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Effects of divalent cations on the ultrasonic absorption coefficient of negatively charged liposomes (LUV) near their phase transition temperature

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The ultrasonic absorption coefficient per wavelength ($\alpha\lambda$), as a function of temperature and frequency, was determined for large unilamellar vesicles (LUV) in the vicinity of their phospholipid phase transition temperature, using a double crystal acoustic interferometer. (The vesicles were composed of a 4:1 (w/w) mixture of dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG)). It has been found that $\alpha\lambda$ reaches a maximum $(\alpha\lambda)_{max}$ at the phase transition temperature (t_m) of the phospholipids in the bilayer, at an ultrasonic relaxation frequency of 2.1 MHz. Divalent cations (Ca^{2+} and Mg^{2+}), added to LUV suspensions, shifted $(\alpha\lambda)_{max}$ to higher temperatures, dependent upon the concentration of divalent cation. It was also found that the shape of the $\alpha\lambda$ versus t curve was significantly changed, representing changes in the Van't Hoff enthalpy of the transition, and therefore, the cooperative unit of the transition. This suggests that divalent cations interact individually with the negatively charged phospholipid headgroups of DPPG and with DPPC headgroups, thus decreasing the cooperative unit of the transition. The observed upward shift in t_m suggests an interaction that increases the activation energy and, therefore, the temperature of the phase transition. However, $\alpha\lambda$ as a function of frequency did not change with the addition of divalent cations and, thus, the relaxation time of the event responsible for the absorption of ultrasound is not changed by the addition of divalent cations.

Introduction

Ultrasound is often employed in science and technology [1], and is applied as a diagnostic and therapeutic tool in medicine [2,3]. The observed rate of absorption of ultrasound in biological systems is relatively high, is largely determined by the macromolecular content [2], and is not accounted for entirely by theories of classical ultrasound propagation in bulk media [4]. The fate of the absorbed energy as heat dissipation or as contributions to conformational or chemical changes in, for example the cell membrane, is poorly understood [2]. A deeper understanding of ultrasound absorption in biological media could aid in the identification of the events contributing to ultrasonic interaction with the membrane. Liposomes, specifically large unilamellar vesicles (LUVs) in aqueous suspension, have been considered as model cell systems for studying these phenomena [5,6,7]. These vesicles are assumed to be spherical,

Abbreviations: LUV, large unilamellar vesicle; MLV, multilamellar vesicle; SUV, small unilamellar vesicle.

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with diameters in the range of 0.2 to 0.8 μm, and are composed of natural or synthetic phospholipids [5]. The relatively simple structure and composition of these vesicles, and their association with the suspending medium, could aid in identifying the events contributing to ultrasonic interaction with membranes. The LUV system can be made more complex by, for example, incorporating proteins and other biomolecules to model living cells more closely.

Previous studies

Ultrasound has been used to investigate the mechanical and thermodynamic properties of liposomes [5–11]. Direct measurement of the speed of sound and determination of the ultrasonic absorption coefficient per wavelength, $\alpha\lambda$ (the exponential reduction in the sound pressure amplitude as the acoustic wave travels the distance of one wavelength), can be made in the suspensions. The first ultrasound studies involving liposomes were performed on small unilamellar vesicle (SUV) and multilamellar vesicle (MLV) suspensions [9–12], and showed that $\alpha\lambda$ exhibited maxima at certain relaxation frequencies [9–12], presumably representing movement of phospholipids in the vesicle membrane. It was observed with MLVs that $\alpha\lambda$ exhibited a large increase near the phase transition temperature (t_m) of the phospholipids in the vesicle membrane [11,12]. LUVs, which are structurally more similar to biological cells than SUVs or MLVs, also exhibit a large increase in $\alpha\lambda$ (in the 1–5 MHz range of ultrasound) near the phase transition temperature of the vesicle membrane. This peak in absorption of ultrasound is correlated with structural changes in the membrane which lead to dramatic increases in the permeability of LUV membranes in the vicinity of the phase transition temperature. For the membrane composition used in these experiments, this increase in permeability (as measured by the release of tritiated Ara-C), occurs 2°C below the t_m of the phospholipid bilayer [13]. Further ultrasonic studies using LUVs have shown that, at the phase transition temperature of the liposomes, a maximum in $\alpha\lambda$ at 2.1 MHz occurs and represents a relaxation frequency of the medium [5]. Such a frequency dependence is also seen in NMR studies of MLVs, which show correlation times of 10^{-6} to 10^{-8} s for the methylene groups nearest to and furthest from the headgroups [14].

