) represents the real absorption of membranes.

The accuracy was consequently $\pm 0.7$ cm/s for the velocity measurement and $\pm 10^{-3}$ cm$^{-1}$ for the absorption measurement. The temperature of sample cells was controlled within $\pm 0.001$°C by a large temperature bath. Since the sedimentation rate of the multilamellar liposomes was large, the sample was circulated by a peristaltic pump.

### 2.3. Limiting numbers of suspension properties

The ultrasonic velocity $V$ and absorption $\alpha$ are effectively expressed by the mechanical properties of the medium:

$$V = (K/\rho)^{-1/2}, \quad (5)$$

$$\alpha = (\omega^2/2\rho V^3)(\kappa + \tfrac{4}{3}\eta), \quad (6)$$

in which $K$ is the bulk modulus, $\kappa$ and $\eta$ are the bulk and shear viscosities, respectively, $\rho$ is the density and $\omega$ is the angular frequency of sound. Since the change in density is usually smaller than that of the mechanical properties, we may roughly assume that the ultrasonic velocity and absorption vary according to the change in the bulk modulus and viscosity (internal friction).

In the case of suspensions, however, the propagation constants depend on the mechanical properties of both solute and solvent as well as on the concentration of the suspensions. Therefore, we derive limiting numbers of velocity and absorption for a quantitative discussion [15]:

$$[V] = \lim_{c\to 0}(V - V_s)/V_s c, \quad (7)$$

$$[\alpha\lambda] = \lim_{c\to 0}(\alpha\lambda - \alpha_s\lambda_s)/c, \quad (8)$$

where $c$ is dry weight concentration and the subscript s denotes the solvent. These quantities are similar to the limiting viscosity number which is introduced for the quantitative discussion of suspension viscosity. Using $[V]$ and $[\alpha\lambda]$, we can compare samples of different composition and concentration. We can also estimate the mechanical properties of a membrane from the limiting numbers under appropriate conditions, which is shown in section 5. Since $(V - V_s)/V_s$ and $(\alpha\lambda - \alpha_s\lambda_s)$ are proportional to the concentration $c$, we

regard $(V-V_s)/V_s c$ and $(\alpha\lambda-\alpha_s\lambda_s)/c$ as true limiting numbers.

## 3. Results

In order to investigate the effect of mixing of two miscible lipids, we have measured the ultrasonic velocity and the absorption of two-component liposomes of dipalmitoyl- and dimyristoylphosphatidylcholine. The results are summarized in figs. 2 and 3, in which the limiting numbers of velocity and absorption are plotted as a function of temperature. The temperature dependence of the ultrasonic behavior is consistent with previous experiments [2,4]. The transition temperature increases as the dipalmitoylphosphatidylcholine content is raised, as listed in table 1. The phase transition becomes less sharp when the concentration of a minor component is increased. In addition to these general tendencies, we can point out several detailed features.

(1) Shape of transition anomalies. All samples show an anomalous dip of the velocity and an anomalous absorption peak in the vicinity of the transition temperature. A sharp decrease in the velocity at the transition point that is found in pure phosphatidylcholine bilayers disappears in composite lipid bilayers. Correspondingly, the temperature dependence of the absorption is asymmetric in the pure systems and symmetric in the composite ones. A lipid bilayer with a dipalmitoylphosphatidylcholine content of 87% is characterized by a sharp cusp of the ultrasonic velocity and of the absorption. On the other hand, composite lipid bilayers with a smaller di-

