Interaction of Ultrasound and Model Membrane Systems: Analyses and Predictions

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(Received: July 26, 1991; In Final Form: November 13, 1991)

Low-intensity ultrasound (approximately 10^{-6} W/cm^2) in the frequency range 0.5–5.0 MHz was employed to investigate biomembrane structural relaxation kinetics via absorption and velocity dispersion spectroscopy. The multilamellar vesicles utilized in this investigation were composed of either pure phospholipids or mixtures of phospholipids and small molar fractions of protein gramicidin. The experimental findings reveal enhanced ultrasound interactions near the lipid phase transition temperature. The enhanced ultrasound absorption spectra closely resemble single-relaxaton spectra, suggesting that the membrane constituents undergo a simple two-state transition. The temperature dependence of the relaxation frequency is followed with the combined aid of the absorption and velocity dispersion spectrum. Thermodynamic and electrical capacitor two-state transition models are developed to help describe the observed phenomena and to predict to a reasonable degree of accuracy the enhanced findings promoted by ultrasound.

I. Introduction

Numerous experimental techniques such as differential scanning calorimetry (DSC),[1] steady-state and time-resolved fluorescence depolarization spectroscopy,[2] NMR,[3] ESR,[4] and ultrasound absorption spectroscopy[5-7] have been utilized to characterize quantitatively the membrane state of constituents and their self local environment dynamical interactions. One method of interrogating systems in thermodynamic equilibrium is to perturb suddenly a thermodynamic parameter, such as temperature or pressure, and to measure the relaxation characteristics of the system toward its new equilibrium value. Alternatively, the linear response of a system subjected to small harmonic perturbations of a thermodynamic parameter provides for determination of the relaxation characteristics;[8] e.g., an ultrasonic wave is accompanied by periodic temperature and pressure perturbations.[8,9] A single complete theory describing the mechanisms of plasma membrane sound absorption does not exist. For investigations of such underlying mechanisms, investigators have, over the past two decades, studied simple model membrane systems, such as liposomes, which serve as a first approximation to the in vitro biological cell plasma membrane. Different research groups have investigated the sound absorption properties of liposomes (usually consisting of phosphatidylcholine (PC) lipids such as dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC)) and have observed large deviations from the classical theory of sound absorption in the proximity to the lipid thermotropic phase transition temperature. Such deviations have been postulated and inferred to arise from a structural relaxation process within the lipid bilayer.[10]

Permeability measurements of ions, such as Na^+,[11] and small anticancer drug compounds[12] of PC liposomes have revealed maximal enhancements in the vicinity of the phospholipid phase transition temperature and have been postulated to arise from the interfacial regions between the solid and liquid domains where macromolecular packing defects are most likely. Wu and McConnell[13] have suggested that the coexistence of two lipid phases near the phase transition region could facilitate passive transport and membrane protein activities due to the measured existence of enhanced lateral compressibility. Motivated by this suggestion, Kanehisa and Tsong[14] developed a two-state lipid coexistence model which allows for a pure lipid membrane system to coexist in domains, or "clusters", of lipids in a nondominant phase within a pool of dominant phase lipids. Kanehisa and Tsong also presented a phenomenological description of the enhanced passive permeation of molecules and the structural relaxation characteristics of clusters as featured in liposome temperature jump experiments. Recently, Parasassi et al.[15] presented experimental evidence on the phase fluctuations in phospholipids with the fluorescence probe laurdan, which yields different excitation and emission spectra in gel and liquid crystalline phases. The difference in the excitation spectra allows the photoselection of laurdan molecules in each of the two phases, and utilizing these differences in the emission spectra indirectly measures the interconversion rates between the two phases. Thus, for the membrane system in thermodynamic equilibrium with its surroundings, its constituent clusters, composed of either gel or fluid lipids, have been hypothesized, and indirectly observed, to fluctuate rapidly in energy and volume due to their interactions with the immediate surroundings.

It seems likely that the enhancement in ultrasound absorption could arise due to structural relaxation of lipid fatty acyl chains since it is reversible and does not exhibit hysteresis.[16] Determination of the lipid structural relaxation frequencies can be of theoretical and clinical importance. Maximal coupling between the (biomembrane) system and the perturbing modality is to be expected when the perturbation frequency equals the natural relaxation frequency of the system, thereby resulting in greatest energy deposition and possibly leading to a maximal bioeffect. Results of such structural coupling with the modality may possibly lead to further enhancements in permeation of specific ions and small drug compounds.

For the study reported herein, it is postulated that ultrasound perturbs harmonically the concentration equilibrium of the phospholipid molecules occupying the two dominant states of the membrane, viz., the crystalline and fluid states near the phase transition temperature. Low-intensity continuous wave ultrasound ($\sim 10^{-6}$ W/cm^2) is employed in the frequency range 0.5–5.0 MHz to investigate the multilamellar vesicle (MLV) liposome structural

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relaxation kinetics of several neutral PC lipids via absorption spectroscopy. Although the molecular mechanistic details regarding how this equilibrium becomes perturbed by ultrasound are not understood, it is considered that the temperature and pressure perturbations induced by the ultrasound wave play significant roles. An understanding is obtained of the measured absorption and relaxation characteristics through empirical findings and through two empirical models.

II. Brief Overview of Sound Absorption

The mechanical energy in the propagating pressure wave is absorbed by the fluid medium it traverses by two mechanisms, viz., the classical absorption processes and the physical relaxation processes.[17] Classical absorption arises as a consequence of the medium having a finite viscosity and a finite thermal conductivity. This results in shearing motions between the fluid molecules, leading to viscous energy losses and to thermal energy being transported along temperature gradients. Nonclassical absorption mechanisms result due to thermal, chemical, and/or structural relaxation processes whenever the chemical or structural equilibrium is perturbed by the changes in pressure and temperature induced by the sound wave. The culmination of the two classical and the many possible nonclassical absorption processes comprises the pressure amplitude absorption coefficient α in $P(x) = P_0 \exp(-\alpha x)$[17]

$$\alpha = \alpha_{\text{classical}} + \alpha_{\text{nonclassical}} \tag{1}$$

$$\alpha_{\text{classical}} = \frac{\omega^2}{2\rho v^3}\left[(4/3)\eta_{\text{shear}} + \frac{(\gamma - 1)}{C_p}K\right] \tag{2}$$

where ω is the angular frequency of the pressure wave and v is the velocity of sound propagation through the medium of density ρ having a shear viscosity η_{shear}, thermal conductivity K, and heat capacities at constant pressure C_p and constant volume C_v, with $\gamma = C_p/C_v$.

