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Analyses and predictions of ultrasound interactions with model biomembrane systems via two-state transition model

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ANALYSES AND PREDICTIONS OF ULTRASOUND INTERACTIONS WITH MODEL BIOMEMBRANE SYSTEMS VIA TWO-STATE TRANSITION MODEL

BY

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Abstract

Low intensity ultrasound (approximately 10^{-6} W/cm^2) in the frequency range of 0.5 MHz to 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 either composed of pure phospholipids or mixtures of phospholipids and small molar fractions of protein gramicidin. The experimental findings reveal enhanced ultrasound interactions near to the lipid phase transition temperature. The enhanced ultrasound absorption spectra closely resemble single relaxation 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.

This dissertation is affectionately dedicated to the Love and Joy of my life.

ACKNOWLEDGEMENTS

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Chapter 1

Introduction

1.1 Introduction

The fundamental unit of life, the biological cell, is well designed for communicating with its exterior by its dynamic membrane system. The plasma membrane, which defines the boundaries of the cells and ensures that its contents are confined and responsive to environmental changes, consists predominantly of lipids, proteins and cholesterol. At normal physiological temperatures it has been well ascertained, through a variety of experimental techniques, that lipids, proteins and cholesterols are highly interactive with one another. Typical eukaryotic cell membranes are known to contain highly diversified lipids and proteins to perform specialized functions such as active receptors and transport channels. Invariably, any investigative undertakings on the modes of interactions of a typical cytoplasmic membrane become exceedingly difficult to analyze and quantify. 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 [5,6,7] have been utilized to help characterize thermodynamic parameters, membrane fluidity and to elucidate the lipid and protein interactions with their surrounding environment. A large bulk of the thermodynamic research efforts have been directed towards gaining a better understanding of the physical behavior of solid to liquid crystalline state phase transition and the influence of incorporated proteins, and cholesterol [8].

One of the oldest and most common method of interrogating systems in thermodynamic equilibrium is to perturb suddenly one of the thermodynamic parameters, such as temperature or pressure, and to measure the relaxation characteristics of the system towards its new equilibrium value. To this end, an acoustical wave is a good modality of choice, inducing periodic temperature and pressure perturbations [9,10].

To date, no single acceptable theory exists to explain the mechanisms of sound absorption arising form the plasma membrane. To investigate such underlying mechanisms, there has been an inclination over the past two decades to experiment with simple model membrane systems, such as liposomes, which serve as a first approximation to the *in vitro* plasma membrane of cells. Different research groups have investigated 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's thermotropic phase transition temperature. Such enhanced deviations have been postulated and inferred to arise from a structural relaxation process within the lipid bilayer [11]. Recently, Saad et al. [12] and Yumita et al. [13] have reported on their observations of therapeutic ultrasound to activate cell killing for a few lines of tumor cells, *in vitro* . The precise modes of cell killing is not fully understood by these investigators. However, since the plasma membrane is the first component of the cell to encounter the pressure wave, it is may be speculated to be a likely site of interaction resulting in structural and functional modifications of transport systems and to an increase in plasma membrane permeability

[14]. It is unclear as yet whether such enhancements in permeability arise due to permanent membrane structural alterations or due to structural relaxations of proteins and lipid fatty acyl chains induced by rapid temperature and pressure fluctuations. If the latter is responsible, it then becomes important to determine the natural relaxation frequencies of the plasma membrane in order to yield an optimal rate of molecules and anti-cancer drug influx. Similar remarks may be applicable to drug efflux from vesicle systems within the ultrasound field.

Permeability measurements of certain ions, such as K^+, Na^+, and small molecules, such as Tempo [4], from pure PC liposomes have revealed enhancements in the vicinity of the phospholipid phase transition temperature. Such enhancements in permeation are found to be maximal at the phase transition temperature and have been postulated to arise from the interfacial regions between the solid and liquid domains where the macromolecular packing defects are most likely. Wu et al. [15] have suggested that the coexistence of two lipid phases near the phase transition region, or in a mixture of two lipid components, could facilitate passive transport and membrane protein activities due to the measured existence of enhanced lateral compressibility. Motivated by this suggestion, Kanehisa et al. [16] 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 non- dominant phase within a pool of dominant phase lipids. Tsong et al. have also presented a phenomenological description of the enhanced passive permeation of molecules and the structural relaxation characteristics of clusters as featured in the liposome temperature jump experiments. Recently, Parasassi et al. [17] have

presented the strongest experimental evidence on the phase fluctuations in phospholipids with the help of the unique fluorescence probe, laurdan, which offers a different excitation and emission spectra in gel and liquid crystalline phases. The difference in the excitation spectra allows the photoselection of laurdan molecules in one of the two phases and, utilizing these differences in the emission spectra, they indirectly measured the interconversion rates between the two phases. Their experiments were preformed with DPPC multilamellar vesicles (MLV) near the phase transition temperature.

Thus, even when the membrane system is in thermodynamic equilibrium with its surroundings, its constituent clusters, composed either of gel or fluid lipids, have been hypothesized and noted to fluctuate rapidly in energy and volume due to their dynamic interactions with their immediate surroundings.

The primary objective of this study is to develop a mechanistic understanding, through a working model, that can explain and predict to a reasonable degree of accuracy the enhanced ultrasound absorption characteristics attributed to structural relaxations of modelled biological membrane systems. The study presented here is intended to contribute to a better understanding of the interactions between ultrasound and membrane constituents in general. It is hoped that the gain in knowledge and insight acquired from this study may serve as a foundation in understanding and controlling ultrasound induced reversible effects on biological membranes, and may contribute in application to the field of medical ultrasound.

1.2 A succinct overview on sound absorption

The classical theory of sound propagation and absorption in fluids involves the three fundamental relations, namely, the equation of continuity of mass flow, Newton's second law of motion, and the equation of state of the fluid. The second order linear differential wave equation can be obtained through the linearization of these fundamental relations when only small perturbations of density and pressure changes are involved.

Let's consider the one dimensional case. The equation of continunity is,

$$\frac{\partial s}{\partial t} + \frac{\partial U}{\partial x} = 0 \qquad 1.1$$

with

$$s \equiv \frac{\rho - \rho_o}{\rho_o} \qquad 1.2$$

where ρ and ρ_0 are the perturbed and unperturbed densities, respectively, and U is the particle velocity along the x direction, the direction of wave propagation. The statement of Newton's second law of motion is

$$\rho_0 \left(\frac{\partial U}{\partial t}\right) = -\left(\frac{\partial P}{\partial x}\right). \qquad 1.3$$

Combining Eq. 1.1 and Eq. 1.3 yields

$$\frac{\partial^2 P}{\partial x^2} = \rho_0 \frac{\partial^2 s}{\partial t^2} \qquad 1.4$$

Due to the rapid fluctuations in time, more than $10^6\ \mathrm{sec}^{-1}$, the acoustic disturbance is assumed to be adiabatic, and the simplest equation of state

can be expressed as

$$P = \frac{1}{\beta_{ad}} s \qquad 1.5$$

where β_{ad} is the adiabatic compressibility. Hence, Eq.1.4 may be rewritten to yield the wave equation:

$$\frac{\partial^2 P}{\partial x^2} = \frac{1}{V^2} \frac{\partial^2 P}{\partial t^2} \qquad 1.6$$

with

$$V^2 = \frac{1}{\rho \beta_{ad}} \qquad 1.7$$

By inspection, the solution of this wave equation is $P=P_0 \exp i(\omega t-\omega x/v)$ for a plane sound wave propagating in the positive x direction, without dissipation. When sound absorption occurs, the simple equation of state needs to be modified [18]. The wave number $k^*= \omega/v$ then becomes a complex containing a real part $2\pi/\lambda$ and an imaginary part, α, describing absorption. Thus, the modified solution to the wave equation due to absorption processes is

$$P = P_0 \exp(-\alpha x) \exp i(\omega t-kx) \qquad 1.8$$

where k is the wave number ω/v.

The mechanical energy in the propagating pressure wave is absorbed by the medium it is traversing via two significant mechanisms, the so-called classical absorption processes and the physical relaxation processes [18]. Classical absorption arises as a consequence of the medium having a

finite viscosity and a finite thermal conductivities. This results in shearing motions, predominantly between solvent molecules, leading to viscous energy losses and to thermal energy being transported along temperature gradients within the medium. Non-classical absorption mechanisms, result due to thermal, chemical, and/or structural relaxations within the medium, whenever the chemical or structural equilibrium is perturbed by the changes in pressure or temperature induced by the sound wave. The culmination of these two classical and many the possible non-classical absorption processes comprise the pressure amplitude absorption coefficient α [18]:

$$\alpha = \alpha_{\text{classical}} + \alpha_{\text{non-classical}} \qquad 1.9$$

$$\alpha_{\text{classical}} = \frac{\omega^2}{2\rho c^3}\left[\, 4/3\, \eta_{\text{shear}} + \frac{(\gamma - 1)}{C_p} K\right] \qquad 1.10$$

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

A significant "nonclassical" source of absorption is the perturbation of molecular equilibria caused by the pressure and temperature alternations of the sound wave. This process is relaxational in character and leads to what is called the excess absorption, i.e., in excess of the classical mechanisms of viscosities and heat conductivities. The excess absorption contributions can often be greater than that due to classical absorption. Examples of such relaxational processes are the perturbation of the structural equilibria of associated liquids [19], conformational changes such

as boat-chair isomerization in cyclohexanes and helix-coil transitions in polypeptides [20].

The anomalous, or excess, absorption which cannot be accounted for by the classical theory of sound absorption have come to be predominantly attributed to relaxation processes, i.e.,

$$\alpha_{excess} = \alpha_{experiment} - \alpha_{classical} \cong \alpha_{relaxation} \qquad 1.11$$

Frequently a dimensionless absorption coefficient is employed in characterizing the sound absorption in the medium, $\alpha\lambda$, the absorption per wavelength. $\alpha\lambda$ is a quantitative measure of the degradation in the pressure amplitude as the wave travels a distance of one wave length.

It is to be recognized that typical excess sound absorption spectra of biochemical solutions are usually not single processes, but may involve up to several processes, which may be described in terms of linear superposition of several independent single relaxations, each having their characteristic relaxation time, τ [10].

$$(\alpha\lambda)_{excess} = \sum_{i=1}^{n} 2\,(\alpha\lambda_i)_{max} \frac{\omega\tau_i}{1 + (\omega\tau_i)^2} \qquad 1.12$$

1.3 Survey of past ultrasound absorption studies of simple biomembranes

All past 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. Indeed, 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 exist a particular driving frequency at which the absorption reaches a maximum value. See Fig.1.1 and Fig 4.1 a, b.

Early ultrasonic absorption studies of DPPC liposomes by Gamble and Schimmel [21] involved the measurement of relaxation spectra over the frequency range 14 - 265 MHz and exhibited a relaxation process with the τ of10 ns, or the relaxation frequency of 15.9 MHz. The relaxation amplitude was found to be a function of temperature and was maximal at the mid-point of the thermal phase transition, viz., T_m=41.3 ^oC. Harkness and White [22] compared the ultrasonically irradiated vs. unirradiated suspensions of DPPC in the frequency range 0.6 to 4.0 MHz, as a function of temperature, and found the temperature of maximum absorption for the irradiated suspension to be 2 to 3 ^oC below that found for the unirradiated ones. They concluded that the absorption coefficient, as a function of temperature for a fixed driving frequency, passes "through a maximum at a temperature which is dependent upon the sample preparation and its history". They also pointed out that more than a single relaxation time was required to characterize the observed absorption spectra. Ultrasound absorption studies on DMPC dispersions were carried out by Eggers and Funck [5] in the vicinity of the T_m range and three sets of relaxation components with relaxation times of 3.4 ns, 20 ns, and 200 ns were

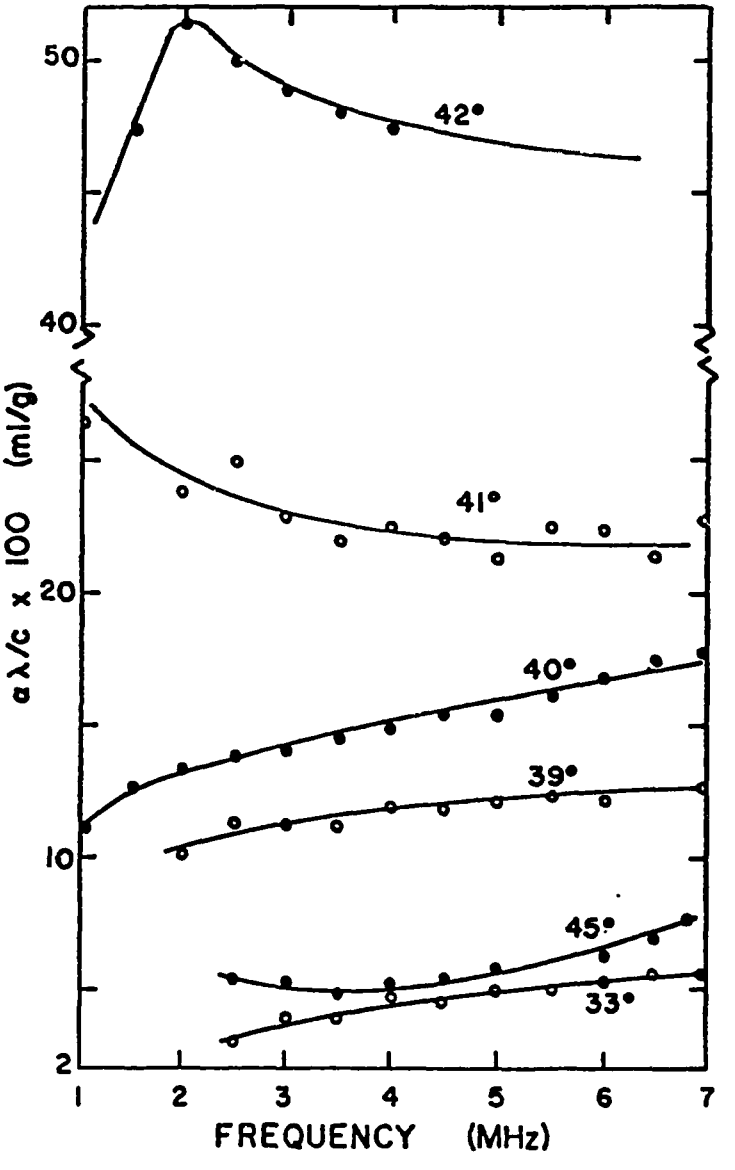

Fig. 1.1 Dependence of ultrasound absorption on pure lipid Large Unilamellar Vesicle suspension on frequency. Obtained from Ref. 11

observed with the 20 ns (f_{relax} = 8 MHz) component exhibiting a definite absorption maxima at the T_m.

