Characterization of Surfactant Vesicles as Membrane Mimetic Agents. 2. Temperature-Dependent Changes of the Turbidity, Viscosity, Fluorescence Polarization of 2-Methylanthracene, and Positron Annihilation in Sonicated Dioctadecyldimethylammonium Chloride

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Abstract: Sonic dispersal of dioctadecyldimethylammonium chloride (DODAC) resulted in the formation of stable bilayer vesicles. Electron micrographs showed the presence of bilayer DODAC vesicles with radii of 150 ± 10 Å and bilayer thickness of 50 ± 5 Å. Turbidity, viscosity, fluorescence polarization of 2-methylanthracene, and positron annihilation established phase transitions at 30.0 and 36.2 °C. DODAC vesicles containing 17.2% cholesterol (w/w) had analogous phase transitions at 23.0 and 34.7 °C. Placing DODAC vesicles in hyperosmolar solutions elicited osmotic shrinkage. The extent and initial rates of osmotic shrinkage have been determined as functions of the electrolyte gradients and temperature. At 25 °C, DODAC vesicles act as ideal osmometers in a relatively narrow electrolyte range. At 0.10 M osmotic gradient ions permeate the vesicle. Increasing the temperature and adding cholesterol increases the extent of ion influx. DODAC vesicles are chemically the simplest functional membrane mimetic agents.

Introduction

Biological membranes provide compartments for given reactive systems and maintain concentration gradients by their selective uptake of appropriate reagents. Chemical modeling of membrane functions is not only inherently interesting but may lead to practical utilizations. Although liposomes, smectic mesophases of phospholipid bilayers, have been extensively investigated as membrane models,[3-5] their complexities and chemical instabilities have necessitated the development of simpler yet functional membrane mimetic agents. Formation of closed bilayer particles, ufasomes, have been reported upon shaking thin films of oleic and linoleic acids in aqueous buffers.[6-8] Disappointingly, ufasomes did not live up to their expectations as membrane models. Their formation was found to be inhibited by electrolytes, and they were unstable outside the pH 6–8 range, did not concentrate on centrifugation, and retained substrates poorly.[7] Vesicle formation from fatty acids, containing a single chain of 8–18 carbon atoms, has also been reported.[8b]

Surfactant vesicles, formed upon the sonic dispersal of dialkyldimethylammonium salts[9-14] and dihexadecyl phosphate,[15] proved, however, to be the simplest functioning membrane models. In contrast to ufasomes, surfactant vesicles can be prepared in the pH 1–13 range, remain stable for months, and entrap and retain substrates in substantial amounts.[12,15] In our laboratories we have characterized preliminarily dioctadecyldimethylammonium chloride (DODAC) surfactant vesicles.[12,16] Sonic dispersal of DODAC initially formed multicompartment and subsequently single-compartment bilayer vesicles. Single-compartment DODAC vesicles entrapped large molecules but were relatively permeable to proton and hydroxide ion. Recently, we have investigated sodium 2,2,5,5-tetramethyl-1-pyrrolidinyloxy-3-carboxylate spin probes in DODAC vesicles.[16] This radical is distributed between bulk water and the outer surface of the vesicle. Electron paramagnetic resonance spectroscopy and scavenging studies have indicated the longer term permeation of the spin probe to the inner surface and to the interior of the DODAC vesicles. The entirely analogous behavior of spin probes in liposomes substantiates further the utilization of DODAC vesicles as effective membrane models.

Thermotropic phase transition is an important property of membranes and liposomes.[17-20] A variety of techniques have been employed in the present work for investigating the phase transition temperature and osmotic behavior of DODAC vesicles. The obtained results provide additional much needed information on fluidities and allow further assessments of cationic surfactant vesicles as membrane mimetic agents.

Experimental Section

The preparation, purification, and characterization of DODAC have been previously described.[16] Cholesterol (MCB) was recrystallized twice from ethanol. 2-Methylanthracene (Aldrich) was recrystallized from methanol. Reagent grade inorganic salts were used as received. Synthetic dipalmitoyl-DL-α-phosphatidylcholine (Sigma, Grade I) and octadecylamine (Aldrich) were used as received. Water was purified by deionization and subsequent distillation in an all-glass apparatus.

The typical vesicle preparation involved the dispersal of 12.0 mg of solid DODAC in 2.0 mL of water at 50–60 °C by means of the microprobe of a Braunsonic 1510 sonifier set at 70 W. Cholesterol-containing vesicles were prepared by cosonicating cholesterol with DODAC. Sonication times were 15 and up to 45 min in the absence and in the presence of cholesterol, respectively. Passage through a Sepharose column (Sigma 4B-200, 40–190 μ, 185 × 16 mm) separated vesicles from free DODAC. The presence of single-compartment vesicles have been previously demonstrated.[12] 2-Methylanthracene containing DODAC vesicles was prepared as follows. Solid 2-methylanthracene was deposited onto the wall of a test tube by evaporating chloroform solutions of it by a steady stream of nitrogen. Typically, 0.1 mL of 1.0×10^{-3} M 2-methylanthracene was used. Dispersal of 12.0 mg of DODAC in 2.0 mL of water was carried out in the test tube containing solid 2-methylanthracene as described above. Separation of free 2-methylanthracene from that bound to or entrapped by the DODAC vesicles was accomplished by a passage through a Sephadex column (Sigma G50-80, 20–80 μ, 16 × 178 mm) using water as eluent. Passage through the Sepharose or Sephadex column diluted the vesicles two- to fivefold. Dipalmitoyl-DL-α-phosphatidylcholine liposomes were prepared by dissolving a thin film of lipid in 2.0 mL of

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aqueous 5.5×10^{-3} M phosphate buffer containing 0.10 M NaCl. The lipid film was deposited on the wall of a round-bottom flask by rotary evaporating chloroform solutions of the lipid, 6.0 mg (8.2 μmol), the cholesterol, 2.0 mg (5.2 μmol), and the appropriate amounts of octadecylamine. The aqueous liposome dispersion was sonicated for 15 min at 55–60 °C by means of the microprobe of the Braunsonic 1510 sonifier set at 70 W. Subsequent to sonication, the 2.0-mL liposome solutions were made up to 10.0 mL by the same phosphate buffer. These solutions were used for the osmotic shrinkage experiments.

Bulk viscosities were measured using an Ostwald viscometer. The time required for a bulk of liquid to discharge through a capillary under the force of its weight was measured at different temperatures in a thermostat. The viscometer was calibrated by determining the viscosity of distilled water at each temperature.