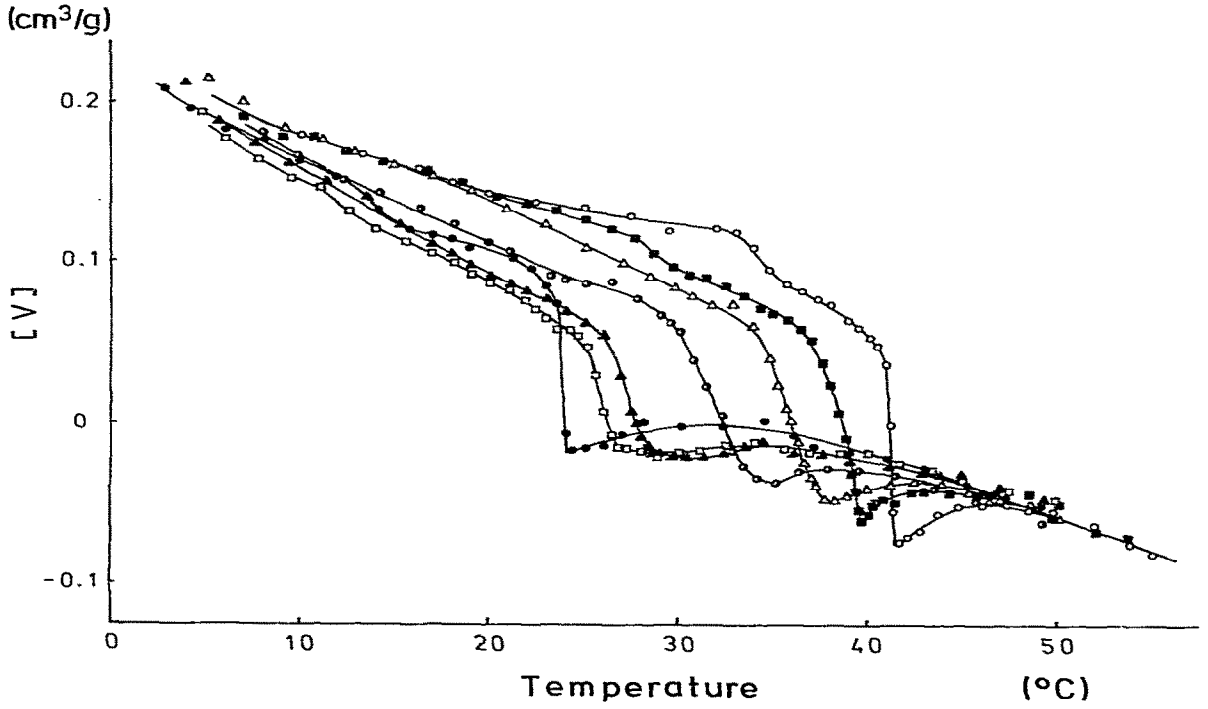


Fig. 2. The limiting number of the velocity as a function of temperature for two-component lipid bilayers of dipalmitoylphosphatidylcholine and dimyristoyl-phosphatidylcholine. The dipalmitoylphosphatidylcholine content is 0% (●), 13.4% (□), 31.6% (▲), 48.0% (◑), 64.9% (△), 86.6% (■) and 100% (○).

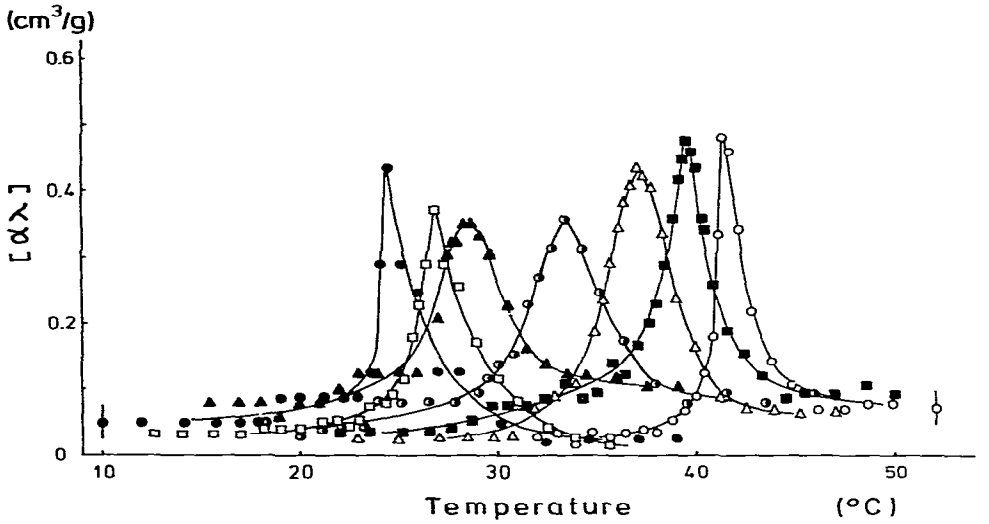


Fig. 3. The limiting number of the absorption per wavelength as a function of temperature for two-component lipid bilayers of dipalmitoylphosphatidylcholine and dimyristoylphosphatidylcholine. The dipalmitoylphosphatidylcholine content is 0% (●), 13.4% (□), 31.6% (▲), 48.0% (◑), 64.9% (△), 86.6% (■) and 100% (○).

palmitoylphosphatidylcholine content show a smooth and broad dip of the velocity and an absorption peak.