However, as pointed out by Sano et al. [7], the kinetic behavior, as well as the static behavior, of the phase transitions were later found by the above mentioned investigators [5,22] to be dependent upon the structure (multilamellar or unilamellar) and on size of the liposomes. Hence, a reasonable explanation, offered by Sano [7] to the above observed multi-relaxation processes, by Harkness and White and Eggers and Funck, may in fact be attributed by the presence of liposomes with various sizes and structures.

A careful 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]. They reported that near the thermotropic phase transition only a single relaxation absorption peak was observed, within their frequency range of investigation of 1 to 100 MHz. The relaxation time and amplitude exhibited a maximum at the T_m with a τ of 20ns, which they claim to be relatively insensitive to the liposome size.

Strom-Jenson et al. [11] and Maynard et al. [23], studied the ultrasound absorption properties of 4:1 (weight/weight) DPPC:DPPG mixtures of large unilamellar vesicles, average diameter of 0.2μm, in the frequency range of 0.5 to 5 MHz about the liposome phase transition temperatures T_m of 42 ^oC. As expected, the absorption per wavelength ($\alpha\lambda$) reached its maximum value at T_m with a characteristic relaxation time of 76ns or a relaxation frequency of 2.11 MHz. In addition, Strom-Jensen et

al. [11] also studied small perturbations placed within the phospholipid bilayer of this model membrane system by incorporating small amounts of gramicidin and cholesterol. It was found that the addition of either gramicidin or cholesterol into the bilayer broadens the ultrasonic absorption at T_m. More significantly, the addition of 5 mol% of gramicidin to the lipid bilayer is observed to increase the average relaxation time from 76ns (=2.11 MHz) to 211ns (=0.75 MHz) with an unchanged phase transition temperature T_m of 42 °C. See Fig. 1.2.

1.4 A simple two-state transition hypothesis

In response to the above described findings of enhanced ultrasound absorption [7,11,23], it may be postulated that the equilibrium which the pressure wave is perturbing is the concentration equilibrium of the phospholipid molecules in the crystalline state to those in the fluid state of liposomes near the T_m, i.e., concentration of lipid molecules in crystalline state <---------> concentration of lipid molecules in fluid state. The precise molecular mechanistic details as to how this equilibrium becomes perturbed by ultrasound is not understood. In cognizance of the two-state transition theory (found in Chapter 2) it may be conjectured that this harmonic shift in the concentration may be accomplished via thermal as well as pressure perturbations induced by the harmonic ultrasound wave.

As pointed out by Sano et al. [7], when care is exercised in preparing a uniform structure and size of the DPPC liposome population, the relaxation absorption spectra of the preparation can be analyzed in terms of a single relaxation process. This simple and appealing observation provides motivation to analyze other more complex systems, eg., mixtures

of phospholipids and small protein molecules the in bilayer, to a first order approximation, in a simple coexisting two state - relaxation model of lipids in thermodynamic equilibrium near the liposome phase transition temperature.

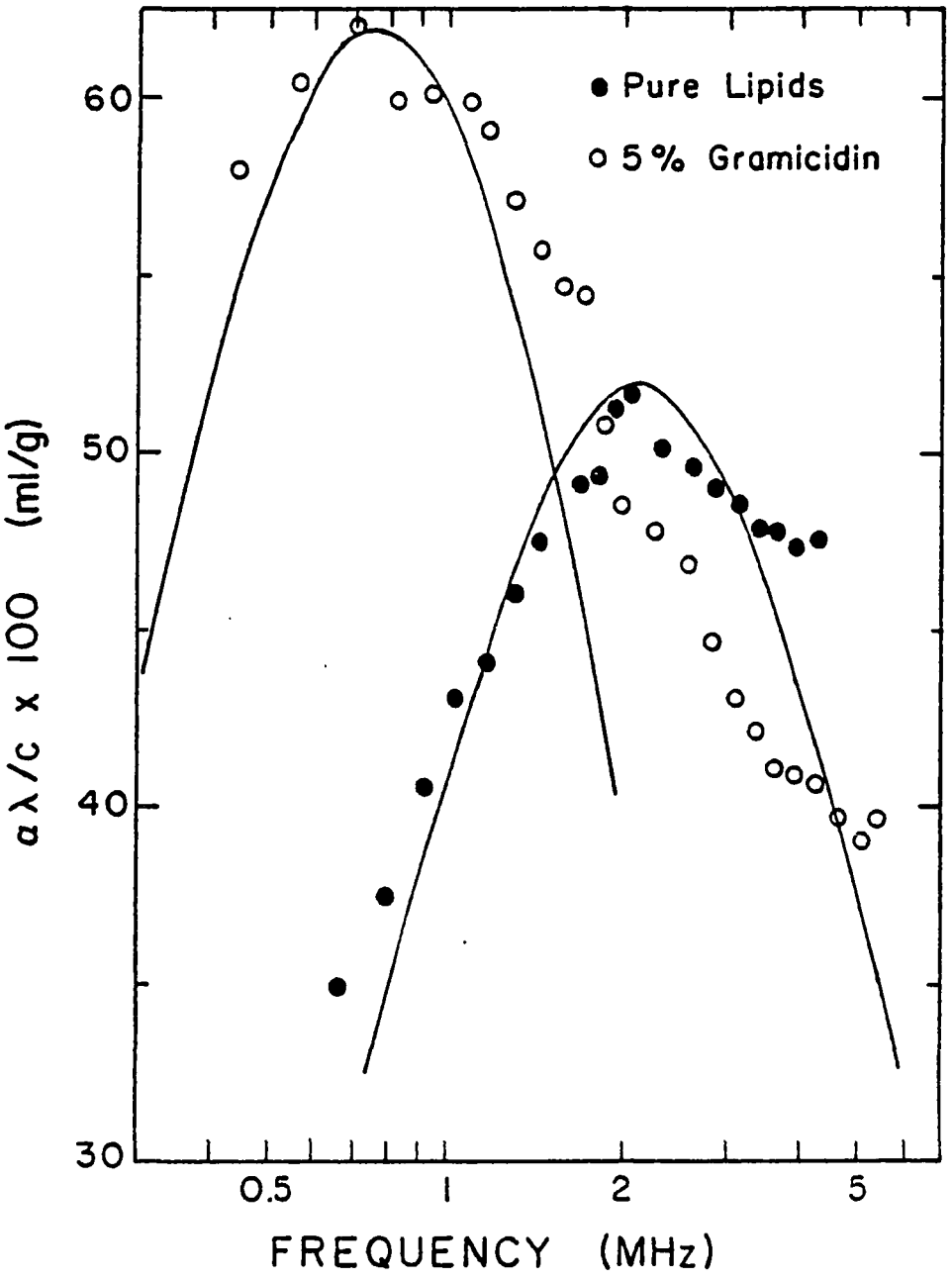

Fig. 1.2 Dependence of ultrasound absorption in pure lipid DPPC/DPPG (4:1 weight/weight) and 5 mol% gramicidin LUV suspension. Obtained from Ref. 32.

Chapter 2

Theory of sound absorption due to a single relaxation process

Consider the simplest case, viz., perturbations induced by the pressure wave between the equilibrium of two states:

$$A <-----> B$$

That is, attention is focussed primarily upon the structural relaxation kinetics predominantly arising from the trans-gauche transitions in the fatty acyl chains of the membrane lipids which interact with the acoustic wave. Here, let A be the molecules in trans (ground) state and B be the molecules in gauche (excited) state. Let K_f and K_b be the forward and backward rate constants, respectively.

At equilibrium $K_f\, C^{eq}{}_A = K_b\, C^{eq}{}_B$ where C_A and C_B are the concentrations of molecules in the respective states A and B. Further, the relative distribution of molecules in state A and B is governed by Boltzmann statistics,

$$\frac{C_B}{C_A} = \mathrm{EXP}\left[-\frac{\Delta G^{o}}{RT}\right] = K = \text{Equilibrium Constant.} \qquad 2.1$$

If the pressure is now suddenly changed from P_0 to $P_0 + \Delta P$, the rate at which C_B and C_A approach their new equilibrium positions $C^{eq'}{}_B$ and $C^{eq'}{}_A$ (provided ΔP is small in comparison to P_0) is described by the first order rate theory. Specifically, the time evolution of molecules in state B is

$$\frac{dC_B}{dt} = K_f C_A - K_b C_B \qquad 2.2$$

which has as solution $(C_B - C^{eq'}_B) = (C^{eq}_B - C^{eq'}_B)\, e^{-t/\tau}$, where $1/\tau$ is the angular relaxation frequency $\omega_{relaxation} = K_f + K_b$. 2.3

Hence, a finite amount of time is required to reach a new equilibrium. In addition, associated with such a shift in the equilibrium state is a finite volume change. Application and interpretation of Le Chatelier's principle [30] suggest that a pressure increase drives the system towards a state of lesser volume, as

$$\frac{\partial \ln K}{\partial P} = -\frac{\Delta V}{RT} \qquad 2.4$$

In place of a static pressure perturbation, consider now a sinusoidally varying pressure perturbation with angular frequency, ω. Consider two extreme cases. First, let the period of the pressure wave be much less than the relaxation time, i.e., $\omega\tau >> 1$. In this case, fluctuations in pressure are so rapid that the equilibrium has no chance to react to the pressure changes, and the volume of the system remains constant. See Fig. 2.1. Second, consider the case for which the period of the pressure wave $T>>\tau$, i.e., $\omega\tau << 1$. At very low frequencies the equilibrium adjusts itself readily to the pressure fluctuations during the wave period. The energy extracted by the system in the first half of the cycle is returned by the system to the wave during the second half of the cycle. See Fig. 2.2. In the intermediate case, where $\omega\tau \cong 1$, the work done by the pressure wave in the first half of the cycle is greater than the work returned by the system

to its surroundings during the second half of the cycle, resulting in a net energy extraction from the wave. See Fig. 2.3.

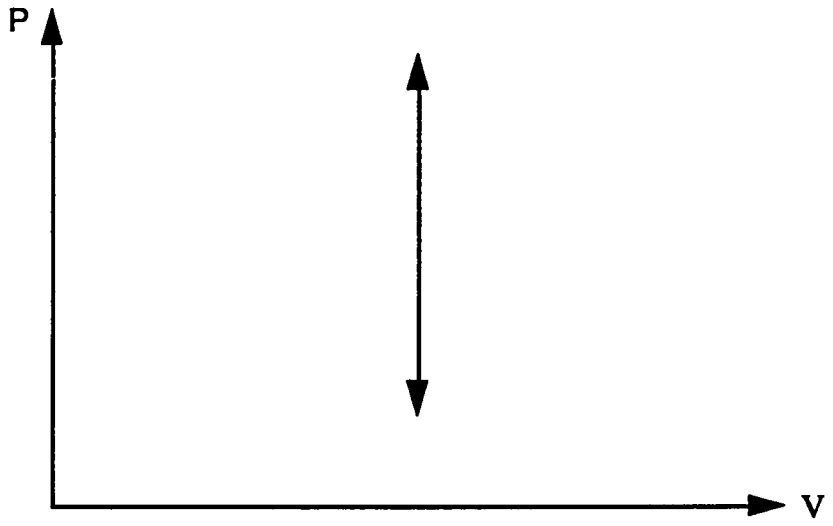

Fig. 2.1 No work is done on the system by the pressure wave. The mechanical energy in the sound wave remains constant.

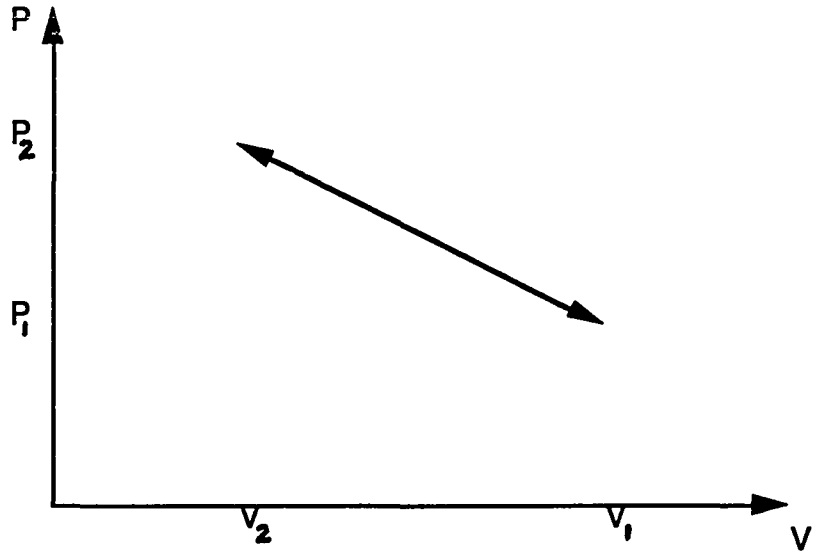

Fig. 2.2 **No work is done by the acoustic wave. Response is perfectly in phase with the perturbation**

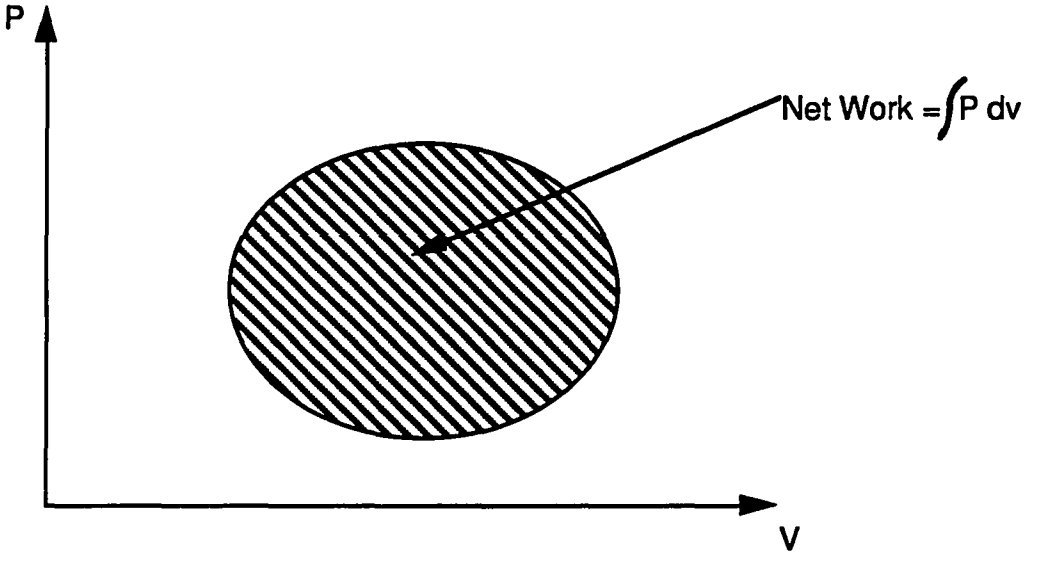

Fig. 2.3 Energy is removed from the acoustic wave due to a finite phase lag.