The anomalous absorption in excess of that predicted by classical theory has been attributed to relaxation processes.

$$\alpha_{\text{excess}} = \alpha_{\text{experiment}} - \alpha_{\text{classical}} \cong \alpha_{\text{relaxation}} \tag{3}$$

Frequently the dimensionless absorption per wavelength, $\alpha\lambda$, which is a quantitative measure of the degradation in the pressure amplitude as the wave travels a distance of one wavelength, is employed. Typical excess sound absorption spectra of solutions of biomolecules are not single-relaxation processes but may be described in terms of linear superposition of several independent single relaxations, each having their characteristic relaxation time, τ.[9]

$$(\alpha\lambda)_{\text{excess}} = \sum_{i=1}^{n} 2(\alpha\lambda_i)_{\max}\frac{\omega\tau_i}{1 + (\omega\tau_i)^2} \tag{4}$$

Eigen and de Maeyer[8] have pointed out that it is possible to obtain $\alpha\lambda$ and the dispersion in sound velocity through the linearized sound wave equation when the adiabatic compressibility is modified and written as a complex function

$$\beta_{\text{ad}} = \beta_{\text{ad}}^{\infty} + \frac{\beta_{\text{relax}}}{1 + i(\omega\tau)} \tag{5}$$

where β^{∞} is a real quantity and corresponds to the adiabatic compressibility at frequencies well above the relaxation frequency and β_{relax} is a real quantity and corresponds to the relaxing part of the adiabatic compressibility of the membrane. After a lengthy manipulation procedure, it is shown that[8]

$$\alpha\lambda \cong 2\,\frac{\pi\beta_{\text{relax}}}{\beta_{\text{ad}}^{\infty}}\,\frac{\omega\tau}{1 + (\omega\tau)^2} \tag{6}$$

and

$$v \cong (\rho\beta_{\text{ad}}^{\infty})^{-1/2}\left(1 - \frac{\beta_{\text{relax}}}{2\beta_{\text{ad}}^{\infty}}\,\frac{1}{1 + (\omega\tau)^2}\right) \tag{7}$$

Eigen and de Maeyer have also shown the relaxing part of the adiabatic compressibility to be given by

$$\beta_{\text{relax}} = \frac{\Gamma}{RT}\left\{\Delta V - \frac{\Delta H\Theta}{\rho C_p}\right\}^2 \tag{8}$$

where ΔV is the difference in the molar volume between the two states of a fundamental transition unit, e.g., a lipid cluster, ΔH is the molar enthalpy of reaction, Θ is the thermal expansion coefficient at constant pressure, C_p is the specific heat capacity at constant pressure, ρ is density, R is the gas constant, T is the surrounding temperature in degrees Kelvin, and Γ is the "proportionality constant". For a two-state system, the proportionality constant is[8]

$$\Gamma = C_0K/(1 + K)^2 \tag{9}$$

where the equilibrium constant K is given by the ratio of the lipid concentrations in the fluid state B to the concentration in the crystalline state A, i.e., $K = C_B^{eq}/C_A^{eq}$ and $C_A^{eq} + C_B^{eq} = C_0$. The equilibrium constant may also be expressed in terms of the fractional population of molecules in state B, f_β, relative to those in state A, f_α. Substituting K into eq 9 and making use of the identity $f_\alpha + f_\beta = 1$ yields $\Gamma = C_0f_\alpha f_\beta$, and the final result for a two-state transition is

$$\alpha\lambda = 2\frac{\pi C_0f_\alpha f_\beta}{(\beta_{\text{ad}}^{\infty} + \beta_{\text{relax}})RT}\left(\Delta V - \frac{\Delta H\Theta}{\rho C_p}\right)^2\frac{\omega\tau}{1 + (\omega\tau)^2} \tag{10}$$

Typically $\beta_{\text{relax}} \ll \beta_{\text{ad}}^{\infty}$ and $\Delta H\Theta/\rho C_p \ll \Delta V$ for biochemical solutions,[5,8] so to a good approximation

$$\alpha\lambda = 2\frac{\pi C_0f_\alpha f_\beta}{\beta_{\text{ad}}^{\infty}RT}\{\Delta V\}^2\frac{\omega\tau}{1 + (\omega\tau)^2} \tag{11}$$

III. Experimental Section

Sample Preparation. The 14–17 carbon chain neutral phospholipids, DMPC, $DC_{15}PC$, DPPC, and $DC_{17}PC$, employed in this investigation were obtained in 10-mL chloroform aliquots from Avanti Biochemical Co. (Birmingham, AL) and were used without further purification. Gramicidin was obtained from Sigma Chemical Co. (St. Louis, MO) and was stored in a chloroform suspension at a concentration of 10 mg/mL until use. Hepes was obtained from Sigma. Hepes buffer was made of 10 mM Hepes, 139 mM NaCl, 6 mM KCl, and distilled water at pH 7.4 and was utilized as the reference fluid in the ultrasound measurements.

To make MLVs, lipid aliquots containing the same type of lipid were opened at room temperature and transferred into the rotary evaporator. The chloroform was then removed by the evaporator at a pressure of one Torr at 50 °C. The lipids were dried thoroughly in approximately 30 min under these conditions. The dried lipids were redissolved with the Hepes buffer to a final concentration of 10 mg/mL, at a temperature 10 °C above the lipid phase transition temperature T_m (see Table III). The single lipid component MLV liposomes were then formed in a vortex mixer, at a temperature 10 °C above the lipid T_m, for 5 min and then cooled to room temperature. The suspension was used immediately in the measurement procedure.

The same procedure was used in making the slightly perturbed MLVs containing 2.5 and 5.0 mol % gramicidin. Appropriate quantities of lipid and gramicidin in chloroform solution were measured and transferred into the rotary evaporator flask where they were continually mixed within the same organic phase at room temperature and atmospheric pressure. The procedure outlined above was then implemented in making slightly modified MLVs containing gramicidin.

Measurement Method. The ultrasound absorption and velocity dispersion measurements performed in this investigation on liposome suspensions were made with a conventional Eggers and

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Funck type cylindrical resonator.[18] The end walls of the resonant cavity are formed by two piezoelectric quartz transducers separated the distance d (=5.5 cm), by a hollow plexiglass cylinder, providing a sample volume of approximately 3 mL. One transducer is excited by a Hewlett-Packard 8660B synthesized signal generator at a predetermined frequency and produces longitudinal plane waves in the fluid medium within the cavity while the other transducer acts as a receiver. The amplitude of the resulting standing wave at the receiving transducer surface is monitored by a Hewlett-Packard 8552A, 8553B spectrum analyzer. The amplitude of the standing wave peaks at resonance frequencies when the standing wave boundary conditions are fulfilled, i.e., when the separation distance of the two piezoelectric transducers comprising the end walls of the resonator is an odd number of half-wavelengths.

$$d = n\lambda_n/2 \tag{12}$$

Since the speed of sound, v, is given by $v = f_n\lambda_n$, the nth resonance frequency mode is given by

$$f_n = v/\lambda_n = nv/2d \tag{13}$$

Thus, v can, in principle, be calculated from the difference between successive resonance frequencies. The accuracy of such a measurement is limited mainly by the accuracy to which d is known, viz., 0.001 in.