Ultrasonic studies employing LUVs have shown that the incorporation of certain substances into the membrane bilayer could affect the temperature dependence of $\alpha\lambda$, as well as its frequency dependence. Specifically, the incorporation of cholesterol or gramicidin into the phospholipid bilayer broadened the phase transition, possibly by decreasing the cooperativity of the phase transition [5], and the addition of gramicidin also shifted the relaxation frequency of the suspension from 2.1 to 0.75 MHz at t_m [5]. This suggests that certain substances that insert into the membrane are able to affect the event responsible for the absorption of ultrasonic energy.

For the case of the biological cell, the situation of an entire bilayer undergoing a gel to liquid-crystalline phase transition is not seen, as most cell membranes are in the liquid-crystalline state [15]. However, regions of cell membranes, in undergoing compositional and chemical changes at physiological temperature, may still undergo phase transitions [15], so that the study of the lipid bilayer phase transition with liposomes, besides being theoretically challenging, is biologically relevant.

Divalent cations

The importance of divalent cations, especially Ca^{2+}, in biological systems is well known, as many biological processes (e.g., exocytosis and neuronal transmission processes) involve Ca^{2+} interactions with cell membranes [16]. Such Ca^{2+} interactions may involve Ca^{2+}-induced changes in the permeability of the membrane. In LUV suspensions (dipalmitoylphosphatidylcholine (DPPC)/ dipalmitoylphosphatidylglycerol (DPPG) (4:1, w/w ratio)), the addition of Ca^{2+} produced a Ca^{2+} concentration-dependent increase of the temperature at which a significant rise in permeability occurs [13]. That the Ca^{2+} interacts specifically with negatively charged liposome membranes, rather than with neutrally charged liposome membranes, has been supported [16]. We hypothesize that since the phospholipids of vesicle membranes in suspension contribute to the temperature-dependent ultrasound absorption coefficient, the addition of cations to a liposome

suspension should influence that absorption coefficient. The peak of $\alpha\lambda$ (indicative of t_m) may be shifted to a higher temperature, as indicated by permeability studies. If the Ca^{2+} interacts with individual phospholipids, then the cooperativity of the phase transition, as observed by ultrasound, may also be altered, as well as the frequency dependance of $\alpha\lambda$. In this paper, the results of a set of experiments of ultrasound absorption in liposome suspensions, as a function of temperature, frequency, and divalent cation concentration in the liposome preparation, are presented.

Materials and Methods

All liposome preparations were made from mixtures of dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG), in a 4:1 (w/w) DPPC/DPPG ratio. All lipids were obtained from Avanti Biochemical Co. (Birmingham, AL, U.S.A.). The DPPG, whose headgroup has a net negative charge at pH 7.4, is thought to inhibit liposome aggregation and fusion due to surface repulsion of liposomes [7]. Samples of DPPC and DPPG (100 μg) gave single spots on thin-layer chromatograms (silica gel G developed with $CHCl_3/CH_3OH/H_2O$ (64:24:4, v/v) visualized with I_2 vapor). Crystalline 1-β-D-arabinofuranosylcytosine (Ara-C) and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Tritiated cytosine arabinoside, [^{3}H]Ara-C, (64 mCi/mg, 98% pure by thin-layer chromatography) was purchased from Amersham Corporation (Arlington Heights, IL, U.S.A.). Ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA) was purchased from Sigma Chemical Co. Hepes buffer was composed of 10 mM Hepes, 139 mM NaCl, 6 mM KCl, and distilled water, using 10 M NaOH to adjust the pH to 7.4 at room temperature. Liposomes were prepared by the reverse phase evaporation method [17], resulting in distributions of liposomes having an average diameter of 0.19 ± 0.05 (S.E.) μm and ranging in size from 0.06 to 0.25 μm (as determined from electron micrographs of suspensions). The average concentration of phospholipid in the resulting suspensions was 21.0 ± 0.1 mg/ml (S.E.), using the phosphorus assay of Bartlett [18].

Samples of the original suspensions were diluted to 2 mg/ml phospholipid in hepes buffer for the acoustic measurements. This is hereafter referred to as a standard LUV preparation. Approx. 3–4 ml of this solution were used in the acoustic interferometer, although a reservoir of 6–7 ml of the sample solution is attached to the interferometer.