Rewrite Eq. 2.2 and observe the time evolution of molecules in a particular state, say B relative to an equilibrium point in response to periodic perturbation. In so doing, also infer the time evolution of the volume and adiabatic compressibility, etc., of the simple two state system.

$$-\frac{dC_B}{dt} = K_b C_B - K_f C_A \qquad 2.5$$

Making use of the fact that $C_0 = C_A + C_B$ yields

$$-\frac{dC_B}{dt} = (K_f + K_b)\, C_B - K_f C_0 = (K_f + K_b)\left[C_B - \frac{1}{1 + \frac{K_b}{K_f}}\, C_0 \right] \qquad 2.6$$

and with the help of Eq. 2.3 gives

$$\frac{dC_B}{dt} = -\frac{1}{\tau} C_B + \frac{1}{\tau} C^{eq} \qquad 2.7$$

Since C^{eq}_B is invariant in time, the above equation can be written as

$$\frac{dX}{dt} + \frac{1}{\tau} X = 0 \qquad \text{Where } X = C_B - C_B^{eq} \qquad 2.7\ a$$

The homogeneous solution is $X = X_0 e^{-t/\tau}$, with $X_0 = C^o_B - C_B{}^{eq}$. Where C^o_B is the initial concentration in state B. The effect of the harmonic pressure wave is to drive the population of molecules in state B harmonically away from, and closer towards, the equilibrium value. Thus the pressure wave induces a perturbation in the above homogeneous differential equation and is represented by a forcing function term f(t) as

$$\frac{dX}{dt} + \frac{1}{\tau} X = \frac{f(t)}{\tau} \qquad 2.8$$

The general solution to this inhomogeneous equation is given by [9]

$$X(t) = \frac{1}{\tau}\, e^{-t/\tau} \int_0^t \exp(t'/\tau)\ f(t')\ dt' \ . \qquad 2.9$$

Set the forcing function to be the simple harmonic relation with a driving frequency ω,

$$f(t') = A \exp(i\omega t') \qquad 2.10$$

which has the solution

$$X(t) = A \frac{e^{i\omega\tau}}{1 + i\omega\tau} \qquad 2.11$$

This can be rewritten as

$$X(t) = \frac{A}{\sqrt{1 + (\omega\tau)^2}} \exp i\,(\omega t - \Phi) \qquad 2.12$$

where $\tan \Phi = \omega\tau$.

The linear response lags behind the driving force. Since the volume, heat capacity, adiabatic compressibility, etc., of the system depends directly upon the population distribution among the two states A and B, solutions analogous to Eq. 2.11 can be obtained exhibiting their specific response to lag behind the perturbation. Hence, due to this finite phase difference between pressure and the volume response of the system, there results a net mechanical energy extraction out of the pressure wave in each complete cycle.

Eigen and De Mayer have pointed out [9] that it is possible to obtain $\alpha\lambda$ and the dispersion in sound velocity through the linearized wave equation Eq.1.6 when the adiabatic compressibility is written is written as a complex function

$$\beta = \beta^{\infty} + \frac{\beta_{relax}}{1 + i(\omega\tau)} \qquad 2.13$$

where β^{∞} is real quantity and corresponds to the adiabatic compressibility of the membrane at an extremely high ultrasound frequency. β_{relax} is real quantity as well, and corresponds to the relaxable part of the adiabatic compressibility of the membrane. Equation 2.13 implies a complex propagation velocity

$$\frac{1}{v} = \frac{1}{v_{RE}} - i\frac{\alpha}{\omega} \qquad 2.14$$

Through Eq. 1.7 the β is related to α as

$$\rho\beta = \{ (\frac{1}{v_{RE}})^2 - (\frac{\alpha}{\omega})^2 \} - i\frac{2\alpha}{\omega v_{RE}} \qquad 2.15$$

Thus the real and imaginary parts of β are related to the velocity and absorption as

$$v_{RE}^2 = \frac{-2\,\mathrm{Re}\,\beta}{\rho\,(\mathrm{Im}\,\beta)^2 \{ 1 - [1 + (\mathrm{Im}\,\beta / \mathrm{Re}\,\beta)^2]^{1/2} \}} \qquad 2.16$$

$$\frac{\alpha v_{RE}}{\omega} = \frac{\mathrm{Re}\,\beta}{\mathrm{Im}\,\beta \{ 1 - [1 + (\frac{\mathrm{Im}\,\beta}{\mathrm{Re}\,\beta})^2]^{1/2} \}} \qquad 2.17$$

where Re and Im denote the real and imaginary parts of β :

$$\mathrm{Re}\,\beta = \beta_{ad}^{\infty} + \frac{\beta_{Relax}}{1 + (\omega\tau)^2} \qquad 2.18$$

$$\mathrm{Im}\,\beta = -\frac{\omega\tau\,\beta_{Relax}}{1 + (\omega\tau)^2} \qquad 2.19$$

Following Eigen and De Mayer [9] for most relaxations $\beta << \beta^{\infty}$ which implies Im $\beta <<$ Re β. Thus,

$$\frac{\alpha\lambda}{2\pi} \cong -\frac{\mathrm{Im}\,\beta}{\mathrm{Re}\,\beta} = \frac{\beta_{Relax}}{\beta_{ad}^{\infty}} \frac{\omega\tau}{1 + (\omega\tau)^2} \qquad 2.20$$

$$v_{RE} \cong [\rho\,\mathrm{Re}\,\beta]^{1/2} = (\rho\beta^{\infty})^{-1/2} \left(1 - \frac{\beta_{Relax}}{2\beta^{\infty}} \frac{1}{1 + (\omega\tau)^2}\right) \qquad 2.21$$

After a very lengthy thermodynamic manipulation process involving the thermodynamic Maxwell's equations and the First and Second laws of thermodynamics the relaxable part of the adiabatic compressibility is found to be

$$\beta_{relax} = \frac{\Gamma}{RT} \left\{ \Delta V - \frac{\Delta H\,\Theta}{\rho C_p} \right\}^2 \qquad 2.22$$

Where ΔV is the molar reaction volume, ΔH is the molar enthalpy of reaction, Θ is the thermal expansion coefficient at constant pressure, Γ is the "proportionality constant", C_p is the specific heat capacity of the medium at constant pressure, ρ is the density of the medium, R is the gas

constant, and T is the surrounding temperature measured in degrees kelvin. For a complete deriviation of Eq. 2.22 see Andrew J. Matheson's text [20].

For a two state system A <-------> B, and recalling $C^{eq}_B/C^{eq}_A = K$, with $C^{eq}_A + C^{eq}_B = C_o$, gives

$$\Gamma = \frac{C_0 K}{(1+K)^2} \qquad 2.23$$

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. Substitution of K into Eq. 2.23 and making use of the identity $f_\alpha + f_\beta = 1$ yields $\Gamma = C_o f_\alpha f_\beta$. Hence, the final result for a two state transition is:

$$\alpha\lambda = 2\frac{\pi C_o f_\alpha f_\beta}{(\beta^\infty_{ad} + \beta_{relax})RT}\left(\Delta V - \frac{\Delta H\Theta}{\rho C_p}\right)^2 \frac{\omega\tau}{1+(\omega\tau)^2} \qquad 2.24$$

Typically $\beta_{relax} \ll \beta^\infty_{ad}$ and $\Delta H\Theta/\rho C_p \ll \Delta V$ for biochemical solutions [5,9], so to a good approximation

$$\alpha\lambda = 2\frac{\pi C_o f_\alpha f_\beta}{\beta^\infty_{ad} RT}\{\Delta V\}^2 \frac{\omega\tau}{1+(\omega\tau)^2}. \qquad 2.25$$

Figure 2.4 shows the behavior of $\alpha\lambda$ vs. frequency after normalizing the prefactor.

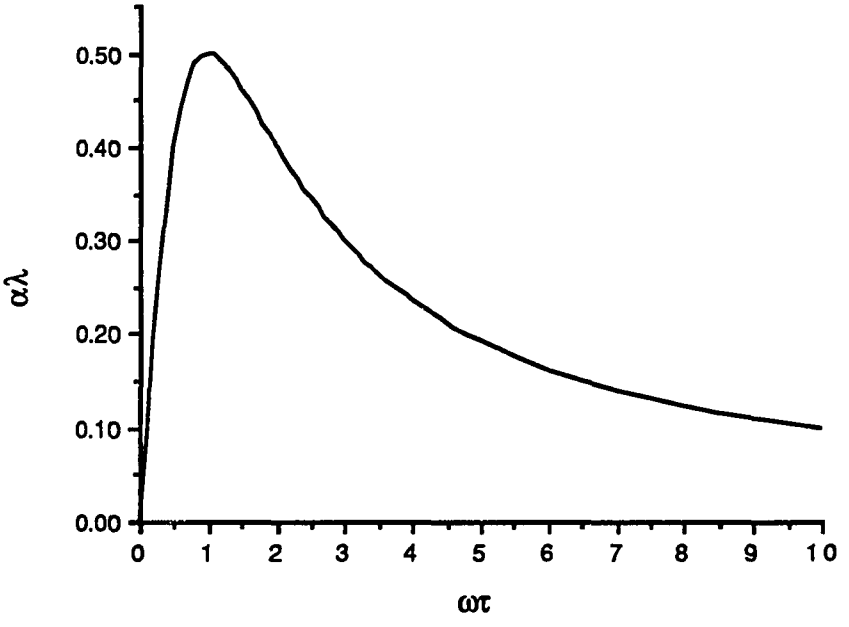

Fig. 2.4 Theoretical absorption per wavelength due to a single relaxation process

Chapter 3

Sample preparations and measurement techniques

3.1 Sample Preparations

The fourteen to seventeen carbon chain neutral phospholipids, DMPC, DCPC, DPPC, and DCPC employed in this investigation were all 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 further use. Hepes were purchased from Sigma Chemical Company (St. Louis, MO). Hepes buffer was made of 10 mM Hepes, 139 mM NaCl, 6mM KCl, distilled water at pH 7.4, and was utilized as a reference solvent in the ultrasound measurement.

MLV suspensions were employed in this investigation due to (1) the available abundance of their transition thermal properties [8] which are necessary for predicting their ultrasound behavior; (2) their calorimetric equivalence to Large Unilamellar Vesicles (LUV) [31], which have served as simplified in-vitro membrane models; (3) their equivalence to the ultrasound absorption behavior to LUVs [32], (4) and the relative ease in making MLVs.

To make MLVs, lipid aliquots containing the same type of lipid were opened at room temperature and transfered into the rotary evaporator. The chloroform was then removed by the evaporator at a pressure of one torr at 50 ^{o}C. The lipids were thoroughly dried in approximately 30 minutes 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's T_m. The single lipid type MLV liposomes were then formed in a vortex mixer, at a temperature 10 °C above the lipid's T_m for 5 minutes and then rapidly cooled to room temperature. The suspension was immediately used in the measurements.

Same procedure was used in constructing a 50 mol% mixture of DMPC/DPPC MLV liposomes. Appropriate quantities of lipid in the chloroform solutions were measured and removed from the aliquots and transfered 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 the MLVs. Similar remarks apply to the construction of slightly perturbed MLVs containing of 2.5 and 5.0 mol% gramicidin proteins.

3.2 Measurement Techniques

The ultrasound absorption and velocity dispersion measurements preformed in this investigation (on phospholipid aqueous suspensions) were made with a conventional Eggers and Funck type cylindrical resonator [25]. The resonant cavity (sometimes referred to as the "cell") is formed by two piezoelectric quartz transducers held a distance d, apart by a hollow plexiglass cylinder which is filled with the sample solution. See Fig 3.1. The experimental schematics is shown in Fig 3.2. One transducer Q_1, generates longitudinal plane waves within the cavity while the other transducer Q_2, acts as a receiver and monitors the amplitude of the resulting standing wave pattern at its face end. This amplitude reaches

peaks at resonance frequencies when the standing wave boundary condition are fulfilled, i.e., when

$$d = n\frac{\lambda_n}{2} \qquad 3.1$$

Since $v = f_n\lambda_n$, the n^{th} resonant frequency mode is given by

$$f_n = \frac{v}{\lambda_n} = \frac{n\,v}{2\,d} \qquad 3.2$$

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

The resonance theory supporting the findings in this investigation are based upon the assumption of an ideal resonator characterized by lossless reflection at the transducers and by plane wave propagation. As pointed out by Eggers and Funck, under this assumption of one dimensional wave propagation the measured output voltage of Q_2 near a resonance frequency f_n can be described by

$$U_n^{out} = U_n^{max}\left(1 + \frac{\sin^2(\pi\frac{f}{f_o})}{\sinh^2(\alpha d)}\right)^{-(1/2)} \qquad 3.3$$

where the maximum output voltage is proportional to the driving voltage at Q_1 and a function of the specific acoustic impedance of the fluid media within the cell ρv. a d is the ultrasound absorption per transducer distance, d. The fundamental resonance frequency of the liquid column f is