The absorption per wavelength of the media for the nth resonance is directly related to the half-power bandwidth of the resonance Δf_n,[18] i.e.

$$\alpha\lambda = \pi\Delta f_n/f_n \tag{14}$$

The excess absorption per wavelength due to the liposome presence is

$$(\alpha\lambda)^{\text{lipids}}_{\text{excess}} = \pi(\Delta f_{\text{solution}} - \Delta f_{\text{solvent}})/f_n \tag{15}$$

assuming the component absorption (and velocity) magnitudes to be additive.[19] The excess absorption is frequently reported in units of excess absorption per wavelength per concentration of lipid, and this quantity is known as the specific absorption per wavelength. When the concentration of the "solute" (lipid) is taken in the limit to approach zero, the specific absorption is called the limiting excess absorption per wavelength.

Although the accuracy of the sound propagation speed of the liquid within the resonant cavity is limited by the value available for d, fractional changes in sound propagation can be obtained with greater precision assuming the speed of the solution is the linear combination of that of the solvent and solute. Thus

$$\frac{v_{\text{solute}}}{v_{\text{solvent}}} = \frac{v_{\text{solution}} - v_{\text{solvent}}}{v_{\text{solvent}}} \tag{16}$$

That is

$$\frac{v_{\text{solute}}}{v_{\text{solvent}}} = \frac{f_{\text{solution}}\lambda_{\text{solution}} - f_{\text{solvent}}\lambda_{\text{solvent}}}{f_{\text{solvent}}\lambda_{\text{solvent}}} \tag{17}$$

or from eq 12 and the nth resonance peak

$$\frac{v_{\text{solute}}}{v_{\text{solvent}}} = \frac{f_{\text{solution}}(2d_{\text{solution}}/n) - f_{\text{solvent}}(2d_{\text{solvent}}/n)}{f_{\text{solvent}}(2d_{\text{solvent}}/n)} \tag{18}$$

which reduces to

$$\frac{v_{\text{solute}}}{v_{\text{solvent}}} = \frac{f_{\text{solution}} - f_{\text{solvent}}}{f_{\text{solvent}}} \tag{19}$$

as d is unchanged for all samples. The limiting fractional sound speed of the solute relative to the solvent at zero concentration is then given by

$$[v]_{\text{solute}} = \lim_{C_0 \to 0} \frac{v_{\text{solution}} - v_{\text{solvent}}}{C_0 v_{\text{solvent}}} = \frac{f_{\text{solution}} - f_{\text{solvent}}}{C_0 f_{\text{solvent}}} \tag{20}$$

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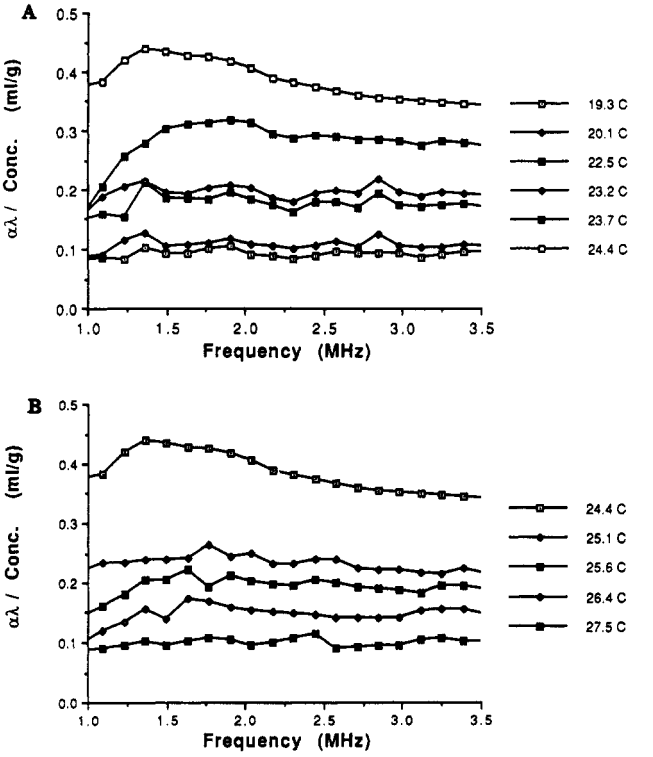

Figure 1. (A) DMPC MLV absorption spectra at several temperatures increasing toward T_m = 24.4 °C. (B) DMPC MLV absorption spectra at several temperatures increasing from T_m = 24.4 °C.

The enhanced dispersion in the ultrasound velocity arises due to the change in the membrane adiabatic compressibility[19]

$$[v]_{\text{solute}} = [v]_\infty - \Delta\frac{1}{1 + (\omega\tau)^2} \tag{21}$$

and

$$[\alpha\lambda]_{\text{excess}} = 2\pi\Delta\frac{(\omega\tau)}{1 + (\omega\tau)^2} + 2\pi B\omega \tag{22}$$

where $[v]_\infty$ is the limiting number of the velocity at very high frequencies, Δ is the relaxation strength of the membrane given by

$$\Delta = \beta_{\text{relax}}/2\beta_{\text{solvent}} \tag{23}$$

and B corresponds to the contribution of the classical sound absorption mechanisms occurring within the membrane. If the classical sound absorption of the membrane is small in comparison to the nonclassical part (as is experimentally found near the phase transition temperature for the PC liposomes as seen in Figures 2 and 4), eq 21 can be rewritten in terms of $[\alpha\lambda]$ to yield

$$[v]_\infty - [v] = \frac{[\alpha\lambda]}{2\pi(\omega\tau)} = \frac{[\alpha\lambda]}{2\pi}\frac{f_{\text{relax}}}{f} \tag{24}$$

where

$$f_{\text{relax}} = \frac{2\pi([v]_\infty - [v])f}{[\alpha\lambda]} \tag{25}$$

IV. Results

The enhanced ultrasound absorption per wavelength behavior of the pure PC, as well as that of small molar quantity mixtures of gramicidin with DPPC MLV liposome systems, studied in this investigation is typified in the pure DMPC absorption spectra at various temperatures (see Figure 1). Each spectrum is an average of three distinct sample measurements. Figure 1A shows the enhancement of ultrasound absorption as the temperature is increased in steps toward the DMPC phase transition temperature T_m of 24.4 °C. When the sample temperature is several degrees Celsius below T_m, the deviation from classical theory of sound

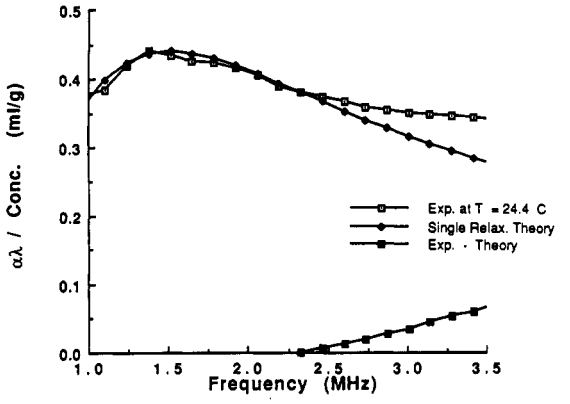

Figure 2. Comparison of the DMPC absorption spectrum at T_m with that determined by a single-relaxation process.

