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The effects of ^2H_2O on the phase transition of large unilamellar vesicle (LUV) suspensions as detected by ultrasound spectroscopy and electron spin resonance

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The importance of water in the molecular dynamics of large unilamellar vesicle (LUV) suspensions, in which increasing portions of the water were replaced by ^2H_2O, was investigated. Determinations of the ultrasonic absorption coefficient per wavelength, $\alpha\lambda$, were performed as a function of temperature and frequency for LUVs (LUVs: 4:1 (w/w) mixture of dipalmitoylphosphatidylcholine, DPPC, and dipalmitoylphosphatidylglycerol, DPPG) in the vicinity of their phospholipid phase transition, using a double crystal acoustic interferometer. Electron spin resonance (ESR) and differential scanning calorimetry (DSC) were also employed to probe this system. When increasing portions of the aqueous content of the LUV suspensions were replaced by ^2H_2O the phase transition temperature increased from 42.0 °C to 42.9 °C (indicating an increase in the activation energy of the transition), and the amplitude of $\alpha\lambda$ at the phase transition increased. However, $\alpha\lambda_{max}$ as a function of frequency at the phase transition did not change with the addition of ^2H_2O, indicating that the relaxation time of the event responsible for the absorption of ultrasound was unaffected. The increase in the activation energy of the transition with the addition of ^2H_2O suggested that the mobility of phospholipids near the membrane/aqueous interface was changed. Electron spin resonance (ESR) experiments on LUVs with nitroxide spin probes positioned at the membrane/aqueous interface (5-doxyl stearate and CAT_{16}) showed that LUVs in ^2H_2O have a broader splitting, A_{max}, at the membrane/aqueous interface than do LUVs in H_2O. These results suggest that ^2H_2O changes the mobility and/or structure of the phospholipids in the region of the membrane/aqueous interface. This difference in A_{max} was not seen for the probe PC-12-doxyl stearate, which resides at the C-12 position of the bilayer.

Introduction

Ultrasound is a useful diagnostic and therapeutic tool in medicine [1,2]. However, the observed rate of ultrasonic absorption in biological systems is relatively high and is not accounted for entirely by theories of classical ultrasound propagation in bulk media [3]. It is therefore important to understand the mechanisms of ultrasonic absorption in biological systems to determine the safety of diagnostic and therapeutic ultrasound. In addition, ultrasound may be used as a probe of molecular, cellular, and cell membrane events. Ultrasound can make unique contributions to the study of biological membranes because it provides information about the mechanical and thermodynamic properties of the cell membrane. Direct measurement of the speed of sound and determination of the ultrasonic absorption coefficient per wavelength, $\alpha\lambda$, (defined as the exponential reduction in the sound pressure amplitude as the acoustic wave travels the distance of one wavelength) can be made in biological suspensions and may be analyzed to investigate mechanisms of absorption of ultrasound in such suspensions. As biological membranes are complex, the understanding of ultrasonic interaction with such membranes may be conveniently investigated by using models of cell membranes. Liposomes, including MLVs (multilamellar vesicles), SUVs (small unilamellar vesicles) and especially LUVs (large unilamellar vesicles), in aqueous suspension have been considered as model cell systems for studying these phenomena [4,5,6]. Large unilamellar vesicles, formed by the reverse

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phase evaporation method [7] are assumed to be spherical, with diameters in the range of 0.2 to 0.8 μm and are composed of natural or synthetic phospholipids [4]. 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.

Previous studies

Ultrasound has been used to investigate the mechanical and thermodynamic properties of liposomes [4–6,8–11]. Previous ultrasound studies involving liposomes showed that $\alpha\lambda$ exhibited maxima at specific relaxation frequencies [9–11]. The relaxational absorption of ultrasound in the vesicle suspensions is presumably due to ultrasound coupling to movement of phospholipids in the vesicle membrane. In MLV and LUV suspensions, $\alpha\lambda$ exibited a large increase near the phase transition temperature (T_m) of the phospholipids in the vesicle membrane [11,12]. In LUV suspensions this peak in $\alpha\lambda$ (in the 1–5 MHz range) 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. Further ultrasonic studies using LUVs composed of DPPC/DPPG (4 : 1, w/w) have shown that, at the phase transition temperature of the LUVs, a maximum in $\alpha\lambda$ at 2.1 MHz occurs, probably identifying a relaxation frequency of the medium [4].

In this study ^2H_2O was used to replace H_2O in the aqueous buffer (in specified proportions) of LUV suspensions in order to investigate the importance of water in the interaction(s) of ultrasound with LUV membranes. ^2H_2O was used as a specific variation on water structure (see Table I) in order to determine the sensitivity of ultrasonic absorption to perturbations of the membrane/water interface.

In order to study ^2H_2O perturbation of LUV suspensions with a static thermodynamic measure, differential scanning calorimetry (DSC) was employed. DSC was performed because the temperature dependence of $\alpha\lambda$ at ultrasonic frequencies near the relaxation frequency in LUV suspensions is qualitatively like that of excess specific heat, C_p, as determined by DSC. It is important to note however, that DSC measures only the static thermodynamic variables of this system while ultrasound measurements provide information on the relaxation times and therefore the kinetics of the system under study.

Electron spin resonance (ESR) was used as an additional technique to probe changes in the membrane caused by ^2H_2O. ESR spin probes, such as nitroxides attached to a specific carbon in a stearate molecule, can provide information about the membrane structure and mobility at different depths [13]. The spectral features of different nitroxides were used here to probe differences in LUV membranes in the phospholipid headgroup region, at the water-hydrocarbon interface and deep inside the hydrocarbon region.

