Compressibility of Lipid Mixtures Studied by Calorimetry and Ultrasonic Velocity Measurements

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Ultrasonic velocity and heat capacity temperature profiles of various lipid mixtures have been recorded with high accuracy. This included mixtures of phophatidylcholines with different chain length as well as phosphatidylcholine mixtures with diacyl glycerides. Following previous studies relating the heat capacity to the isothermal compressibility of lipids close to the chain melting transition, we found that the measured ultrasonic velocities are very similar to those calculated from the heat capacity. This implies that we are able to determine the compressibility changes from the excess heat capacity and the heat capacity changes from ultrasonic velocity measurements. The sound velocity and heat capacity traces are discussed with respect to the phase diagrams of the lipid mixtures.

1. Introduction

Membrane lipids melt in a temperature regime from −20 to +60 °C, which is in a broader sense of biological relevance. The chain melting is accompanied by a pronounced maximum in the specific heat capacity. In previous publications it was shown that the enthalpy change in these transitions is proportional to the change in volume.[1−3] According to the well-known fluctuation/dissipation theorem, the heat capacity is proportional to enthalpy fluctuations, whereas the compressibility is proportional to volume fluctuations. Thus, if enthalpy and volume changes are proportional functions, excess heat capacity and isothermal compressibility are also proportional functions. Furthermore, we demonstrated that the ratio between enthalpy and volume changes is roughly the same for all lipids, which we have investigated, including complex biological membranes as lung surfactant.[3] This lead to the conclusion that the lipid compressibility changes in the melting transition are generally proportional to the excess heat capacity. These findings are of quite some interest because they allow us to relate the heat capacity to the changes in the elastic constants even if the composition of the membranes is unknown.

It has been shown previously that the bulk ultrasonic velocity of lipid dispersions displays a minimum at the melting transition.[4−6] The ultrasonic velocity is a function of the density and the adiabatic compressibility. The adiabatic volume compressibility, however, is related to the isothermal compressibility via the Maxwell relations.[7] Evaluation of experimental ultrasonic velocity profiles can therefore serve as a tool to obtain information about the compressibility of lipid dispersions.[4−6] It has been shown that the ultrasonic velocity profiles of pure DMPC and DMPC-cholesterol mixtures can correctly be predicted from the heat capacity,[6] if a proportional relationship between heat capacity and isothermal compressibility is assumed.

The intention of this paper is to demonstrate the close coupling of heat capacity and adiabatic compressibility for various binary lipid system. To do so, we measured heat capacities with differential calorimetry and ultrasonic velocities with a high sensitivity differential ultrasonic cell[8] and compared them with a straightforward theoretical analysis based on the concepts outlined above.

2. Materials and Methods

1,2-Dilauroyl-3-*sn*-phosphocholine (DLPC), 1,2-dimyristoyl-3-*sn*-phosphocholine (DMPC), 1,2-dipalmitoyl-3-*sn*-phosphocholine (DPPC), 1,2-dioleoyl-3-*sn*-phosphocholine (DOPC), and 1,2-dimyristoyl-3-*sn*-glycerol (DMG) were purchased from Avanti Polar Lipids (Birmingham, AL,USA), Fluka (Taufkirchen, FRG), and Sigma (Deisenhofen, FRG). All phospholipids were used without further purification. To produce lipid mixtures, lipids were dissolved in an organic solvent (usually in a dichloromethane-methanol 50−50 mixture) and mixed in the appropriate ratios. The solvent was removed with a nitrogen stream. Remainders of the solvent in the membranes were removed by deposition in a desticator under high vacuum for several hours. Aqueous lipid dispersions were prepared by weighing appropriate amounts of the lipids and water (Millipore) into suitable flasks. Gentle shaking of the sample at temperatures above the melting transition produces multilamellar vesicles. Unilamellar vesicles of uniform shape and size were made by extrusion, utilizing a Lipofast extruder (Avestin Inc., Ottawa, Canada) and a polycarbonate filter with pore diameters of 100 nm.