(2) Pretransition. A small bro. : point about ten degrees below the gel-to-liquid crystal transition is distinctly observed in the lipid bilayers without contamination as well as with a dipalmitoylphosphatidylcholine content of 87%. In other samples, the ultrasonic velocity in the gel phase steadily decreases as the temperature increases.

(3) Magnitude of velocity and absorption. The limiting number of the velocity of all smaples converges to the same curve in the temperature region far above and below the transition temperature, suggesting that the bulk modulus is almost independent of the composition. The limiting velocity number is $\approx 0.2$ cm³/g at 5°C and $-0.05$ cm³/g at 50°C. The limiting number of the absorption per wavelength scatters around 0.05 cm³/g when the temperature is sufficiently different from the transition temperature.

## 4. Phase diagram of two miscible lipids

In order to discuss the phase diagram of two miscible lipids, we first classify the phase diagrams of two-component systems in general according to Landau and Lifshitz [12]. The phase boundary of a two-component system forms a plane in a three-dimensional phase space due to Gibbs' phase rule. Therefore, the cross-section of the phase boundary with the temperature composition ($T-X$) plane has to be a line or some combination of lines. Three typical cases of the $T-X$ phase diagrams are shown in fig. 4, in which two components are assumed to be miscible in either of the two phases and not to form any stoichiometric compounds:

(a) The phase boundary does not have any maximum or minimum.

(b) There is an isoconcentration point.

(c) There are one or two critical points.

Since dipalmitoylphosphatidylcholine and dimyristoylphosphatidylcholine are considered to be

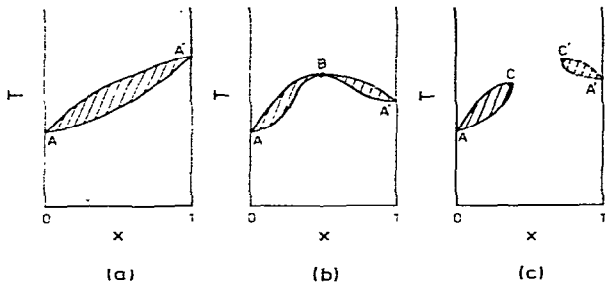


Fig. 4. Three types of phase diagrams for binary mixtures in general, which are miscible in either phase. (a) The boundary has neither a maximum nor a minimum. (b) There is an isoconcentration point B. (c) The phase diagram has critical points C and C'.

miscible in gel as well as liquid crystal phases [2], it should be reasonable to assume that the phase diagram of the present system is equivalent to one of the three cases in fig. 4, although there may be some minor variations. From the viewpoint of this general discussion, most previous work on two-component lipid bilayers appears to be prejudiced, because only the first case is taken into consideration [3–5].

However, we have to elucidate the cause of the transitional anomalies of the ultrasonic velocity and absorption in lipid bilayers before we answer the question as to which phase diagram in fig. 4 is most suitable for describing the two-component systems of miscible lipids. The transitional anomalies of the ultrasonic velocity and absorption are related to the anomalous behaviour of the order parameter in the vicinity of the transition point, which may be discussed conveniently within the framework of the Landau theory of phase transitions [18,19]. Recently, the Landau theory was applied to lipid bilayer systems by Jähnig [20], who introduced a first-order term in the free energy expansion to represent a residual order of hydrocarbon chains in the disordered phase:

$$F = -A_1\langle s\rangle + \tfrac{1}{2}A_2\langle s\rangle^2 - \tfrac{1}{3}A_3\langle s\rangle^3 + \tfrac{1}{4}A_4\langle s\rangle^4, \quad (9)$$

in which $\langle s\rangle$ is an average orientational order parameter. Here, we assume as usual that $A_1$, $A_3$ and $A_4$ are positive and $A_2$ is linearly dependent upon the temperature: $A_2 = a_2(T - T^*)$. Jähnig showed that this type of free energy expansion describes well the results of experiments as well as microscopic theories of lipid membranes [20,21].