Osmotic shocks were provided by rapidly injecting 0.25-mL solution of DODAC vesicles or liposomes (prepared as described above) into 3.0-mL thermostated osmolar solutions, contained in a thermostated spectrophotometry cell. Rates of turbidity changes were determined at 400 nm by the Cary 118C spectrophotometer. Osmotic rates faster than 3 min were followed in a Durrum-Jasco stop-flow spectrophotometer.

Fluorescence polarizations were obtained in the thermostated cell compartment of a SPEX Fluorolog spectrofluorometer in the E/R mode. Generally, 2.5-mm slits with 10-nm band path were used. Fluorescence polarization was determined by placing a single Glan-Thompson polarizer in the excitation beam and an UV polacoat film in the emission beam. All fluorescences were determined in air-saturated solutions. The excitation and emission wavelengths used were 350 and 410 nm.

Samples for the electron micrography were prepared by sonicating 20 mg of DODAC in 2.0 mL of water containing 1.0% (by weight) phosphotungstic acid for 25 min in a Braunsonic 1510 sonicator at 70 W. The undispersed DODAC was removed by centrifugation at 5000 rpm in an International Clinical centrifuge for 10 min. One drop of the supernatant was placed on the grid of a Hitachi HU 11-E electron microscope.

Positron lifetime measurements[21] were carried out by the usual delayed coincidence method as previously described.[22] The resolution of the system, as measured by the fwhm of the prompt coincidence spectrum of a ^{60}Co source without changing the 1.27- and 0.511-MeV bias, was found to be less than 0.36 ns fwhm. Specially designed cylindrical sample vials (Pyrex glass 100 mm long and 10 mm i.d.) were filled with about 2 mL of the appropriate solution. The positron sources consisted of 3–5 mCi of ^{22}Na diffused into a thin foil of soda lime glass.

The relative amount of positron annihilation occurring in the glass was found to be less than 2%, for which corrections were made. The radioactive glass sources were suspended in the center of the ampule and all solutions were carefully deoxygenated by bubbling argon through the solutions and carrying out all operations in an argon atmosphere. The vials were subsequently sealed off and the measurements carried out at the various temperatures in a specially designed thermostat, which allowed to control the temperature within ±0.5 °C.

The aqueous DODAC solutions were prepared as described above. Sonication for the positron annihilation experiments utilized, however, a Branson Sonifier Cell Disruptor (power setting 45 W) at 56 °C for 30 min, unless specified otherwise in the Results.

Results

Electron Microscopy. Electron micrographs of a typical DODAC vesicle preparation are shown in Figure 1. Formation of closed compartment bilayers is clearly seen. There is a distribution of vesicle sizes ranging between 250 and 500 Å in diameter. Shorter sonication times resulted in the formation of considerably larger multicompartment vesicles. We note that the addition of phosphotungstic acid necessitated longer sonication times.

Turbidity Measurements. Changes of absorbances at 400 nm as a function of temperature were used to monitor the morphological changes of the DODAC surfactant–DODAC vesicle system. Turbidity changes have been previously utilized for investigating lipid phase transitions.[23,25] DODAC (12.0 mg) was sonicated in 2.0 mL of water for 20 s and placed in the

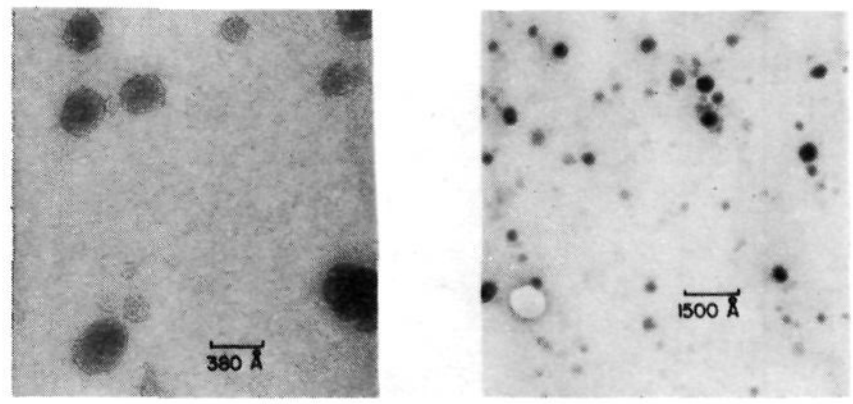

Figure 1. Electron micrographs of sonicated DODAC vesicles at 258 000- and 66 600-fold magnifications.

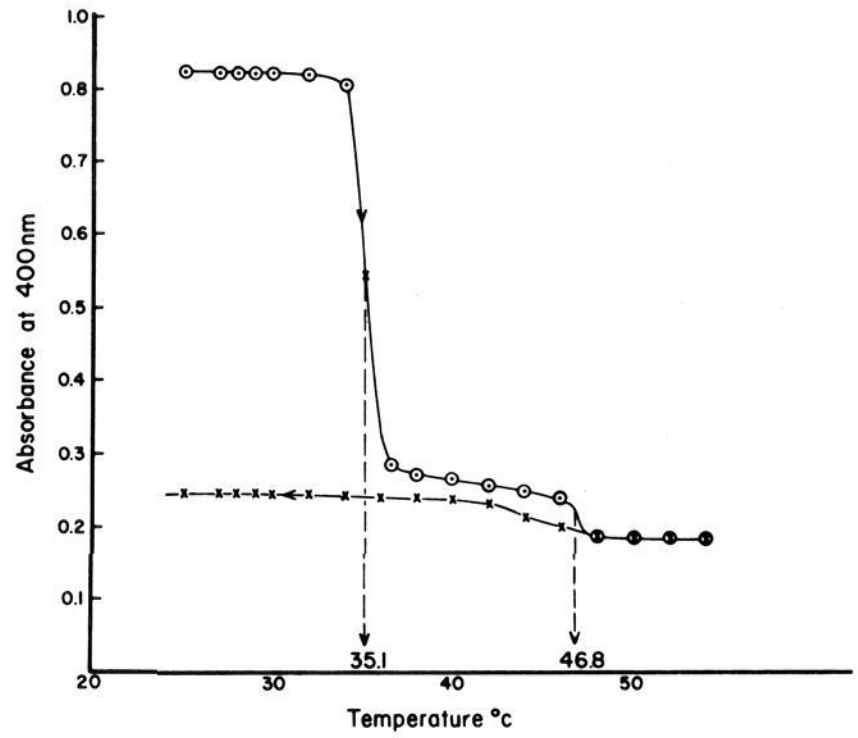

Figure 2. Plot of absorbance changes of DODAC dispersions, following 20-s sonication as a function of increasing (heating cycle) and decreasing temperature (cooling cycle).