Apparatus

The acoustic interferometer used in this study was based on the design of Labhardt and Schwarz [19] and consists of two identical X-cut quartz transducers (diameter = 1 inch, $f_{fund} = 4.0$ MHz), positioned coaxially and parallel 5.1 mm apart [6], forming the end walls of the measuring cell. One of the transducers is excited electrically at a predetermined CW frequency to transmit ultrasonic waves through the fluid medium. The other transducer receives the sound wave and converts it into an electrical signal. The electrical input to the transmitting transducer is obtained from a synthesized signal generator (HP 8660B, Hewlett-Packard, Palo Alto, CA, U.S.A.), and the stable power drive is maintained by the HP 86601A RF section. The electrical output from the receiving transducer is monitored by a spectrum analyzer (HP 8553B). Ultrasonic intensities of less than 1 $\mu W/cm^2$ were used [7], which is many orders of magnitude below that needed to produce cavitation or a significant temperature increase [7]. The entire system resonates acoustically at certain input signal frequencies. The mechanical quality factor Q of this resonance is related to the acoustic absorption per wavelength by [6]

$$\pi/Q = \pi\Delta f/f_0 = \alpha\lambda$$

where α is the absorption coefficient per unit path length, $\alpha\lambda$ is the absorption per wavelength, λ is the wavelength, Δf is the 3 dB bandwidth of the resonance, viz., the difference in the two frequencies for which the output power of the signal is one-half that at the resonance frequency f_0, and Q is the quality factor $f_0/\Delta f$.

The excess absorption due to the presence of LUVs in the suspension is obtained by subtracting the absorption coefficient of the reference buffer from that of the entire ensemble (reference buffer

plus LUVs). For the situation pertaining herein, for which the suspension acoustic velocity and impedance are virtually the same as that of the reference buffer, diffraction correction is unnecessary [3]. The excess absorption is

$$(\alpha\lambda)_{\text{excess}} = \pi(\Delta f - \Delta f_{\text{ref}})/f_0$$

Procedure

In this study, the excess absorption coefficient of LUV suspensions, with and without divalent cation added, was determined as a function of temperature mainly at the frequency of 3.1 MHz, although a determination of $\alpha\lambda/c$ versus frequency (0.7–5.1 MHz) was also made at the phase transition temperature of the LUV suspension. This was done only at t_m because only at t_m is ultrasound absorbed greatly and at a characteristic frequency. The temperature of the interferometer was maintained to within $\pm 0.05\,^\circ$C during data collection by immersion of the interferometer ensemble in a temperature controlled water bath (Exacal 500 with Endocal 350 refrigeration unit, and DCR-4 temperature Digital Controller, Neslab, Portsmouth, NH, U.S.A.). At least 30 min were allowed for stabilization of the interferometer after each temperature change. The calorimetric transition enthalpy was determined by measuring the area under the $\alpha\lambda$ versus temperature curve and comparing this area with the area of a transition of known calorimetric enthalpy, as determined by differential scanning calorimetry (DSC). It must be noted that this assumes a correlation between the enthalpy of the static thermodynamic transition and that of the ultrasonic transition. The Van't Hoff enthalpy, ΔH_{VH} (cal/mol), of the transition was calculated using the thermal width of the ultrasonic absorption coefficient by [6]

$$\Delta H_{\text{VH}} = 6.9 T_m^2/\Delta T_{1/2}$$

These enthalpy values may be used to determine the cooperative unit of the transition, equal to the ratio of the Van't Hoff enthalpy to the calorimetric enthalpy [5].