Following Jähnig's discussion, let us consider the fluctuation of the order parameter. We replace $\langle s\rangle$ by $\langle s\rangle + \delta s$, and obtain the fluctuation of the free energy in the form,

$$\delta F = \left(\tfrac{1}{2}A_2 - A_3\langle s\rangle + \tfrac{3}{2}A_4\langle s\rangle^2\right)\delta s^2. \quad (10)$$

The first-order term vanishes because of the definition of the equilibrium state. Due to the equipartition theorem, the strength of the fluctuation in the disordered phase is obtained as

$$\langle \delta s^2\rangle = kT/A_2 = kT/a_2(T - T^*). \quad (11)$$

As for the relaxation of the order parameter fluctuation, the usual assumption of the equation $\mathrm{d}\delta s/\mathrm{d}t = \zeta\,\mathrm{d}\delta F/\mathrm{d}\delta s$ leads to the relaxation time

$$\tau = 1/\zeta A_2 = 1/\zeta a_2(T - T^*). \quad (12)$$

So the magnitude of the fluctuation and the relaxation time anomalously increases in the vicinity of $T^*$ together with the coherence length. When the transition is first order, the transition temperature parts from $T^*$, and the anomalies of $\langle \delta s^2\rangle$ and $\tau$ become less distinct. Therefore, the anamalous enhancement of $\langle \delta s^2\rangle$ and $\tau$ is characteristic of second-order phase transitions.

This anomalous enhancement of the order parameter fluctuation as well as the relaxation time leads to the anomalous dip of the ultrasonic velocity and the absorption peak. The ultrasonic relaxation with a single relaxation time $\tau$ is described by the equations [13,22]

$$V^2 = V_0^2 + \left(V_\infty^2 - V_0^2\right)\frac{\omega^2\tau^2}{1 + \omega^2\tau^2}, \quad (13)$$

$$\alpha\lambda = \frac{\pi\left(V_\infty^2 - V_0^2\right)}{V^2}\,\frac{\omega\tau}{1 + \omega^2\tau^2}, \quad (14)$$

where $V_0$ is the ultrasonic velocity at frequencies lower than $1/\tau$ and $V_\infty$ is the corresponding quantity at higher frequencies. The orientational relaxation time of hydrocarbon chains is reported to be $\approx 10^{-9}$ s [23], and our recent measurement of the frequency dependence of the ultrasonic velocity

and absorption revealed a relaxation time of $\approx 10^{-8}$ s [24]. Therefore, $\omega\tau$ has to be much smaller than unity at the measuring frequency of 3 MHz. So eqs. (5) and (13) lead to the relation

$$V^2 = K_0/\rho, \tag{15}$$

where $K_0$ is the bulk modulus at frequencies much lower than $1/\tau$. Since the isothermal bulk modulus $K_T$ is inversely proportional to the fluctuation of the number of molecules in a semimicroscopic volume $v$ [25],

$$kT/vK_T = \langle \delta n^2 \rangle / \langle n^2 \rangle, \tag{16}$$

the enhancement of the density fluctuation coupled with the order parameter fluctuation causes the anomalous decrease in the isothermal bulk modulus $K_T$. Although $K_0$ is not necessarily equal to $K_T$, the anomalous dip of the ultrasonic velocity has to be due to the anomalous increase in the fluctuation of the order parameter. The ultrasonic absorption per wavelength in eq. (14) is proportional to the relaxation time under the condition that $\omega\tau \ll 1$. Hence the anomalous enhancement of the ultrasonic absorption has to be due to the increase in the relaxation time, namely the critical slowing down [18,19], which is predicted by eq. (12).

Here, our discussion is only qualitative and we have not referred to the nature of the coupling between the order parameter fluctuation and the ultrasonic propagation, which is necessary for the above-mentioned mechanism of the ultrasonic anomalies. Since the ultrasonic anomalies of liquid crystals in the vicinity of the nematic-isotropic transition were successfully explained by a theory of Imura and Okano [26,27] which was analogous to Fixman's theory of critical liquid mixtures [28], the coupling in the lipid bilayer may also be associated with the anomaly of the dynamic heat capacity. In such a case, the single relaxation process assumed in eqs. (13) and (14) is an oversimplification of the phenomena. However, if we restrict ourselves to a qualitative discussion, it appears certain that the anomalous dip of the velocity was well as the anomalous peak of the absorption indicates the second-order character of the lipid bilayer transition.

On the other hand, the very abrupt change of the velocity observed in single-component liposomes has to be due to the isothermal structural change in the first-order transition, as discussed by many investigators [29]. The phase transition is second order if the coefficients of the free energy in eq. (15) have the relationship,

$$A_3 = 3\left(A_1 A_4^2\right)^{1/3}. \tag{17}$$

When $A_3$ is larger than this value, however, the phase transition becomes a first order, and the order parameter abruptly jumps at the transition temperature. Therefore, the abrupt change of the velocity together with very sharp changes in the heat capacity [29] and density [30] must indicate the first-order character of the lipid bilayer transition.