Heat capacities were mostly recorded in an upscan mode on a VP calorimeter from Microcal, Inc. (Northampton, MA) at scan rates of 5 deg/h. Downscan measurements in a four cell

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calorimeter (Hart Scientific, Provo, Utah, USA) have been performed with the DLPC/DPPC samples. Ultrasonic velocity measurements were performed with the aid of a twin-cell instrument, as described in detail recently.[8] Applying this method, the sound velocity of the lipid dispersion, with concentration of 4 or 5 mg/mL was determined relative to that of water at the same temperature. For this purpose, both the sample and the reference lipid were placed in circular cylindrical cavities which were contained in the same metallic block, to guarantee optimum heat contact between the cells. The sound velocity of the sample at around 2.5 MHz was derived from the resonance frequencies of the cell, which in turn were determined from the continuously recorded resonator transfer functions. The frequency of measurements corresponds with a wavelength around 0.6 mm within the sample. On this scale, the suspensions may be considered homogeneous. The sound velocity of water as the reference was taken from the literature.[9] The phospholipid vesicle solutions were measured in 1° temperature intervals at a scan rate of about 3 deg/hr.

3. Results and Discussion

Theoretical Considerations. Let us separate the enthalpy of a hydrated lipid into a bulk term that is independent of the melting process, H_0, and a differential term, ΔH, describing the change in enthalpy as a function of temperature, T, during the melting transition:

$$H^{\text{lipid}}(T) = H_0^{\text{lipid}}(T) + \Delta H^{\text{lipid}}(T),$$
$$\frac{\mathrm{d}H^{\text{lipid}}}{\mathrm{d}T} = \frac{\mathrm{d}H_0^{\text{lipid}}}{\mathrm{d}T} + \frac{\mathrm{d}(\Delta H^{\text{lipid}})}{\mathrm{d}T} = c_{\text{p,0}}^{\text{lipid}} + \Delta c_{\text{p}}^{\text{lipid}} \quad (1)$$

Whereas H_0^{lipid} is mainly due to bond vibrations and headgroup hydration, the latter term, ΔH_{lipid}, describes the changes in chain isomerization and the change in dispersive interactions between chains. c_{p} is the heat capacity, and $\Delta c_{\text{p}}^{\text{lipid}}$ is the excess heat capacity. Similarly, one can describe the lipid volume and volume change by the relations

$$V^{\text{lipid}}(T) = V_0^{\text{lipid}}(T) + \Delta V^{\text{lipid}}(T),$$
$$\frac{\mathrm{d}V^{\text{lipid}}}{\mathrm{d}T} = \frac{\mathrm{d}V_0^{\text{lipid}}}{\mathrm{d}T} + \frac{\mathrm{d}(\Delta V^{\text{lipid}})}{\mathrm{d}T} \quad (2)$$

We have found experimentally for a various lipids, lipid mixtures and biological membranes that[2,3]

$$\frac{\mathrm{d}(\Delta V^{\text{lipid}})}{\mathrm{d}T} = \gamma \Delta c_{\text{p}}^{\text{lipid}} \rightarrow \Delta V^{\text{lipid}}(T) = \gamma \Delta H^{\text{lipid}}(T) \quad (3)$$

with a proportionality constant $\gamma = 7.8 \times 10^{-4}$ cm^3/J.[3] The factor γ was found to be within experimental error the same for all lipids and mixtures investigated.

According to a well-known statistical thermodynamics theorem, the heat capacity is given by the fluctuations of the enthalpy, and the isothermal compressibility is given by the fluctuations of the volume:

$$c_{\text{p}} = \frac{\overline{H}^2 - \overline{H^2}}{RT^2}, \quad \kappa_{\text{T}} = \frac{\overline{V}^2 - \overline{V^2}}{\overline{V} \cdot RT} \quad (4)$$

where R is the gas constant, and the oberbar denotes thermodynamic averaging. In the following, we omit the overbar, because all experimental observables represent averaged values. Because of eq 3, it follows[2] that

$$\Delta \kappa_{\text{T}}^{\text{lipid}}(T) = \frac{\gamma^2 T}{V^{\text{lipid}}(T)} \Delta c_{\text{p}}^{\text{lipid}} \quad (5)$$

and the total isothermal lipid compressibility is given by

$$\kappa_{\text{T}}^{\text{lipid}}(T) = f_{\text{gel}} \kappa_{\text{T,gel}}^{\text{lipid}}(T) + f_{\text{fluid}} \kappa_{\text{T,fluid}}^{\text{lipid}}(T) + \Delta \kappa_{\text{T}}^{\text{lipid}}(T) \quad (6)$$

