Fluorescence and Calorimetric Studies of Phase Transitions in Phosphatidylcholine Multilayers: Kinetics of the Pretransition†

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ABSTRACT: Discrepancies between calorimetric and fluorescence depolarization monitoring of the pretransition in multilamellar vesicles of synthetic phosphatidylcholines are shown to result primarily from the slow rate of this transition. The depolarization of fluorescence of the membrane-associated dye 1,6-diphenyl-1,3,5-hexatriene was used to determine the temperature of the pretransition for a series of heating and cooling scan rates. These temperatures, when plotted vs. scan rate, extrapolated linearly to the transition temperature at zero-scan rate, T_m = 29.8 ± 0.8 °C. The slopes obtained from these plots yielded characteristic times for the transition of 8 to 30 min. In addition, analysis of temperature-jump experiments, assuming first-order kinetics, gave characteristic times in the range 4–8 min. The data are taken to suggest a most likely value for the pretransition characteristic time of 5 ± 2 min, with larger values possibly explainable by supercooling effects. Slight differences between the calorimetrically and fluorimetrically determined main transition temperatures appear to result from perturbation of the phosphatidylcholine bilayer by the fluorescent probe.

In a previous study of the thermotropic behavior of dipalmitoylphosphatidylcholine (DPPC[1]), large, multilamellar vesicles (Suurkuusk et al., 1976), a disagreement was noted between the pretransition temperature, as monitored by differential scanning calorimetry and by scanning fluorescence depolarization using diphenylhexatriene (DPH) as a hydrophobic probe. Since moderate concentrations of DPH were shown not to significantly affect the calorimetric transition temperature, it was suggested that hysteresis may have been the source of the difference between our heating, calorimetric and cooling, fluorimetric scans. Subsequently, several reports have confirmed our suspicions of hysteresis in this transition (Sklar et al., 1977; Gaffney and Chen, 1977; Luna and McConnell, 1977; Tsong and Kanehisa, 1977). As yet, no attempt has been made to determine the source of this hysteresis.

In this paper, we report measurements of the rate of the DPPC pretransition. We have chosen the fluorescence polarization of DPH as a means of monitoring the phase transition, since these measurements can be made rapidly, and DPH has previously been shown to equilibrate between bilayer regions of vastly different fluidity quite rapidly ($t_{1/2} < 6$ s, Lentz et al., 1976b). Our results are consistent with a slow rate of the pretransition being the source of the hysteresis in DPPC vesicles.

Finally, we define other small discrepancies between the calorimetrically and fluorimetrically determined phase behavior of phosphatidylcholine multilamellar vesicles. Specifically, we report slight differences in the peak position and breadth of the main phase transition, as observed by these two techniques. In contrast to the pretransition results, these differences are shown not to be due to hysteresis but rather to perturbation of the bilayer by the probe.

Experimental Procedure

Materials. 1,2-Diacyl-3-*sn*-phosphatidylcholines (DMPC, DPPC, DSPC) were synthesized as described previously (Lentz et al., 1976a; Suurkuusk et al., 1976) by the methods of Robles and Van den Berg (1969). 3-*sn*-Glycerophosphorylcholine, needed for this synthesis, was prepared from hen egg yolk phosphatidylcholine (Chadha, 1970), and fatty acids were obtained from Sigma (>99% pure). The purity of the synthesized phosphatidylcholines was established by thin-layer chromatography (Quantum Q5 plates; chloroform–methanol–water, 65:25:4, as solvent), using 1 or 2 μmol of phosphatide per spot (Lentz et al., 1976a). All the phosphatides were judged to be better than 98% pure by this measure. DPH (Aldrich, zone-purified) was a gift from M. Shinitzky and Y. Barenholz.