$$f_o = \frac{v}{2\,d.} \qquad 3.4$$

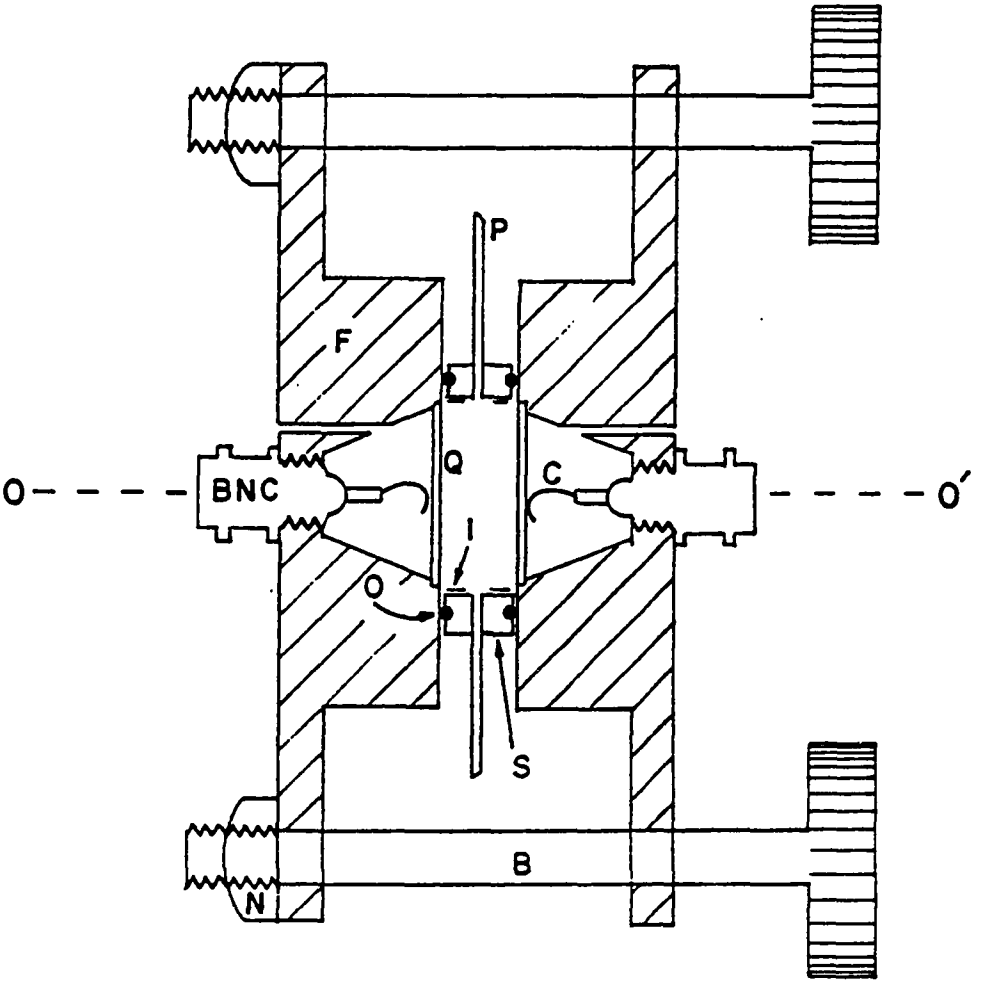

Fig. 3.1 Cross-section of cylindrical resonator

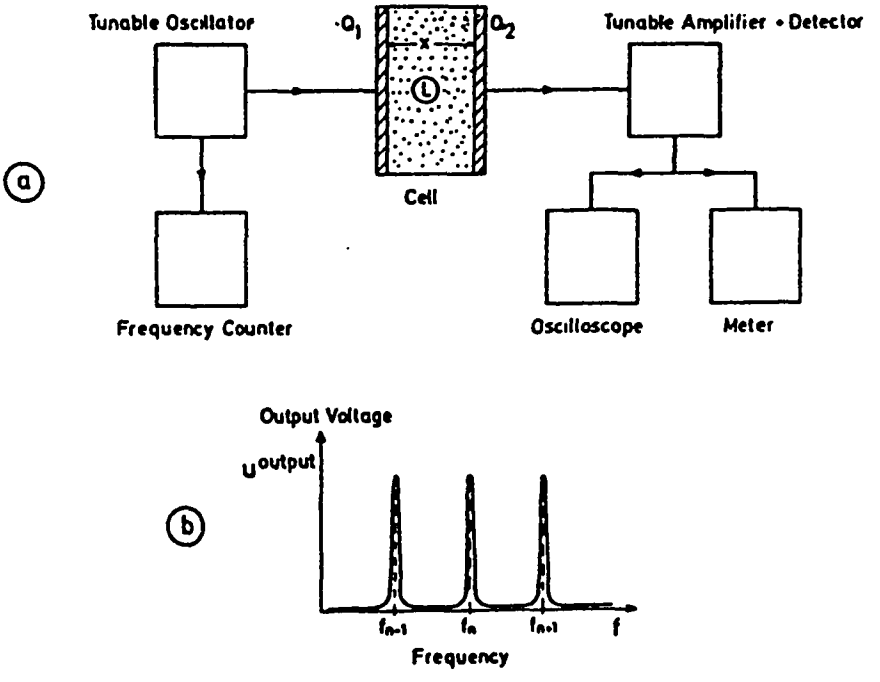

Fig. 3.2 Schematic diagram of the experimental arrangement. (a) Block diagram for the resonator method. Q_1-input transducer (terminated by 50 W); Q_2-output transducer; L-liquid in the cell. (b) Output voltage from the cell as a function of frequency.

The acoustic power within the cell is directly proportional to the square of the measured output voltage. Following Eggers and Funck, when considering small attenuation, i.e., $\alpha d << 1$, Eq. 3.3 is linearized near a resonant frequency, i.e., sine and hyperbolic sine functions are approximated by their arguments, to yield a relationship between absorption per unit wavelength and the half-power bandwidth Δf of a particular peak and the frequency f_n of that peak,

$$\frac{\alpha\lambda}{\pi} = \frac{\Delta f}{f} = \frac{1}{\text{Quality factor}} . \qquad 3.5$$

Eq. 3.5 yields the quality factor Q of an "ideal" resonator with only the sample solution's attenuation. The quality factor of a "real" system is inversely proportional to the total energy loss of the resonator system, and contains additional contributions from various energy losses, such as, beam divergence, scattering, imperfect reflections at the transducer surfaces and mechanical coupling losses. Following Eggers and Funck, [25] assume that all these losses are additive. Hence, the resulting over all quality factor Q is given by

$$\frac{1}{Q_{real}^{solution}} = \frac{1}{Q_{ideal}^{solution}} + \frac{1}{Q_{extra}} . \qquad 3.6$$

It is realized that the ultrasound absorption of the solute can now be effectively determined if $1/Q^{solvent}_{real}$ is measured separately within the same cell, retaining all identical parameters. Hence, the so-called excess absorption attributed to the lipids is obtained through

$$\frac{1}{Q^{lipid}} = \frac{1}{Q_{real}^{solution}} - \frac{1}{Q_{real}^{solvent}} . \qquad 3.7$$

Consequently, Eq 3.5 yields,

$$(\alpha\lambda)^{lipids}_{excess} = \frac{\pi(\Delta f_{solution} - \Delta f_{solvent})}{f_n} \quad . \qquad 3.8$$

In literature the excess absorption of lipids are frequently reported in units of excess absorption per wavelength per concentration of lipids, known as specific absorption. When the concentration of the "solute" is taken in the limit to approach zero this specific absorption is called the limiting excess absorption per wavelength.

Although it is difficult to measure the exact sound propagation velocity of a liquid within the resonant cavity, due to the required knowledge of a precise d value, fractional changes in sound propagation, however, are easily attainable. Thus,

$$\frac{v_{solute}}{v_{solvent}} = \frac{v_{solution} - v_{solvent}}{v_{solvent}} \quad . \qquad 3.9$$

That is,

$$\frac{v_{solute}}{v_{solvent}} = \frac{f_{solution}\,\lambda_{solution} - f_{solvent}\,\lambda_{solvent}}{f_{solvent}\,\lambda_{solvent}} \qquad 3.10$$

or through Eq. 3.1 at some n^{th} resonance peak

$$\frac{v_{solute}}{v_{solution}} = \frac{f_{solution}\,(2d_{solution} / n) - f_{solvent}\,(2d_{solvent}/n)}{f_{solvent}\,(2d_{solvent} / n)} \quad . \qquad 3.11$$

When great care is exercised in keeping the same configuration of the resonant cell during both suspension and reference experiment, so that $d_{solution} = d_{solvent}$ we get

$$\frac{v_{solute}}{v_{solution}} = \frac{f_{solution} - f_{solvent}}{f_{solvent}} . \qquad 3.12$$

In keeping with the nomenclature developed by Mitaku et al. [26], the limiting sound velocity is given by

$$[v] = \lim_{C_o \to 0} \frac{v - v_{solvent}}{C_o v_{solvent}} = \frac{f - f_{solvent}}{C_o f_{solvent}} . \qquad 3.13$$

In knowledge of the ultrasound relaxation theory developed in Chapter 2, Mitaku et al. [26] pointed out that the enhanced dispersion in the ultrasound velocity arises mainly due to the change in the membrane's adiabatic compressibility, given by

$$[v] = [v]_\infty - \Delta \frac{1}{1 + (\omega\tau)^2} \qquad 3.14$$

and

$$[\alpha\lambda]_{excess} = 2\ \pi\Delta \frac{(\omega\tau)}{1 + (\omega\tau)^2} + 2\pi B \omega \qquad 3.15$$

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

$$\Delta = \frac{\beta_{relax}}{2\beta_{solvent}} . \qquad 3.16$$

B corresponds to the sum total of all the classical sound absorptive mechanisms occuring within the membranes. If the net classical sound absorption of the membrane is small in comparison to the non-classical part we may rewrite Eq. 3.14 in terms of $[\alpha\lambda]$ to yield,

$$[v]_{\infty} - [v] = \frac{[\alpha\lambda]}{2\pi\,(\omega\tau)} = \frac{[\alpha\lambda]}{2\pi}\,\frac{f_{relax}}{f} \qquad 3.17$$

and obtain the f_{relax} parameter,

$$f_{relax} = \frac{2\pi(\,[v]_{\infty} - [v]\,)\,f}{[\alpha\lambda]}\,. \qquad 3.18$$

For cylindrical resonators to operate at an optimal degree of efficiency so as to yield the largest possible Q value, a high degree of planarity and parallelism of the transducers' surfaces is required so as to minimize energy losses due to redirection of multiply reflected partial waves out of the resonator's axis. As pointed out by Eggers and Funck, the total number of reflections that a partial wave must undergo before being reduced to 1/e of its original value is given by

$$m = \frac{1}{\alpha L} = \frac{Q\lambda}{\pi L}\,. \qquad 3.19$$

For distilled water $m \approx 2{,}000$ at $f = 1$ MHz.

A factor which governs the accuracy of an absorption and a velocity measurement is the temperature stability of the cell while the peak frequency location and the half power frequency bandwidth are being measured. An estimate of the uncertainty in the measurement due to

temperature fluctuation is acquired by considering changes in sound velocity with respect to temperature

$$\frac{\delta v}{\delta T} = f_n \frac{\delta \lambda_n}{\delta T} + \lambda_n \frac{\delta f_n}{\delta T}. \qquad 3.20$$

Taking the temperature derivative of Eq. 3.1 and substituting back into Eq. 3.21, yields

$$\frac{\delta v}{\delta T} = f_n \lambda_n \frac{1}{d} \frac{\delta d}{\delta T} + \lambda_n \frac{\delta f_n}{\delta T} \qquad 3.21$$

or

$$\frac{1}{v} \frac{\delta v}{\delta T} = \xi + \frac{1}{f_n} \frac{\delta f_n}{\delta T} \qquad 3.22$$

where ξ is the linear thermal expansion coefficient of the resonant cavity. Thus,

$$\delta f_n = \frac{f_n}{v} \frac{\delta v}{\delta T} \delta T - \xi f_n \delta T. \qquad 3.23$$

The second term on the right hand side of the equal sign is quite small in comparison with the first since $\xi \approx 10^{-6}$ °C for the plexiglass cell utilized in this investigation. Consequently,

$$\delta f_n \cong \frac{f_n}{v} \frac{\delta v}{\delta T} \delta T. \qquad 3.24$$

As pointed out by Eggers and Funck [25], at room temperature

$$\delta f_n \equiv (1.8 \times 10^{-3}) f_n (\delta T). \qquad 3.25$$

A temperature drift of 10^{-3} °C at f = 3 MHz will yield a frequency drift of 5.4 Hz.

3.3 Cell Design

The small volume cylindrical resonant cell employed . in this investigation was guided by the design of Eggers and Funck in 1973 as well as by previous work preformed here at University of Illinois Bioacoustics Research Laboratory. The cell was design to operate with small sample volumes of less than 5 ml, in the frequency range of 0.5 to 6 MHz.

Fig. 3.1 exhibits a figure of revolution about the O - O' axis of the assembled cell. The frame F, and most of the cell's mechanical components were made from plexiglass. Plexiglass material was selected over brass or steel so as to greatly reduce the possibility of corrosion as well as the thermal linear expansion coefficient. The two plexiglass halves F are held together by three plexiglass bolts, B, and associated brass nuts, N, which were machined to have 64 threads per inch so as to permit fine adjustments and achieve a high degree of parallelism of the quartz transducers, Q, required for the resonator operation. The Qs were fixed within an accurately machined recess within the end walls of the cylindrical cavity by an RTV sealant and flat neoprene rings, O. The lateral cylindrical wall of the cavity was formed by plexiglass spacers, S, which were fitted with O to make a liquid tight seal against F. The two vertical diametrically opposite ports, P, on S were used to fill and empty the cell with the sample fluid. A standard male BNC connector was threaded into

the frame and a light pressure from the spring contact, C, made the electrical contact to the quartz crystal.

A pair of one inch diameter, x - cut, quartz crystals purchased from Valpey-Fisher Corporation having a fundamental resonance frequency of 4 MHz were employed in this study. The surface of each transducer was highly polished by the manufacturer and provided the necessary smoothness and flatness to maintain numerous multiple reflections without significant beam spreading.

3.4 Cell Utilization

A primary step in optimizing the performance of the cell was made in establishing a high degree of parallelism between the two transducers. In this investigation, as with many others [23,32] this was simply accomplished by tuning the bolts of the cell's cavity containing only distilled water at room temperature until an optimum resonance output signal was measured. Starting at a resonance frequency near 1 MHz, the bolts were turned by small amounts and the amplitude of resonance signal was compared to the prior measured amplitude. This general procedure was repeated until no further amplitude increase could be achieved. This fine tuning procedure was carried on to higher resonant frequencies near the 5 MHz where only the slight adjustments were necessary. The cell was in near perfect tuning after 3 MHz.