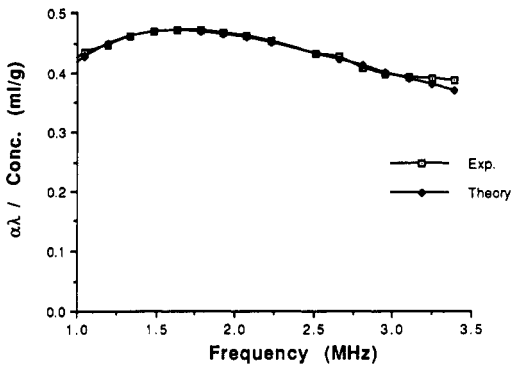

Figure 3. $DC_{15}PC$ absorption spectrum at T_m = 33.5 °C compared with single-relaxation theory.

absorption is relatively small and uniform over the frequency range of measurement. As the temperature is increased toward T_m, the uniform sound absorption spectra are gradually transformed into spectra containing evidence of a broad peak. When the temperature is further increased toward T_m, the broad peak is observed to become more clearly defined and is displaced toward lower frequencies. When the temperature equals the DMPC phase transition temperature, maximal anomalous sound absorption occurs with a single well-defined sound absorption peak centered near 1.4 MHz. Figure 1B shows that as the temperature is further increased beyond T_m, the nonclassical sound absorption weakens and the amplitude of the spectrum is noted to diminish progressively. The single peak is noted to lose definition and to be displaced toward higher frequencies.

Figure 2 compares the well-defined DMPC absorption spectrum at T_m with the spectrum of a single-relaxation process having the same frequency and magnitude. The difference spectrum, viz., the experimentally observed spectrum minus the theoretical spectrum, is also shown in Figure 2 and reveals a linear difference at the higher frequencies. No significant deviations are observed at frequencies below f_{relax}. According to the sound absorption theory for single-relaxation processes, eq 22, such deviations are expected to result from the presence of classical absorption processes within the lipid bilayer system. Similar remarks can be made for Figures 3, 4, and 5 which display, respectively, $DC_{15}PC$, DPPC, and $DC_{17}PC$ absorption spectra at their respective T_m values. Single-relaxation spectra are also shown in these figures.

Figure 6 shows the temperature dependence of the $\alpha\lambda$ parameter of DPPC MLV liposomes at the sample resonance frequency of 3 MHz.

The temperature dependence of the sound velocity of the DPPC lipid was also measured at the sample resonance frequency of 3 MHz and is shown in Figure 7. A line is drawn tangent to the limiting velocity curve, below the T_m, to represent the temperature dependence of the limiting sound velocity for the case when the membrane system *had not* undergone relaxation in the neighborhood of T_m. According to relaxation theory, viz., eq 21, such a condition would prevail when the perturbation frequency is much

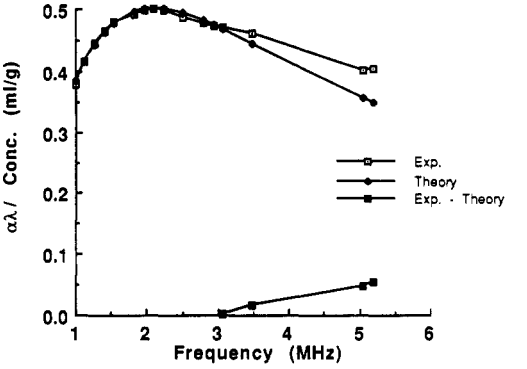

Figure 4. DPPC absorption at T_m = 42.0 °C compared with single-relaxation theory.

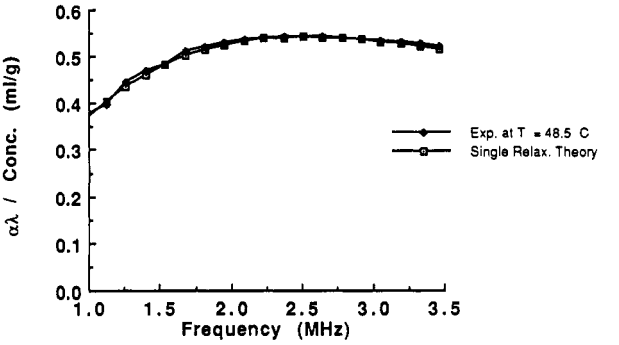

Figure 5. $DC_{17}PC$ absorption at T_m = 48.5 °C compared with single-relaxation theory.

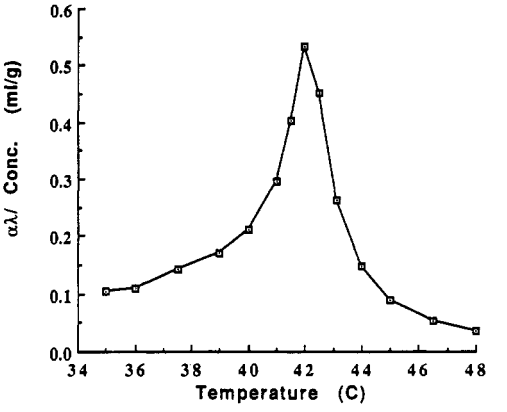

Figure 6. DPPC absorption vs temperature at 3 MHz.

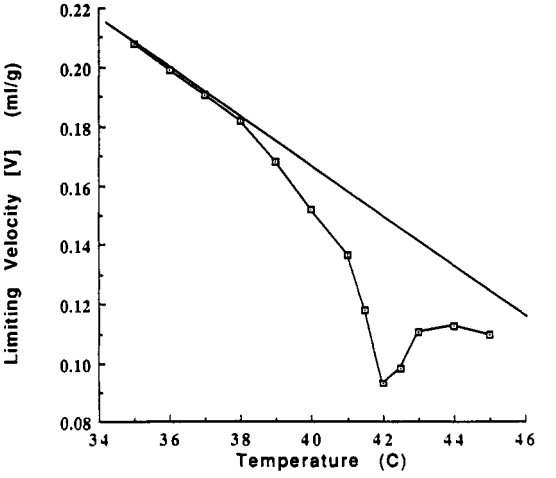

Figure 7. DPPC limiting velocity dispersion at 3 MHz.

greater than the natural relaxation frequency of the membrane system, f_{relax}. Hence, the tangent line is interpreted to represent the temperature dependence of the sound velocity in the limit,

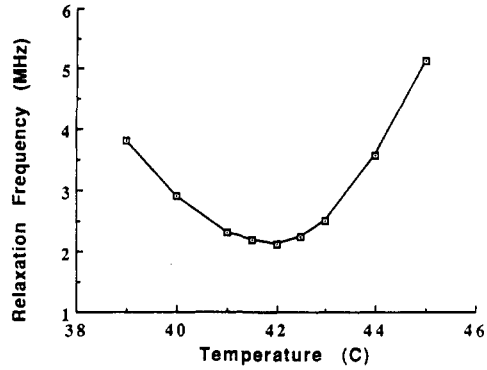

Figure 8. DPPC relaxation frequency temperature dependence as determined through Figures 6 and 7 and eq 25.