Now, we may distinguish the three types of phase diagrams in fig. 4 by the dependence of the transitional anomalies on the composition. For cases (a) and (b), two singular points will be observed at any composition except for the isoconcentration point due to two branches in the coexistence regions. In contrast, the abrupt change at the transition point disappears when the composition approaches the critical concentration in the case of (c). Moreover, the cusp-like curve of various physical properties such as heat capacity and ultrasonic velocity at the critical point is replaced by a smooth curve when the concentration is in a super-critical region [13]. Typical ultrasonic behavior near a critical point is reported for a gas-liquid transition [31]: the abrupt change in the ultrasonic velocity below the critical temperature vanishes at the critical point and there is no discontinuous change in a super-critical region.

Fig. 5 shows the temperature dependence of the ultrasonic velocity of five samples with higher dipalmitoylphosphatidylcholine content. The velocity is plotted as a function of the temperature difference from the absorption peak on an expanded scale. Fig. 5 clearly shows that only a single-component lipid bilayer is characterized by a sharp decrease in the velocity at the transition temperature which indicates a first-order phase transition. However, the distinct dip of the velocity indicates that the transition of a pure di-

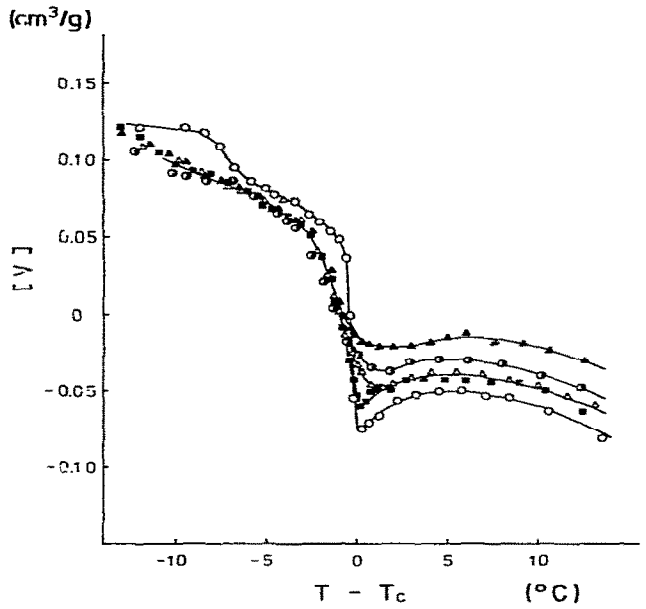


Fig. 5. Transition anomalies of the ultrasonic velocity for two-component lipid bilayers on an expanded temperature scale. The abscissa is the temperature difference from the maximum of the ultrasonic absorption. The dipalmitoylphosphatidylcholine content is 31.6% (▲), 48.0% (◑), 64.9% (△), 86.6% (■) and 100% (○).

palmitoylphosphatidylcholine bilayer is very near the second-order transition. As the dipalmitoylphosphatidylcholine content is decreased to 86.6%, the sharp change at the transition point vanishes and the dip of the velocity forms a sharp cusp. Further decrease in the dipalmitoylphosphatidylcholine content replaces the cusp by a broad and smooth dip. As a consequence, fig. 5 is very similar to the anomalies of the ultrasonic velocity in the vicinity of a critical point of a gas-liquid transition [31] and strongly suggests that the phase diagram has a critical point near the dipalmitoylphosphatidylcholine content of 87%. This conjecture is supported by the fact that the temperature dependence of the ultrasonic absorption does not have two singular points but a single symmetric peak in the mixed lipid bilayers. Particularly, the lipid bilayer of 86.6% in dipalmitoylphosphatidylcholine content shows a critical exponential relation to the temperature with the exponent 0.7, as shown in fig. 6. This critical exponential form of the ultrasonic absorption also suggests that a critical point is located very near the concentration of 87%.