thermostated cell compartment of the spectrophotometer. The temperature was raised gradually from 25 to 56 °C and then lowered gradually to 25 °C. The absorbance in the DODAC was measured at frequent intervals at known temperatures (Figure 2). Initially, below 33 °C there was only a heterogeneous dispersion of solid DODAC as manifested by the relatively high turbidity. On raising the temperature most of the surfactants went into solution with the concomitant decrease of turbidity. In the region of 35–45 °C, there was only a small decrease of absorbance. Raising the temperature further resulted in a second transition in the 46–48 °C region. Above this temperature, large multicompartment vesicles are formed. There is a pronounced hysteresis on lowering the temperature. The transition now occurs between 40 and 42 °C. More significantly, further lowering of the temperature causes only a small increase in the turbidity. These results are entirely consistent with the formation of vesicles above the phase transition temperature of DODAC. Once the vesicles are formed, they remain stable even if the temperature is lowered well below the phase transition temperature. The assumed heterogeneity did not allow analogous turbidity determinations for cholesterol containing DODAC vesicles.

Bulk Viscosities. Increasing the temperatures results in a decrease of the bulk viscosities of DODAC vesicles. Experiments were carried out only "on the way up" in temperature. Figure 3 shows logarithmic plots of the viscosities of water and surfactant vesicles, prepared by sonicating DODAC above 50 °C in the absence and in the presence of cholesterol, as functions of reciprocal temperatures. Interestingly, viscosities of

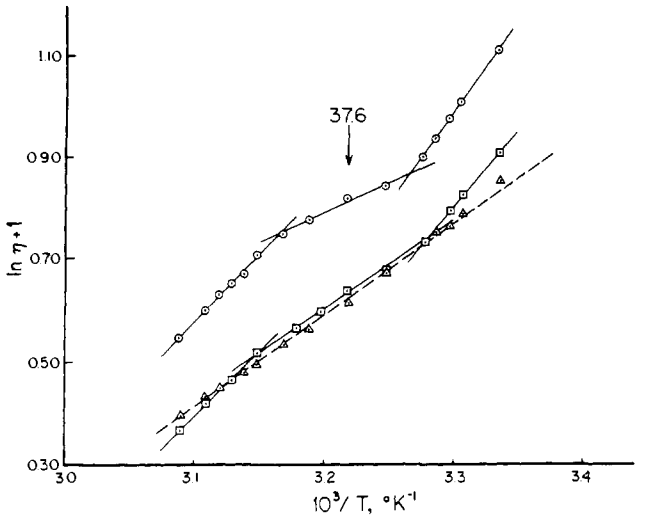

Figure 3. Plots of bulk viscosities of water (△, broken line) and of DODAC vesicles in the absence (○) and in the presence of 17.2% cholesterol (w/w) (□) as a function of temperature.

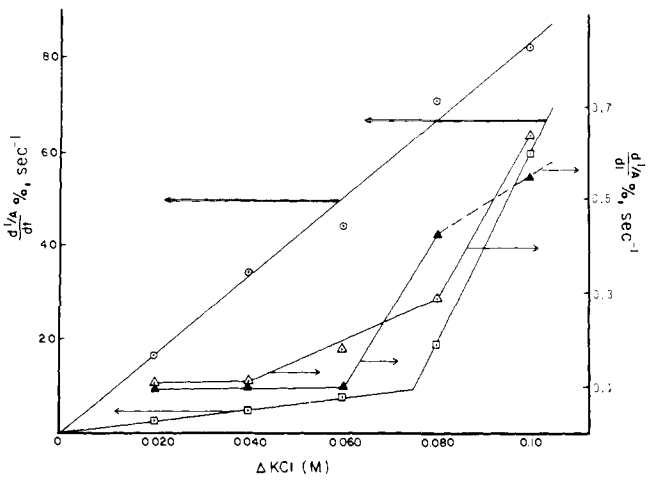

Figure 5. Changes of initial shrinkage velocities of DODAC vesicles in the absence of cholesterol at 25.0 (○) and at 33.0 °C (□), and those in the presence of 3.8 (▲) and 17.2% (w/w) (△) cholesterol at 25.0 °C, as a function of KCl gradient across the bilayer.

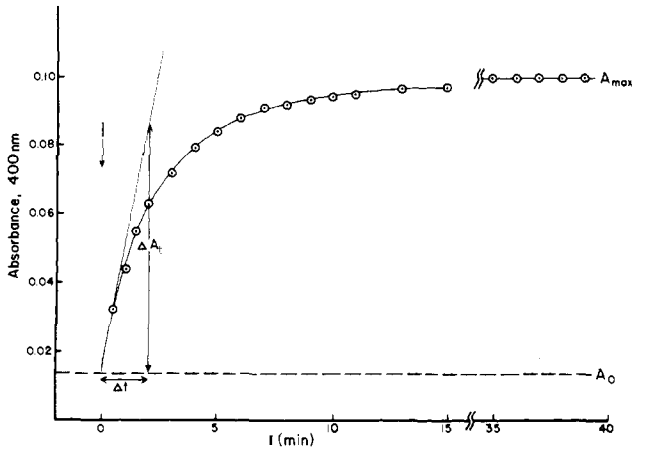

Figure 4. Typical time-dependent absorbance change of DODAC vesicles following an osmotic shock (see Results for explanations of the symbols used).