where f_{gel} and f_{fluid} are the temperature dependent fractions of gel and fluid lipid, respectively, (with $f_{\text{gel}} + f_{\text{fluid}} = 1$). The fluid fraction can be obtained from integrating the heat capacity profile $f_{\text{fluid}} = \int_{T_0}^{T} c_{\text{p}}\, \mathrm{d}T / \int_{T_0}^{T_1} c_{\text{p}}\, \mathrm{d}T$. Here T_0 is a temperature below, and T_1 is a temperature above the melting events. $\kappa_{\text{T,gel}}^{\text{lipid}}(T)$ and $\kappa_{\text{T,fluid}}^{\text{lipid}}(T)$ are the compressibilities of the pure gel phase and the pure fluid phase. For these functions, we assumed a linear temperature dependence which is obtained from fitting the ultrasonic experiments. Because fluctuations from different subensembles of a lipid dispersion are additive, the isothermal compressibility of a lipid dispersion is given by the weighted average of the compressibilities of each component of the system

$$\kappa_{\text{T}}(T) = f^{\text{H}_2\text{O}} \kappa_{\text{T}}^{\text{H}_2\text{O}} + f^{\text{lipid}} \kappa_{\text{T}}^{\text{lipid}} \quad (7)$$

where $f^{\text{H}_2\text{O}}$ is the volume fraction of water and f^{lipid} is the volume fraction of lipid. Using the Maxwell relations one can derive the adiabatic compressibility from isothermal compressibility, volume expansion coefficient, heat capacity, and volume:[7]

$$\kappa_{\text{S}}(T) = \kappa_{\text{T}}(T) - \frac{T}{V c_{\text{p}}(\omega)} \left(\frac{\mathrm{d}V}{\mathrm{d}T}\right)^2 \quad (8)$$

The heat capacity is a function of frequency. The frequency dependence arises from the time dependence of heat exchange within the sample, i.e., absorption of heat by chain vibrations, changes in chain conformation, and exchange of heat between lipid and aqeous medium. The latter exchange rates have been measured by volume perturbation calorimetry[10,11] and pressure jump calorimetry[12] and were found to be on the second to minute time scale within lipid transitions. This is much slower than the time scale of the ultrasonic experiment described below (2.5 MHz or microsecond time regime).

An additivity of compressibilities as for the isothermal case is not generally fulfilled for the adiabatic compressibility, because it depends on heat exchange between different compartments of the ensemble. However, if we assume for the time being that there is no heat exchange between the hydrated lipid and the aqueous buffer in the microsecond time regime, we can write the adiabatic compressibility of the lipid dispersion as the weighted average of the adiabatic compressibility of the water and of the hydrated lipid:

$$\kappa_{\text{S}}(T) = f^{\text{H}_2\text{O}} \kappa_{\text{S,H}_2\text{O}} + f^{\text{lipid}} \kappa_{\text{S,lipid}} \quad (9)$$

Here the adiabatic compressibility $\kappa_{\text{S,H}_2\text{O}}$ of water has been calculated[2] from the volume and the heat capacity of water[13] and the isothermal compressibility.[14] We will discuss below the justification of the above assumption. The adiabatic compressibility of the lipid dispersion is now given by

$$\kappa_{\text{S}}(T) = f^{\text{H}_2\text{O}} \kappa_{\text{S,H}_2\text{O}}(T) + f^{\text{lipid}} \left(\kappa_{\text{T,lipid}}(T) - \frac{T}{V_{\text{lipid}} c_{\text{P,lipid}}(\omega)} \left(\frac{\mathrm{d}V^{\text{lipid}}}{\mathrm{d}T}\right)^2 \right) \quad (10)$$

with $c_P^{lipid}(\omega) = c_P^0(\omega) + \Delta c_P$ (see results section), $V^{lipid} = V_0^{lipid}(T) + \gamma f_{fluid}\Delta H$. Because heat conduction in water is very high, the adiabatic compressibility of water is nearly identical to its isothermal compressibility in the frequency regime used by us (around 5 MHz). The ultrasonic velocity, c, of the lipid dispersion is now given by

$$c = \sqrt{\frac{1}{\rho\kappa_S}} \tag{11}$$

This velocity depends on the lipid fraction f^{lipid}. It can be translated into a function, u, which is independent of lipid concentration through

$$u = \frac{c - c_{H_2O}}{c_{H_2O}[L]} \tag{12}$$

where $[L]$ is the lipid concentration in mg/mL.