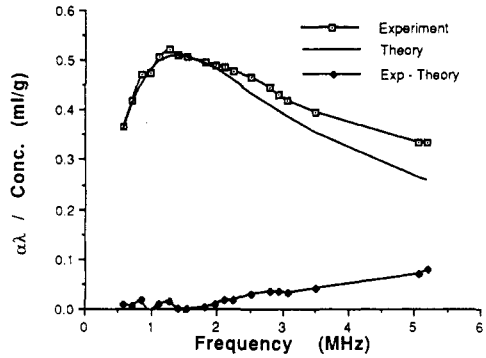

Figure 9. Absorption spectrum of DPPC with 2.5 mol % gramicidin at T_m = 42.0 °C.

as the ultrasound driving frequency f approaches infinity. As stated above, it becomes increasingly difficult to resolve accurately and to follow the frequency position of the absorption maxima when the temperature is progressively moved away from T_m. Such difficulties can be overcome if it is assumed that the main absorption peak is well described by a single-relaxation process. Through this assumption, the measured $\alpha\lambda$ and the difference $[v]_\infty - [v]$ as a function of temperature of pure DPPC MLVs, at the frequency of 3 MHz, were utilized in the single-relaxation theory eq 25 to yield the temperature dependence of the f_{relax} parameter (see Figure 8). Ultrasound absorption between pure and a slightly modified or "perturbed" membrane system with 2.5 mol % gramicidin, at the T_m of 42 °C, is compared with the single-relaxation theoretical dependence in Figure 9.

V. Discussion

All previous findings of ultrasound absorption in which liposome preparations have been employed as membrane models have shown a common result of enhanced ultrasound absorption, as the system's temperature is brought in proximity to the thermotropic phase transition temperature T_m of the liposomes; i.e., $(\alpha\lambda)_{max}$ is a strongly dependent function of temperature.[7,10,16] At a fixed driving frequency, this absorption is found to attain its largest value at the phase transition temperature of the liposome system, T_m. Furthermore, the absorption is also noted to be a function of the driving frequency of the ultrasound wave; i.e., there exists a particular driving frequency at which the absorption reaches a maximum value.

An investigation focusing attention on the effects of the size of the unilamellar liposomes of DPPC on the relaxation of the ultrasound absorption near T_m was carried out by Sano et al.,[7] who reported that only a single-relaxation absorption peak was observed near the thermotropic phase transition, within their frequency range of investigation of 1–100 MHz. The relaxation time and amplitude exhibited a maximum at the T_m with a τ of 20 ns, which they claim to be relatively insensitive to the liposome size.

Strom-Jenson et al.[10] and Maynard et al.[16] studied the ultrasound absorption properties of 4:1 (w/w) DPPC/DPPG mixtures of large unilamellar vesicles, average diameter of 0.2 μm, in the frequency range 0.5–5 MHz about the liposome phase transition temperatures T_m of 42 °C. The absorption per wavelength ($\alpha\lambda$) was found to reach its maximum value at T_m with a characteristic relaxation time of 76 ns or a relaxation frequency of 2.11 MHz. Strom-Jensen et al.[10] also studied small perturbations placed within the phospholipid bilayer of this model membrane system by incorporating small amounts of gramicidin and cholesterol and found that the ultrasonic absorption broadens at T_m. More significantly, the addition of 5 mol % of gramicidin to the lipid bilayer was observed to increase the average relaxation time from 76 (2.11 MHz) to 211 ns (0.75 MHz) with the phase transition temperature T_m unchanged at 42 °C.

TABLE I: Summary of the Pure Lipid Ultrasonic Absorption Findings

lipid	T_m, K	$(\alpha\lambda)_{max}/C_0$, mL/g	f_{relax}, MHz
DMPC	297.4	0.44	1.42
$DC_{15}PC$	306.5	0.47	1.62
DPPC	315.0	0.50	2.11
$DC_{17}PC$	321.0	0.54	2.50

TABLE II: Summary of the Steady-State Molar Volume Changes of Pure Lipids at T_m Obtained through Nagle and Wilkinson[20]

lipid	steady-state vol change at T_m, mL/mol of lipid
DMPC	18.30
$DC_{15}PC$	21.88
DPPC	27.15
$DC_{17}PC$	31.24

The ultrasound absorption measurements of aqueous dispersions of DMPC, $DC_{15}PC$, DPPC, and $DC_{17}PC$, described earlier, are summarized in Table I. These findings support the two-state transition hypothesis due to the observed absorption spectra line shaped features consistent with eq 11, at the respective lipid T_m values.

It is interesting to note from Table I that the ratio of $(\alpha\lambda)_{max}$ between any two neighboring PC lipids, say 1 and 2, is nearly unity. Since $T_{m1} \cong T_{m2}$, in view of the single-relaxation sound absorption theory, viz., eq 11, it follows that $\Delta V_1 = \Delta V_2$. Evidently, the change in the volume of an average size cluster undergoing phase fluctuation appears to be a constant in the PC lipid family investigated. Steady-state changes in volume of several PC lipids at their phase transition temperatures have been measured by volume dilatometry techniques[20] and are summarized in Table II.

If it is assumed that the change in volume of an individual lipid ΔV^*, within the fluctuating cluster, is the same as that of the measured steady-state or time-average volume change listed in Table II, then

$$\Delta V = Q\Delta V^* = \text{const} \tag{26}$$

where Q is the cooperative unit size, i.e., the number of lipids undergoing phase change within a cluster. It then follows that

$$Q_1\Delta V_1^* \approx Q_2\Delta V_2^* \tag{27}$$

Furthermore, if it is assumed that the rate of the cluster size growth (or collapse), i.e., $dQ/dt \approx \Delta Q/\Delta t \sim Q/t$, is independent of the PC lipids, then

$$Q_1/\tau_1 \approx Q_2/\tau_2 \tag{28}$$

or

$$Qf_{relax_1} \approx Q_2 f_{relax_2} \tag{29}$$

$$Q_1/Q_2 = \Delta V_2^*/\Delta V_1^* = f_{relax_2}/f_{relax_1} \tag{30}$$

It can be readily verified from the f_{relax} findings reported herein that this line of reasoning leads to an excellent agreement with eq 30.

It is recognized that the work done in cluster growth by thermal energy may be modeled as the work done by a battery in elec-

(20) Nagle, J.; Wilkinson, D. *Biophys. J.* **1978**, *6*, 159.