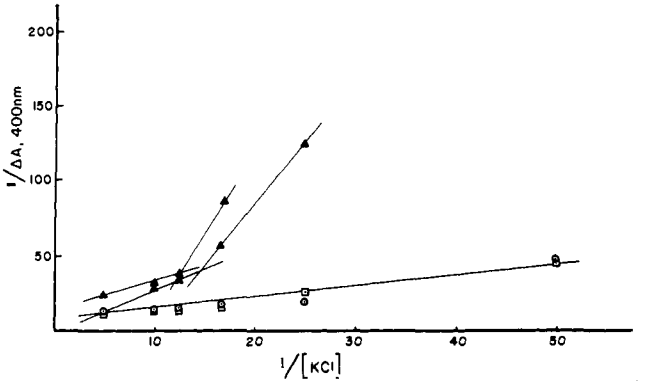

Figure 6. Plots of absorbance difference following osmotic shocks of DODAC vesicles in the absence of cholesterol at 25.0 (○) and 33.0 °C (□), and those in the presence of 3.8 (▲) and 17.2% (w/w) (△) cholesterol.

cholesterol containing vesicles are quite similar to that of water. Viscosities of DODAC vesicles prepared in the absence of cholesterol are much higher, however, than that of water. There are pronounced changes in the slopes of these plots at 32.8 and 42.4 °C in the absence and at 31.7 and 44.5 °C in the presence of cholesterol, respectively. Midpoints of the range of changed slopes, 37.6 and 38.0 °C, are taken to be the phase transition temperatures, T_C values, of the DODAC vesicle in the presence and in the absence of cholesterol, respectively.

Osmotic Shocks. A typical time-dependent increase of the absorption at 400 nm is illustrated in Figure 4 for DODAC vesicles in a 0.10 M hyperosmolar KCl solution. Using previous notations[19] (see Figure 4), the percent of initial shrinkage velocity, $d(1/A)/dt\%$, is given by $(\Delta A_i/\Delta t)100/A_0$. Similarly, the extent of shrinkage, $1/\Delta A$, is determined by $1/(A_{max} - A_0)$. Two types of experiments were performed. In the first type, osmotic shrinkage was examined as a function of the KCl gradient across the vesicle. In the second set of experiments, osmotic shocks at a given electrolyte gradient were examined as a function of increasing temperature. Experiments were carried out only "on the way up" in temperature. Changes in the initial shrinkage velocities for DODAC vesicles as functions of ΔKCl are shown in Figure 5. At 25.0 °C, the initial shrinkage velocities for DODAC vesicles are seen to be appreciably greater in the absence of cholesterol than in its presence. For DODAC vesicles, in the absence of cholesterol initial shrinkage velocities increase linearly with increasing concentrations of KCl outside the vesicles up to 0.10 M electrolyte at 25.0 °C. The range linearity is shorter for cholesterol-containing vesicles than those for DODAC in the absence of this additive. A similar trend is seen in plots of $1/\Delta A$ vs. 1/[KCl] (Figure 6).

Initial shrinkage rates of DODAC vesicles were also determined by using LiCl and NaCl in addition to KCl as hyperosmolar solutions. The data are given in Table I. At each temperature, the initial shrinkage rates of DODAC is seen to decrease with increasing size of the alkali cation. The shrinkage rate is much slower at 40 °C than at 25 °C.

Initial shrinkage rates have also been determined for phospholipid liposomes as a function of added octadecylamine concentrations (Table II). Since the conditions were quite different from those used for DODAC, comparison of absolute shrinkage rates between these two kinds of vesicles is meaningless. The important fact to observe is the linear increase of the initial shrinkage rate for phospholipid liposomes with increasing concentrations of added octadecylamine.

Initial rates of osmotic shrinkage are sensitive to temperature changes. Figure 7 shows logarithmic plots of dA/dt against the reciprocal temperature for the initial shrinkage rates of DODAC vesicles in the absence and in the presence

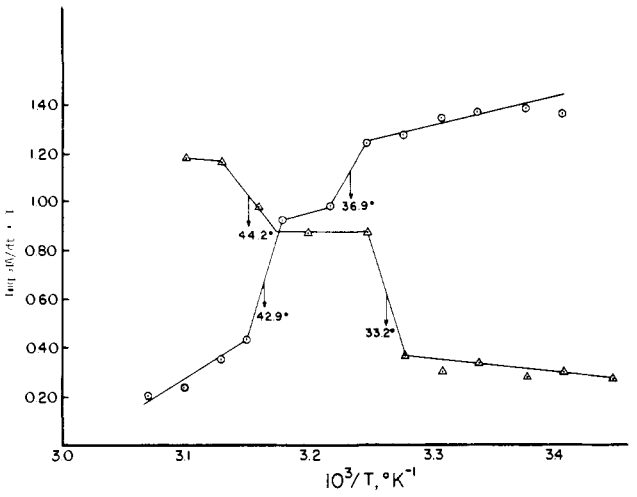

Figure 7. Initial shrinkage rates of DODAC vesicles in the absence (○) and in the presence of 17.2% (w/w) (△) cholesterol as a function of reciprocal temperature.

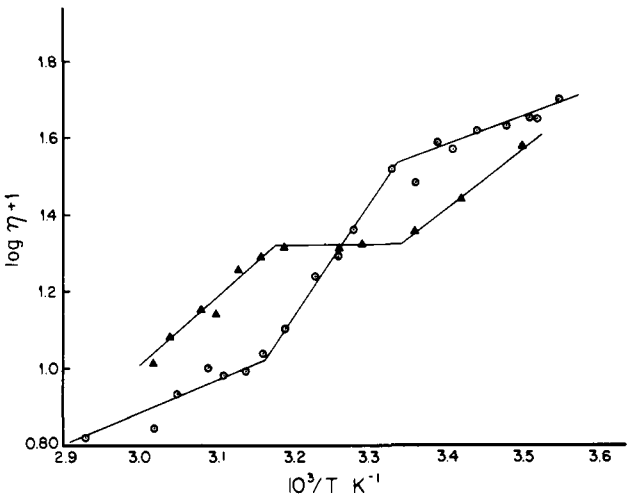

Figure 8. Plots of microviscosities of DODAC vesicles in the absence (○) and in the presence of 17.2% (w/w) cholesterol (△) as a function of reciprocal temperature.

Table I. Initial Shrinkage Rate of DODAC Vesicles[a]

electrolyte	$10^4 dA/dt$, s^{-1} 25.0 °C	$10^4 dA/dt$, s^{-1} 40.0 °C
LiCl	3.83 ± 0.22	0.59 ± 0.04
NaCl	2.93 ± 0.07	0.34 ± 0.04
KCl	2.78 ± 0.10	0.25 ± 0.01

[a] 12.0 mL of DODAC in 2.0 mL of H_2O sonicated at 55–60 °C for 15 min. The sonicated solution was diluted to 4.0 mL by H_2O. This solution (0.25 mL) was rapidly injected to 3.0 mL of thermostated hyperosmolar solution. Turbidity changes were followed at 400 nm. Initial shrinkage rates were obtained as shown in Figure 4. Osmotic gradient in each case was 9.23×10^{-2} M.