The cell was filled using a 25 ml glass syringe supported vertically with its outlet connected to the lower filling port P of the cell by a polyethylene tubing. The fluid was then carefully pipetted into the syringe body and very slowly gravity feed (typically at a rate of 1 ml/min) at

atmospheric pressure into the cell's cavity. Slow introduction of the fluid into the cavity is essential so as to avoid unwanted gaseous cavities within fluid during the filling process. The effects of such bubbles is to drastically reduce the quality factor of the cell and lead to significant errors in [$\alpha\lambda$] and [v] measurements. After the cell was filled and electrical connections made by a 50 ohm BNC coaxial cables, the cells and its plexiglass support were placed into two plastic bags and the entire assembly was submerged into the water bath. As judged by the frequency stability of cell resonances, this technique provided excellent temperature stability. A constant temperature was always achieved within one hour.

The actual measurements was made with the schematic instrumentation shown in Fig. 3.3. The continuous driving sinusoidal voltage frequency at Q_1 was controlled by a Hewlett-Packard 8660 B frequency synthesizer. The output voltage from Q_2 was connected directly to a Hewlett-Packard 8552A spectrum analyzer which measured the amplitude of the received voltage signal. The input frequency was swept manually until a resonance was observed in the form of a voltage peak across the frequency analyzer display. The resonance frequency was then noted, and the half power bandwidth frequency measured through manual adjustments in input frequency, until the frequency analyzer display drops to $\frac{1}{\sqrt{2}}$ its V_{max} value on both sides of the display peak. It typically took under seven seconds to "hop" from f_n to f_{n+1} or f_{n-1}, and approximately under two seconds to measure Δf.

It is important to state that for all measurements preformed in this investigation, the root mean square driving voltage on Q_1 was held at the

same value of 0.2V. Assuming a 100% conversion of electrical power into acoustical power yields a constant acoustic intensity of $I \approx 10^{-6}\ W/cm^2$. Thus, a constant, and a very low ultrasound intensity was employed through out this investigation.

Chapter 4

Experimental Results

The enhanced ultrasound absorption behavior of pure, as well as mixtures of PC lipid, protein, and cholesterol of the MLV liposome systems studied in this investigation is typified in the pure DMPC absorption frequency spectra at various temperatures. See Fig. 4.1a and Fig. 4.1b. Each spectrum presented in this investigation is an average of three distinct sample measurements. Figure 4.1a exhibits the enhancement of ultrasound absorption as the temperature is increased in finite steps towards the DMPC phase transition temperature T_m at 24.4°C. When the sample temperature is several degrees Celsius below T_m, the deviation from the classical theory of sound absorption is relatively small and uniform over the frequency range of measurement. As the temperature is increased towards T_m the uniform sound absorption spectra are gradually transformed into spectra containing evidence of a broad peak. When the temperature is further increased towards T_m this broad peak is observed to become more clearly defined and it is noted to drift towards lower frequencies. When the temperature equals the DMPC phase transition temperature of T_m=24.4°C, maximal anomalous sound absorption occurs with a single well defined sound absorption peak centered near 1.4 MHz. Figure 4.1b shows that as the temperature is further increased away beyond T_m, the non-classical sound absorption weakens and the amplitude of the spectrum is noted to progressively diminish. The single peak is noted to lose definition and drifts back to higher frequencies.

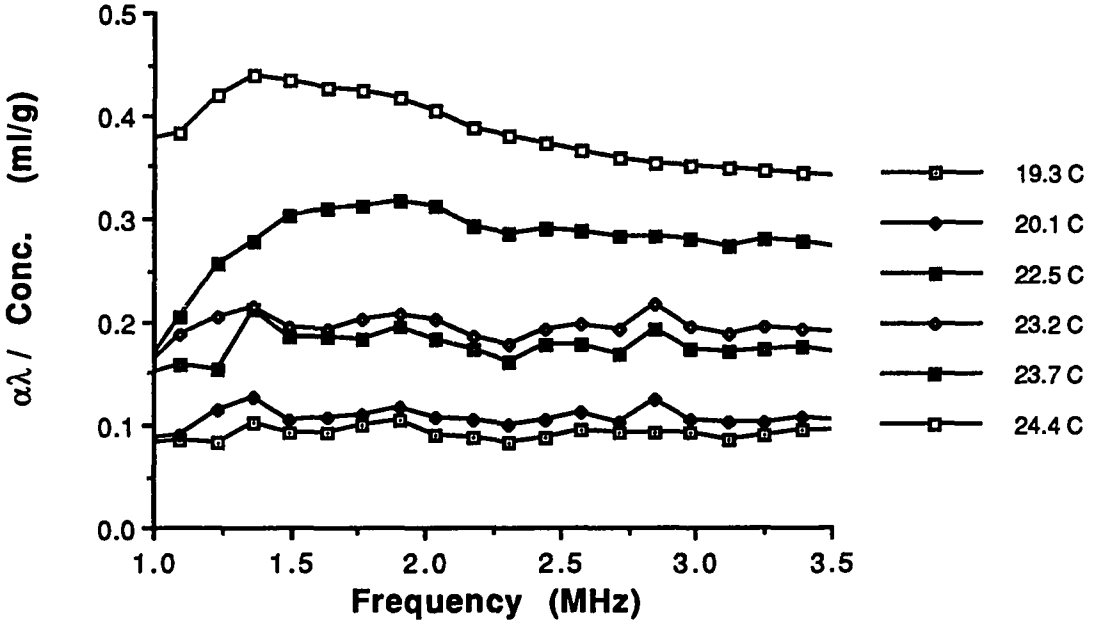

Fig. 4.1 A - Pure DMPC MLV absorption at several temperatures

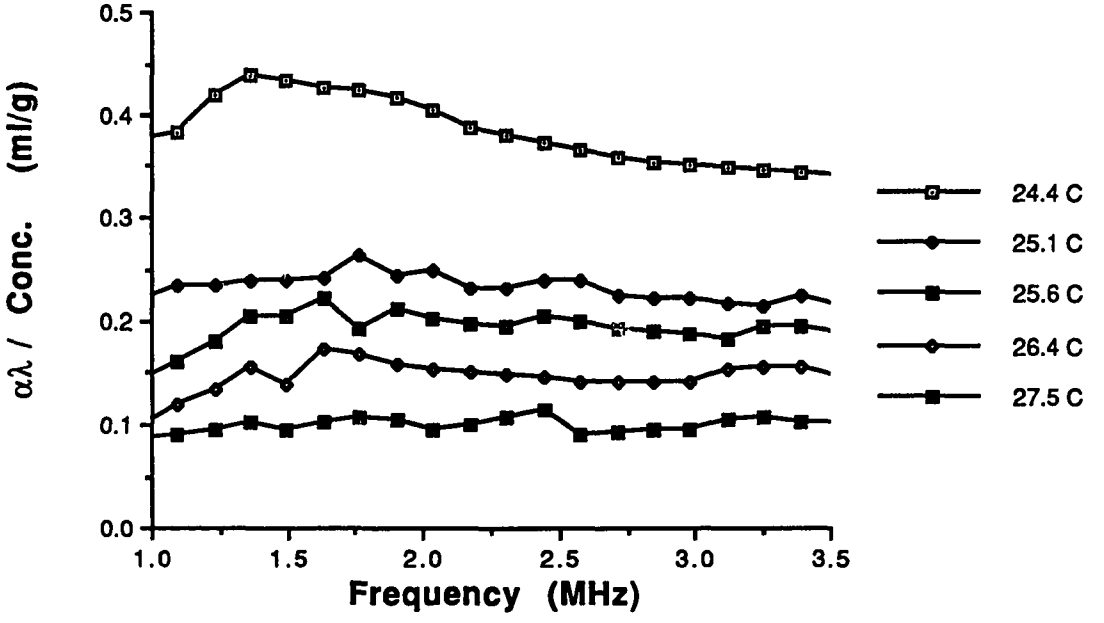

Fig. 4.1 B - Pure DMPC MLV absorption at several temperatures

Figure 4.2 compares the well defined DMPC absorption spectrum at Tm with the theoretical spectrum of a single relaxation process having the same frequency and magnitude. The difference spectrum, viz, experiment spectrum minus the theoretical spectrum is also shown in Fig.4.2, and reveals a linear deviation at higher frequencies. No significant deviations are observed at frequencies below f. According to the sound absorption theory for a single relaxation processes, Eq 3.15, such deviations are expected to result from the presence of classical absorption processes within the lipid bilayer system. Similar remarks can be made for Figs. 4.3, 4.4, and 4.5 which display, respectively. DC15PC, DPPC, and DC17PC absorption spectra at their respective T_m values. Theoretical single relaxation spectra are also shown in these figures.

Figure 4.6 shows the temperature dependence of the $\alpha\lambda$ parameter of pure DPPC MLV liposomes, all measured at the resonance frequency of 3 MHz.

The temperature dependence of the sound velocity of the DPPC lipid was also measured at the resonance frequency of 3MHz and is shown in Fig. 4.7. A line is drawn tangent to the limiting velocity curve below the T_m, to represent the temperature dependence of the limiting sound velocity if the membrane system had not undergone relaxation in the neighborhood of T_m. According to relaxation theory, such a condition would prevail when the perturbation frequency is much greater than the natural relaxation frequency of the membrane system, f_{relax}. Hence, the tangent line is interpreted to represented the temperature dependence of the

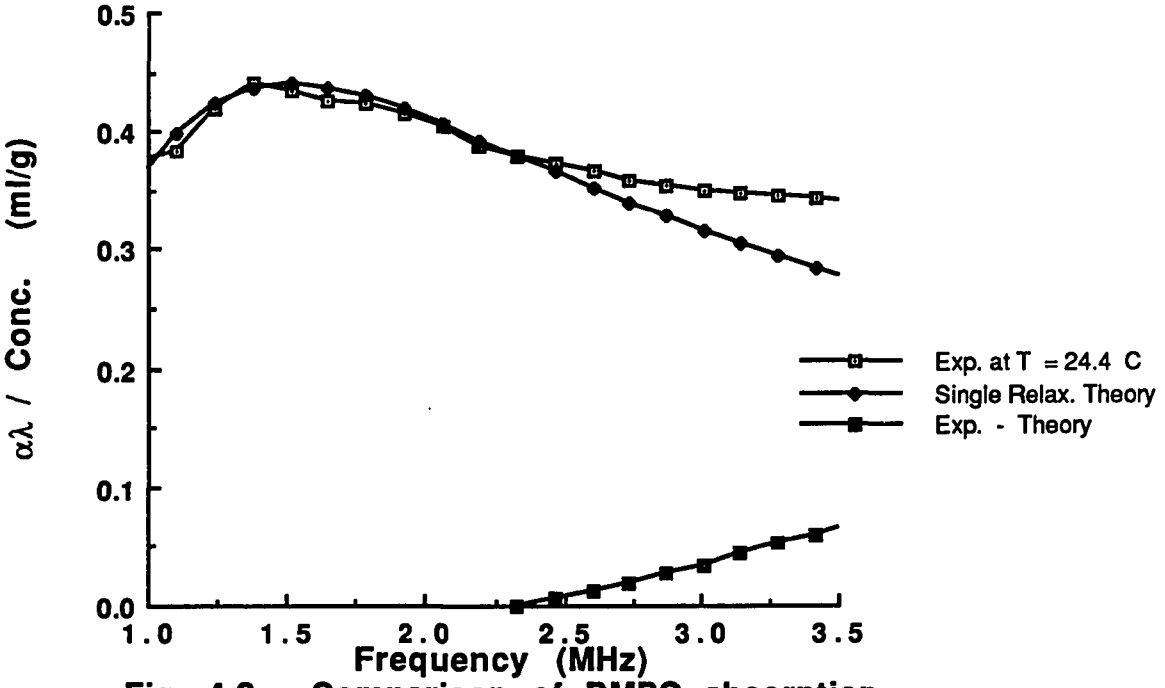

Fig. 4.2 - Comparison of DMPC absorption with single relaxation theory at T_m

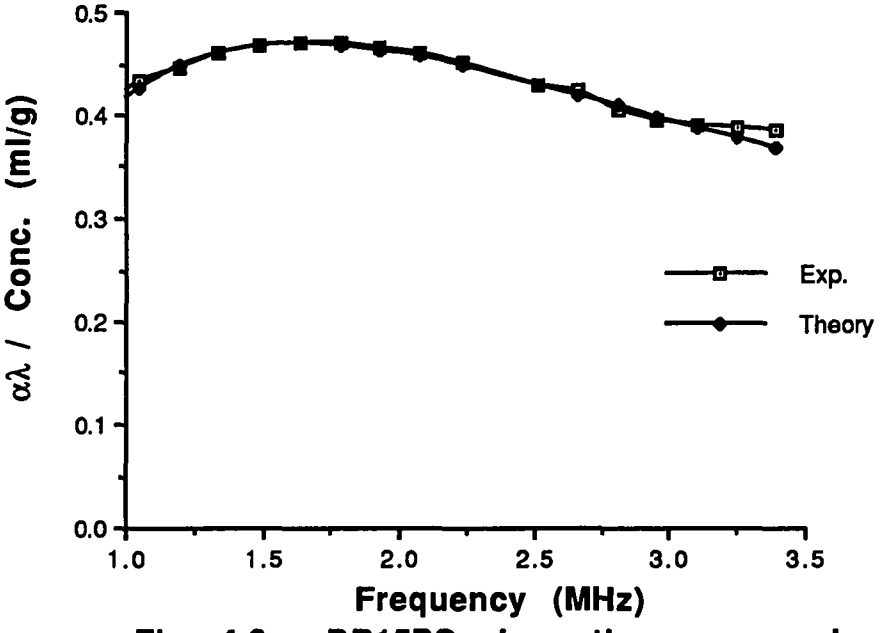

Fig. 4.3 - DP15PC absorption compared with single relaxation theory at Tm = 35.5 C

