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ULTRASONIC EVIDENCE FOR STRUCTURAL RELAXATION IN LARGE UNILAMELLAR LIPOSOMES

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The ultrasonic absorption of large unilamellar vesicles (average diameter 0.2 μm) was determined in the frequency range 0.5–5 MHz. The liposomes were composed of a 4:1 mixture by weight of dipalmitoyl phosphatidylcholine and dipalmitoyl phosphatidylglycerol. They were studied with and without cholesterol or gramicidin incorporated into the bilayer. A large increase in absorption occurs at the solid to liquid-crystalline phase transition temperature (42°C) of the pure lipid vesicles. This increase in absorption is interpreted as a structural relaxation of the 'melting' fatty acid chains occurring with an average relaxation time of 76 ns. The liposomes were also found to be extremely permeable near the transition temperature. Essentially complete release of cytosine arabinoside, a small water-soluble molecule, occurred at 42°C. Addition of cholesterol or gramicidin to the bilayer of the liposomes broadened the ultrasonic absorption and reduced the efflux of cytosine arabinoside at the phase transition. No increase in absorption was observed at the transition temperature in the presence of 50 mol% of cholesterol. Gramicidin, in addition to broadening the transition, slows the isomerization of bonds in the hydrocarbon chains of the lipids. A concentration of 5 mol% gramicidin increased the average relaxation time to 211 ns.

Introduction

The physical properties of liposomes have received considerable attention during the past 15 years [1]. Methods used to characterize the structure and thermodynamic parameters of liposomes include differential scanning calorimetry [2], electron paramagnetic resonance, nuclear magnetic resonance [3,4] and ultrasound [5,6]. Ultrasound is well suited for such studies because the propagation velocity and absorption coefficient provide information on the mechanical properties of the suspension. Ultrasound can also be used to probe liposome membrane phase equilibria by the measurement of the absorption coefficient over the appropriate frequency range [6].

Previous ultrasonic studies of liposomes have considered their behavior in the vicinity of the solid to liquid-crystalline phase transition [6–10]. Acoustic absorption measurements on multilamellar vesicles (outer diameter 1–19 μm) and small unilamellar vesicles (outer diameter 20–50 nm) reveal a significant increase in absorption as the transition temperature is approached. The absorption per wavelength shows a temperature dependence for both of these types of liposome which is similar to that observed using differential scanning calorimetry [2]. For example, multilamellar vesicles

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Abbreviations: DPPC, dipalmitoyl phosphatidylcholine; DPPG, dipalmitoyl phosphatidylglycerol.

exhibit a sharp, tall peak in absorption versus temperature with a 1–2deg. C width at half-maximum height, while small unilamellar vesicles have a broad (5–7deg. C), short peak [8]. In addition, the temperatures of maximum acoustic absorption in multilamellar and small unilamellar vesicles suspensions occur at the phospholipid's solid to liquid-crystalline transition temperature (T_m) [8–10].

Large unilamellar vesicles (outer diameter 0.1–0.8 μm), are intermediate in size between small unilamellar and multilamellar vesicles, and appear to be calorimetrically equivalent to multilamellar vesicles [2]. Large unilamellar vesicles provide a more realistic model of natural membranes than small ones or multilamellar vesicles, and may be of use in elucidating mechanisms of ultrasonic absorption in tissues. In the present investigation, ultrasonic absorption measurements were made on large unilamellar vesicles suspensions near T_m. At this temperature the acoustic absorption was found to increase sharply, and the liposomal membrane became leaky to small water-soluble molecules.

Materials and Methods

DPPC and DPPG were purchased from Avanti Biochemical Co. (Birmingham, AL, U.S.A.). Samples of 100 μg produced single spots on thin-layer chromatograms (silica gel G; developed with $CHCl_3/CH_2OH/H_2O$ (64 : 24 : 4), visualized with I_2 vapor). Tritiated cytosine 1-β-D-arabinofuranoside ([^{3}H]cytosine arabinoside) (approx. 64 mCi/mg, 98% pure by TLC) and [^{14}C]DPPC (approx. 156 μCi/mg, 98% pure by TLC) were purchased from Amersham (Arlington Heights, IL, U.S.A.). Cholesterol was purchased from Nu Chek Prep. (Elysian, MN, U.S.A.). Crystalline cytosine arabinoside, Hepes, and gramicidin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The commercial preparation of gramicidin is a mixture of four different peptides known as gramicidins A, B, C and D. Types A, B and C are each composed of 15 amino acids and differ only in the aromatic residue at position 11. Gramicidin D may contain several more amino acids. Commercial gramicidin is composed of 85%A, 9%B, 6%C, and trace amounts of D [11].