TABLE I

Physical properties of H_2O and ^2H_2O

	H_2O	^2H_2O	
Molecular mass (daltons)	18.016	20.028	[20]
C_p (cal/g) (4–25 °C)	1.000	1.028	[20]
H_f (kcal/mol)	1.436	1.501	[20]
Dielectric constant	78.39	78.06	[20]
m.p. (°C)	0.0	3.82	[20]
b.p. (°C)	100.0	101.42	[20]
Critical temperature (°C)	374.2	371.5	[20]
Density at 25 °C (g/ml)	0.997	1.1044	[20]
Velocity of sound (m/s) at 20 °C	1482.9	1384.2	[24]
Velocity sound (m/s) at 40 °C	1529.3	1433.1	[24]
Index of refraction	1.333	1.3384	[20]
Dipole moment (deBye)	1.76	1.78	[20]
Viscosity (25 °C) (cP)	0.8903	1.107	[25]

Materials and Methods

All standard 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 Polar Lipids (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 [6]. 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 Company. Hepes-buffered saline (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. Deuterium oxide (99.8 mol%) was purchased from Sigma Chemical Company. Nitroxides, 5- and 12-DS (4,4-dimethyloxazolidine-*N*-oxyl derivative of 5- and 12-ketostearic acid); and CAT_{16} 4-(*N*,*N*-dimethyl-*N*-hexadecyl)amino-2,2,6,6-tetramethylpiperidine-*N*-oxyl were purchased from Molecular Probes (Junction City, OR, U.S.A.) and PC-12-DS (1-palmitoyl-2-(12-doxyl stearoyl)phosphatidylcholine) was purchased from Avanti Polar Lipids. Fig. 1 shows the structures of these molecules.

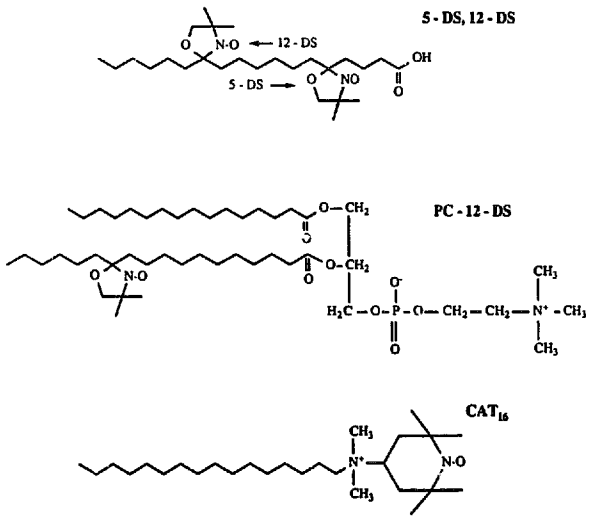

Fig. 1. The structures of the nitroxide-labelled molecules used in this study: 5-DS, 4,4-dimethyloxazolidine-*N*-oxyl derivative of 5-keto-stearic acid; PC-12-DS, 1-palmitoyl-2-(12-doxyl stearoyl)phosphatidylcholine; CAT_{16}, 4-(*N*,*N*-dimethyl-*N*-hexadecyl)amino-2,2,6,6-tetramethylpiperidine-*N*-oxyl.

Liposome preparation

Standard large unilamellar vesicle (LUV) preparations were made from mixtures of DPPC/DPPG in a 4:1 (w/w) ratio, and were prepared by the reverse phase evaporation method based on the work of Szoka and Papahadjopoulos [7]. Dry lipids (100 mg DPPC, 25 mg DPPG) were dissolved in an organic solution of 8 ml isopropyl ether and 4 ml chloroform. At a temperature (50 °C) well above the T_m of 42.0 °C, 4 ml of the buffer dilution (30 mosM Hepes buffer adjusted to 300 mosM with Ara-C) were added. The inclusion of Ara-C resulted in a larger population of LUVs with a narrower size distribution than the same LUVs prepared in Hepes buffer [14]. The mixture was then sealed under N_2 gas and sonicated for approx. 2 min, forming an emulsion. The emulsion was transferred to a rotary evaporator, where reverse phase evaporation, i.e., preferential removal of the organic phase, took place, with vigorous foaming and venting. The aqueous phase which evaporated was then replaced to result in 5.0 ml final volume, and the suspension was dialyzed at approx. 9 °C. This process results in the production of distributions of liposomes having an average diameter of 0.21 ± 0.01 μm (S.E.) and ranging in size from 0.10 to 0.43 μm, as determined from electron micrograph preparations of suspensions. The average concentration of phospholipid in the preparation was 25.0 ± 0.1 mg/ml (S.E.), using the Bartlett phosphorus assay [15]. Samples of the original suspensions were diluted to 2 mg/ml phospholipid in buffer for acoustic measurements. This is referred to as a standard LUV suspension. The only difference in the preparation of ^2H_2O LUV suspensions as compared to standard LUV suspensions is that the H_2O in the aqueous component of the suspension was replaced with deuterium oxide. Electron micrographs showed no significant difference in the size distribution of these two LUV populations. In the subsequent dilution to 2 mg/ml, varying ratios of $^2H_2O/H_2O$ were used.