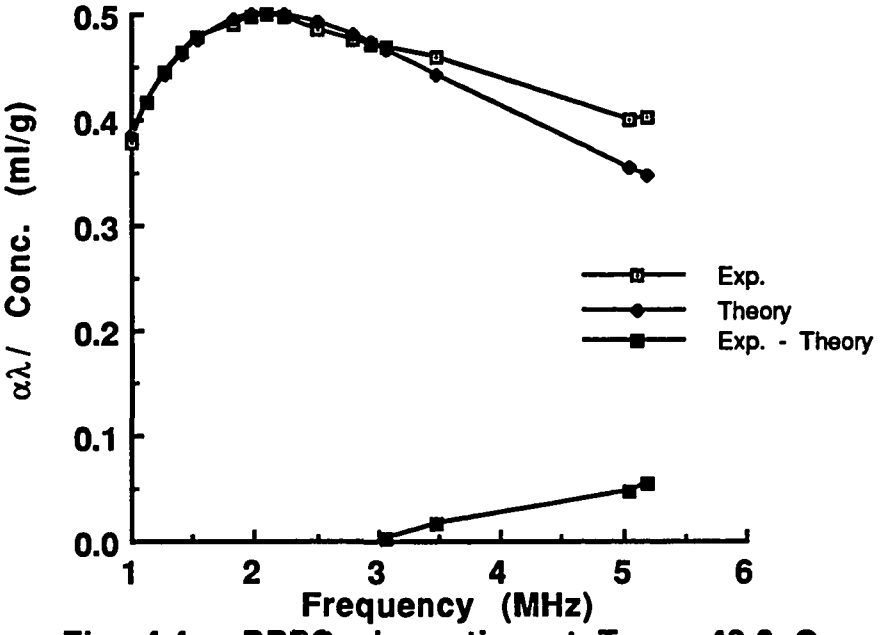

Fig. 4.4 - DPPC absorption at Tm = 42.0 C compared with single relaxation theory

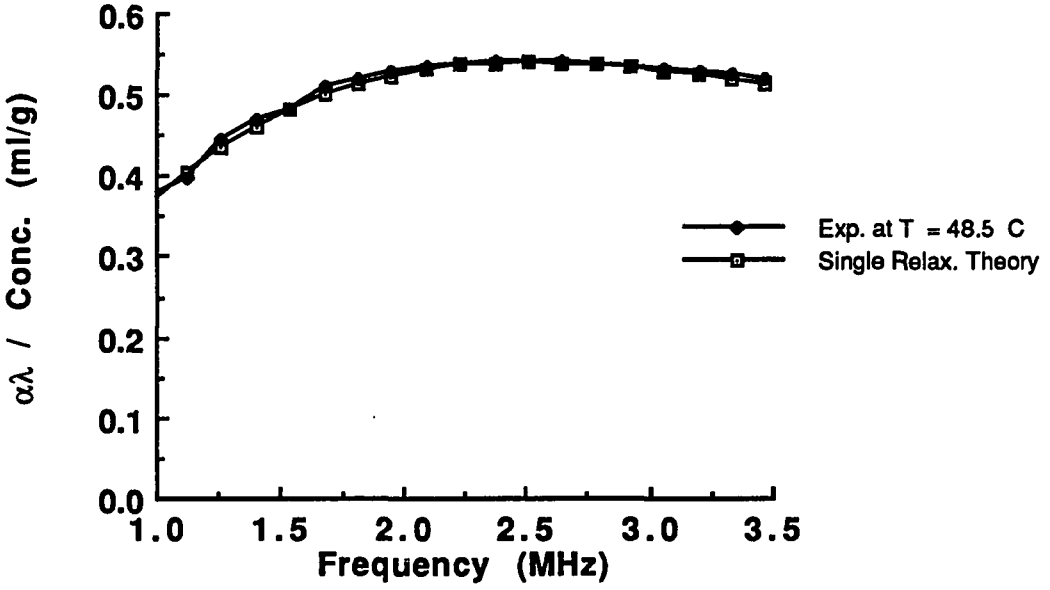

Fig. 4.5 - DC17PC absorption at Tm = 48.5 C compared with single relaxation theory

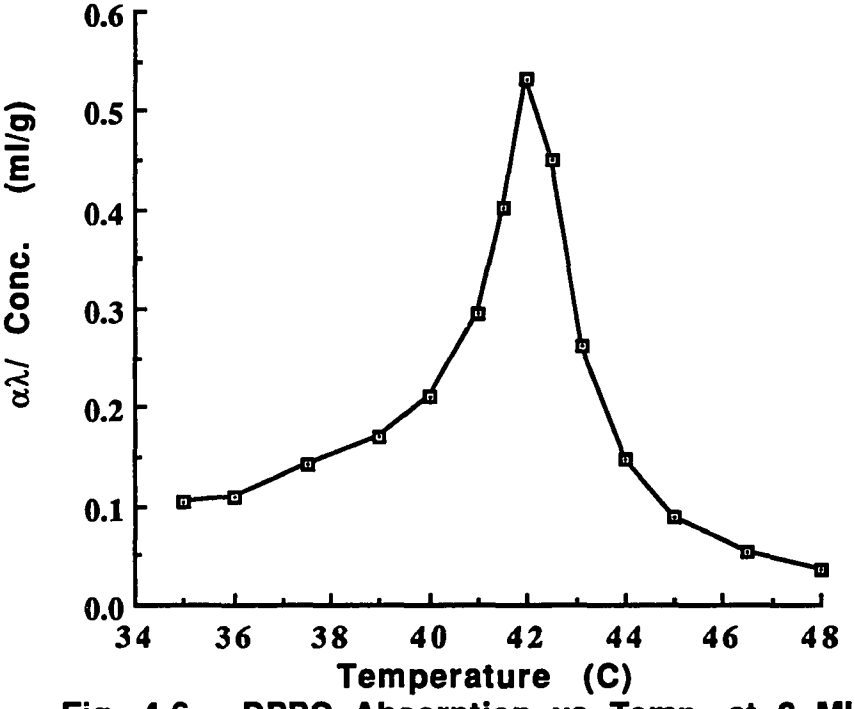

Fig. 4.6 - DPPC Absorption vs Temp. at 3 MHz

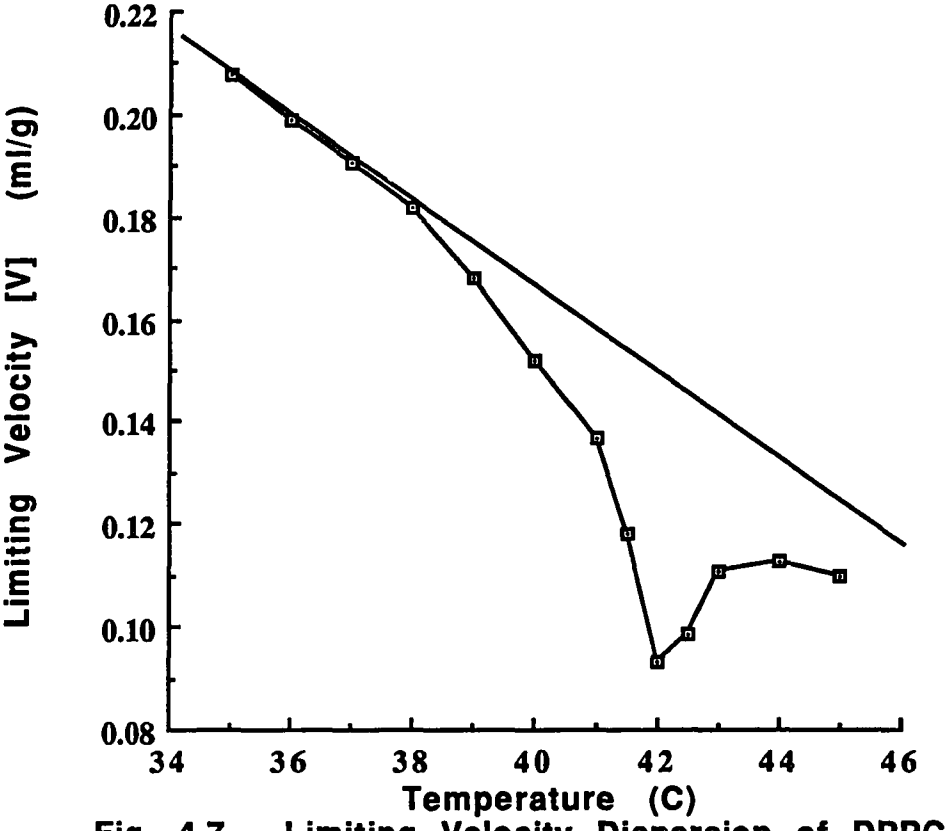

Fig. 4.7 - Limiting Velocity Dispersion of DPPC At 3 MHz

sound velocity in the limit 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 system's temperature is progressively moved away from T_m. Such resolving 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 in $[V]_\infty$ - [V] as a function of temperature of pure DPPC MLVs, at the frequency of 3MHz, were utilized in the single relaxation theory Eq.3.18 to yield the temperature dependence of the f_{relax} parameter. See Fig. 4.8.

Fig. 4.9 shows the ultrasound absorption frequency spectrum of a 50 mol% mixture of DMPC:DPPC at the T_m of 33.8 °C. As evident, the f_{relax} approximately occurs midway between f^{DMPC}_{relax} and f^{DPPC}_{relax}. Ultrasound absorption between pure and a slightly modified or "perturbed" membrane system with a 2.5 mol% gramicidin at the T_m of 42°C is compared with the single relaxation theoretical dependence in Fig. 4.10.

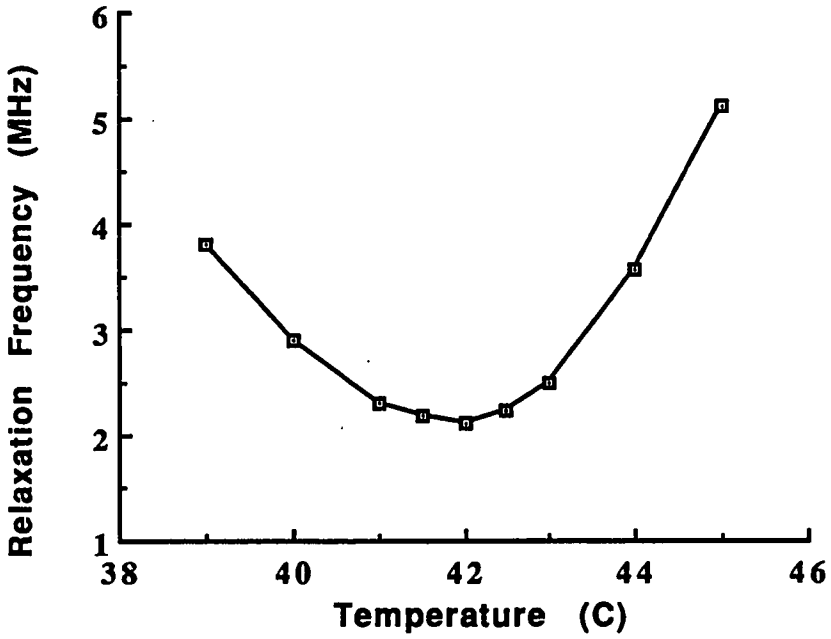

Fig. 4.8 - DPPC relaxation frequency temperature dependence through Fig. 4.6 and Fig.4.7

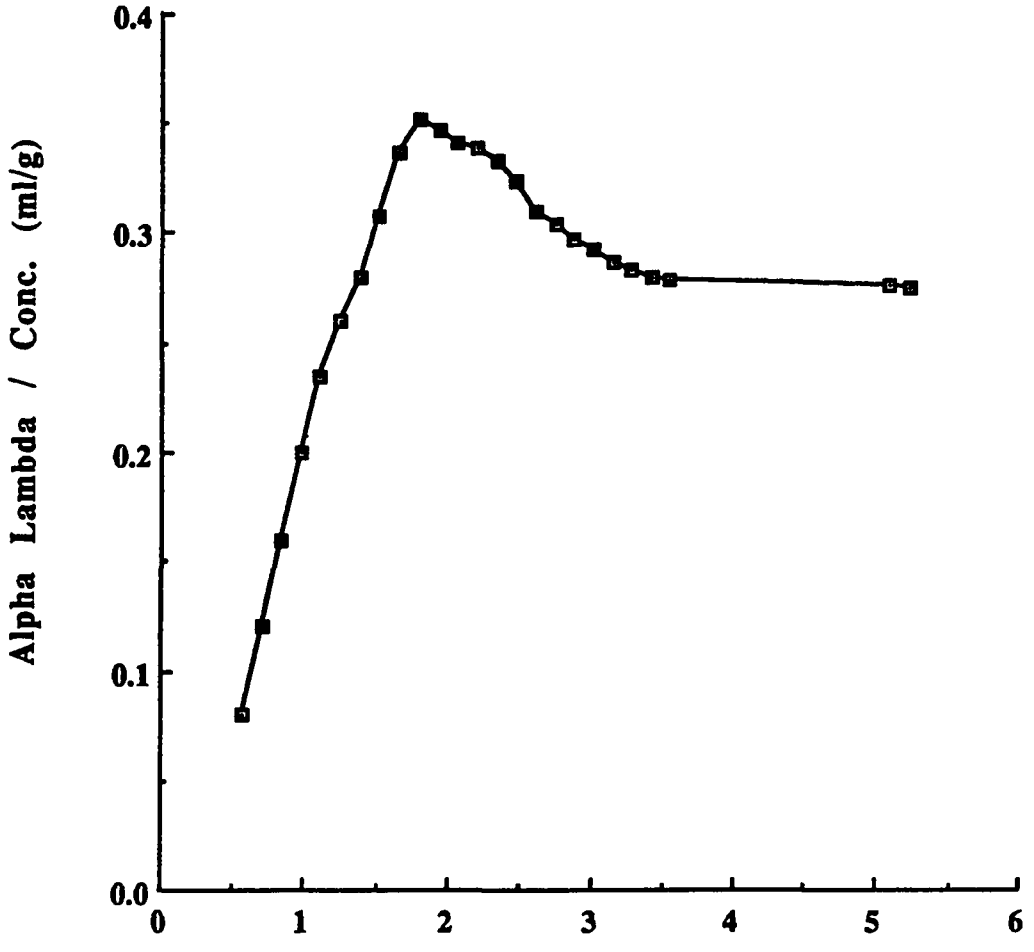

Fig. 4.9 - DMPC : DPPC 50 mol% mixture

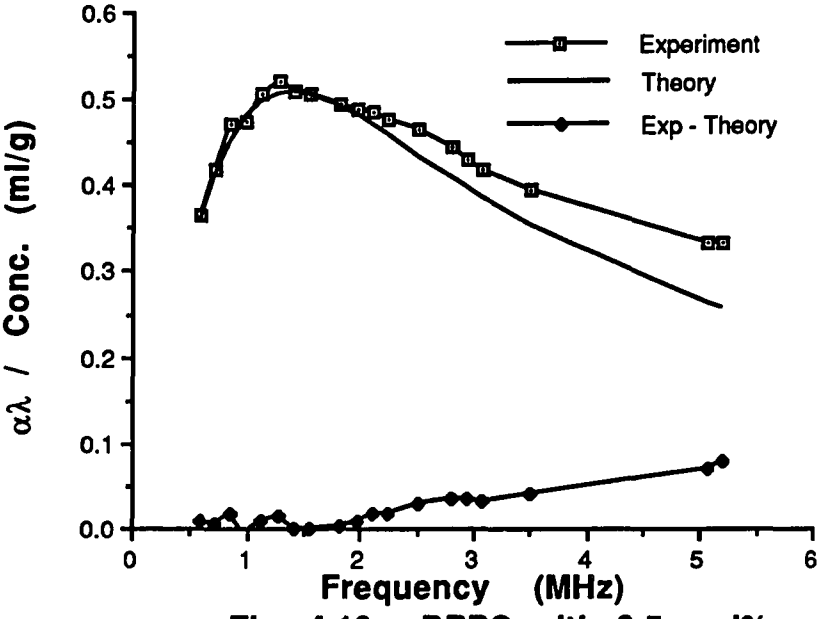

Fig. 4.10 - DPPC with 2.5 mol%

Chapter 5

Data Analysis and Discussion

No presently available theory is capable of accurately predicting the phase transition temperature and the Gibb's free activation energy ΔG^{+}_{α} and ΔG^{+}_{β} of any lipid species. Thus, it is interesting to test the two-state transition hypothesis via Eq. 2.24. A straight forward way of testing the transition hypothesis is by way of inspection of the absorption spectra of different phospholipid liposome systems. By the two-state hypothesis, the absorption spectrum is expected to follow Eq 2.24 closely and to exhibit a line feature having a single maxima at the characteristic relaxation frequency of the lipid system. See Fig. 2.4

The ultrasound absorption measurements of aqueous dispersions of DMPC, $DC_{15}PC$, DPPC, and $DC_{17}PC$, described earlier, are summarized in Table 5.1. These findings strongly support the two-state transition hypothesis due to the observed absorption line shaped features consistent with Eq. 2.24, at the respective lipid's T_m value.