Large unilamellar vesicles were prepared by the reverse-phase evaporation process of Szoka and Papahadjopoulos [12], as modified by Magin and Weinstein [13]. Briefly, 100 mg DPPC and 25 mg DPPG were mixed in chloroform and evaporated to dryness on a rotary evaporator (Pope Scientific, Menomonee Falls, WI, U.S.A.). An organic phase of 8 ml isopropyl ether and 4 ml chloroform was added to dissolve the lipids. Then, an aqueous phase containing 300 mM cytosine arabinoside in a 1–10 dilution of 300 mosM Hepes buffer (139 mM NaCl/6 mM KCl/10 mM Hepes; pH 7.4) was heated to 50°C and added to the organic phase. This mixture was placed in a cylindrical bath sonicator (Laboratory Supplies Co., Hicksville, NY, U.S.A.) for 5 min at 45–50°C until it formed a homogeneous emulsion. Then, after transferring the emulsion to a rotary evaporator, the organic phase was slowly drawn off at reduced pressure. At this point, caution had to be exercised because the emulsion foamed extensively. When the foaming was complete, the liposome suspension was kept at 50°C for 30 min and then cooled rapidly by immersion in an ice bath. The liposomes were then completely dialyzed against 300 mosM Hepes buffer at 5°C until use, always within one day. These liposomes captured approx. 25% of the cytosine arabinoside originally present in the aqueous phase. Samples for ultrasonic study were prepared by dilution to 2 mg lipid/ml in degassed Hepes buffer.

For measurement of cytosine arabinoside release, the liposomes were diluted to approx. 10 mM lipid with Hepes buffer. The suspension was transferred to a small glass bottle and placed in a circulating water-bath at 35°C. The temperature of the bath was increased in regular increments of 1°C, allowing 5 min at each temperature. Aliquots (100 μl) were removed at each temperature and placed in 5×20 mm cellulose proportionate centrifuge tubes (Beckman Instruments, Palo Alto, CA, U.S.A.). The tubes were centrifuged for 5 min at $178\,000 \times g$ (Beckman Airfuge) to pellet the liposomes. A 50 μl-sample of the clear supernatant was tested for the presence of [^{3}H]cytosine arabinoside, using a liquid scintillation counter (Beckman LS-7500). The percentage of the total cytosine arabinoside released as a function of temperature was determined from these data. The [^{14}C]DPPC in the supernatant was simultaneously

measured to confirm that all the liposomes were pelleted.

Ultrasonic absorption measurements were made with a cylindrical resonator based on the design of Labhardt and Schwarz [14]. The resonator was excited by a Hewlett-Packard 8660B synthesized signal generator, and the standing wave amplitude was monitored with a Hewlett-Packard 8552A, 8553B spectrum analyzer. In this method, the absorption per wavelength is directly related to the bandwidth of the longitudinal resonances. Excess absorption per wavelength due to the liposomes was calculated as the difference between absorption per wavelength in liposome suspensions and absorption per wavelength in the Hepes buffer. A constant temperature was obtained by immersing the resonator in a Neslab Exocal 500 water-bath, and allowing at least 30 min for stabilization of the resonator temperature after each temperature change.

The absorption parameter determined from these measurements is the specific absorption per wavelength, denoted as $\alpha\lambda/c$. It expresses the exponential reduction in the pressure amplitude as the acoustic wave travels through a distance of one wavelength, divided by the lipid concentration in grams per milliliter. When plotted against frequency, the excess specific absorption per wavelength for a single relaxation process exhibits a maximum at the relaxation frequency.

Results

Absorption measurements at 4 MHz on large unilamellar vesicles as a function of temperature are shown in Fig. 1. The specific absorption per wavelength for liposomes composed of DPPC/DPPG (4 : 1 weight ratio) exhibits a peak at 42°C, the transition temperature for these lipids. At temperatures 5deg. C away from the transition, the ultrasonic absorption by the liposome suspension was only slightly greater than that by the Hepes buffer. The effect of cholesterol is also shown in Fig. 1. The addition of 20 mol% cholesterol greatly broadened the absorption peak and reduced its amplitude. At 50 mol% cholesterol, all evidence of a transition had disappeared. The effect, on the acoustic absorption, of incorporation of gramicidin into the bilayer is similar to that of cholesterol.