Experimental Results. In Figures 1−4, the heat capacity c_p and sound velocity number u are displayed as a function of temperature for suspensions of large unilamellar liposomes from DMPC/DPPC, DLPC/DPPC, DMPC/DMG, and DOPC/DPPC lipid mixtures, respectively. Each figure shows, around the gel/fluid phase transition temperature T_m of the lipid systems, the experimental c_p scans (top), the measured sound velocity numbers u (middle), as well as the u data, which, according to the above theoretical model, result from the c_p data (bottom). In the formulation of the sound velocity as a function of the heat capacity, it was tacitly assumed that the lipid and the water in the vesicle suspensions are adiabatically decoupled, meaning that no heat transfer takes place on the time scale of the sound velocity experiment (0.4 μs per period). This seems surprising because the heat transfer in water is very fast (0.6 W/mK at 20 °C). However, this assumption is justified by the experimental observation that relaxation processes in membranes close to transitions are very slow (1−60 s).[10,11] Grabitz et al.[12] have directly measured the heat transfer between lipid membranes and water in the phase transition regime using a calorimetric pressure jump technique. Relaxation times in the second to minute time regime were found. The calorimetric perturbation technique is performed on aqueous dispersions. The heat released by the lipid membranes after a pressure jump is transferred to the aqueous medium and then recorded by the temperature sensors of the calorimeter. Thus, the relaxation processes measured in these experiments are actually monitored via the heat transfer between lipid and water, and it is thus shown to be slow. The finding of such slow heat transfer rates still requires a theoretical explanation, but it clearly is an experimental fact. Therefore, it can be assumed that in the MHz frequenvy regime no heat exchange takes place between lipid and water.

Additionally, calculating the u values from the c_p profiles around the transition temperature T_m, the linear relationships

$$\kappa_{T,gel}^{lipid} = \kappa_{0,gel} + \Delta\kappa_{gel}(T - 303.15K) \tag{13}$$

and

$$\kappa_{T,fluid}^{lipid} = \kappa_{0,fluid} + \Delta\kappa_{fluid}(T - 303.15K) \tag{14}$$

have been assumed to consider the temperature dependence in the background compressibility of the membranes in their gel and fluid state, respectively. We basically adjusted these two functions to obtain the experimental baselines in the ultrasonic velocity profiles (Figures 1−4).

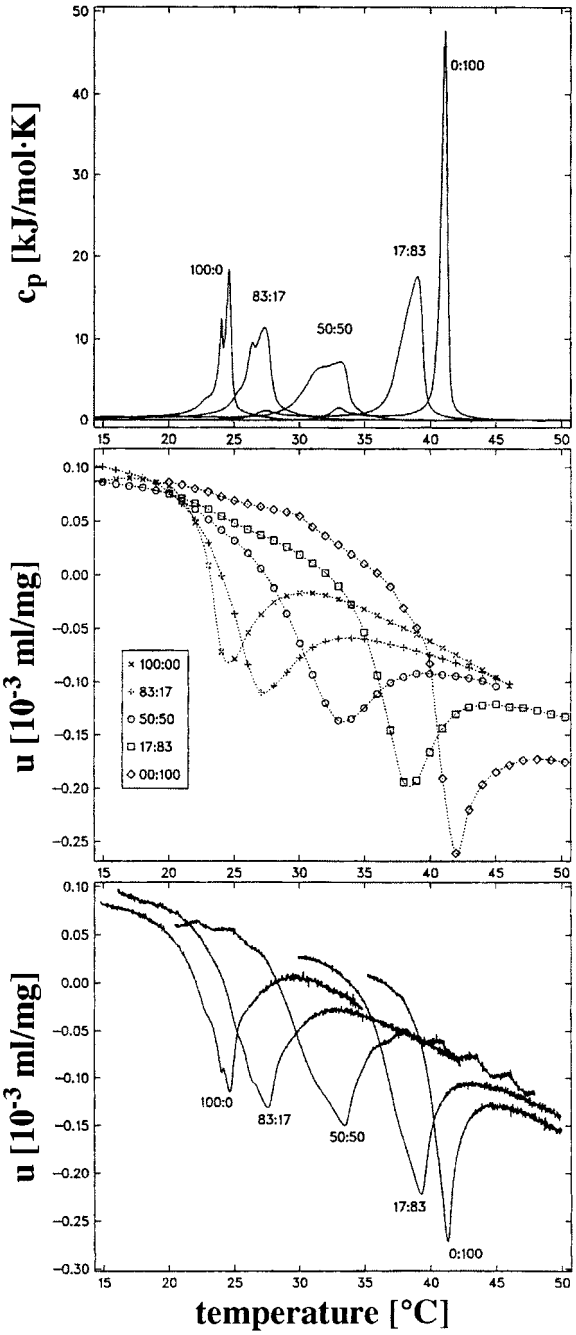

Figure 1. Temperature scans of the heat capacity c_p(top) and the sound velocity number (12, middle) as well as sound velocity number profiles as calculated from the c_p traces for suspensions of unilamellar vesicles made of DMPC and DPPC with different mole fractions.