Table II. Initial Shrinkage Rates of Dipalmitoyl-DL-α-phosphatidylcholine Liposomes as a Function of Added Octadecylamine at 24.0 °C[a]

[octadecylamine], μmol	dA/dt, s^{-1}
0	0.000 59 ± 0.000 04
1.1	0.070 00 ± 0.004 00
2.8	0.180 00 ± 0.010 00
5.6	0.450 00 ± 0.030 00
8.3	0.660 00 ± 0.020 00

[a] See Experimental Section and Results for details. The osmotic gradient in each case was 1.9 M NaCl.

of cholesterol at ΔKCl = 0.10 M. In the absence of cholesterol, initial shrinkage rates increase steadily with decreasing temperature in the 53–44.5 °C range. There is a sharp increase of dA/dt in the range of 44.5–41.5 °C. The midpoint of this sharp increase, 42.9 °C, indicates a phase transition. At lower temperatures, there is a further increase of dA/dt, and a second transition occurs at 36.2 °C. Significantly, in the presence of cholesterol the opposite trend was observed. Increasing the temperatures resulted in faster osmotic shrinkages. The dA/dt vs. $1/T$ plot consisted of two sigmoidal curves. Midpoints of the most sensitive temperature ranges, 33 and 44 °C, correspond to phase transitions of cholesterol-containing DODAC vesicles.

Fluorescence Polarizations of 2-Methylanthracene. The degrees of polarization, P, were calculated by[26,27]

$$P = \frac{I_{\parallel\parallel} - I_{\parallel\perp}(I_{\perp\parallel}/I_{\perp\perp})}{I_{\parallel\parallel} + I_{\parallel\perp}(I_{\perp\parallel}/I_{\perp\perp})} \quad (1)$$

where ($I_{\perp\perp}$, $I_{\parallel\parallel}$) and ($I_{\perp\parallel}$, $I_{\parallel\perp}$) mean fluorescence intensities at parallel and crossed positions of the polarizers, respectively. They can be correlated with the temperature, T, lifetime of the fluorescence probe, τ, and viscosity, η, by the equation[26-28]

$$\frac{\frac{1}{P} - \frac{1}{3}}{\frac{1}{P_0} - \frac{1}{3}} = 1 + \frac{kT\tau}{\eta v_0} \quad (2)$$

where v_0 is the effective volume of the fluorescent probe. Polarizations of 2-methylanthracene in the DODAC vesicles were converted to microviscosities by using the previously determined relationship between fluorescence polarization (and lifetimes) and viscosity for 2-methylanthracene in glycerol-water.[28]

Microviscosities, as reported by 2-methylanthracene, of DODAC in the absence and in the presence of cholesterol as functions of the temperature are shown in Figure 8. Experiments were only carried out "on the way up" in temperature. Since 2-methylanthracene is intercalated in the hydrophobic regions of vesicles,[28] microviscosities at each temperature are greater than bulk viscosities (compare Figures 3 and 8). Arrhenius plots for DODAC vesicles show two abrupt changes in slope (Figure 8). For DODAC vesicles in the absence of cholesterol, the midpoint between these abrupt changes, 35.5 °C, corresponds to the phase transition temperature. It is worth mentioning that the range of this transition, 26.8–42.5 °C, is rather broad. Similar broad phase transitions have been observed for single-compartment liposomes.[31] Conversely, fluorescence polarizations reported sharp phase transitions for multicompartment liposomes.[31]

Fluorescence polarizations of 2-methylanthracene in DODAC vesicles are quite different in the presence of cholesterol. The reported microviscosities are higher than in the absence of cholesterol (Figure 8). Macroviscosities showed the opposite trend (Figure 3). Equally importantly, in the transition range for cholesterol-containing DODAC, 26.2–42.0 °C, microviscosities remain unaltered. Thus, microviscosity determinations in cholesterol-containing DODAC cannot be utilized for assessing phase transition temperature. This presumably is due to the interaction of cholesterol with 2-methylanthracene.[28] The temperature dependence of the microviscosities is essentially identical below and above the phase transition temperatures for DODAC vesicles both in the ab-

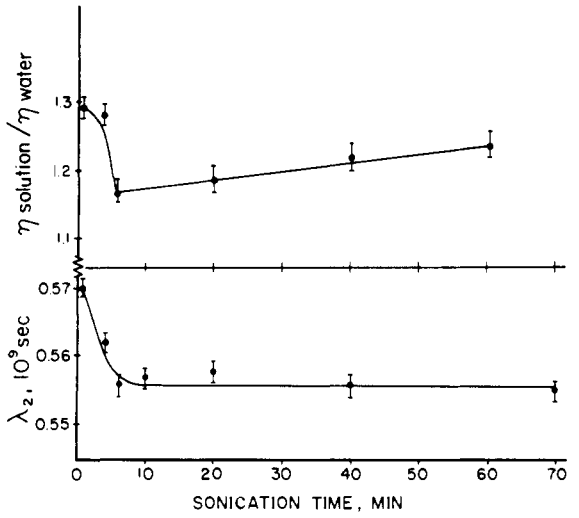

Figure 9. Plots of viscosities (upper curve) and positronium annihilation rates (lower curve) against sonication time for DODAC vesicles.

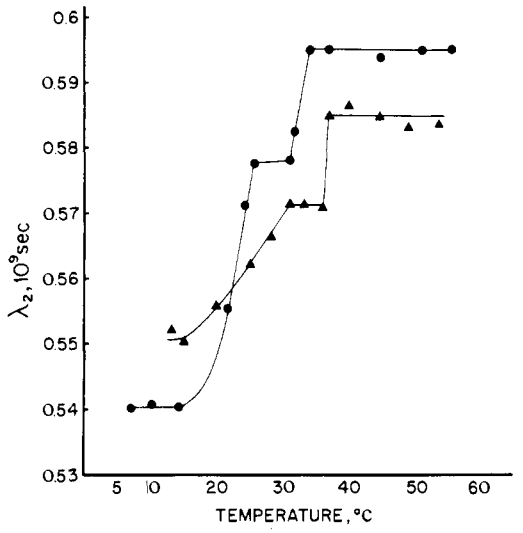

Figure 10. Plots of positronium annihilation rates against temperature for DODAC vesicles in the absence (▲) and in the presence of 17.2% (w/w) (●) cholesterol.