It is interesting to note that the ratio of $(\alpha\lambda)_{max}$ between any two PC lipids is nearly unity. Since $T_{m1} \cong T_{m2}$, in view of the single relaxation sound absorption theory, 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 parameter in the PC lipid family investigated. Steady state changes in volume of several PC lipids at their phase transition temperature have been measured by volume dilatometry techniques [27] , and are summarized in the Table 5.2 below. If it is boldly 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 5.2, then

Table 5.1

Summary of the pure lipid ultrasonic absorption findings

Lipid	T_m (K)	$\frac{\alpha\lambda}{[C_o]}^{max}$ (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 5.2

Summary of the steady state molar volume changes of pure lipids at T_m obtained through Nagle and Wilkinson [27]

Lipid	Steady State Volume Change At T_m (ml/mole of lipid)
DMPC	18.30
$DC_{15}PC$	21.88
DPPC	27.15
DC17PC	31.24

$$\Delta V = Q\,\Delta V^{*} = \text{Const.}, \qquad 5.1$$

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

It then follows that

$$Q_1\,\Delta V_1^{*} = Q_2\,\Delta V_2^{*}. \qquad 5.2$$

Furthermore, if it is assumed that the rate of cluster growth (or collapse) is independent of the PC lipid type, then

$$\frac{Q_1}{\tau_1} = \frac{Q_2}{\tau_2} \qquad 5.3$$

or

$$Q_1\, f_{relax_1} = Q_2\, f_{relax_2} \qquad 5.4$$

$$\frac{Q_1}{Q_2} = \frac{\Delta V_2^{*}}{\Delta V_1^{*}} = \frac{f_{relax_2}}{f_{relax_1}} \qquad 5.5$$

It can be readily verified from the f_{relax} findings reported herein that this line of reasoning leads to an excellent agreement with Eq. 5.5.

It is recognized that the work done in cluster growth by thermal energy may be modelled as the work done by a battery in electrically 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

$$\tau = \gamma C / D \qquad 5.6$$

where γ is a proportionality factor.

Thus, $\tau = (\gamma/D) (Q/ k_B T)$ and $D/\gamma = (1/\tau) (Q/ k_B T)$. If it is assumed that the ratio between the thermal diffusion coefficient and the proportionality factor is approximately the same for all the pure lipids investigated in this study, then

$$\text{Constant} = \frac{1}{\tau_1} \frac{Q_1}{k_B T_{m_1}} = \frac{1}{\tau_2} \frac{Q_2}{k_B T_{m_2}} \qquad 5.7$$

Since, $k_B T_{m1} \approx k_B T_{m2}$, equations 5.4 and 5.5 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 a present lack of available ΔV data. If all is consistent, the above model predicts ΔV = 18 ml/mol and 9.65 ml/mol for 2.5 mol% and 5 mol% gramicidin composition, 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 Fig. 5.1. [α] is allowed to be equal to the concentration of lipid in the crystalline state and [ß] 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 $K_{forward}$

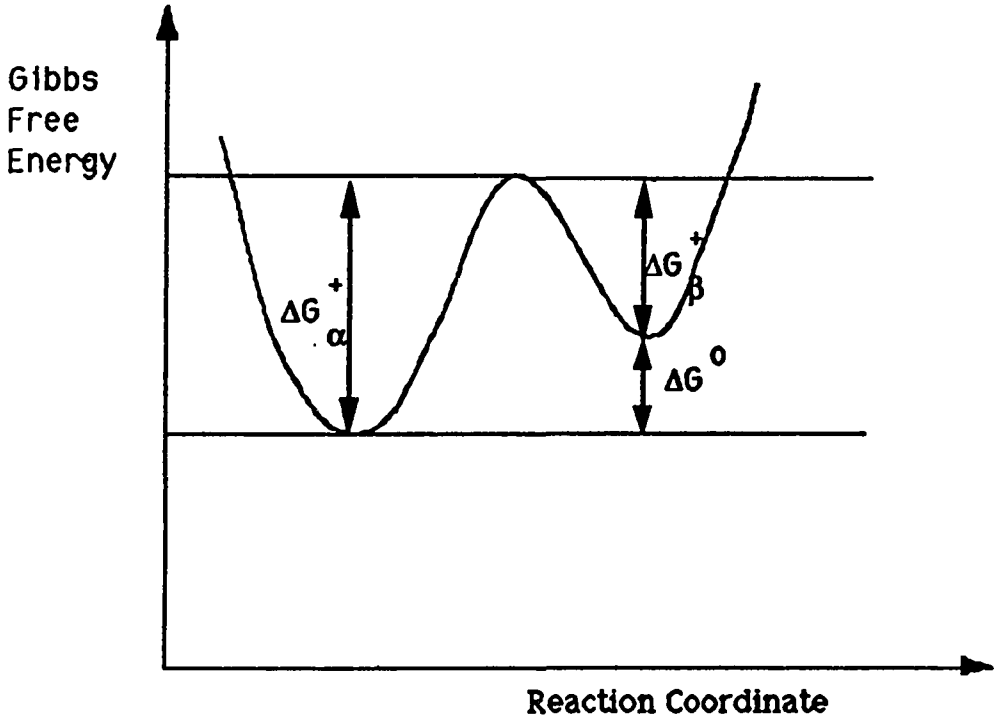

Fig. 5.1 Two-state energy profile of a simple modelled membrane

is the rate of lipid becoming fluid (sec.$^{-1}$) and $K_{backward}$ is the rate of lipid falling back into the crystalline state.

The ratio [ß] / [α] = k_f / k_b for a two state model obeys the Boltzmann distribution, i.e., [ß] / [α] = exp (- ΔG^o/ RT) where, ΔG^o is the difference in the Gibbs free energy between the bottom of the two wells and comprises the differences in van't Hoff enthalpy ΔH^o_{vH} , and differences in entropy ΔS^o; i.e.,

$$\Delta G^o = \Delta H^o_{vH} - T\Delta S^o \qquad 5.8$$

From the fluctuation - dissipation theorem [24], 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 [24], e.g.,

$$f_{fluctuation} = f_{dissipation} = \frac{K_f + K_b}{2\pi} \qquad 5.9$$

The $K_{forward}$ and $K_{backward}$ rate constants are obtained from the transition state theory (TST) [9,20] . The rate at which the membrane lipid surmounts the potential barrier depends upon the fractional 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 (-ΔG^+/RT) where ΔG^+ is the activation Gibbs free energy. The frequency of attack on the barrier is given through ($K_{Boltzmann}$ T) = h f. Therefore, the rate of transition is given by

$$K_{rate} = \frac{k_B T}{h} \exp\left(-\frac{\Delta G^+}{RT}\right) \qquad 5.10$$

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

Hence,

$$K_{forward} = \frac{k_B T}{h} \exp\left(- \frac{\Delta G_{\alpha}^{+}}{RT} \right) \text{ and } K_{backward} = \frac{k_B T}{h} \exp\left(- \frac{\Delta G_{\beta}^{+}}{RT} \right) \qquad 5.11$$

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

$$f_{fluctuation} = f_{dissipation} = \frac{k_B T}{2\pi h}\left[\exp\left(- \frac{\Delta G_{\alpha}^{+}}{RT} \right) + \exp\left(- \frac{\Delta G_{\beta}^{+}}{RT} \right) \right] \qquad 5.12$$

At T_m , half of the lipid population is in the crystalline state and the other half is in fluid state. Therefore, ΔG^{o} is identically equal to zero at T_m and $\Delta G^{+}_{\alpha} = \Delta G^{+}_{\beta}$. ΔG^{+}_{m} is denoted to be equal to the ΔG^{+}_{α} and ΔG^{+}_{β} only at T_m. Hence, the frequency of relaxation at T_m, is obtained through Eq. 5.12 and rewritten as

$$f_{relax \text{ at } T_m} = \frac{k_B T_m}{\pi h} \exp\left(- \frac{\Delta G_{m}^{+}}{RT} \right) \qquad 5.13$$

From Eq. 5.13 and the observed f_{relax} values from Table 5.1 it is empirically found that

$$\Delta G^{+}_{m} = 4{,}300 \text{ cal/mol} - T_m (-13.65 \text{ entropy units}). \qquad 5.14$$

Figure 5.2 compares the "observed" relaxation frequencies of the PC lipids investigated in this study with the two-state TST predictions from Eq. 5.13 and the above empirical relationship Eq. 5.14.

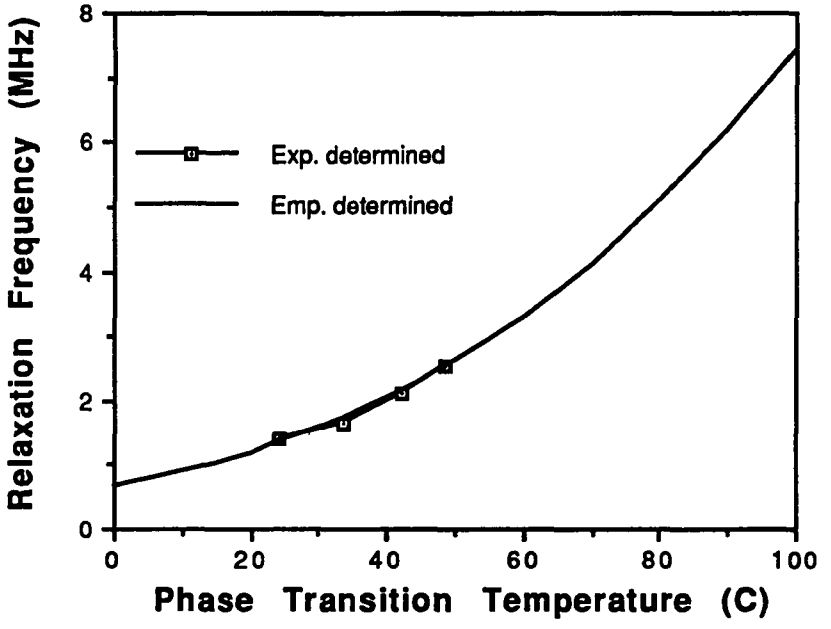

Fig. 5.2 Comparision between Exp. relaxation frequency and the emperical relationship obtained through the two-state theory

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 Fig.5.3. As a necessary consequence of the principle of conservation of mass, and total number of conserved states of the non-degenerative two-state system, it follows that

$$| \delta(\Delta G^{+}_{\alpha}) | = | \delta(\Delta G^{+}_{\beta}) |. \qquad 5.15$$

The change in ΔG^{o} near the T_m is given by

$$| \delta(\Delta G^{+}_{\alpha}) | + | \delta(\Delta G^{+}_{\beta}) | = | \delta(\Delta G^{o}) |. \qquad 5.16$$

Consequently,

$$| \delta(\Delta G^{+}_{\alpha}) | = 1/2 \, | \delta(\Delta G^{o}) |. \qquad 5.17$$

Theoretical prediction of the temperature dependence of the f_{relax} parameter for the two-state transition model is made with the aide of Eqs. 5.15 and 5.16. Rewritting Eq. 5.12 in terms of Eq. 5.13 and 5.15 yields

$$f_{relax} = \frac{k_B T}{2\pi h} \exp\left(- \frac{\Delta G^{+}_{m}}{RT} \right) (2) \cosh \left(\frac{\delta(\Delta G^{+})}{RT} \right) \qquad 5.18$$

at any temperature, T. Through Eq. 5.17, Eq. 5.18 can be rewritten as

$$f_{relax} = \left(\frac{T}{T_m} \right) f_{relax \text{ at } T_m} \exp \left(- \frac{\Delta G^{+}_{m} \chi}{RT} \right) \cosh \left(\frac{\Delta H^{o}_{vH} \chi}{2RT} \right) \qquad 5.19$$

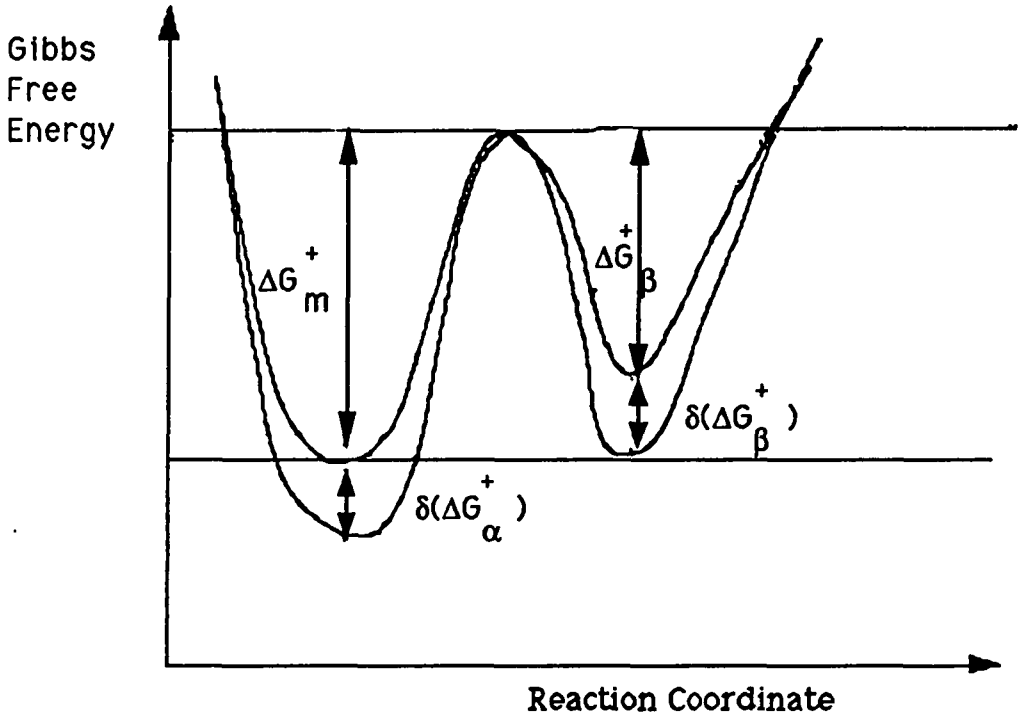

Fig. 5.3 Temperature dependence of the activation Gibbs free energy of α and β states relative to T_m.

where $\chi = (1 - T/T_m)$. From the f_{relax} findings and through Eq. 5.13, ΔG^+_m is determined. $\Delta G^+_m = \Delta G^+_\alpha = \Delta G^+_\beta = 8{,}600$ cal/ mol at T_m for DPPC.