Apparatus

The acoustic interferometer used in this study is based on the design of Labhardt and Schwarz [16] and consists of two identical X-cut quartz transducers (diameter = 1 inch, fundamental frequency, $f_{fund} = 4.0$ MHz), positioned coaxially and parallel 5.5 mm apart 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 85521, HP 8553B). Ultrasonic intensities of less than 1 $\mu W/cm^2$ are used, which is many orders of magnitude below that needed to produce cavitation or a significant temperature increase [6]. 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 [5]:

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

where α is the amplitude absorption coefficient per unit path length, $\alpha\lambda$ is the absorption per wavelength, λ, and 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 this situation, in which the acoustic velocity and impedance of the suspension are virtually the same as that of the reference buffer, diffraction correction is unnecessary [2]. The excess absorption is:

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

ESR measurements were performed at X-band (9.5 GHz) on a Varian E-109 ESR spectrometer. The temperature of the sample was controlled by a Varian gas flow system using nitrogen. Spectra were recorded in a standard first derivative mode with 100 kHz modulation and a microwave power of 5 mW. Data were collected and stored as arrays of 1024 points per spectrum with a

PC computer [17]. The hyperfine splittings, i.e. the positions of the peaks were determined by expanding the spectral feature of interest using the computer (estimated error ± 0.3 G for 100 G scans).

Ultrasound measurement procedure

LUV suspensions were made with varying percentages of H_2O in the aqueous portion replaced by ^2H_2O. With each of the mixtures, ^2H_2O constituted (molar volume) 0, 20, 50, 80, or 100% of the aqueous portion of the suspension. In these experiments LUVs were prepared in ^2H_2O or H_2O buffer and were subsequently diluted into mixtures of ^2H_2O and H_2O buffer to give the final $^2H_2O/H_2O$ ratios. The results for these experiments depended only upon the final $^2H_2O/H_2O$ ratios, and were not significantly affected by the solvent used (^2H_2O or H_2O buffer) for LUV preparation. The excess absorption coefficients, $\alpha\lambda/c$, (c = concentration in g/ml; estimated $\alpha\lambda/c$ error = 0.01 ml/g) of these LUV suspensions were determined as a function of temperature and frequency. The temperature was varied from 35°C to 46°C, while at each temperature the measurement frequency was varied from 0.64 to 5.2 MHz.

The temperature of the interferometer was maintained to within ±0.05 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 the thermal stabilization of the interferometer after each temperature change. During the experiment remixing was performed to ensure that the LUV suspension was not experiencing liposome aggregation or fusion. Settling was not observed, as evidenced by the lack of change in $\alpha\lambda/c$ over time at any one temperature, and the lack of change in $\alpha\lambda/c$ after remixing of the suspension.

The calorimetric transition enthalpy (ΔH_{cal}) was determined by comparing the area of the $\alpha\lambda/c$ versus temperature curve with the area under a standard LUV $\alpha\lambda/c$ curve, whose calorimetric enthalpy had been determined by differential scanning calorimetry (DSC). The Van't Hoff enthalpy ΔH_{VH} (cal/mol) of the transition may be calculated using the thermal width of the ultrasonic absorption coefficient from [6]:

$$\Delta H_{VH} = 6.0\, T_m^2/\Delta T_{1/2}$$

These enthalpies may be used to determine the cooperative unit of the transition $\Delta H_{VH}/\Delta H_{cal}$ [4].

DSC procedure

The DSC-2 (Perkin-Elmer, St. Louis, MO, U.S.A.) was used to obtain the specific heat at constant pressure, C_p, versus temperature on 50 μl samples of LUV suspensions. The LUV suspensions in this study were 25.0 mg/ml phospholipid and the temperature range studied was 22°C to 52°C.

ESR procedure

In order to study more closely the dynamic interaction of ^2H_2O in LUV suspensions with the phospholipid bilayer, LUVs made in ^2H_2O (where ^2H_2O is the only aqueous component of the buffer solution) were investigated with ESR spin label probes. Lipid soluble nitroxides (5-DS, PC-12-DS, 12-DS or CAT_{16}) were added to the ^2H_2O LUV suspensions at a phospholipid : probe ratio of 100 : 1 to avoid spin-spin broadening. This was achieved by drying the probe on the bottom and sides of a culture tube (with N_2 gas), and then adding the freshly prepared liposomes and vortexing to ensure complete mixing. The probe partitioned into the organic phase of the LUV suspension, as was seen in the observed ESR signal, indicative of a probe incorporated into the hydrophobic phospholipid bilayer [13,18]. The resulting LUV suspensions were then separated into two groups. One group was kept stored under nitrogen (these are referred to as ^2H_2O LUVs). The second group was dialyzed against ordinary buffer to replace the ^2H_2O with H_2O (referred to as H_2O (LUVs). Determinations of the anisotropic maximal splitting, A_{max}, (see Fig. 7) as a measure of nitroxide mobility were made both as a function of aqueous composition (H_2O versus ^2H_2O in the buffer) and temperature from 20°C to 48°C.

Results

Ultrasound and DSC results

Differential scanning calorimetry measurements of LUV suspensions prepared in 95–100% ^2H_2O buffer showed a thermotropic phase transition qualitatively similar to that of standard LUV suspensions, with the exception that the phase transition temperature was 43°C, compared to 42°C for standard LUVs, as seen in Fig. 2.