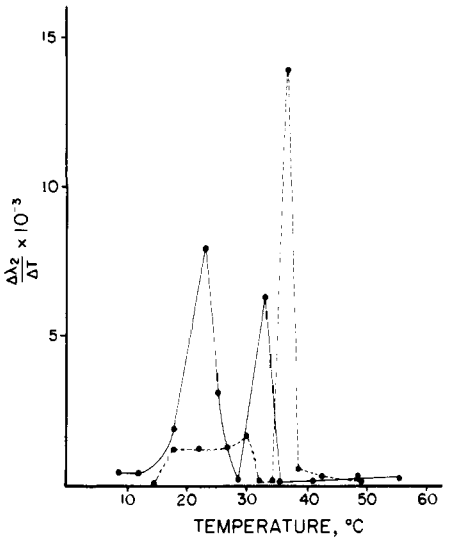

Figure 11. Differential plots of positronium annihilation rates as a function of temperature for DODAC vesicles in the absence (broken line) and in the presence (solid line) of 17.2% (w/w) cholesterol.

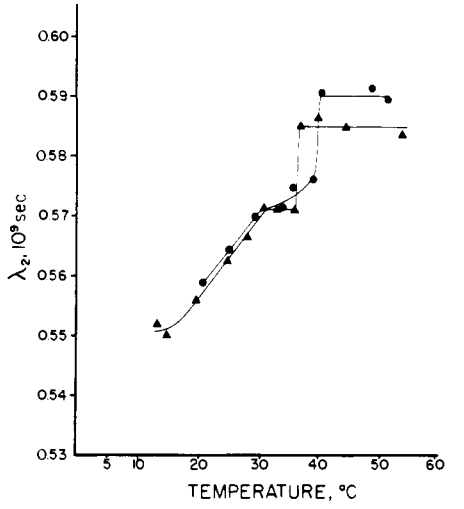

Figure 12. Plots of positronium annihilation rates against temperature for DODAC vesicles. In the heating cycle the temperature of the sample was gradually increased (▲), whereas in the cooling cycle the temperature was gradually decreased (●).

sence and in the presence of cholesterol (Figure 8). As expected, there is a greater temperature dependence for cholesterol-containing vesicles.

Positron Annihilation. The general method of positron lifetime data analysis has been described in great detail previously,[29,30] and will not be, therefore, reiterated here. Important parameters are λ_2 and I_2. The former is the slope of the long-lived component of the positron lifetime and it indicates the rate of reactions of thermal *o*-positronium atoms (Ps) in the solution. I_2 represents the intensity of the long-lived component of the positron lifetime and it can be correlated to the number of Ps atoms formed.[21]

Positron annihilation parameters were determined as a function of sonication time. DODAC (0.30 g) in 50.0 mL of triply distilled water was sonicated at 56 °C for different times. Bulk viscosities and λ_2 and I_2 values were determined on aliquots withdrawn at appropriate times. Although I_2 remained unaltered, λ_2 values decreased markedly with increasing sonication time in the 1–10-min period. Further sonication did not alter, however, the positronium reactivities. This behavior paralleled nicely the corresponding bulk viscosities of the DODAC-water dispersions (Figure 9). Vesicles were apparently formed after 10 min sonication of DODAC dispersions.

In a second series of experiments, λ_2 and I_2 values were determined for DODAC vesicles, formed by 30-min sonication of 12.0 mg of DODAC in 2.0 mL of H_2O, as a function of increasing temperature. No significant changes in I_2 could be observed. However, λ_2 values increased with increasing temperature in the 15–31 °C region, and exhibited a well-defined plateau in the 31–36 °C region. This plateau was followed by a steep increase of λ_2 values, which leveled off above 37 °C (Figure 10). Positronium lifetimes in cholesterol-containing DODAC vesicles as a function of increasing temperature are also shown in Figure 10. In the presence of cholesterol, the λ_2 vs. temperature curve is seen to shift to a lower temperature.

Temperature dependence of positronium lifetimes in DODAC vesicles became more apparent on plotting $\Delta\lambda_2/\Delta T$ against temperature. As shown in Figure 11, clear peaks for $\Delta\lambda_2/\Delta T$ emerge at 24 and 37 °C for the aqueous solution containing DODAC vesicles and at 23 and 33 °C for the DODAC vesicles, which were formed by cosonicating DODAC and cholesterol. It should be pointed out that in both systems λ_2 seems to level off below 15 °C (Figure 10).

Table III. Phase Transition Parameters of DODAC Vesicles in the Absence of Cholesterol

method	transition I, °C		transition II, °C		transition X, °C	
	range	T_C	range	T_C	range	T_C
turbidity			34.0-36.2	35.1	46.0-48.0[a]	46.8[a]
bulk viscosity			32.8-42.4	37.6		
microviscosity			26.8-42.5	35.5		
			(29.7-40.6)[b]	(36.4)[b]		
osmotic shrinkage			35.0-38.0	36.2	41.0-44.0[c]	42.9[c]
positron annihilation	27.0-32.0	30.0	34.0-38.5	37.0		

[a] Temperature changes due to phase transition of DODAC. [b] Value for single compartment dipalmitoyl-DL-α-phosphatidylcholine liposome, taken from ref 31. [c] Transition due to altered electrolyte permeation.

Table IV. Phase Transition Parameters of DODAC Vesicles in the Presence of 17.2% Cholesterol (w/w)

method	transition I, °C		transition II, °C		transition X, °C	
	range	T_C	range	T_C	range	T_C
bulk viscosity			31.7-44.5	38.0		
osmotic shrinkage			31.4-34.7	33.2	41.5-46.5	44.2[a]
positron annihilation	13.0-28.0	23.0	31.5-34.5	33.0		

[a] Transition due to altered electrolyte concentration.

If the positron lifetime measurements are carried out by decreasing the temperature (cooling cycle) as shown in Figures 12 and 13, in both systems λ_2 exhibits a sharp drop at considerably higher temperatures than in those experiments where the temperature was increased (heating cycle), e.g., about 42 °C compared with 37 °C in aqueous DODAC vesicle solutions, and 37 °C compared with 33 °C in aqueous DODAC–cholesterol vesicle solutions. In both cases, the plateaus in the 31–36 and 26–32 °C region, respectively, observed with increasing temperatures are considerably less well defined and also shifted to higher temperatures.

Since each measurement took 2–4 h, and most of them were duplicate or triplicate, the good reproducibilities implied lack of time-dependent (other than hysteresis) morphological changes of DODAC vesicles.