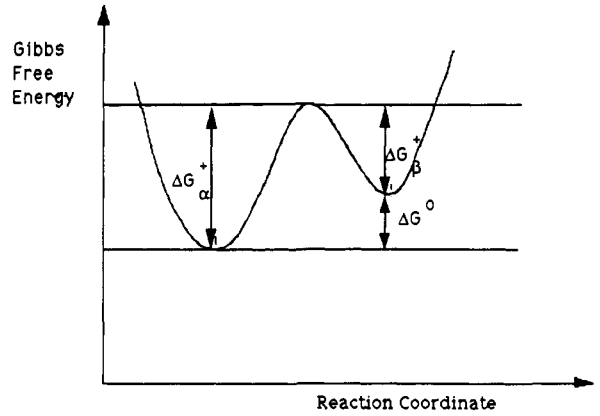

Figure 10. Two-state energy profile of a simple modeled membrane.

trically charging a capacitor. If Q represents the average number of lipids fluctuating within a cluster and V represents the thermodynamic potential per lipid molecule, i.e., $V = k_BT$/molecule, then the capacitance $C = Q/V$. From elementary physics, the time for charging and discharging a capacitor is given by $\tau = RC$. In this case R may be viewed as the thermal resistance of heat flow. Since R is inversely proportional to the thermal diffusion coefficient, D, this yields

$$\tau = \gamma C/D \tag{31}$$

where γ is a proportionality factor. Thus, $\tau = (\gamma/D)(Q/k_BT)$ and $1/R = D/\gamma = (1/\tau)\ (Q/k_BT)$. If it is assumed that the thermal resistance R is invariant over all PC lipids investigated herein, then it follows that

$$\text{const} = \frac{1}{\tau_1}\frac{Q_1}{k_BT_{m1}} \tag{32}$$

Since, $k_BT_{m1} \approx k_BT_{m2}$, eqs 29 and 30 are recovered.

It is difficult to remark on the applicability of this "capacitor" model to membrane systems containing small perturbations, such as gramicidin proteins, due to the present lack of ΔV data. If all is consistent, the above model predicts $\Delta V = 18$ and 9.65 mL/mol for the 2.5 and 5 mol % gramicidin compositions, respectively, within the DPPC lipid bilayer. Nevertheless, as pointed out below, accurate f_{relax} predictions on pure, as well as perturbed, membrane systems can be made through thermodynamic considerations.

The two-state model of the liposome system is depicted in the energy diagram shown in Figure 10. $[\alpha]$ is allowed to be equal to the concentration of lipid in the crystalline state, and $[\beta]$ is allowed to be equal to the concentration of lipid in the fluid state. Under the thermodynamic equilibrium condition at any temperature T, $[\alpha]K_f = [\beta]K_b$, where $K_{f(orward)}$ is the rate of lipid becoming fluid (s^{-1}) and $K_{b(ackward)}$ is the rate of lipid falling back into the crystalline state.

The ratio $[\beta]/[\alpha] = k_f/k_b$ for a two-state model obeys the Boltzmann distribution, i.e., $[\beta]/[\alpha] = \exp(-\Delta G°/RT)$ where, $\Delta G°$ is the difference in the Gibbs free energy between the bottom of the two wells and comprises the differences in van't Hoff enthalpy $\Delta H°_{vH}$, and differences in entropy $\Delta S°$, i.e.

$$\Delta G° = \Delta H°_{vH} - T\Delta S° \tag{33}$$

From the fluctuation–dissipation theorem,[21] the rate at which the system fluctuates about its equilibrium value is precisely the same rate at which the system dissipates energy (ultimately as heat) into the surrounding aqueous environment when perturbed from its equilibrium value,[21] e.g.

$$f_{fluctuation} = f_{dissipation} = (K_f + K_b)/2\pi \tag{34}$$

The $K_{f(orward)}$ and $K_{b(ackward)}$ rate constants are obtained from the transition-state theory (TST).[8,22] The rate at which the membrane lipid surmounts the potential barrier depends upon the fractional

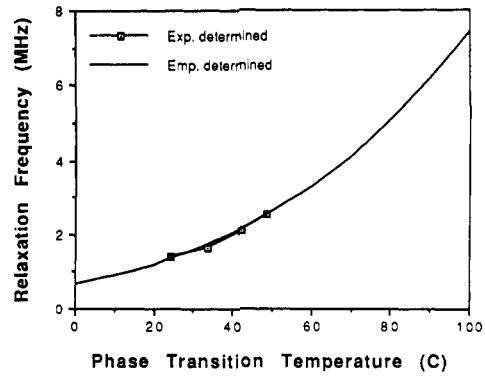

Figure 11. Comparison between the experimentally determined relaxation frequency parameter and the empirical relationship obtained through the two-state theory.

population of lipid at the top of the barrier multiplied by their frequency of attack on the barrier. The fractional population just at the top of a particular well's barrier is given by $\exp(-\Delta G^+/RT)$, where ΔG^+ is the activation Gibbs free energy. The frequency of attack on the barrier is given through $(K_{Boltzmann}T) = hf$. Therefore, the rate of transition is given by

$$K_{rate} = \frac{k_BT}{h}\exp\left(-\frac{\Delta G^+}{RT}\right) \tag{35}$$

where $\Delta G^+ = G_{intermediate} - G_{initial}$. Hence

$$K_f = \frac{k_BT}{h}\exp\left(-\frac{\Delta G^+_\alpha}{RT}\right) \text{ and } K_b = \frac{k_BT}{h}\exp\left(-\frac{\Delta G^+_\beta}{RT}\right) \tag{36}$$

Consequently, the frequency of relaxation of a two-state system is given as

$$f_{fluctuation} = f_{dissipation} = \frac{k_BT}{2\pi h}\left[\exp\left(-\frac{\Delta G^+_\alpha}{RT}\right) + \exp\left(-\frac{\Delta G^+_\beta}{RT}\right)\right] \tag{37}$$

At T_m, half of the lipid population is in the crystalline state and the other half is in the fluid state. Therefore, $\Delta G°$ is identically equal to zero at T_m and $\Delta G^+_\alpha = \Delta G^+_\beta$. ΔG^+_m is denoted to be equal to ΔG^+_α and ΔG^+_β only at T_m. Hence, the frequency of relaxation at T_m is obtained through eq 37 and rewritten as

$$f_{relax\ at\ T_m} = \frac{k_BT_m}{\pi h}\exp\left(-\frac{\Delta G^+_m}{RT_m}\right) \tag{38}$$

From eq 38 and the observed f_{relax} values from Table I, it is found empirically that

$$\Delta G^+_m = 4300 \text{ cal/mol} - T_m\ (-13.65 \text{ entropy units}) \tag{39}$$

Figure 11 compares the "observed" relaxation frequencies of the PC lipids investigated in this study with the two-state TST predictions from eq 38 and the empirical relationship eq 39.