The results of ultrasound measurements in LUV suspensions with varying percentages of ^2H_2O buffer (where H_2O buffer is replaced with ^2H_2O buffer) are given in Table II. Some interesting points were noted. For an LUV suspension whose aqueous component consisted of 95–100% ^2H_2O (Fig. 3), the $\alpha\lambda/c$ versus temperature curve exhibited a shape qualitatively similar to that of a standard LUV curve, with a sharp peak of $\Delta T_{1/2}$ of 1.8 ± 0.1 C° (as is seen in LUV suspensions). However, two differences were noted. First, the phase transition temperature, T_m, was increased to 42.9 ± 0.1°C in ^2H_2O preparations, compared to 42.0°C seen in standard LUV suspensions. Second, ΔH_{cal} of this measurement, as determined by the area beneath the $\alpha\lambda/c$ versus temperature curve, was approx. 10–20%

TABLE II

Characteristics of the lipid phase transition determined from ultrasound absorption coefficient measurements in LUV suspensions with varying percentages of ^2H_2O

ΔH_{cal} was obtained by comparison of the area under an $\alpha\lambda/c$ versus T (°C) curve of known enthalpy. The Van't Hoff enthalpy was calculated from the width of the ultrasound $\alpha\lambda/c$ peak at one-half its maximum height. The cooperative unit was estimated from the ratio of the Van't Hoff enthalpy to the calorimetric enthalpy. Estimates of error are given for each value.

LUV composition DPPC/DPPG (4:1, w/w) ^2H_2O or H_2O preparation	0% ^2H_2O H_2O	20% ^2H_2O ^2H_2O	50% ^2H_2O ^2H_2O	50% ^2H_2O H_2O	50% ^2H_2O H_2O	80% ^2H_2O H_2O	100% ^2H_2O ^2H_2O
T_m (°C)	42.0± 0.1	42.8± 0.2	42.0±0.2 43.0±0.1	42.0±0.2 43.0±0.1	43.0±0.2	42.8± 0.2	42.9± 0.1
$\Delta T_{1/2}$ (C°)	1.6± 0.2	2.3± 0.2	-	-	-	2.0± 0.2	1.7± 0.2
ΔH_{VH} (kcal/mol)	428 ±21	299 ±15	-	-	-	344 ±17	405 ±21
ΔH_{cal} (kcal/mol)	7.5± 0.2	6.3± 0.6	6.2±0.6	7.6±0.8	7.6±0.8	9.4± 0.9	9.7± 0.9
Cooperative unit size	57 ± 4	48 ± 4	-	-	-	37 ± 3	42 ± 3

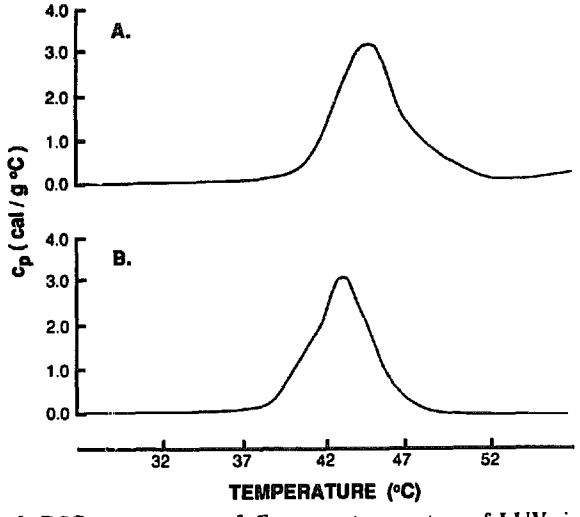

Fig. 2. DSC measurement of C_p versus temperature of LUVs in: (A) 100% ^2H_2O buffer (T_m = 43° C); (B) H_2O buffer (T_m = 42° C).

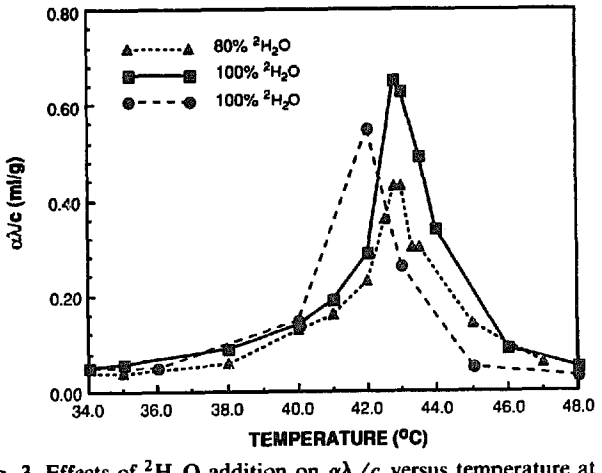

Fig. 3. Effects of ^2H_2O addition on $\alpha\lambda/c$ versus temperature at 3.1 MHz of LUVs in 100% H_2O, 100% ^2H_2O and 80% ^2H_2O. The estimated error in $\alpha\lambda/c$ is ±0.01 ml/g..

greater than that normally seen in standard LUV suspensions (see Table II). This was not in agreement with DSC measurements which showed a non-significant difference in enthalpy between the two types of LUVs (Fig. 2). This suggests some difference in the enthalpy determined by ultrasonic absorption measurements and by DSC measurements.

Standard LUVs diluted into ^2H_2O buffer (resulting in at least an 80% ^2H_2O aqueous component) showed a curve close to that of LUV suspensions with 95–100% ^2H_2O, with a transition temperature of 42.9 ± 0.1° C. However, the curve showed a decreased amplitude and a slightly broadened peak, suggesting a biphasic nature in the curve with some of the phospholipid undergoing transition below 42.9° C ($\Delta T_{1/2}$ = 2.0 C°) (Fig. 3).