Before adding divalent cations to the LUV suspension, $\alpha\lambda$ was determined over the temperature range 37 °C to 45 °C, at 3.1 MHz, and was compared with that of standard LUV suspensions to insure that the suspension consisted of LUVs (as opposed to being qualitatively similar to an SUV or MLV curve). This is the most convenient method to determine the composition of the suspension. The temperature of the specimen was then set to its measured phase transition temperature, t_m, and the fluid in the cell was forced out of the acoustic interferometer and into the attached reservoir, which is also immersed in the temperature bath. 4–40 μl of 250 mM $CaCl_2$ solution were then added to the suspension, resulting in the final sample $CaCl_2$ concentrations of 0.1 mM and 1.0 mM. (Similar amounts of $MgCl_2$ were added to LUV suspensions when later measurements were made in which Mg^{2+} was studied.) The mixture was then allowed to equilibrate thermally for five minutes in the reservoir and to allow complete mixing of the suspension. Through gravity feed, the fluid was allowed (by release of a piston) to return into the cell, and another $\alpha\lambda$ determination, over approximately the same temperature range, was made. Once the t_m values of such suspensions were found, a determination of $\alpha\lambda/c$ versus frequency was made in order to determine the characteristic frequency of the ultrasonic absorption. The addition of such small quantities of (divalent cation) solutions of greater than 300 milliosmolarity is not calculated to increase the osmolarity of the solution outside the liposomes by more than 5%, and thus should not disturb the isosmolarity condition across the lipid bilayers. It is important to note here that other investigators have reported that the addition of divalent cations causes aggregation, and subsequently fusion, of LUVs and that sufficiently large additions of these cations causes membrane disruption [20]. However, except for the 10 mM $MgCl_2$ solution, the concentrations of divalent cations used in this study were below those for which fusion has been observed [20]. Also, some aggregation of the LUVs may have occurred, as evidenced by (1) the observed decrease in $\alpha\lambda$ over the temperature range and (2) the necessity for remixing after the addition of the divalent cations, approximately every six hours (by flushing out the contents of the cell and gravity-feeding the contents back into the cell). Nonetheless, after mixing the observed $\alpha\lambda$ versus temperature curve was still characteristic of

LUVs. After $\alpha\lambda$ was determined, a Ca^{2+} chelator (EGTA) was added to the LUV suspensions containing $CaCl_2$, at the t_m of the suspension. In each case $\alpha\lambda$ as a function of temperature (35°C–45°C) was determined in order to observe the effect of the divalent cation chelator on the system. Data presented below show that, under suitable conditions, the LUVs can be returned to their standard state and this is considered evidence that intact LUVs were employed in the present studies.

The DSC-2 (Perkin-Elmer, St. Louis, MO, U.S.A.) was used to obtain the heat capacity at constant pressure C_p versus temperature on 50 μl samples of LUVs. This is done because the temperature dependence of $\alpha\lambda$ at ultrasonic frequencies near the relaxation frequency in LUV suspensions is qualitatively like that of excess C_p, as measured by DSC, a static thermodynamic technique [2]. It is important to note, however, that DSC measures only the static thermodynamic variables of this system while ultrasound measurements provide information of the relaxation times and, therefore, the kinetics of the system.

The LUV suspensions ranged in phospholipid concentration from 25 mg/ml to 60 mg/ml. A first set of experiments employed $CaCl_2$ and $MgCl_2$ concentrations of 0, 2.5 and 25.0 mM. A second set of DSC measurements involved LUVs with internal aqueous phase of 50 mM EGTA, while maintaining isosmolarity and pH across the bilayer. The latter set of experiments employed $CaCl_2$ concentrations of 0, 2.5, 8.0 and 20 mM. The conditions of isosmolarity across the lipid bilayer, in each of these suspensions, were not violated, as determined by the criteria stated in Materials and Methods.

Results

Effects of $CaCl_2$

Fig. 1 shows the acoustic absorption per wavelength per unit concentration as a function of temperature, for suspensions of LUVs. The curve is that of the standard preparation LUVs. With the addition of $CaCl_2$ to the LUV suspension, the peak is shifted toward higher temperatures, decreases in amplitude, and exhibits broadening. However, the relaxation frequency of the ultrasonic absorption did not change with increasing

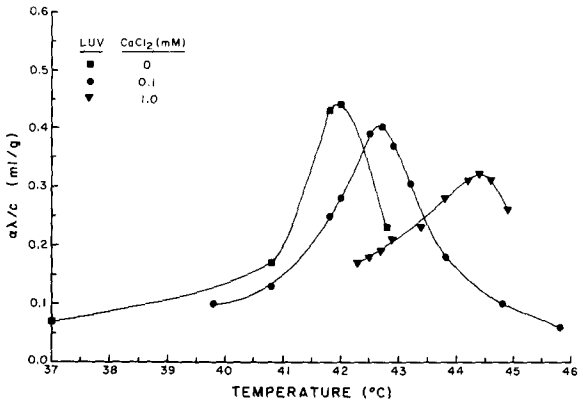

Fig. 1. Effects of $CaCl_2$ (0 mM, 0.1 mM, and 1.0 mM) on $\alpha\lambda/c$ versus temperature.

concentrations of divalent cations. That is, $\alpha\lambda/c$ versus frequency always had a peak at 2.1 MHz even with the addition of $CaCl_2$.