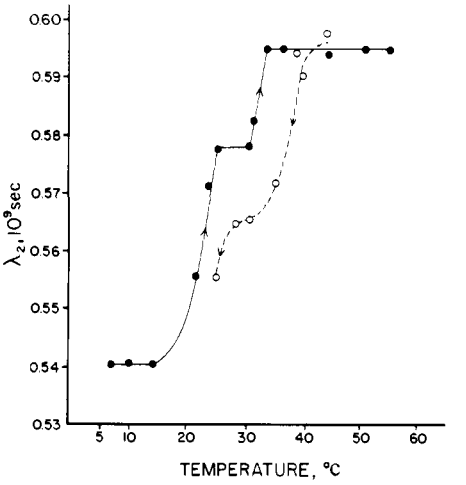

Figure 13. Plots of positronium annihilation rates against temperature for DODAC vesicles containing 17.2% (w/w) cholesterol. In the heating cycle the temperature of the sample was gradually increased (●), whereas in the cooling cycle the temperature was gradually decreased (○).

Discussion

Formation of closed bilayer DODAC vesicles is evident from the electron micrographs (Figure 1). Most of the vesicles had 300-Å diameters, but there were some with diameters as small as 250 Å and as large as 500 Å. The highest magnification picture showed bilayer vesicles with radii of 150 ± 10 Å and bilayer thickness of 50 ± 5 Å. These dimensions resemble very closely those observed for phospholipid liposomes[3-5] and are in complete accord with those reported previously for DODAC vesicles.[11]

Phase transitions of DODAC vesicles have been unequivocally demonstrated by several methods. Tables III and IV summarize the data for vesicles prepared in the absence and in the presence of cholesterol. As expected, temperature ranges of the transition and sensitivities depend on the method utilized. Positron annihilation is considered to be the most sensitive method.[21,29] Perturbations are minimal since no reagent is added (unlike in microviscosity determinations) and the size of the vesicles remains unaltered (unlike in the osmotic shrinkage experiments). Furthermore, positron annihilation measures a microscopic property (unlike that obtained in bulk viscosity measurements) efficiently on the subnanosecond time scale when all macroscopic motions and morphological changes are frozen. Annihilation rates of positroniums in pure water and in pure octadecane have been determined to be 0.57×10^9 and 0.32×10^9 s^{-1}, respectively.[21] The determined positronium annihilation rates in DODAC represent the weighed average of the rates for annihilation in water and in a hydrophobic environment. This method probes, therefore, hydrophobicity changes. The decrease of positronium annihilation rates with increasing sonication times of DODAC dispersions (Figure 9) supports this interpretation. Once the vesicles are formed, increasing the temperature results in greater positronium annihilation rates. This is a consequence of increased fluidities and water penetration between the bilayers and hence of increased probabilities of positron annihilation in an apparently aqueous environment.

Several aspects of phase transition temperatures, determined by positron annihilation, merit discussion. The phase transition temperature of single compartment bilayer DODAC vesicles, 37 °C, is essentially identical with that observed for dipalmitoyl-DL-α-phosphatidylcholine liposomes (36.4 °C).[31] Significantly, pretransition changes are clearly seen in DODAC vesicles (Figure 11). The appearance of pretransition temperature has been attributed to specific interactions between the choline moieties of the phospholipid and the water hydrating them.[32] The presence of the choline group was considered, in fact, essential for the pretransition to occur.[32] Observation of pretransition temperatures implies either a dif-

ferent behavior of DODAC and liposome vesicles or the higher sensitivity of our method or both. Even more surprising are the observed sharp pretransition and transition temperatures for cholesterol-containing vesicles. Conversely, addition of cholesterol initially broadens and ultimately eliminates the phase transition temperature of phospholipid liposomes.[33] Pronounced hystereses of the higher temperature transition were observed on determining positronium lifetimes in DODAC vesicles by decreasing the temperature (Figures 12 and 13). Concomitantly, the lower temperature phase transition disappeared. Hystereses have also been observed for the lower transition of multicompartment liposomes.[25] In contrast to DODAC, equilibrium cooling curves for liposomes were displaced to lower temperatures.

Thermal transitions of phospholipid liposomes have been rationalized in terms of morphological changes.[32] At temperatures below the pretransition, vesicles consist of one-dimensional lamellas with the hydrocarbon chains fully extended and somewhat tilted. Transformations from one- to two-dimensional structures occur at the pretransition temperature. The fully extended lipid molecules are distorted by periodic undulations. At the transition temperature, the hydrocarbon chains "melt" by assuming mobile liquid-like arrangements and revert to one-dimensional lattices. Phase transitions determined for DODAC vesicles can also be accommodated by analogous morphological changes. Detailed understanding of the temperature-dependent structural changes of DODAC vesicles will become available upon additional experimentation.

Only the main transition temperature was detected by macro- and microviscosity measurements (Figures 3 and 7). Addition of cholesterol decreases the bulk viscosities (Figure 3) by virtue of forming firmer and smaller DODAC vesicles which discharge faster through the capillary of the Ostwald viscometer. Interaction of 2-methylanthracene with cholesterol (vide supra) does not allow the comparison of temperature-dependent micro- and macroviscosity changes as a function of this additive.

Occurrence of osmotic shrinkage in hyperosmolar solution can be taken as substantial evidence for the presence of closed DODAC vesicles. Osmotic shrinkage is the consequence of much faster water effusion from the vesicles than solute infusion into them. Under ideal conditions, vesicles behave like perfect osmometers, i.e., they are completely impermeable to solute but are completely permeable to water. Experimental manifestation of this phenomenon is the linearity of reciprocal absorbances (or shrinkage rates) with the reciprocal (or linear) concentration gradients across the bilayer covering reasonable ranges of solute concentrations. Applying this criterion, multicompartment phosphatidylcholine liposomes were shown to be ideal osmometers.[34] Observed linearities of the extent (Figure 6) and initial shrinkage rates (Figure 5) of DODAC vesicles at given temperatures and conditions suggest ideal behavior in the observed concentration range of electrolytes. At 25 °C in the absence of added cholesterol DODAC vesicles are seen to behave like an ideal osmometer up to 0.10 M electrolyte gradient. Marked dependence of the initial shrinkage rate on the cation (Table I), even at 9.23×10^{-2} M osmotic gradient, implies the permeation of ions into DODAC vesicles.[35] Increasing the temperature or adding cholesterol to DODAC vesicles increases the extent of ion permeation. This is manifested in substantial decrease in the linearities of the plots in Figures 5 and 6 as well as in the slower initial shrinkage rates in the presence of cholesterol and at higher temperatures. The negative temperature dependence of the initial shrinkage rates of DODAC vesicles (Figure 7) is in accord with the composite ion-water permeabilities. Accordingly, the higher temperature phase transitions (42.9 and 44.2 °C in the absence and in the presence of cholesterol) are attributed to altered electrolyte permeation. Transitions occurring at 36.2 and at 33.2 °C represent, by analogy to other independent values (Tables III and IV), the melting of the hydrocarbon chains of DODAC bilayers.