The $\alpha\lambda/c$ versus temperature curve for LUVs diluted into a $^2H_2O/H_2O$ buffer mixture so as to produce a 1:1 $^2H_2O/H_2O$ (±5%) ratio for the aqueous compo-

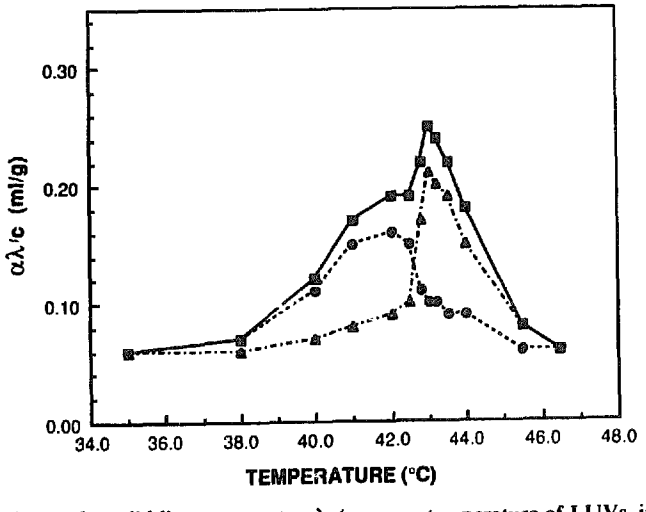

Fig. 4. The solid line represents $\alpha\lambda/c$ versus temperature of LUVs, in 1:1 $^2H_2O/H_2O$ buffer. The broken lines represent two simulated peaks, at 42.0° C and 43.0° C, respectively.

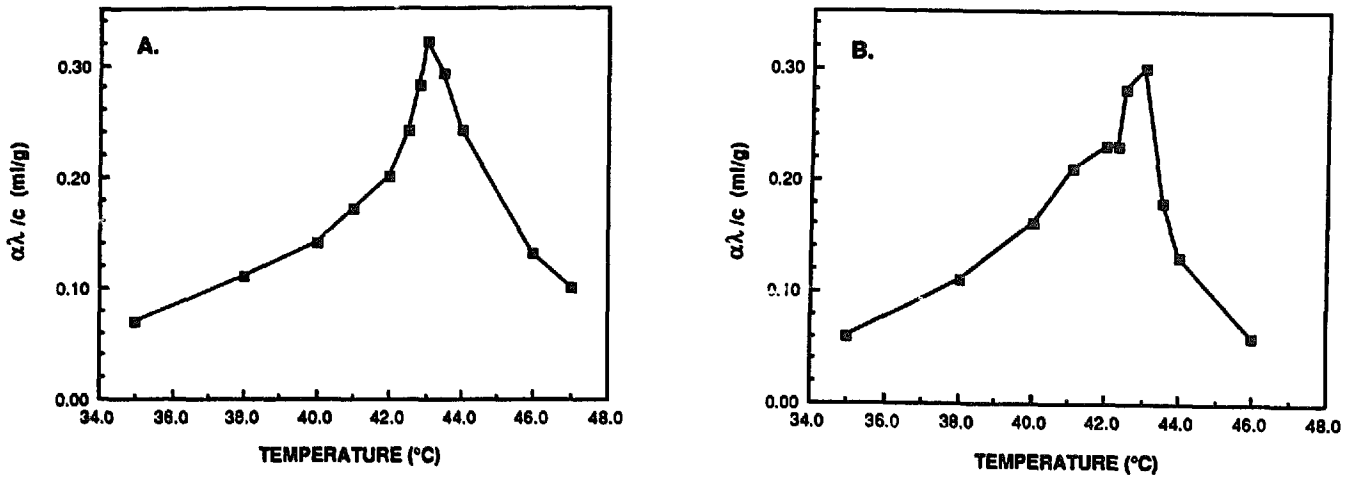

Fig. 5. $\alpha\lambda/c$ versus temperature in: (A) 0.1 μm filtered LUVs in 1 : 1 $^2H_2O/H_2O$ buffer; (B) 0.2 μm filtered LUVs in 1 : 1 $^2H_2O/H_2O$ buffer.

nent of the suspension, is seen in Fig. 4. ΔH_{cal} was less than that of standard LUV suspensions. The clearly biphasic nature of this curve suggested two superimposed curves, with peaks at 42.0 °C and 43.0 °C, each representing decreased cooperativity (compared to standard LUV curves). This may be due to the interaction of a smaller cooperative unit of phospholipids with a given distinct aqueous domain. This curve has been decomposed into two curves as is shown in Fig. 4. These two peaks may represent the phase transitions of single phospholipids or groups of phospholipids interacting with groups of H_2O or ^2H_2O molecules, respectively. While the timescale of the interaction of single water molecules and phospholipids is too short to be detected by ultrasound in the megahertz frequency range, it is possible that the LUV membranes cause a difference in the partitioning of the two aqueous types, and that these domains at the membrane surface may have lifetimes observable by ultrasound. However, two other possible explanations for this phenomenon were considered.

The possibility exists that the inside and outside bilayers may have experienced different effects in $^2H_2O/H_2O$ mixtures. While the ^2H_2O concentration inside the LUVs was not different from that outside the LUVs, it is possible that some feature of the charge distribution on the inside of the bilayer may have been different than that on the outside bilayer. This is remote given that the inside/outside phospholipid ratio for bilayers of this diameter is nearly unity. The main evidence against this is that the two peaks, covering roughly equal areas, were only seen with the 1 : 1 $^2H_2O/H_2O$ mixture. As noted before, the LUV suspensions in 80% ^2H_2O showed a main peak at 42.9 °C with the suggestion of some phospholipid undergoing transition below 42.9 °C. This suggests that the biphasic nature of the $\alpha\lambda/c$ versus temperature curve is dependent upon the $^2H_2O/H_2O$ ratio, and not on the presence of an inside/outside difference in the LUV bilayers.