Figs. 3 and 4 show that under suitable conditions, the LUVs can be returned to close to their standard t_m, upon addition of EGTA.

Effects of $MgCl_2$

The addition of $MgCl_2$ to LUV suspensions yields similar results, though with some important exceptions (Fig. 2). The shift seen in t_m as a function of divalent cation concentration was not as great as that for $CaCl_2$ additions. Also, the decrease in amplitude of the $\alpha\lambda/c$ versus temperature peak was much less than for $CaCl_2$ addition. Convenient extrapolations of these curves show

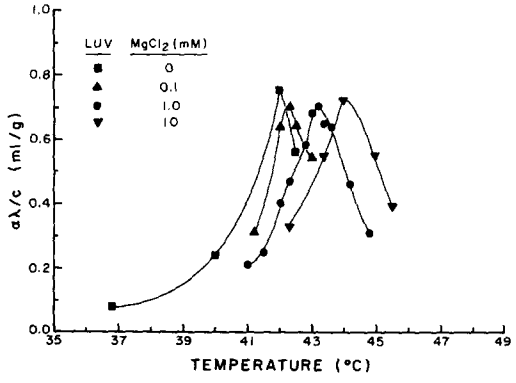

Fig. 2. Effects of $MgCl_2$ (0 mM, 0.1 mM, 1.0 mM, and 10.0 mM) on $\alpha\lambda/c$ versus temperature.

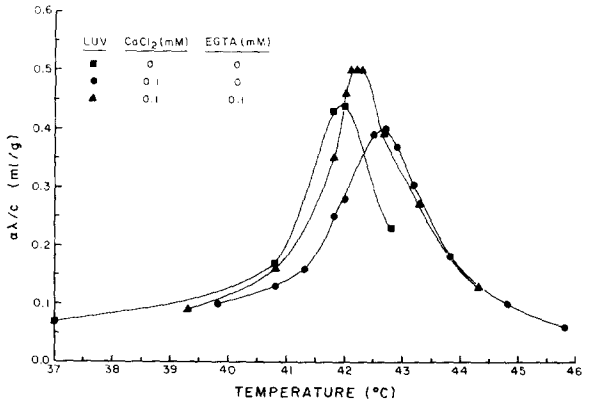

Fig. 3. Addition of EGTA partially offsets the effects of $CaCl_2$ on LUV suspensions (0.1 mM $CaCl_2$, 0.1 mM EGTA).

that the ΔH of the transitions remained approximately the same at $CaCl_2$ concentrations of 0.1 mM and 1.0 mM, as did the ΔH at $MgCl_2$ concentrations of 0.1 mM and 1.0 mM. However, at 10 mM Mg^{2+} concentration, the ΔH of the phase transition does increase (see Table I).

DSC measurements of LUV suspensions with divalent cations

DSC measurements exhibit a peak in the heat capacity of the suspension which appears qualitatively to be similar to the ultrasound results. However, the DSC temperature measurement is done on a time scale or minutes, compared with the time scale of hours required for the ultrasound measurement to be accomplished. This may be of importance in studying the kinetics of Ca^{2+} interaction with the LUV bilayers as is seen in Fig. 5.

While the ultrasound $\alpha\lambda$ versus temperature measurements exhibit a single peak shifting to a higher t_m when divalent cations are added, DSC measurements exhibit two peaks (Fig. 5B). One peak is characteristic of the t_m of standard preparation LUVs, while the other peak has a t_m shifted toward higher temperatures, depending upon the divalent cation concentration in the suspension. Each of these peaks on the first heating is approximately half the magnitude of the peak seen with a normal LUV suspension. However, as successive temperature cycles are conducted on the same sample, the first peak decreases in magnitude, eventually becoming insignificant, and finally leaving only a single peak at the higher t_m (Figs. 5C and 5D). A slower rate of heating the sample may have overcome this effect, as equi-