The resolution in the sound velocity measurements is indeed as high as 10^{-5}. Depending on the wetting behavior of the samples and also due to the presence of microscopic gas bubbles in the samples, however, the experimental error in the absolute sound velocity values may be higher.[8] The data for the background compressibility of the membranes, therefore, should not be overemphasized. However, for the DMPC/DPPC system, one consistent set of parameters ($\kappa_{0,gel}$, $\Delta\kappa_{gel}$, $\kappa_{0,fluid}$, and $\Delta\kappa_{fluid}$) was sufficient for all mixing ratios (Table 1). Tosh and Collings[15] investigated the isothermal compressibilites of DPPC and found values of $5.2 \pm 0.8 \times 10^{-4}$ cm^3/J in the P_β' phase (35 °C), whereas our value for the gel phase extrapolated to 35

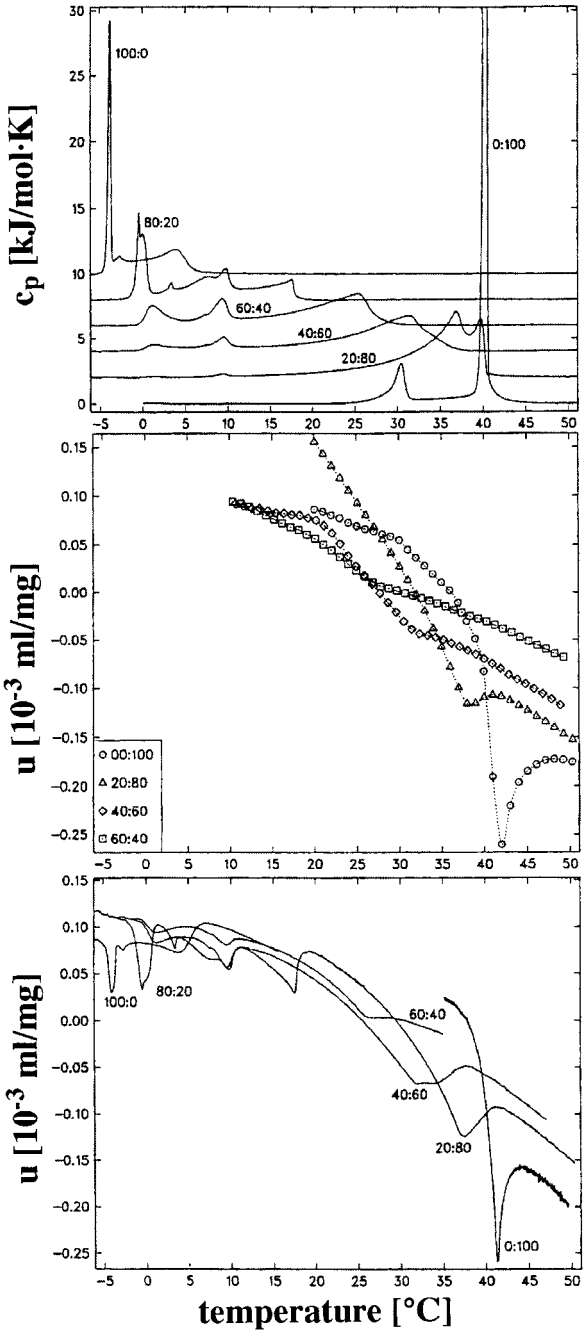

Figure 2. Same plots as Figure 1 but for mixtures of DLPC and DPPC. For clearness, heat capacity traces have been shifted.