The possibility also exists that the biphasic nature of the curve in the 1 : 1 mixture of H_2O and ^2H_2O was dependent upon a partitioning of the two aqueous types to distinct subpopulations of the liposomes. As the liposome preparations were homogeneous in all aspects except for size (as a range of sizes exists for a given liposome suspension), size distributions may have accounted for the biphasic nature of the curves. In order to study this possibility, LUV suspensions of different size populations were investigated. LUV suspensions were made and were then filtered into two groups, those extruded through a 0.2 μm filter and those extruded through a 0.1 μm filter. These suspensions were then (in different experiments) diluted into a mixture of $^2H_2O/H_2O$ buffer to give, within 5%, a 1 : 1 $^2H_2O/H_2O$ ratio. The results seen in Fig. 5 and Table II show that although the $\alpha\lambda/c$ versus temperature curves of these different size LUV populations were slightly different, the clearly biphasic nature of the

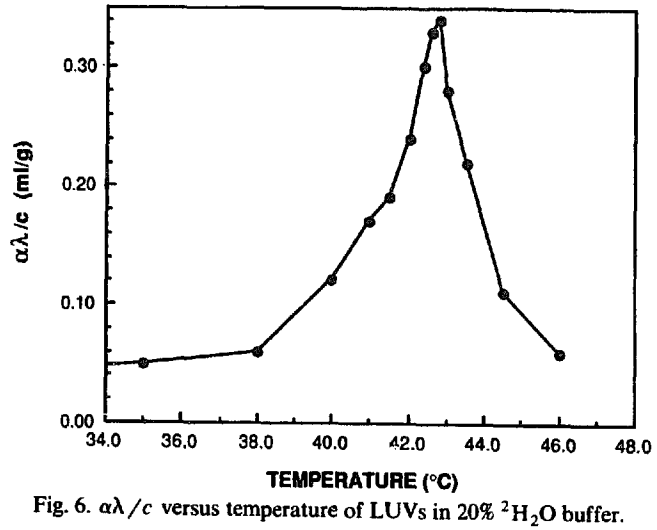

Fig. 6. $\alpha\lambda/c$ versus temperature of LUVs in 20% ^2H_2O buffer.

curve, with a broad maximum at 42.0 °C and a sharper peak at 43.0 °C was still seen. This suggested that it was ^2H_2O and/or H_2O interacting with individual phospholipids or small groups of phospholipids, that caused a biphasic curve to be seen. However, the distinct peak observed at 42.0 °C in the first two preparations was not seen in the 0.1 μm filtered LUVs, suggesting perhaps that some feature of the LUV size may have affected slightly their interaction with water or deuterium oxide.

LUV suspensions prepared such that ^2H_2O made up at least 20% of the aqueous component of the suspension produced ultrasonic $\alpha\lambda/c$ values nearly that of LUVs in 80% ^2H_2O buffer, with a T_m of 42.8 ± 0.2 °C (Fig. 6) and $\Delta T_{1/2} = 2.3$ C°, which was slightly broader than that seen in 100% or 80% ^2H_2O buffer LUV suspensions. The enthalpy, however, was not as large as that seen in pure ^2H_2O LUV or standard LUV suspensions (see Table II). It is important to note that in all of these experiments the relaxation frequency of the phase transition, as determined by ultrasound, remained 2.1 MHz. This result suggests that ^2H_2O does not change the kinetics of the molecular event that is perturbed by ultrasound.

ESR results

The broad spectra in Figs. 7B–D are typical for a nitroxide probe immobilized within the membrane [18] while the spectrum in Fig. 7A is characteristic for rapidly tumbling nitroxides in water. The anisotropic maximal splitting, A_{max}, for different probes within the membrane of LUVs is often used as a measure of the fluidity of the bilayer at different fatty acyl positions [13]. The A_{max} of the labels is greater for labels whose nitroxide group is placed at the membrane surface than for those labels whose nitroxide group is located within the hydrophobic region of the phospholipid bilayer, in the order CAT_{16} > 5-DS > PC-12-DS, in agreement with observations for many other membranes [13,18]. Thus if ^2H_2O affects membrane structure and/or mobility in LUV suspensions, ESR probes at different membrane levels may be used to determine the site(s) of that perturbation.

The results of 5-DS experiments at 20 °C are given in Figs. 7C and 7D. The experiments at 20 °C showed that A_{max} for 5-DS was increased in ^2H_2O LUV suspensions compared to H_2O LUV suspensions. The ESR results showed this trend also with increasing temperature, although the difference between the A_{max} in ^2H_2O versus H_2O did decrease as the temperature was increased until, at 48.0 °C (5 C° above T_m), no significant difference in A_{max} was observed between the two samples (see Table III). These results suggest that aqueous structure is important in the phospholipid dynamics near the membrane/aqueous interface, even to the level of the fifth carbon atom of the fatty acyl chains.