Although the critical point at lower dipalmitoylphosphatidylcholine content is not distinguished in the present results, we suppose that there is a critical point at a dipalmitoylphosphatidylcholine content between 0% and 13%, because the first-order character observed in the pure dimyristoylphosphatidylcholine bilayer disappears when the dipalmitoylphosphatidylcholine content is raised to 13%. Consequently, the transition anomalies of the ultrasonic velocity as well as the absorption suggest a phase diagram as shown in fig. 7. This phase diagram appears to explain reasonably well not only the broadening of the phase transition but also the appearance of sharp and

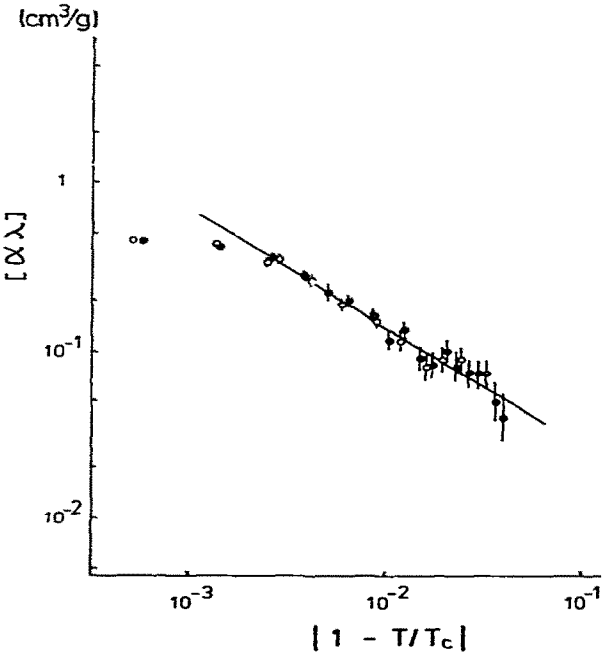


Fig. 6. The ultrasonic absorption of a sample with a dipalmitoylphosphatidylcholine content of 86.6% plotted against temperature in a double logarithmic scale for $T<T_c$ (●) and $T>T_c$ (○).

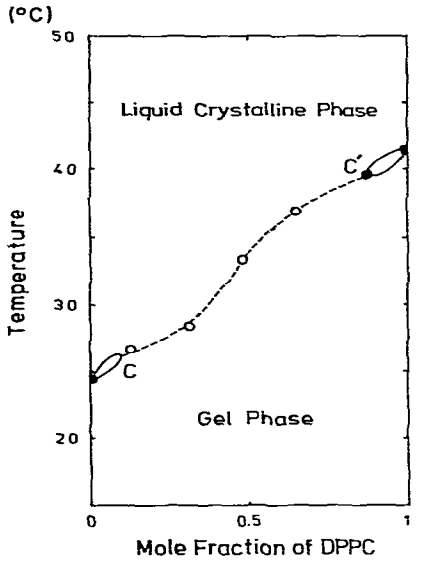


Fig. 7. The most plausible phase diagram of binary mixtures of dipalmitoylphosphatidylcholine and dimyristoylphosphatidylcholine in a temperature-composition space. C and C' are critical points. The solid lines represent the phase boundary and a dashed line is drawn along the absorption maximum.

broad components of the transition anomalies. As Mabrey et al. [6] pointed out, the two components of the transition anomalies may not be understood by a phase diagram like fig. 4a by which previous data were analyzed [6].

However, we have to bear in mind that the present result is not a decisive proof of critical points. We suppose that at least the following points should be studied and discussed further. First, there seems to be a particular case of first-order transition in which the abrupt change in the ultrasonic velocity is not observed. For example, the ultrasonic velocity abruptly increases at a nematic-isotropic transition [19,32], while it decreases at the gel-to-liquid crystal transition of pure dipalmitoylphosphatidylcholine bilayers as shown in fig. 2. These systems are very similar in that the transitions are weakly first order and associated with the orientational order of anisotropic molecules. Therefore, it seems that the magnitude and even the sign of an abrupt change in the velocity at a first-order transition depend upon some molecular properties like the elasticity of molecules. Second, the present measurement of ultrasonic propagation constants at 3 MHz gives only limited information about the critical dynamics of membrane structure. So, we cannot quantitatively discuss the critical exponents of the relaxation time and strength. Third, the contamination level is rather high, $\approx 1\%$ according to the specification of the supplier, and it is difficult to study the composition dependence in so much detail.

As for the first point, calorimetry is the most reliable method for distinguishing a first-order transition. Mabrey et al. reported the transition anomalies of the same binary system as ours [4]. Their data apparently showed a broadening of the endothermic peak, which they explained by the phase separation mechanism of fig. 4a. However, we suppose that their heat capacity data are rather favorable to the broadening mechanism of critical points or at least do not contradict it. Lipid bilayers with dipalmitoylphosphatidylcholine contents of 37.2% and 64.4% showed a rather broad peak of $\approx 3$°C in half width with only a slight shoulder. There is neither a sharp peak of the endotherm, which suggests latent heat, nor two distinct singular points. Therefore, the latent heat seems to disappear at intermediate dipalmitoylphosphatidylcholine content consistent with the disappearance of the abrupt change in the ultrasonic velocity. More careful thermal analysis will clarify this point.