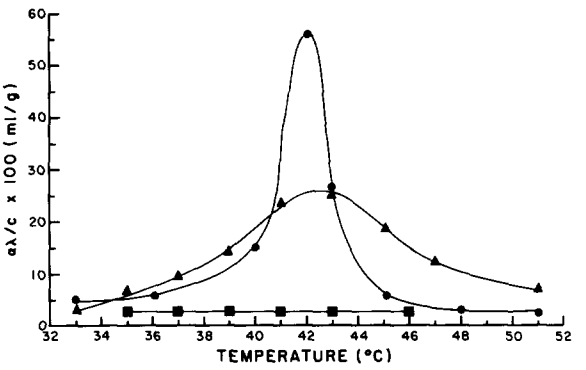

Fig. 1. Specific absorption per wavelength at 4 MHz as a function of temperature. Liposomes in suspension were composed of DPPC/DPPG (4:1, w/w), with no cholesterol (●), with 20 mol% cholesterol (▲), and with 50 mol% cholesterol (■).

As shown in Fig. 2, for gramicidin concentrations of 5 and 10 mol%, the absorption peak at transition was broadened in comparison with that of large unilamellar vesicles composed only of phospholipids.

The release of cytosine arabinoside, a small water-soluble molecule (M_r 243), from liposomes of various compositions is shown in Fig. 3. The leakage of cytosine arabinoside occurs almost entirely within 1°C of the transition temperature for liposomes composed of DPPC/DPPg (4 : 1 weight ratio). Addition of 20 mol% cholesterol to the liposomes resulted in significant release of cytosine arabinoside well below T_m. Unlike the pure phos-

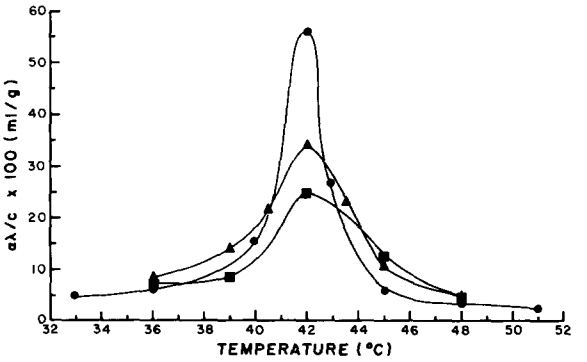

Fig. 2. Specific absorption per wavelength at 4 MHz as a function of temperature. Liposomes in suspension were composed of DPPC/DPPG (4:1, w/w) with no gramicidin (●), with 5 mol% gramicidin (▲), and with 10 mol% gramicidin (■).

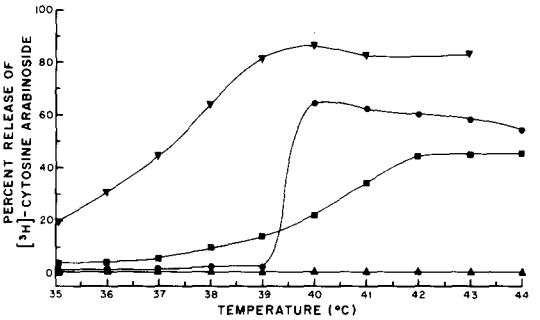

Fig. 3. Release of [^{3}H]cytosine arabinoside from liposomes as a function of temperature. Liposomes in suspension were composed of DPPC/DPPG (4:1 w/w) with no cholesterol (●), with 20 mol% cholesterol (■), with 50 mol% cholesterol (▲) and with 5 mol% gramicidin (▼). Each preparation was heated in a stepwise manner over the indicated temperature range with 5 min periods at each sampling point.

pholipid preparation, this leakage extends over a 4deg. C range in temperature. No substantial leakage of cytosine arabinoside was seen from liposomes containing 50 mol% cholesterol between 35 and 45°C. Addition of 5 mol% gramicidin also decreases the initial temperature at which cytosine arabinoside is released, and increases the temperature release range.