The frequency dependence obtained in Fig. 4.8 is compared with the predicted frequency dependence for the two-state model theory, i.e., Eq. 5.19 in Fig. 5.4. A strong correlation between the "measured" frequency dependence and the two-state transition theory is noted when the ΔH^o_{vH} is fitted to a value of 160,000 cal/mol in Eq. 5.19. It is noted that $\Delta H^o_{vH}/RT \approx 260$. From previous DSC studies the cooperative unit of melting for DPPC aqueous dispersions is determined to be around 260 [29]. 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 [33].

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 Fig. 5.5. Thus, the transition of

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

is viewed as

$$\langle\alpha\rangle_{unknown} \rightarrow\rightarrow \langle\alpha\rangle_{Ref} \rightarrow\rightarrow \langle Intermediate\rangle \rightarrow\rightarrow \langle\beta\rangle_{Ref} \rightarrow\rightarrow \langle\beta\rangle_{unknown}$$

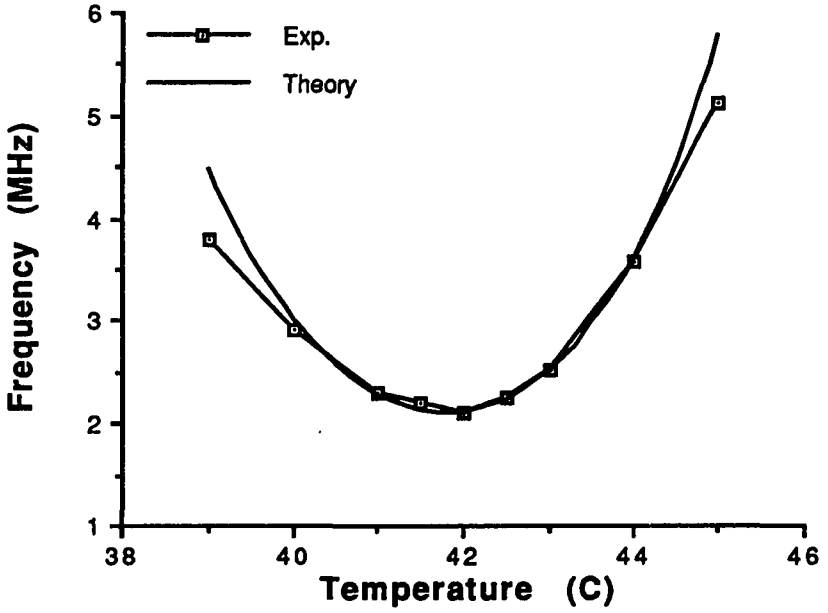

Fig. 5.4 Comparision of determined relaxation frequency with the two-state theory

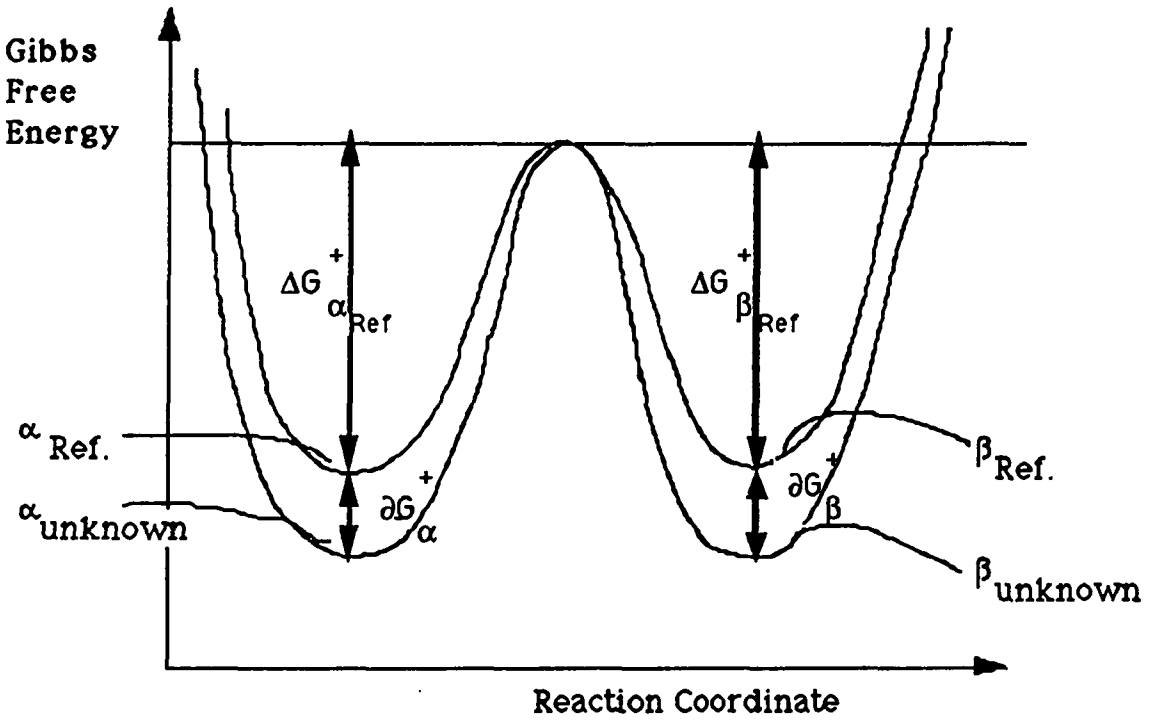

Fig. 5.5 Two-state energy profiles of the reference and the unknown lipid at their respective T_m.

with

$$\left|\partial(\Delta G_{\alpha}^{+})\right| = \left|\partial(\Delta G_{\beta}^{+})\right| \qquad 5.20$$

and

$$\Delta G^{+}_{\alpha\ \text{unknown}} = \Delta G^{+}_{\alpha\ \text{Ref}} + \partial(\Delta G_{\alpha}^{+}) \qquad 5.21$$

Thus, it becomes necessary to find $\partial(\Delta G^{+}\alpha)$ so that $f_{\text{relax unknown}}$ can be predicted. Through the same line of reasoning used in deriving Eq. 5.17, it follows that

$$|\partial(\Delta G^{+}\alpha)| = 1/2\ |\ \partial(\Delta G^{o})\ | \qquad 5.22$$

where

$$\partial(\Delta G^{o}) = \Delta G^{o}_{\text{unknown}} - \Delta G^{o}_{\text{Ref}} \approx (\ \partial(\Delta H^{o}) - T_{m\ \text{Ref}}\ \partial(\Delta S^{o})\ ) \qquad 5.23$$

and $\partial(\Delta H^{o}) = \Delta H^{o}_{\text{unknown}} - \Delta H^{o}_{\text{Ref}}$ and $\partial(\Delta S^{o}) = \Delta S^{o}_{\text{unknown}} - \Delta S^{o}_{\text{Ref}}$. Arbitrarily treating the reference lipid as DPPC, the $\Delta G^{+}\alpha\ \text{Ref}$ can be readily determined through Eq. 5.13 to yield $\Delta G^{+}\alpha\ \text{Ref}$ = 8,600 cal/ mol. The pure and "perturbed" membrane lipid relaxation frequencies at T_ms are obtain through Eq. 5.23. These predications are compared with the experimental findings and summarized in Table 5.3 below.

The experimental data is 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

$$-\frac{\partial(\Delta G^{\circ})}{\partial n} = \left(\frac{\partial(\Delta H^{\circ})}{\partial n} - T_m \frac{\partial(\Delta S^{\circ})}{\partial n}\right) \qquad 5.24$$

with

$$\partial(\Delta G_{\alpha}^{+}) = -\left(\frac{1}{2}\right)\partial(\Delta G^{\circ})\ \Delta n \qquad 5.25$$

Table 5.3

Comparison between Exp. and Thermodynamic Predictions

MLV	T_m (K)	$\delta(\Delta G^{+}_{\alpha})_{predicted}$ cal / mol	f^{Theory}_{relax} MHz	$f^{Measured}_{relax}$ 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

where Δn is the protein concentration. $\partial(\Delta H^o)/\partial n$ is an experimentally determined function through previous DSC studies preformed by Chapman et al. [28]. See Fig. 5.6. It is encouraging to find that f_{relax} is totally accounted for by this function at these low concentrations. Apparently, $\partial(\Delta S^o)/\partial n \approx 0$ 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_o due to absorption after the longitudinal wave travels a distance x from the source is [18]:

$$I = I_o \exp(-\alpha x). \qquad 5.26$$

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,

$$\Delta I = (I_o - I) = I_o (1 - \exp(-\alpha\lambda)) \approx I_o \alpha\lambda, \qquad 5.27$$

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 average energy per molecule to undergo the transition, ΔE.

Hence,

$$I_o \alpha\lambda A = N (\Delta E). \qquad 5.28$$

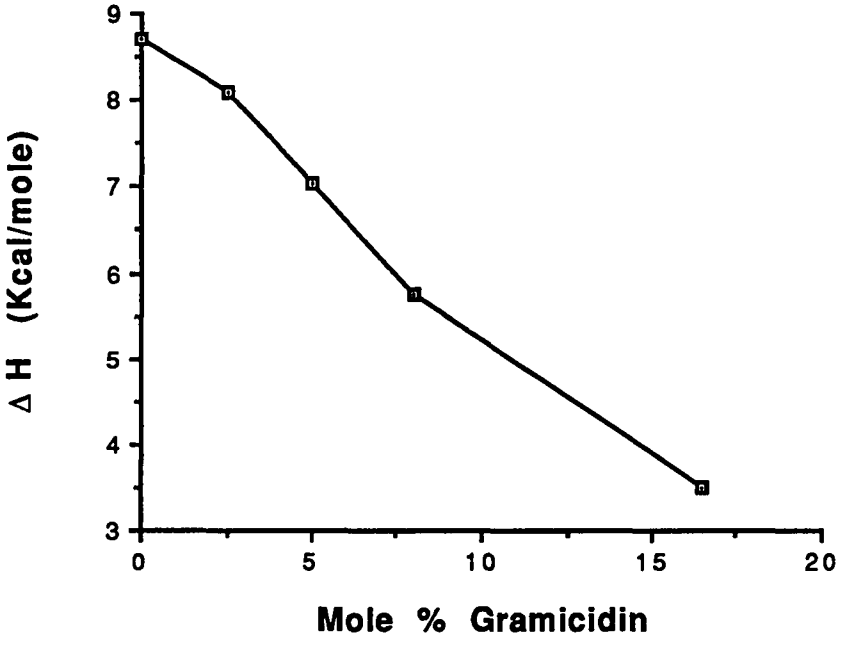

Fig. 5.6 Effect of Gramicidin on lipid transition enthalpy. Obtained from Chapman and Cornell [28]

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 $I \sim 1 \times 10^{-6}$ 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,

$$Q\Delta E = \Delta H_{vH}, \qquad 5.29$$

Eq. 5.28 can be rewritten as

$$I_o (\alpha\lambda) A = (\text{number of clusters promoted}) \Delta H_{vH} \qquad 5.30$$

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 are postulated to depend upon the number and size of fluctuating clusters [16], it may be hypothesized that the enhanced rates in permeations should display an analogous linear functional dependence on the intensity of ultrasound radiation.

Chapter 6

Summary and Concluding Remarks

The results from this investigation have confirmed that low intensity ultrasound absorption and velocity dispersion of modelled biomembranes could adequately be characterized to a two-state system interacting with ultrasound. 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. This coupling is found to be greatest at the constituent lipid T_m. Linear deviations between the absorption findings and the two-state relaxation theory are seen to exist (in Fig. 4.2 and 4.4) at frequencies well above the f_{relax}. Such a behavior could adequately be explained by the possible existence of dissipative mechanisms leading to classical ultrasound absorption within the biomembranes. See Eq. 3.15.

The empirical findings from this study have motivated the "capacitor" and a thermodynamic model. These models are seen to be successful in predicting the relative changes in the frelax parameters of different lipid chain lengths employed in this investigation.

This study has also revealed that a strong correlation between the experimentally determined relaxation frequency temperature dependence with the predicted thermodynamic two-state temperature behavior within the range of $| T - T_m | \leq 2^oC$. See Fig. 5.4. Deviations from the thermodynamic two-state predictions are expected to arise due to the

break down in the assumption used to determine f_{relax} through Eq. 3.18 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's prediction, viz, Eq. 5.19, that an induced cluster (or an equilibrium) fluctuation exhibit 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 equation, viz, Eq. 5.30, it is realized 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 significant role in enhancing ion and drug influx (or efflux) permeation rates, it is postulated that ultrasound would affect these permeation rates. It is hoped that the gain in knowledge and insight acquired from this study may serve as a foundation in understanding and controlling ultrasound induced reversible effects on biological membranes, and in application to the field of medical ultrasound.

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Vita

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