## 5. Estimation of mechanical properties

We may estimate the bulk modulus as well as the bulk viscosity of lipid bilayers from the ultrasonic velocity and absorption. First, we briefly summarize the theory of sound propagation in a spherical particle suspension which was originally proposed by Okano [17,33]. This theory deals with the sound scattering by viscoelastic particles in a viscoelastic solvent and includes former theories as limiting cases [34,35]. Although this theory was recently developed by Sakanishi et al. for the case of spherical shell suspensions [36], multilamellar

liposomes seem to be spherical particles rather than single shelss. Anyway, the final results of these theories coincide under reasonable assumptions.

Suppose that a spherical particle of radius $a$ is embedded in an isotropic medium (solvent). The quantities with subscripts m and s refer to the particle and the medium, respectively. The particle is characterized by the complex bulk modulus $K_m^*$, rigidity $G_m^*$ and density $\rho_m$, and the correspond quantities of the medium are $K_s^*$, $G_s^*$ and $\rho_s$. The wave number is $k$ for longitudinal waves and $\kappa$ for shear waves. We further assume that the incident wave is a plane longitudinal wave and that multiple scattering as well as transport phenomena are negligibly small. Then, if $|k_s a| \ll 1$, $|k_m a| \ll 1$ and $|\kappa_m a| \ll 1$, we can solve the equations of motion of viscoelastic media under appropriate boundary conditions at the particle surface. We finally obtain the propagation constant $\Gamma^*$, the total extinction cross-section $C_{ext}$ of longitudinal waves and the extinction cross-section $C_{sca}$ due to scattering alone,

$$\Gamma^* = \Gamma_s^* \left(1 - 2\pi N \mathrm{k}_s^{-2} \sum_{m=0} j^m A_m\right), \tag{18}$$

$$C_{ext} = 4\pi \, \mathrm{Re}\left(\mathrm{k}_s^{-1} \sum_{m=0} j^{m-1} A_m\right), \tag{19}$$

$$C_{sca} = \sum_{m=0} \frac{4\pi}{2m+1} \left(|A_m|^2 + m(m+1)|B_m|^2 \frac{\mathrm{Re}\, Z_t}{\mathrm{Re}\, Z_f}\right) \tag{20}$$

in which $A_m$ and $B_m$ are coefficients of the polynomials in the expression of scattered longitudinal and shear waves, respectively [17,33]. The number of particles in a unit volume is represented by $N$, $Z_f$ and $Z_t$ are mechanical impedances for longitudinal and shear stress, and $\Gamma_s^*$ is the propagation constant of the solvent. The first three terms, $A_1$, $A_2$ and $A_3$, are the dominant terms of eqs. (18)–(20); $A_1$, $A_2$ and $A_3$ are mainly related to the bulk modulus, density and rigidity, respectively.

Since the rigidity of the membrane is much smaller than the bulk modulus [37] and the density term $A_1$ is usually smaller than $A_0$ in membrane suspensions [8,14], the summation in eqs. (18)–(20) may be replaced by $A_0$ as a rough approximation,

$$A_0 = \frac{1}{3}\left(1 - \frac{3(K_s^* + \frac{4}{3}G_s^*)}{3(K_m^* + \frac{4}{3}G_m^*) - 4(G_m^* - G_s^*)}\right)\frac{(k_s a)^3}{k_s} \tag{21}$$

Hence, simple calculation shows that $C_{sca}$ is smaller than $C_{ext}$ by a factor of $\approx (k_s a)^3$ which is less than $10^{-4}$ for multilamellar liposomes. This fact justifies our assumption that the apparent absorption $\Delta\alpha_{sca}$ due to scattering is much smaller than the real absorption $\Delta\alpha_{abs}$ in eq. (4). If we neglect $G_m^*$ and $G_s^*$ as compared to $K_m^*$ and $K_s^*$ [37], eqs. (18) and (21) lead to the formula

$$\Gamma^* = \Gamma_s^* \left[1 - \tfrac{1}{2}\phi(K_m^* - K_s^*)/K_m^*\right], \tag{22}$$