The frequency dependence of the specific absorption per wavelength in (DPPC/DPPG) large unilamellar vesicles is shown in Fig. 4. A large change in absorption versus frequency is seen only near T_m. At other temperatures further from T_m, $\alpha\lambda/c$ is smaller and does not change with frequency. Similar behavior was observed in liposomes containing cholesterol or gramicidin, with the exception of those incorporating 50 mol% cholesterol, which exhibited no frequency dependence at any temperature. The abrupt change in frequency dependence near the transition temperature suggests the presence of a relaxation process occurring at lower frequencies. Therefore, the ultrasonic absorption was measured over an extended frequency range at 42°C, and the results for DPPC/DPPG and 5 mol% gramicidin large

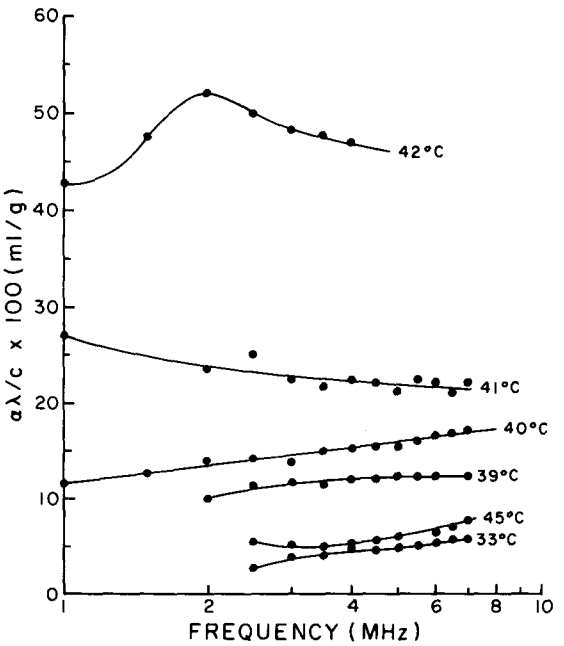

Fig. 4. Specific absorption per wavelength as a function of frequency at various temperatures in DPPC/DPPG (4:1, w/w) liposome suspensions. Temperatures are indicated in °C.

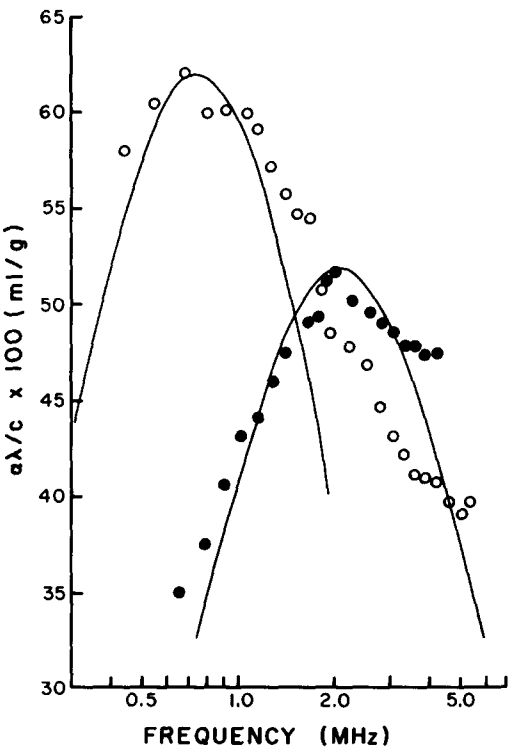

Fig. 5. Specific absorption per wavelength as a function of frequency at 42°C. Liposomes in suspension were composed of DPPC/DPPG (4:1, w/w) with no gramicidin (●) and with 5 mol% gramicidin (○). Solid curves are based on theoretical single relaxation process of Eqn. 1.

unilamellar vesicles are shown in Fig. 5. Each suspension exhibited a peak in specific absorption per wavelength, the DPPC/DPPG large unilamellar vesicles at 2.1 MHz and the 5 mol% gramicidin large unilamellar vesicles at 0.75 MHz. Also drawn on this figure are solid curves corresponding to the theoretical absorption due to a single relaxation process.

Discussion

Heating liposomes composed of well-defined mixtures of pure phospholipids causes an order-disorder phase transition [2]. This transition involves a change in the planar bilayer packing of the lipids and the polar headgroup organization, as well as a type of melting of the fatty acyl side-chains. During the melting, the all-*trans* low temperature structure of the fatty acyl side-chains is replaced by one with several *gauche* bonds in the hydrocarbon chain. The ultrasonic absorption shown in Figs. 1 and 2 appears to be a maximum at this structural phase transition. This conclusion is supported by several facts. First, for these lipids there is a coincidence of the temperature of maximum absorption with the temperature of the transition (42°C) as measured by differential scanning calorimetry [8]. Second, inclusion of cholesterol reduces the magnitude of the ultrasonic absorption in a manner similar to its effect when the phase transition is studied calorimetrically. Third, there is a correspondence between the increase in ultrasonic absorption and the leakage of cytosine arabinoside from the liposomes near this temperature [13].