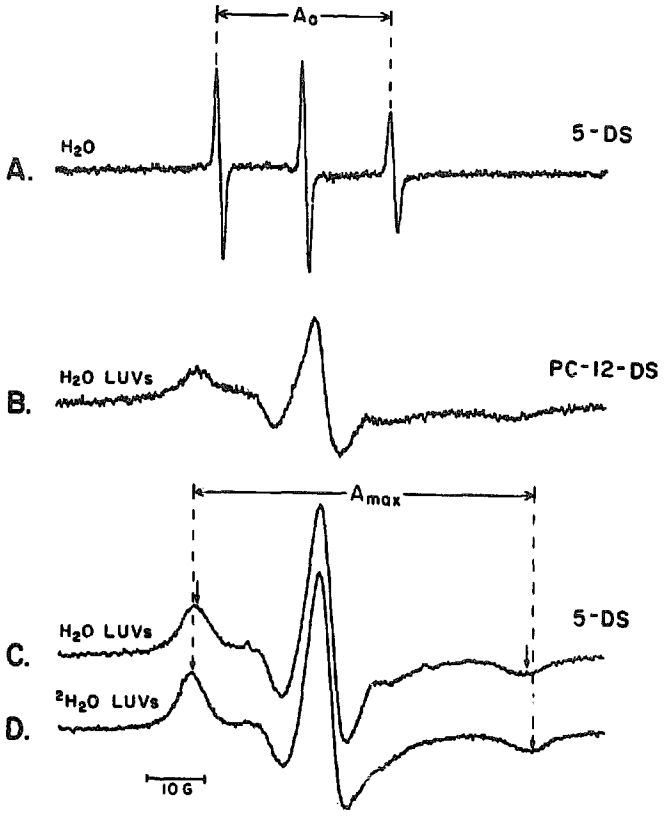

Fig. 7. ESR spectra showing: (A) A_0 of free nitroxides in solution; (B) A_{max} of nitroxides in membranes; (C) A_{max} of 5-DS in LUVs at 20 °C in H_2O-Hepes buffer; (D) A_{max} of 5-DS in LUVs at 20 °C in ^2H_2O-Hepes buffer.

The nitroxide portion of CAT_{16} is located in the aqueous compartment, at the membrane-aqueous interface. CAT_{16} experiments showed a small difference in A_{max} between ^2H_2O and H_2O LUVs at 20 °C but showed no significant difference in A_{max} in ^2H_2O aqueous phase versus H_2O aqueous phase at higher temperatures, suggesting that the difference in phospholipid

TABLE III

ESR data

A_{max} of LUVs in ^2H_2O buffer are compared to LUVs in H_2O buffer, as a function of temperature, below and above the phase transition temperature of the LUV suspensions. The estimate of error of these measurements is 0.3 gauss.

T (°C)	A_{max} (gauss)	
	H_2O LUVs	^2H_2O LUVs
22	59.7	62.3
25	58.0	60.0
29	57.8	59.1
33	55.9	57.6
39	48.6	49.5
42	43.2	43.9
43	–	43.0
44	41.7	42.2
48	41.3	41.3

mobility at higher than room temperature is not significant at the phospholipid headgroup region of the membrane/aqueous interface.

In contrast to the 5-DS, the same experiment using PC-12-DS showed no significant difference in the A_{max} of PC-12-DS in ^2H_2O versus H_2O buffer, suggesting that although aqueous structure is important in determining lipid dynamics and structure near the membrane/water interface, it is not important (on ESR timescales of 10^{-8} s) for lipid dynamics deeper in the lipid bilayer. Previously, 12-DS was incorporated into the LUV bilayer. However, at temperatues below T_m, 12-DS in LUV suspensions partitioned in both the membrane and in water, giving the free (aqueous) nitroxide spectrum superimposed on the spectrum from the probe in the membrane. These lines caused problems in the estimation of maximum splitting. Moreover, partitioning of 12-DS was different for ^2H_2O compared to H_2O. Therefore, PC-12-DS was used instead as a probe of the hydrophobic bilayer, and gave no visible free nitroxide signal.

Discussion

In order to determine the site of ultrasound interaction in LUV suspensions, different regions of LUV structure are perturbed, and the corresponding changes to the frequency dependence of $\alpha\lambda$ at T_m are observed. Three general aspects of the LUV suspension are easily modified: (1) the aqueous component of the suspension, (2) the polar headgroup region of the phospholipid bilayer, and (3) the hydrophobic region of the bilayer. Perturbation of these regions, and any corresponding changes in $\alpha\lambda$ as a function of temperature and especially frequency, may indicate effects on the molecular event to which ultrasound couples. Ultrasound studies employing LUVs have shown that the incorporation of substances such as gramicidin into the LUV membranes changes the temperature and frequency dependence of $\alpha\lambda$ [4], while addition of other substances, such as divalent cations, affects the phospholipid phase transition but does not change the frequency dependence of $\alpha\lambda$. These findings suggest that only substances that interact with the LUV suspension at a specific site or sites may directly perturb the rate of the molecular event responsible for the absorption of ultrasonic energy [4,19].

The physical properties of ^2H_2O are only slightly different from those of H_2O (see Table I). However, small differences in molecular properties may cause a perturbation in the normal aqueous interaction with individual phospholipids. ^2H_2O is more structured than H_2O and it is known that there exists a hydration shell that interacts with phospholipid headgroups at the membrane/water interface [20]; however the molecular dynamics of the interaction between phospholipids and water molecules has not been described and therefore the effects of ^2H_2O on the dynamics of phospholipid molecules and/or their carbon side chains are not known. It is known that replacement of H_2O by ^2H_2O in specific biological systems has a profound effect on those systems. Specifically, ^2H_2O is known to depress the excitation-contraction coupling in frog skeletal muscles and other experiments have suggested the possibility that ^2H_2O influences the mechanism underlying Ca^{2+} release from the sarcoplasmic reticulum induced by depolarization at the transverse tubules [21,22]. If ^2H_2O perturbs the LUV bilayer or the lipid bilayer phase transition and also perturbs $\alpha\lambda$ as a function of frequency, then the membrane/water interface could be a site of interaction of ultrasound and LUVs.