where $\phi$ is the volume fraction of spheres. Finally, the expression of the limiting numbers of velocity and absorption are derived,

$$[V] = \tfrac{1}{2}\left[(K_m - K_s)/K_m\right](1+\delta)/\rho_m, \tag{23}$$

$$[\alpha\lambda] = \pi(\omega\kappa_m/K_m)(1+\delta)/\rho_m \tag{24}$$

[by substituting the equations, $\Gamma^* = \omega/V - j\alpha$, $\Gamma_s = \omega/V_s$, $K_m^* = K_m + j\omega\kappa_m$, $K_s^* = K_s$ and $\phi = c(1+\delta)/\rho_m$, into eq. (22)]. In these equations a factor of $(1+\delta)/\rho_m$ is introduced for the normalization of the concentration in which $\delta$ is the amount of hydrated water in a lipid bilayer of unit weight. The bulk viscosity of the membrane is denoted by $\kappa_m$. Eq. (23) is the same as the modulus term of the previous expression of the limiting velocity number (14). The error of the estimated bulk modulus of the membrane due to the contribution from the other terms in eq. (18) is less than 10% on the assumption of reasonable values of density and rigidity. Eq. (24) is presented for the first time in this work and enables us to estimate the bulk viscosity of membranes.

Since the ultrasonic propagation in the transition regions is associated with the relaxation process of the order parameter, we estimate the bulk modulus as well as the bulk viscosity in a temperature range far removed from the transition temperature. The limiting velocity number is $\approx 0.2$ cm$^3$/g at 5°C and $-0.05$ cm$^3$/g at 50°C, whereas the limiting number of the absorption per wavelength is $\approx 0.05$ cm$^3$/g. Assuming a reasonable value of the normalization factor, $(1+\delta)/\rho_m = 1.4$ [15], we

Table 2
Mechanical properties of lipid bilayers

| | 5°C | 50°C |
|---|---|---|
| bulk modulus (dyne/cm$^2$) | $3.0\times10^{10}$ | $2.2\times10^{10}$ |
| bulk viscosity (P) | 10–20 | 10–20 |

obtain the mechanical properties of the lipid bilayers as listed in table 2.

The values of the bulk modulus are in good agreement with previously reported values, although we neglected the density term. The bulk viscosity from 10 to 20 P is of the same order of magnitude as the shear viscosity which was measured by various techniques [23,38]. The bulk viscosity which may be easily determined from the ultrasonic absorption will provide a very useful measure of the fluidity of membranes.

## 6. Conclusion

We have analyzed the ultrasonic velocity and absorption of two-component liposomes from two points of view: the phase behavior of two miscible lipids and the mechanical properties of membranes.

Concerning the phase behavior of binary lipid bilayers, three kinds of phase diagrams which are typical of binary mixtures of miscible materials were examined by the ultrasonic anomalies in the transition regions. The disappearance of the sharp change in the ultrasonic velocity as well as the critical exponential form of absorption suggested that there is a critical point at a dipalmitoylphosphatidylcholine content of $\approx 87\%$. Another critical point was also suggested at a content lower than 13%. The same kinds of phase diagrams were theoretically proposed by Jähnig for a mixture of phosphatidylcholine and cholesterol [20] based upon calorimetric data by Mabrey et al. [6]. Therefore, it may be suggested that the phase transition of pure phosphatidylcholine is first order but very near a hidden critical point, and that the transition point approaches the critical point when a second component is incorporated.

The bulk modulus of lipid bilayers was $3.0\times10^{10}$ dyne/cm$^2$ at 5°C and $2.2\times10^{10}$ dyne/cm$^2$ at 50°C, which was determined from the ultrasonic velocity. The bulk viscosity was determined from the ultrasonic absorption for the first time and a value of 10–20 P was obtained, except for the transition regions. These mechanical properties are very useful for membrane research, because the compressibility, i.e., the inverse of the bulk modulus, is proportional to the permeability of membranes [39] and the bulk viscosity has to be related to the "fluidity" of membranes.

The ultrasonic technique has several technical advantages: (1) turbid samples may be used, (2) a concentration of a few milligrams per milliliter is sufficient, and (3) the measurement is very easy. In addition, we have shown that it is very sensitive to cooperative phenomena in membranes and also that it enables us to determine the mechanical properties of membranes easily. Using this measuring system we are studying further the phase behavior of lipid bilayers and the mechanical properties of biological membranes.

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