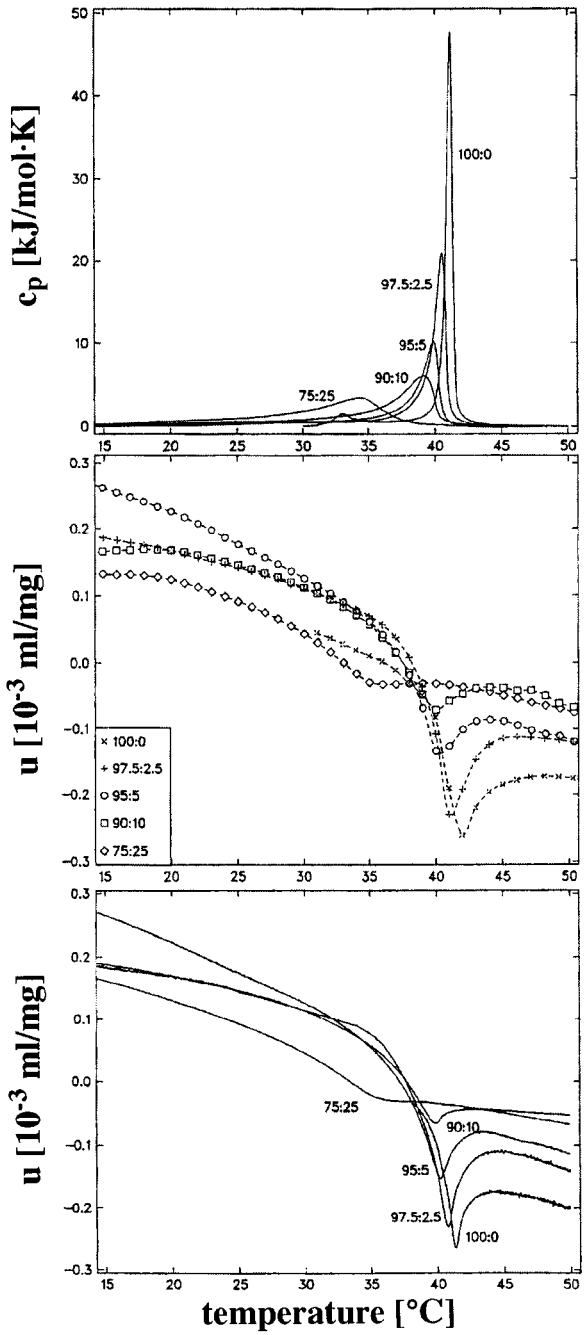

Figure 3. Same plots as Figure 1 but for mixtures of DOPC and DPPC.

°C is 2.3×10^{-4} cm^3/J. Because the pretransition already involves some chain melting,[16] we assume that it is resonable that our value for the gel phase is smaller than the value for the P_β' phase by the above authors. The value given by Tosh and Collings for the fluid phase, $7.8 \pm 1.1 \times 10^{-4}$ cm^3/J, is very close to our value for the fluid phase at 45 °C: 7.2×10^{-4} cm^3/J. We conclude therefore that our method is in principle suitable to obtain accurate values of the isothermal compressibilities of the pure lipid phases. Within the temperature range of interest, the membrane compressibilities of all lipid mixtures shown in Figures 1−4 as well as the slopes in the temperature dependence are found to be significantly higher in the fluid phase than in the gel phase. The absolute values seem to vary with the lipid species, being for instance smaller for DOPG than for DPPC in the fluid phase at 42 °C. In eqs 8 and 10, a frequency dependent heat capacity, $c_P(\omega)$, was used, which expresses the time dependence of heat transfer processes. If we take into account that heat transfer between lipid and water is slow and that heat transfer within the lipid chains is fast on our experimental time scale, the heat capacity of the membranes at 2.5 MHz is given by $c_P(\omega) = c_p^0 + \Delta c_P$. We used $c_p^0 = 1.65$ KJ/mol K, which is the heat capacity of the hydrocarbon chains outside of the transition regime.[17] It should be noted, however, that c_p^0 and therefore the ultrasonic velocity number will be frequency dependent. The dip in the ultrasonic number profiles will be more pronounced at lower frequencies, because c_P assumes higher values, leading to an increase of the adiabatic compressibility at lower frequencies. The sound velocity number profiles calculated from the heat capacity traces agree strikingly well with the corresponding u vs T relations from the c measurements. Thus, the finding that ultrasonic velocities are correctly predicted justifies our assumptions concerning the frequency dependence of the heat exchange and the adiabatic