Electron micrographs, temperature-dependent phase behavior, and osmotic response have provided the required evidence for the presence of closed bilayer DODAC vesicles. They resemble qualitatively the more complex liposomes. An important quantitative difference is the presence of a positively charged nitrogen in DODAC. This will cause electrostatic repulsions of the head groups from each other which, in turn, results in enhanced fluidities. Similar effects have been observed in dipalmitoyl-DL-α-phosphatidylcholine liposomes on adding increasing amounts of octadecylamine (Table II). Cholesterol-containing DODAC vesicles, below their phase transition temperatures, are appreciably less fluid, however, than those in the absence of this additive (Figure 7). A careful control of experimental conditions greatly enhances the utilities of DODAC vesicles as chemically simple yet functional membrane mimetic agents.[36]

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(36) NOTE ADDED IN PROOF. Using low angle light scattering and photon correlation spectroscopy, we have recently determined the molecular weight and hydrodynamic radius of 12 min sonicated DODAC (setting 3 on a Branson B-12 sonifier) vesicles to be 30 million and 309 Å. These values were best accommodated in terms of the formation of single compartment prolate DODAC vesicles (U. Herrmann and J. H. Fendler, to be published in *Chem. Phys. Lett.*, 1979).

Assignments of ^1H Nuclear Magnetic Resonances of the Cystyl, Asparaginyl, and Aromatic Residues of Arginine Vasopressin in D_2O. A Comparison with Lysine Vasopressin and Oxytocin in Terms of Solution Conformation

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Abstract: The resonances of the C^α and C^β protons of the cystyl, asparaginyl, and aromatic residues of [8-arginine]vasopressin (AVP) in D_2O at pD 3.8 and 20 °C were assigned in a rigorous manner by the use of isotopic isomers of AVP that contain specific replacements of protons by deuterons and by comparison of ^1H NMR characteristics of AVP to those of [8-lysine]vasopressin (LVP) and oxytocin (OT). Although there is extensive overlap of resonances of C^β protons even at 360 MHz, all of the chemical shifts of these protons and most of the couplings between them and their vicinal C^α protons could be determined, at least to a first approximation. It was concluded that the cyclic moieties (residues 1-6) of AVP, LVP, and OT possess essentially the same overall backbone conformation, and that the side-chain conformation—or rotamer populations—about the C^α-C^β bonds of the cystyl residue (positions 1 and 6), the tyrosyl residue (position 2), and the asparaginyl residue (position 5) are similar. This study indicates that selective replacements of C^β protons by deuterons are necessary to improve the accuracy of coupling constants extracted from 360-MHz spectra of AVP for use in conformational analysis.

Introduction

The primary structures of some of the naturally occurring neurohypophyseal hormones are shown in Figure 1. The solution conformations of these peptide hormones and their analogues in a variety of solvents have been studied by NMR spectroscopy.[2]

The neurohypophyseal hormone arginine vasopressin (AVP)[3] has been studied by ^{13}C NMR in both $(CD_3)_2SO$ and H_2O[4,5] and by ^1H NMR in $(CD_3)_2SO$[6] and D_2O.[7] In a previous study we reported a comparison of the chemical shifts of the amide protons, the temperature dependencies of these shifts, the exchange rates of these protons for deuterons, and the coupling constants between vicinal amide and C^α protons of AVP, LVP, arginine vasotocin (AVT), and oxytocin (OT) in $(CD_3)_2SO$ by ^1H NMR spectroscopy and concluded that the backbone conformations of these peptides are, to a first approximation, similar.[6] A comparison of these characteristics for these peptides in aqueous solution by ^1H NMR spectroscopy has not been reported. Although few studies of AVP have appeared in the literature,[4-7] several studies of LVP by ^1H NMR in $(CD_3)_2SO$[8,9] and aqueous solution[9,10] and by ^{13}C NMR in both $(CD_3)_2SO$ and H_2O[4,11]—as well as several studies of desaminolysine vasopressin (dLVP) by ^1H NMR in $(CD_3)_2SO$[12] and aqueous solution[13]—have been reported.

Analysis of the C^β proton region of OT in D_2O to yield couplings between vicinal C^α and C^β protons provides information on the conformations about the C^α-C^β bonds (χ^1 values) of some of the amino acid residues.[14-17] A similar analysis of this region of AVP in D_2O is complicated by a high degree of overlap of ^1H resonances in this region.[18] For example, the resonances of the C^β protons of five of the eight residues that have side chains and those of the C^δ protons of Arg^8 overlap between the narrowly spaced limits of 2.8 and 3.5 ppm—i.e., 37 distinct lines are expected to be observed within a region of only 0.7 ppm. This region is of particular interest to us because it contains information on the conformation of the disulfide bridge.

The first step in an NMR analysis is the assignment of resonances. Here we report the use of isotopic isomers (isotopomers)[19] of AVP that contain specific C^α or C^β protons replaced by deuterons to obtain assignments in D_2O for resonances of C^β protons in the region between 2.8 and 3.5 ppm and for those of the corresponding vicinal C^α protons. Assignment of resonances for half-Cys^1, Tyr^2, and Phe^3 are unequivocal, and those for Asn^5 and half-Cys^6 are rigorously justified. We also compare certain ^1H NMR characteristics of AVP, LVP, and OT and interpret these characteristics in conformational terms. We conclude that these three peptides have similar backbone conformations and that side-chain conformations—or rotamer populations—of residues in the cyclic moieties of AVP and OT are similar, with the exception of residue 3 (because of the difference in primary structure at this position) and possibly Gln^4 (which could not be analyzed in detail with the set of isotopomers used in this study).

Materials and Methods

Synthesis of Peptides. Unenriched [8-arginine]vasopressin (AVP) was synthesized by the solid-phase method reported by Live et al.,[20]

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