The cholesterol molecule is thought to insert into the bilayer and associate with two phospholipid molecules [15]. Calorimetric measurements show that as the amount of cholesterol in the bilayer is increased, the enthalpy of the transition decreases in a nearly linear fashion, reaching zero at a mole fraction of one third. The results shown in Fig. 1 agree with this description of cholesterol action. That is, cholesterol at 20 mol% diminishes the specific absorption at 42°C, and eliminates it entirely at 50 mol%. In addition, the effect of cholesterol on the ultrasonic absorption is very similar to its effect on cytosine arabinoside leakage. Based on this analysis, it is concluded that the increase in ultrasonic absorption near the transition temperature is due to an interaction between the ultrasonic wave and the lipid bilayer structural phase change. Previous ultrasonic studies of multilamellar and small unilamellar vesicles have reported similar results [8–10].

The effect of gramicidin on the ultrasonic absorption of liposomes is very similar to that of cholesterol. In other studies of liposomes, both compounds reduced the enthalpy of transition observed by differential scanning calorimetry [16]. Gramicidin, composed primarily of non-polar amino acids, inserts readily into a lipid bilayer, and an association is made between the gramicidin and the hydrophobic chains of nearby phospholipids [17]. In DPPC liposomes, each gramicidin molecule associates with approximately six phospholipids, whereas cholesterol involves only two phospholipids. Incorporation of gramicidin also decreases the enthalpy of transition, but this appears at a lower molar concentration than with cholesterol [17]. The data of Fig. 2 are in qualitative agreement with these calorimetric results. Also, the effect of gramicidin incorporation on leakage of cytosine arabinoside essentially parallels its effect on the ultrasonic absorption. Presumably, gramicidin restricts the configurational freedom of adjacent phospholipids and thereby reduces the number of lipid molecules effectively participating in the phase transition.

This analysis can be established on a more quantitative basis by using the width of the ultrasonic absorption to calculate the Van 't Hoff enthalpy of transition (ΔH_v) for different liposome compositions. The values for these parameters are given in Table I using the assumptions of a one-step relaxation process and a temperature-independent relaxation frequency. Harkness and White [8] have interpreted such enthalpy values as measures of cooperativity between lipid molecules during the phase transition. The number of lipid molecules undergoing the transition as a unit can be estimated from the ratio of the Van 't Hoff enthalpy to the measured calorimetric enthalpy. Thus, according to this analysis, addition of cholesterol or gramicidin decreases the amount of cooperativity in the phase transition of the liposomes. The approximate cooperative unit sizes are 47 for DPPC/DPPG, 12 for 20 mol% cholesterol, 22 for

TABLE I

ENTHALPY OF LIPID PHASE TRANSITION 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 (8.6 kcal/mol) for the solid to liquid-crystalline DPPC transition. Estimates of the error in these measurements are given for each value.

Liposome composition DPPC/DPPG (4:1, w/w)	$t_{1/2}$ (°C)	ΔH_v (kcal/mol)	Cooperative unit size
5% Cholesterol	1.7±0.1	400±25	47±3
20% Cholesterol	6.8±0.3	100± 5	12±1
5% Gramicidin	3.6±0.2	190±10	22±1
10% Gramicidin	4.1±0.2	167± 8	19±1

5 mol% gramicidin, and 19 for 10 mol% gramicidin liposomes.

The ultrasonic absorption observed at the phase transition in liposomes in this study can be modeled as a relaxation process [8]. In such a model an equilibrium is assumed to exist that can be perturbed by the rapid change in pressure which characterizes an ultrasonic wave. Energy is absorbed from the wave as the perturbed equilibrium fails to respond in phase with the ultrasonic pressure changes. For a system exhibiting a single relaxation mechanism, the frequency dependence of the excess absorption per wavelength gives the relaxation time, τ, according to the equation:

$$(\alpha\lambda)_{\text{excess}} = A\frac{f/f_r}{1+(f/f_r)^2} \quad (1)$$

where $\tau = 1/2\pi f_r$, f is the ultrasonic frequency, A is a constant, and f_r is the relaxation frequency. Eqn. 1 exhibits a maximum at $f = f_r$.