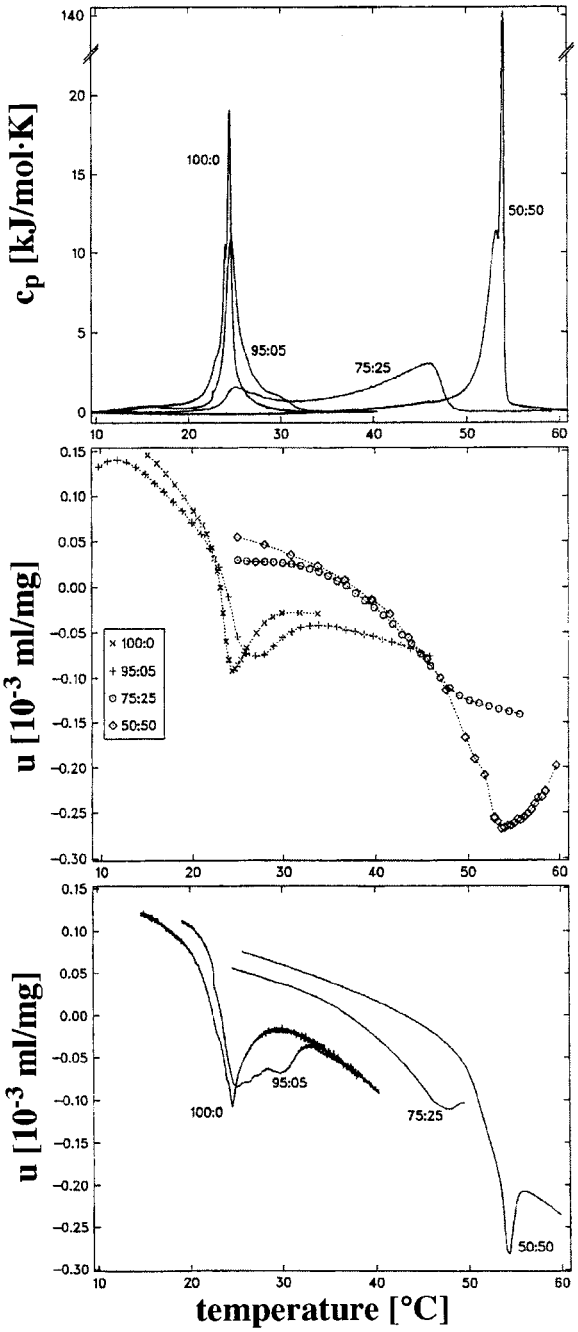

Figure 4. Same plots as Figure 1 but for mixtures of DMPC and DMG.

decoupling of membranes and water in the MHz regime. This has also been discussed by Heimburg.[2] Not only the absolute u values but also the overall shape in the temperature dependence of the sound velocity are correctly predicted by the heat capacity data. This result may be taken as confirmation of both the high accuracy in the sound velocity measurements and the adequate simulation of the sound velocity profiles by the theoretical model. Some fine details are additionally given by the heat capacity curves and by the sound velocity traces derived from them, for instance, the fact that the peaks in the curves of the DMPC/DPPC systems with mole fractions 100:0 and 83:17 (Figure 1) are split is not displayed by the experimental u scans. However, these missing details seem to be mostly due to the mode of sound velocity measurements, sampling the c values at 1° intervals only. For some systems with low transition temperature T_m, the experimental u curves are missing at all (Figure 2), because, in its present form, the twin-cell sound velocity meter is not designed for measurements below 10 °C. Our results confirm the previous conclusion for suspensions of liposomes from DMPC with admixed cholesterol.[6] For those systems, comparison of the sound velocity number with heat capacity traces also displayed a close correlation between these quantities for the bilayer systems, indicating that some of the mechanical properties of membrane systems can be calculated from the specific heat capacity data and vice versa.

TABLE 1: Parameters Used in the Theory Section to Calculate the Ultrasonic Velocity Number from the Heat Capacity[a]

parameter	general expression
$c_{P,0}^{lipid}$	1.65kJ/(molK) [17]
$V^{-1}dV_{gel}^{lipid}/dT$	0.0009/K [2]
$V^{-1}dV_{fluid}^{lipid}/dT$	0.001/K [2]
$\Delta V^{lipid}/V^{lipid}$	0.041 [2]
γ	7.8×10^{-4}cm^3/J [3]
	DMPC/DPPC
$\kappa_{0,gel}$	1.8×10^{-4}cm^3/J
$\Delta\kappa_{gel}$	0.9×10^{-5}cm^3/(JK)
$\kappa_{0,fluid}$	4.0×10^{-4}cm^3/J
$\Delta\kappa_{fluid}$	2.1×10^{-5}cm^3/(JK)

[a] As an example the temperature dependence of the isothermal compressibilities of the DMPC/DPPC mixtures in gel and fluid phase as deduced from the ultrasonic experiment (Figure 1, eqs 13 and 14) are also given.