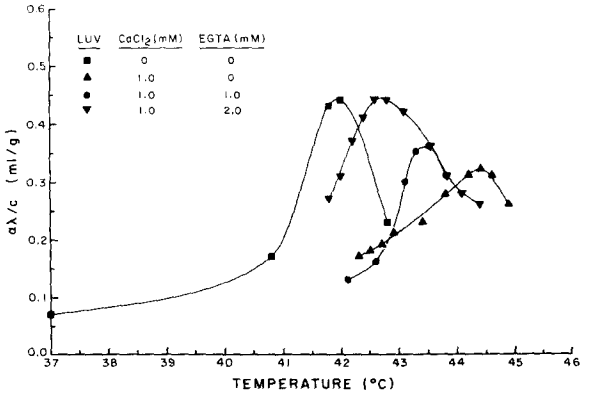

Fig. 4. Addition of EGTA (1.0 mM, 2.0 mM) partially offsets the effects of 1.0 mM $CaCl_2$ on $\alpha\lambda/c$ versus temperature.

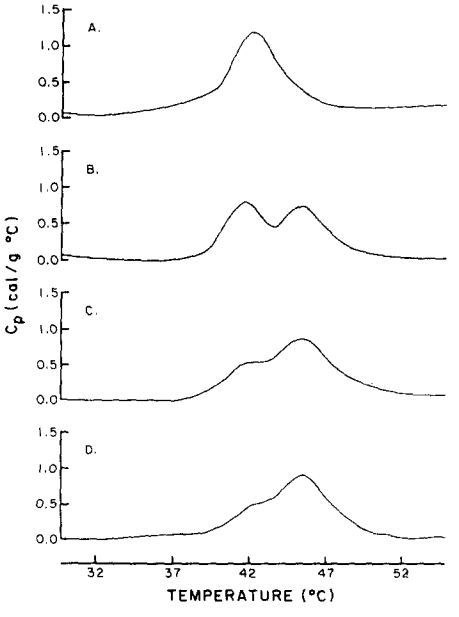

Fig. 5. (A) C_p versus temperature in an LUV suspension. (B) Effects of $CaCl_2$ (2.5 mM) on LUV suspensions, as measured by DSC (Note: two peaks), first heating. (C) The second heating gives a DSC curve where the first peak is reduced in amplitude while the second peak increases in amplitude. (D) The third heating of the sample by DSC exhibits further reduction of the first peak (at the lower temperature) and further increase in amplitude of the peak at higher temperature.

TABLE I

ENTHALPY OF LIPID PHASE TRANSITIONS DETERMINED FROM ULTRASONIC ABSORPTION MEASUREMENTS

The Van't Hoff enthalpy was calculated from the width of the ultrasonic peak at one-half its maximum height. The cooperative unit size was estimated from the ratio of the Van't Hoff enthalpy to the calorimetric enthalpy ΔH_{cal}. Estimates, of error in the measurements are given for each value.

LUV composition DPPC : DPPG (4 : 1, w/w)	0 mM Ca^{2+}	0.1 mM Ca^{2+}	1.0 mM Ca^{2+}	0 mM Mg^{2+}	0.1 mM Mg^{2+}	1.0 mM Mg^{2+}	10.0 mM Mg^{2+}
t_m (°C)	42.0± 0.1	42.7± 0.1	44.0± 0.1	42.0± 0.1	42.3± 0.1	43.2± 0.1	44.0± 0.1
$\Delta t_{1/2}$ (°C)	1.6± 0.2	1.8± 0.2	2.9± 0.2	1.8± 0.2	2.1± 0.2	2.5± 0.2	2.8± 0.2
ΔH_{VH} (kcal/mol)	428 ±21	382 ±19	239 ±12	380 ±19	327 ±16	276 ±14	248 ±12
Cooperative unit size	57 ± 4	51 ± 3	32 ± 2	27 ± 2	23 ± 2	20 ± 1	14 ± 1
ΔH_{cal} (kcal/mol)	7.5± 0.1	7.5± 0.1	7.5± 0.1	13.9± 0.1	13.9± 0.1	13.9± 0.1	18.4± 0.2

libration of Ca^{2+} concentration inside and outside of the LUV bilayer could have occurred. This could indicate that, because the divalent cation is added to the suspension at room temperature for the DSC measurement, the divalent cations first bind to the outer lipid bilayer, and then near t_m, due to increased permeability of the membrane, have access to the inner lipid bilayers of the LUVs as well.