It is shown in these experiments that ^2H_2O, as the aqueous component of LUV suspensions, affects the phospholipid phase transition, as association with ^2H_2O causes at least a subpopulation of the phospholipids to have an increased T_m, to $42.9 \pm 0.1°C$ from $42.0°C$. The 1:1 mixtures of H_2O and ^2H_2O clearly create a biphasic nature in the phospholipid phase transition curve. This may be due to random association of single phospholipids with domains of ^2H_2O or H_2O molecules, which also results in decreased cooperativity of the two components seen in the transition. These results suggest that the replacement of H_2O with ^2H_2O does cause a change in the LUVs, specifically to their phase transition temperature. It is important to note that the $\alpha\lambda_{max}$ of 2.1 MHz for standard preparation LUV suspensions is not changed with the replacement of H_2O by ^2H_2O in the aqueous buffer of LUV suspensions, suggesting that ^2H_2O does not interact with the site of ultrasound absorption in LUV membranes.

As the activation energy of the phase transition is increased by a substance that should only affect the membrane surface, it is hypothesized that the structure and mobility of the phospholipids at the membrane/aqueous interface are changed. In order to study the mobility of the LUV membrane at a specific position at the membrane/aqueous interface, ESR nitroxide probes are used, and should detect any changes in mobility of the phospholipids caused by the addition of ^2H_2O in LUV suspensions.

ESR results indicate that ^2H_2O changes the phospholipid side chain mobility near the membrane/water interface (to the level of carbon-5) but that it does not significantly affect side chain mobility in the hydrophobic (carbon-12) region of the bilayer. The isotropic splitting constant, A_0, of a nitroxide in solvent (Fig. 7A) is dependent only upon the polarity of that solvent, and A_0 for rapidly tumbling nitroxides dissolved in ^2H_2O is the same as in H_2O. Therefore the difference in A_{max} seen in the ^2H_2O and H_2O LUV suspensions is unlikely to originate from the direct interaction of the probe with solvents of different polarity. Furthermore,

in the LUV suspensions, the difference in A_{max} decreases as temperature increases, suggesting a dependence on some factor other than polarity of the solvent. Therefore it seems that the difference in A_{max} reflects changes in the structure of the bilayer. Greater A_{max} for 5-DS and CAT_{16} in ^2H_2O LUV suspensions as compared to H_2O LUV suspensions indicates a more restricted mobility of the probe, i.e., a less fluid membrane in the polar head group regions.

However, the differences in A_{max} of 5-DS and CAT_{16} in the H_2O and ^2H_2O LUV suspensions could be a consequence of the different water penetration in the LUV bilayer for these two aqueous types, which could occur in addition to the differences in phospholipid mobility. It has been shown that water penetrates into lipid bilayers to a significant distance, so that 5-DS is affected by the aqueous solvent [23]. Increased A_{max} in ^2H_2O LUVs might then indicate increased water penetration, i.e. a more polar environment of the probe. PC-12-DS is in the nonpolar environment and is not sensitive to water penetration. These effects can be distinguished by recording the spectra at very low temperatures where the effects of motion disappear [23].

Regardless of the exact origin of the increased A_{max} of nitroxides in the ^2H_2O LUV suspensions, it can be concluded that the increased splitting reflects changes in the structure of the bilayer. Also, these changes are significant in the polar headgroup region and extend a few angstroms into the hydrocarbon region, as is seen in the 5-DS spectra.

Based on this information a model for the perturbation and general interaction of ^2H_2O with phospholipids in the LUV membrane may be formulated: It is given that H_2O is situated at the aqueous/membrane interface to allow the most stable energy configuration of the phospholipids and the water molecules and other solvent molecules. The exact nature of this configuration has not been treated, but it has been assumed that the dipolar water (1.76 deBye) has van der Waals and dipole interaction with the zwitterionic DPPC moiety and with the negatively-charged DPPG headgroups. This configuration allows for a certain mobility (fluidity) of the entire phospholipid moiety, the headgroups and the hydrocarbon chain near the membrane/water interface in particular. When H_2O is replaced by ^2H_2O, a different molecule with an increased molecular weight and increased dipole moment (1.78 deBye), a different low energy configuration at the membrane-water interface must form. It is likely that not only does each ^2H_2O molecule take up a slightly different geometry at the membrane/water interface, but also that the bond strength of the ^2H_2O with those molecules with which it interacts is greater than that of water. This would cause the membrane phospholipids to be more ordered, suggesting a lower mobility at the membrane/water interface (as observed in ESR experiments). It would also suggest that a greater activation energy would be required for the phospholipid gel to liquid-crystalline phase transition to occur. These effects were observed in our experiments.

In order to investigate further the interaction of ^2H_2O with the lipid bilayer a careful study of the effects of ^2H_2O on a series of doxyl stearate nitroxides (e.g. 5-DS, 7-DS, 9-DS, etc....) could be performed to provide an estimation of ^2H_2O penetration into the lipid bilayer (as compared to H_2O) and of the magnitude of its effects at different depths of the bilayer. Another useful technique in the study of ^2H_2O effects on the bilayer would be ^{13}C-NMR, which could provide an estimate of fatty acyl mobility in the bilayer on NMR timescales. As there are ^{13}C-labelled lipids, no probe needs to be inserted into the membrane.

^2H_2O: H_2O mixtures in LUV suspensions might also be studied with ESR and NMR in order to determine whether the effects of ^2H_2O on the phospholipid bilayer are linear with increasing concentration as determined by these techniques. Finally deuterium itself is of interest, as deuterated lipids and proteins are often used in deuterium NMR measurements. It would also be of interest to measure the ultrasonic absorption coefficient per wavelength of specifically-deuterated phospholipids as a function of temperature and frequency and to study these lipids using NMR and ESR as well.

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