It is fruitful to consider the temperature dependence of the ΔG^+_α and the ΔG^+_β near the T_m and then attempt to predict the temperature dependence of the f_{relax} parameter via the two-state model. A two-state system near its T_m is shown in Figure 12. As a necessary consequence of the principle of conservation of mass and total number of conserved states of the nondegenerative two-state system it follows that

$$|\delta(\Delta G^+_\alpha)| = |\delta(\Delta G^+_\beta)| \tag{40}$$

The change in $\Delta G°$ near the T_m is given by

$$|\delta(\Delta G^+_\alpha)| + |\delta(\Delta G^+_\beta)| = |\delta(\Delta G°)| \tag{41}$$

Consequently

$$|\delta(\Delta G^+_\alpha)| = \tfrac{1}{2}|\delta(\Delta G°)| \tag{42}$$

(21) Frauenfelder, H.; Wolynes, P. *Science* **1985**, *229*, 337.
(22) Matheson, A. J. *Molecular Acoustics*; Wiley: New York, 1971; Chapter 12.

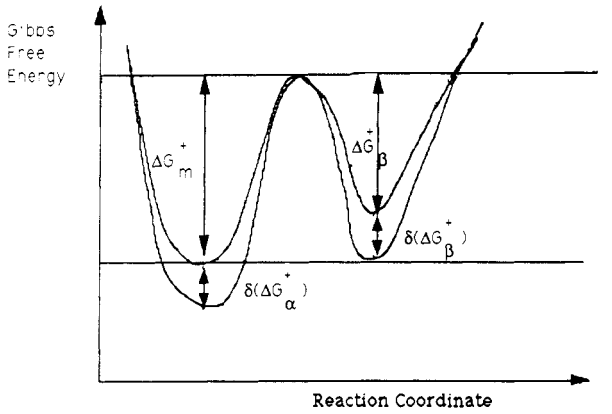

Figure 12. Temperature dependence of the activation Gibbs free energy of α and β states relative to T_m.

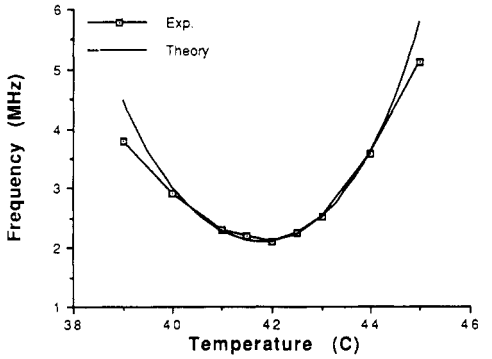

Figure 13. Comparison of the experimentally determined relaxation frequency with the two-state model prediction.

Theoretical prediction of the temperature dependence of the f_{relax} parameter for the two-state transition model is made with the aid of eqs 40 and 41. Rewriting eq 37 in terms of eqs 38 and 40 yields

$$f_{relax} = \frac{k_B T}{2\pi h} \exp\left(-\frac{\Delta G^+_m}{RT}\right)(2) \cosh\left(\frac{\delta(\Delta G^+)}{RT}\right) \quad (43)$$

at any temperature, T. Through eq 42, eq 43 can be rewritten as

$$f_{relax} = \left(\frac{T}{T_m}\right) f_{relax\ at\ T_m} \exp\left(-\frac{\Delta G^+_m \chi}{RT}\right) \cosh\left(\frac{\Delta H^\circ_{vH} \chi}{2RT}\right) \quad (44)$$

where $\chi = 1 - (T/T_m)$. From the f_{relax} findings and through eq 38, ΔG^+_m is determined. $\Delta G^+_m = \Delta G^+_\alpha = \Delta G^+_\beta = 8600$ cal/mol at T_m for DPPC.

The frequency dependence obtained in Figure 8 is compared with the predicted frequency dependence for the two-state model theory, i.e., eq 44, in Figure 13. A strong correlation between the "measured" frequency dependence and the two-state transition theory is noted when the ΔH°_{vH} is fitted to a value of 160 000 cal/mol in eq 44. It is noted that $\Delta H^\circ_{vH}/RT \approx 260$. From previous DSC studies the cooperative unit of melting for DPPC aqueous dispersions is determined to be around 260.[23] Hence, it appears that the average size of the ultrasound promoted cluster fluctuation is approximately the same as the average size of a thermodynamic cooperative melting unit. This finding is in good agreement with the thermodynamic predictions of equilibrium fluctuations of lipid bilayers.[24]

It is realized that an experimentally determined relaxation frequency of a single component phospholipid, e.g., DPPC with $f_{relax} = 2.11$ MHz, can be utilized as a "reference frequency" in predicting relaxation frequencies of other pure, as well as perturbed, membrane systems, whose T_ms are in proximity to $T_{m\,ref}$. The two-state transition of the reference lipid and the unknown lipid membrane systems are represented in the energy diagram in Figure 14. Thus, the transition of

$$\langle\alpha\rangle_{unknown} \rightarrow \langle\beta\rangle_{unknown}$$

is viewed as

$$\langle\alpha\rangle_{unknown} \rightarrow\rightarrow \langle\alpha\rangle_{ref} \rightarrow\rightarrow \langle intermediate\rangle \rightarrow\rightarrow \langle\beta\rangle_{ref} \rightarrow\rightarrow \langle\beta\rangle_{unknown}$$

with

$$|\partial(\Delta G^+_\alpha)| = |\partial(\Delta G^+_\beta)| \quad (45)$$

and

$$\Delta G^+_{\alpha\,unknown} = \Delta G^+_{\alpha\,ref} + \partial(\Delta G^+_\alpha) \quad (46)$$

Thus, it becomes necessary to find $\partial(\Delta G^+_\alpha)$ so that $f_{relax\,unknown}$ can be predicted. Through the same line of reasoning used in deriving eq 42, it follows that

$$|\partial(\Delta G^+_\alpha)| = \tfrac{1}{2}|\partial(\Delta G^\circ)| \quad (47)$$

where

$$\partial(\Delta G^\circ) = \Delta G^\circ_{unknown} - \Delta G^\circ_{ref} \approx (\partial(\Delta H^\circ) - T_{m\,ref}\partial(\Delta S^\circ)) \quad (48)$$

and $\partial(\Delta H^\circ) = \Delta H^\circ_{unknown} - \Delta H^\circ_{ref}$ and $\partial(\Delta S^\circ) = \Delta S^\circ_{unknown} - \Delta S^\circ_{ref}$. Arbitrarily treating the reference lipid as DPPC, the $\Delta G^+_{\alpha\,ref}$ can be readily determined through eq 38 to yield $\Delta G^+_{\alpha\,ref} = 8600$ cal/mol. The pure and perturbed membrane lipid relaxation frequencies at T_ms are obtained through eq 48. These predictions are compared with the experimental findings are summarized in Table III. The experimental data are noted to be in good agreement with the predicted thermodynamic shifts in $\partial(\Delta G^+_\alpha)$. As for the slightly perturbed membranes, $\partial(\Delta G^+_\alpha)$ is predicted through

$$-\frac{\partial(\Delta G^\circ)}{\partial n} = \left(\frac{\partial(\Delta H^\circ)}{\partial n} - T_m \frac{\partial(\Delta S^\circ)}{\partial n}\right) \quad (49)$$

with

$$\partial(\Delta G^+_\alpha) = -\tfrac{1}{2}\partial(\Delta G^\circ)\Delta n \quad (50)$$

where Δn is the protein concentration. $\partial(\Delta H^\circ)/\partial n$ is a function experimentally determined through previous DSC studies per-

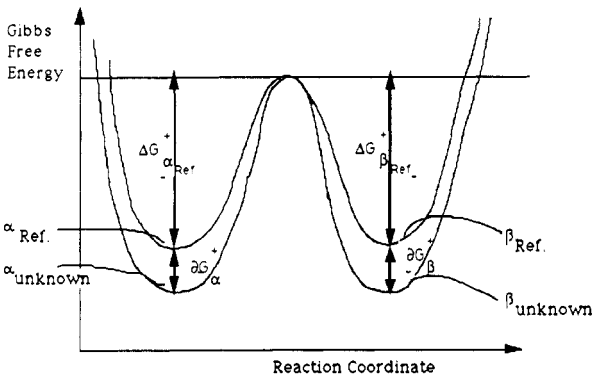

Figure 14. Two-state energy profiles of the reference and the unknown lipid at their respective T_m values.