We attempted to demonstrate that this hypothesis should hold for the removal of calcium as well as for its addition. $CaCl_2$ was added to liposomes whose internal aqueous phase consisted of a high concentration of EGTA (50 mM), while still maintaining conditions of isosmolarity and pH across the bilayer. This experiment could also have been done such that calcium was encapsulated in LUVs. However, calcium tends to change many properties of the lipid bilayer, and may at high concentrations even disturb the bilayer's integrity; therefore, we did not make LUVs in this manner.

It was hypothesized that if calcium was added to such a suspension, in concentrations less than or equal to that of the EGTA, that upon a first heating through the transition, two populations of liposomes would be detected by DSC measurement. One population should be bound to the Ca^{2+}, while another population should not. Subsequent heatings should allow for an equilibrium of EGTA and Ca^{2+} across the bilayer, thus diminishing the DSC peak of the bound lipid and increasing the amplitude of the peak at the normal t_m of the unbound lipid. However, our results suggest that this system is more complicated than was originally believed.

The results show that at a 2.5 mM $CaCl_2$ concentration, the EGTA simply overwhelms the system; two distinct peaks never being seen. This suggests that during the first run through the phase transition temperature (t_m), enough EGTA leaks out effectively to chelate the Ca^{2+}, and effect an equilibrium, as subsequent heatings showed no change in the DSC peak.

At an 8.0 mM $CaCl_2$ concentration very different results are seen (Fig. 6). Two peaks are observed, but the peak representing 'bound' lipid (at a higher t_m) is much smaller than the peak representing 'unbound' lipid. This suggests again that EGTA leaks out of the liposomes at the phospholipid phase transition. Upon subsequent heatings however, an effective chelation of the Ca^{2+} by the EGTA is not seen. In fact, the peak representing phospholipid bound to Ca^{2+} becomes increasingly larger. Therefore, it seems that although EGTA may modulate the effects of Ca^{2+}, it does not negate them, and the normal mode of Ca^{2+} affecting the outside and then the inside bilayers of the LUVs still occurs. The same effects are seen with a 20 mM $CaCl_2$ solution. It is important to note that the temperature at which the second peak occurs, representing bound lipid, increases with the concentration of $CaCl_2$.

Therefore, the DSC measurements suggest that

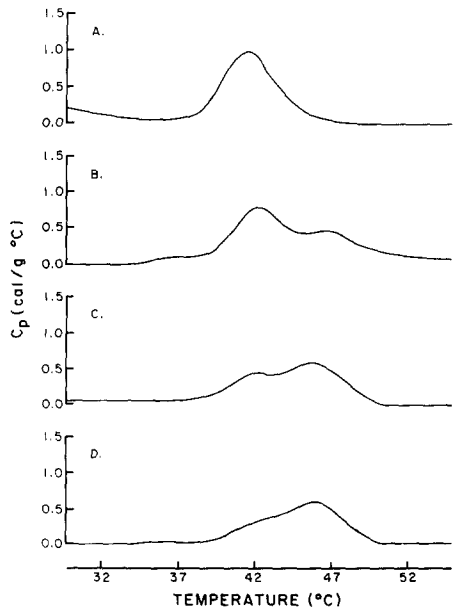

Fig. 6. (A) C_p versus temperature in an LUV suspension whose internal aqueous phase has a 50 mM EGTA concentration. (B) The first heating of the LUV suspension in (A) with 8.0 mM $CaCl_2$, as measured by DSC (Note: two peaks). (C) The fifth heating gives a DSC curve where the first peak is reduced in amplitude while the second peak is increased in amplitude. (Note: the second through fourth heatings indicated the same trend). (D) The seventh heating of the sample by DSC exhibits further reduction of the lower temperature peak, and further increases in amplitude of the higher temperature peak.

Ca^{2+} binding to the membrane may be preferable to its binding with EGTA, and that some rapid equilibration in the system may occur. However, it is not possible to show that the inside/outside effects of Ca^{2+} addition to LUVs can be observed with its removal.

Discussion

The results of this study suggest that the acoustic absorption per wavelength ($\alpha\lambda$), as a function of temperature, in LUV suspensions with added divalent cations was quantitatively different from that seen for standard LUV suspensions, that ultrasound probably interacts with the membrane bilayer, and that ultrasound is sensitive to the divalent cations' interaction with the bilayer. The evidence suggests further that the ultrasound wave is sensitive to movements of the individual phospholipid molecules of the bilayer, since the cooperative unit of the transition, as measured using ultrasound, decreases with increasing divalent cation concentration. However, as the relaxation time of the event responsible for the absorption of ultrasound does not change with the addition of divalent cations, the event absorbing ultrasound energy has not itself been changed, except in the activation energy needed for its occurrence, or more specifically with the activation energy needed for the lipid bilayer phase transition to occur.