A discrepancy arises between measured and calculated u profiles of the DMPC:DMG = 50:50 mixture (Figure 4). The dip in the calculated ultrasound number profile is much narrower than in the experimental data. From the phase diagram of DMPC:DMG, it is known that the 50:50 mixture (in contrast to all other mixtures with lower content in DMG) forms an inverse hexagonal H_{II} phase above the melting transition[18] and the sample tends to aggregate. Inhomogeneous samples, however, cause problems in the analysis of the ultrasonic velocity measurement. On the other hand, aggregation does not cause problems in calorimetric measurements. Thus, we assume that the broadening of the measured ultrasonic velocity profile is an experimental artifact caused by inhomogeneous samples and slow aggregation processes above the melting transition. The DMPC:DMG = 50:50 mixture is the only mixture described in our manuscript that shows this problems. All other mixtures undergo transitions between gel and fluid lamellar phases.

Frequency Dependence. Between 1.3 and 13 MHz Mitaku and Date[19] and between about 1 and 100 MHz Sano et al.[20] have measured the frequency dependence of the ultrasonic absorption coefficient of DPPC suspensions. They descibed their excess absorption data by a relaxation term with discrete relaxation time, displaying some evidence of critical slowing down near the phase transition. More recent broadband ultrasonoic spectra of phospholipid bilayer systems reveal a more complicated characteristics, reflecting at least two relaxation terms.[21,22] The high-frequency term, located in the 100 MHz region is assigned to the rotational isomerization of the lipid hydrocarbon chains. The other relaxation term, which, in principle, had been already found in the previous studies,[19,20] turns out to be a low-frequency broadband relaxation term of which Mitaku and Date[19] have just seen a high-frequency contribution. This term is assumed to be due to the fluctuations in domain sizes of the membranes.

Mitaku and Date[19] have also measured the dispersion in the sound velocity corresponding to the small contribution of the

broadband term in the MHz frequency regime. The effect of dispersion in the sound velocity number was much smaller than the temperature effect near the phase transition temperature found by us. Hence, our temperature profiles are unlikely dominated by the slowing down of the sound velocity dispersion near the phase transition. From a thermodynamic point of view, our assumption that the lipid membrane is adiabatically uncoupled from the aqueous environment depends on the time scale of the experiment. At low frequency, it can be expected that heat transfer takes place between lipids and water. This leads to an effective increase of the heat capacity in eq 8 and consequently to an increase of the adiabatic compressibility. Thus, the reduction of the ultrasonic velocity number at the phase transition should be more pronounced at low frequency, as it was found in experiments.[19] Broadband ultrasonic spectrometry studies are on progress to investigate contributions from critical order parameter fluctuations to the sound velocity more closely.

Biophysical Aspects. The analysis of ultrasound data, presented in this paper, is based on the experimental observation that volume changes and enthalpy changes are proportional functions. This has been described in detail before.[2,3] This finding is not trivial because there is no theoretical necessity for this finding. Therefore, it should be taken as an experimental fact for lipid systems. It has been shown that this relation even holds for complex mixtures as the lung surfactant, which is composed of a variety of lipids as well as of several proteins.[3] If this relation is taken into account as well as the experimental fact that heat exchange between lipid membranes and the aqueous environment is in the millisecond to minute regime,[10,12,23] meaning that buffer and lipid membranes are adiabatically uncoupled in the MHz regime of the experiment, one can derive the ultrasonic velocity profiles from the heat capacity. This finding is quite relevant because ultrasonic velocity profiles provide information about isothermal and adiabatic compressibilities and reveal the deep linkage between the different thermodynamic response functions. The experiments presented here indicate that our analysis is correct for all lipid mixtures investigated. This suggests that heat capacities can generally be related to the compressibilities (and ultrasonic velocities), even in lipid mixtures of unknown composition.

The calorimetric profiles (and in principle the ultrasonic velocity data as well) of Figures 1−4 can be used to construct phase diagrams of lipid mixtures. The phase diagrams of the mixtures investigated in this work agree with those found in the literature (i.e., DMPC/DPPC,[24,25] DLPC/DPPC,[25] DOPC/DPPC,[26] and DMPC/DMG[18]). The increase in heat capacity in the phase coexistence regime is mainly linked to cooperative fluctuations of large numbers of molecules in their physical state. As known from statistical thermodynamics, the sharpness of the transition peak of a single lipid is related to the correlation lengths of the fluctuations, namely of domain sizes. Overall, the heat capacity increase in the transition regime is mainly due to cooperative fluctuations of large numbers of molecules.

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