TABLE III: Comparison between Experimental and Thermodynamic Predictions

MLV	T_m, K	$\delta(\Delta G^+_\alpha)_{predicted}$, cal/mol	f^{theor}_{relax}, MHz	f^{measd}_{relax}, MHz
DMPC	297.4	−240.0	1.35	1.42
$DC_{15}PC$	306.5	−116.0	1.73	1.62
DPPC	315.0	000.0	ref	2.11
$DC_{17}PC$	321.0	+88.0	2.52	2.50
DPPC				
+2.5 mol %	315.0	+267.0	1.40	1.40
+5.0 mol %	315.0	+656.0	0.75	0.75

(23) Hinz, H. J.; Sturevant, J. M. *J. Biol. Chem.* **1972**, *247*, 6071.
(24) Freire, E.; Biltonen, R. *Biochim. Biophys. Acta* **1978**, *514*, 54.

formed by Chapman et al.[25] It is encouraging to find that f_{relax} is totally accounted for by this function at these low concentrations. Apparently, $\partial(\Delta S^\circ)/\partial n \approx 0$ at these low concentrations.

Although the mechanistic details as to how ultrasound promotes cluster fluctuations are presently unknown, it does not preclude the possibility of estimating the fractional number of lipid population affected by the low-intensity ultrasound. The reduction in the ultrasound source intensity I_0 due to absorption after the longitudinal wave travels a distance x from the source is[17]

$$I = I_0 \exp(-\alpha x) \tag{51}$$

The total energy absorbed per unit time across a cross-sectional area A, through a distance of one wavelength λ, is $(\Delta I)A$, where

$$\Delta I = (I_0 - I) = I_0(1 - \exp(-\alpha\lambda)) \approx I_0\alpha\lambda \tag{52}$$

when $\alpha\lambda \ll 1$. Energy absorbed per unit time within volume $A\lambda$ is equated to the product of the number of lipid molecules N promoted in unit time within volume $A\lambda$ and the required energy per molecule to undergo the transition, ΔE. Hence

$$I_0\alpha\lambda A = N(\Delta E) \tag{53}$$

The mass of lipid molecules in volume $A\lambda$ can be calculated from the total concentration of lipid, and the number of molecules in $A\lambda$ can be determined through the molecular weight of lipid. Setting $\Delta E \sim K_B T$ and $I \sim 10^{-6}$ W/cm^2, we find that 2.3×10^{11} DPPC lipid molecules are affected per second out of the 1.25×10^{17}. At this low intensity, ultrasound "probes" the membrane relaxation kinetics without significantly affecting the f_α and f_β equilibrium distributions. Since

$$Q\Delta E = \Delta H_{vH} \tag{54}$$

eq 53 can be rewritten as

$$I_0(\alpha\lambda)A = (\text{number of clusters promoted})\Delta H_{vH} \tag{55}$$

Consequently, the number of clusters promoted by ultrasound is predicted to increase linearly with intensity. Since the rate of enhanced permeation of ions and small drug compounds is postulated to depend upon the number and size of fluctuating clusters,[14] it may be hypothesized that the enhanced rates in permeations should display an analogous linear functional dependence on the intensity of ultrasound radiation.

VI. Conclusions

The results from this investigation confirm that low-intensity ultrasound absorption and velocity dispersion of modeled biomembranes are adequately characterized by a two-state system. It is concluded from the findings, through a two-state relaxation model, that the relaxation strength of the membrane is a strongly dependent function of temperature and gives a qualitative measure of the degree of ultrasound coupling with the membrane. The coupling is found to be greatest at the constituent lipid T_m. Linear deviations between the absorption findings and the two-state relaxation theory occur (in Figures 2 and 4) at frequencies well above the f_{relax}. Such behavior is explained by the existence of classical sound absorption dissipative mechanisms within the biomembranes (see eq 22).

The empirical findings from this study have suggested the "capacitor" and a thermodynamic model, which are successful in predicting the relative changes in the f_{relax} parameters of the different lipid chain lengths employed in this investigation.

This study has also revealed a strong correlation between the experimentally determined relaxation frequency temperature dependence with the predicted thermodynamic two-state temperature behavior within the range $|T - T_m| \leq 2$ °C (see Figure 13). Deviations from the thermodynamic two-state predictions are expected to arise due to the breakdown in the assumption used to determine f_{relax} through eq 25 when $\alpha\lambda_{excess} \approx \alpha\lambda_{cl}$.

It is postulated from the fluctuation–dissipation theorem that the low-intensity ultrasound employed in this investigation promoted cluster fluctuations within the bilayers and were dynamically equivalent to the natural equilibrium thermal fluctuations. It is also concluded from the f_{relax} temperature behavior and the two-state theory prediction, viz., eq 44, that an induced cluster (or an equilibrium) fluctuation exhibits cooperation between the lipid molecules and that its growth period is governed by the average time an individual lipid molecule requires to change its volume, multiplied by the number of lipid molecules involved within the cluster fluctuation. The average cooperative size of the cluster at the phase transition temperature was determined to be the same as the average cooperative size determined through DSC studies.

Through the energy balance eq 55, it follows that the ultrasound promoted cluster density is directly proportional to the intensity of ultrasound radiation and to the absorption coefficient. Since the cluster density function is hypothesized to play a significant role in enhancing ion and drug influx (or efflux) permeation rates, it is postulated that ultrasound would affect these permeation rates. It is believed that the results of this study will contribute to the application of ultrasound induced reversible effects on biological membranes and to medicine.

Acknowledgment. We acknowledge gratefully the partial support of this work by a grant from the National Institutes of Health.

Registry No. DMPC, 18194-24-6; $DC_{15}PC$, 3355-27-9; $DC_{17}PC$, 70897-27-7; DPPC, 63-89-8; gramicidin, 1405-97-6.

(25) Chapman, D.; Cornell, B. A.; Eliasz, A. W.; Perry, A. *J. Mol. Biol.* **1977**, *113*, 517.