The interaction of the divalent cation with the LUVs may, therefore, tentatively be modelled as follows: The divalent cations introduced to the LUV suspensions interact with the negatively charged headgroups of the DPPG molecules and, in some manner as yet undetermined, constrain the movement of DPPG and DPPC molecules, and/or their carbon side chains. While the normal activation energy for the trans to gauche conformational change characteristic of the lipid bilayer phase transition, is 3 kcal/mol [15], the binding of divalent cations to the negatively charged bilayer surface could produce an increase in the activation energy over that characteristic of the phase transition, thus causing the phase transition temperature to be increased. Support for the cations interacting directly with the membrane phospholipids comes from the reduced values of ΔH_{VH}. While the enthalpies, ΔH, of the transitions remain approximately the same for 0.1 mM and 1.0 mM concentrations of the divalent cations, the Van't Hoff enthalpy ($6.9\ T_m^2/\Delta T_{1/2}$), decreases with increasing divalent cation concentration. Therefore, the cooperative unit, $\Delta H_{VH}/\Delta H$, of the transition also decreases as a function of divalent cation concentration. This supports a model of divalent cations interacting with phospholipid headgroups, perhaps even inserting themselves between the headgroups.

The fact that the addition of cation chelators to the suspensions produces a shift of the phase transition temperature back toward that characteristic t_m of LUVs, and reduction in the width of the $\alpha\lambda$ versus temperature, implies that the divalent cation interaction with the negatively charged phospholipid bilayer is not completely irreversible, and that the structure of the LUVs was not altered irreversibly. The DSC measurements suggest that

the binding of the divalent cations to the negatively charged headgroups id dependent upon the temperature at which the cation is added and, thereby, the membrane permeability.

The absorption of ultrasound at a relaxation frequency of 2.1 MHz at t_m indicates a dynamic interaction between the phospholipid bilayer and the ultrasound wave. It is clear that the relaxation frequency is not perturbed by the addition of Ca^{2+}, which interacts with phospholipid headgroups. This indicates that while ultrasonic absorption is sensitive to changes in the lipid bilayer phase transition, the kinetics of its interaction with phospholipid molecules is not directly sensitive to the effects of divalent cations on the bilayer. Therefore, the site of the ultrasonic energy absorption probably is not in the region of the phospholipid headgroups but perhaps lies elsewhere in the bilayer. Such a site may be the phospholipid carbon side chains where, at t_m, the *trans* to *gauche* conformational change occurs, as well as the ultrasonic absorption peak at 2.1 MHz. Support for this exists in which gramicidin, which inserts into the hydrophobic portion of the bilayer, changes the relaxation frequency of the ultrasonic energy absorption [5].

As of yet, the determination of ultrasound interaction with the LUV as a spherical cell, whose volume changes with temperature, has not been satisfactorily separated experimentally from the absorption of the phospholipid molecules. This could be accomplished however, by study of $\alpha\lambda$ as a function of temperature and frequency, using other phases of phospholipid structure. Unfortunately, in certain such phases (H_{II}) hydration of the phospholipid molecules is low [15], and the resulting suspension could not be investigated using the apparatus described herein, although other phases may be more amenable to study. Other aspects to consider in the determination of the nature of ultrasonic absorption by liposomes are certain aspects of movement of the membrane surface, such as molecular rotation, and lateral and transverse diffusion of the phospholipid molecules in the membrane. Dielectric measurements have suggested that rotational relaxation frequencies occur in the 1–5 MHz range [22], while lateral diffusion relaxation frequencies of phospholipids in the bilayer occur in the 2–20 kHz range [22].

The results of this study may also have implications for the use of ultrasound as a probe of the state of biological media. While the ultrasound intensities used in this study are believed to be innocuous regarding the production of reversible or irreversible effects on animals or humans, it is clear that ultrasonic energy is being expended in this system; being dissipated as heat or contributing to conformational or chemical changes of the phospholipids in the cell membrane.

Note added in proof: (Received 9 July 1987)

Recalibration has shown that the ordinate values, C_p, in Figs. 5 and 6 are to be muliplied by 2.86.

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