Referring to Fig. 4, it is clear that no large amplitude relaxations occur at temperatures well away from T_m within the frequency range of this study. However, at the transition temperature, a relaxational absorption appears. This behavior is resolved further in Fig. 5. Theoretical absorption curves based on Eqn. 1 are shown in Fig. 5 for the pure lipid preparation and for the 5 mol% gramicidin preparation. In both cases, the theoretical absorption curve qualitatively describes the experimental data, but the measured values fall on broader curves than predicted by the single relaxation theory. This suggests that more than one relaxation is occurring.

The equilibrium between the *trans* and *gauche* states of bonds in the phospholipid hydrocarbon chains is probably responsible for relaxations shown in Fig. 5. This configurational change in the hydrocarbon chains is the primary event occurring at 43°C in DPPC [2]. A theory developed by Landau and Khalatnikov [18] can be used to describe the frequency dependence of the ultrasonic absorption in terms of a structural relaxation. This theory predicts that the relaxation time should increase sharply in the vicinity of the transition points [9]. This effect, combined with a much greater probability of such conversions near the transition temperature, may account for the frequency and temperature dependence of the specific absorption per wavelength. At temperatures well away from the transition, relaxations that do exist would occur at frequencies too high for observation by the present experimental system.

Curves of ultrasonic absorption versus freqency similar to those in Fig. 4 were reported by Harkness and White [8] for multilamellar and small unilamellar vesicles. While they demonstrated no relaxation in the frequency range 0.4–4 MHz for either type of liposome, there is other evidence that a relaxation may take place in multilamellar vesicles below 3 MHz (White, R., personal communication). A variety of results have been obtained on small unilamellar vesicles. Sano et al. [10] found a relaxation occurring at T_m and centered at 7.2 MHz. Contrarily, Gamble and

Schimmel [7] have reported a relaxation frequency of 16 MHz. The discrepancy among the results of these investigations of small unilamellar and multilamellar vesicles, we believe, is due to differences in the size, structure, and preparation conditions of the liposome samples and illustrates the critical nature of these parameters. These factors are known to have profound effects on the calorimetric properties of liposome suspensions [2] which, as mentioned earlier, parallel the ultrasonic changes.

As this investigation is the only one involving large unilamellar vesicle, direct comparison of data between studies is not possible. While Sano et al. [10] employed liposomes as large as 90 nm in diameter, these are still smaller than the large unilamellar vesicles studied here by almost a factor of 2. Thus it is possible that while these investigators found no dependence of relaxation frequency on size, a strong dependence may exist in the intervening size range. Also, our investigation involved a mixture of phospholipid head groups, while those of other studies have been of a single type. Such a mixture of head groups may also have a significant effect on the relaxation behavior of the liposomes.

The results presented here suggest that large unilamellar vesicles behave more like multilamellar than like small unilamellar vesicles. Differential scanning calorimeter measurements support such a conclusion. They show that small unilamellar vesicles have an approx. 4–6deg. C decrease in T_m compared to multilamellar vesicles of the same composition. In addition, Nagle [19] has listed NMR correlation times of between 10^{-6} and 10^{-8} s, respectively, for the methylene groups nearest and furthest from the headgroup in multilamellar vesicles. Similar NMR measurements on small unilamellar vesicles indicate shorter correlation times. These properties of small unilamellar vesicles are probable due to their small radius, which leads to less efficient hydrocarbon packing and greater fluidity. It is expected, therefore, that a structural relaxation process should occur at different rates in large and small unilamellar vesicles. From the results presented here, the relaxation process in large unilamellar vesicles occurs more slowly than that in small ones.

Finally, it is clear from Fig. 5 that gramicidin has a significant effect on the relaxation characteristics of large unilamellar vesicles at their transition temperature. The most significant change from the pure lipid specimen is a decrease in the average relaxation frequency from 2 to 0.75 MHz. This represents a slowing of the reaction responsible for the relaxational absorption. Such an effect by gramicidin might be expected on the basis of its largely hydrophobic interaction with the bilayer [17]. Through these interactions the gramicidin could restrict the motion of hydrocarbon chains of nearby phospholipids. The result of this restriction is an increase in the relaxation time (decrease in the relaxation frequency), as observed by ultrasonic absorption. Since two gramicidin molecules in an end-to-end arrangement can form a pore in the membrane bilayer through which small cations can pass [20], it may be possible for ultrasound to perturb this process and thus affect ion fluxes through membranes.

Acknowledgements

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