Multilamellar Vesicle Preparation. Multilamellar liposomes were prepared for fluorescence or calorimetric studies by the method of Bangham et al. (1967) using an aqueous phase containing 50 mM KCl (UltraPure, Heico, Inc). The water used in both fluorescence and calorimetric studies was first deionized, then distilled from alkaline $KMnO_4$, and, finally, glass distilled and stored cold in borosilicate glass. For fluorescence studies, a 4-mL portion of suspended multilamellar vesicles (0.5 mM in lipid) was injected with 0.2 μL of 10 mM DPH in tetrahydrofuran, with rapid agitation. This suspension was flushed with argon above 50 °C in order to remove most of the tetrahydrofuran. A portion of the original vesicle suspension (2 mL) was injected with pure tetrahydrofuran and retained as a scattering blank. Samples were then gently swirled for at least 2 h above the transition temperature to allow equilibration of the DPH with the liposome bilayers (Lentz et al., 1976a) and to allow formation of closed vesicles (Bangham et al., 1967). Samples for the calorimetric studies were prepared in a similar fashion, except that they were approximately 10 mM in lipid and no DPH was added. The concentration of lipid in each dispersion was determined as inorganic phosphate, either by the Bartlett procedure (1959) or by a modification of the procedure of Chen et al. (1956).

Fluorescence Depolarization. Fluorescence depolarization

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[1] Abbreviations used: DPH, 1,6-diphenyl-1,3,5-hexatriene; DMPC, DPPC, and DSPC, 1,2-dimyristoyl-, 1,2-dipalmitoyl-, and 1,2-distearoyl-3-*sn*-phosphatidylcholine, respectively; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy.

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of DPH as a function of temperature was measured by a method similar to that described previously (Suurkuusk et al., 1976). A Perkin-Elmer MPF3 spectrofluorometer was modified by the addition of two polarizers (Oriel no. 2734), one in both the emission and excitation beams, and by the installation of a multiwave plate depolarizer in the emission beam (Karl Lambrecht Corp., Chicago). The polarizer in the emission beam was movable from outside the sample compartment by means of a cone-shaped gear and toothed wheel. In addition, a rotating magnet, driven by an electric motor, was mounted directly under the sample stage, in order that vesicle samples could be stirred continuously during a temperature scan. Control experiments confirmed that neither the presence of a rotating stirring flea in the cuvette nor the oscillating magnetic field had any effect on the fluorescence depolarization measurements, except to make them more reproducible. This modification eliminated the previous requirement for high concentrations of sucrose in the lipid dispersion (Lentz et al., 1976a). The Perkin-Elmer power supply was also modified to permit the use of a Hg–Xe 200-W lamp source (Canrad-Hanovia). DPH was excited at the 366-nm Hg band, and DPH fluorescence was measured at 430 nm, using the MPF3 390-nm cutoff filter to reduce scattered light. The intensity of light in the Hg band allowed the use of such narrow excitation slits that the contribution from scattered light was completely eliminated at lipid to dye ratios of 500:1. Depolarization of fluorescence due to light scattering (Teale, 1969) was not detectable above the main phase transition of phosphatidylcholine vesicles. Below the phase transition, a slight depolarization was detected but was not corrected for, since the depolarizing effect was fairly constant with temperature. The temperature of the sample chamber was controlled by a Haake FK-2 thermoregulated bath equipped with a Haake Model PG11 programmable temperature scanning control. A Yellow Springs Instrument thermistor probe connected to a Digitec Model 5810 digital thermometer was used to continuously monitor the temperature of the sample within the cuvette. Controls indicated that, with continuous stirring of the sample, the thermal gradient between the middle and the bottom of the sample was less than 1.5 °C. The temperature was monitored at the middle of the cuvette, while fluorescence was detected from a region slightly above the cuvette bottom. The exact thermal variation along the length of the cuvette was measured at different temperatures and scan rates. The gradient was found to be a linear function of the recorded temperature and displayed no dependence on scan rate. Temperatures reported here have, therefore, been corrected to reflect the temperature in the center of the excitation window of the cuvette. The thermistor was calibrated against a series of standard thermometers with an accuracy of ±0.10 °C, traceable to the National Bureau of Standards (A. H. Thomas). Reported transition temperatures are corrected to these standard thermometers.

A general treatment of the fluorescence probe depolarization technique as applied to membrane structure has been given by Shinitzky et al. (1971), while the use of DPH as a specific probe for the hydrophobic region of a lipid bilayer has been described by Shinitzky and Barenholz (1974). In this method, the anisotropy of probe fluorescence, r, is related to the rotational diffusion rate of the probe molecule, which, in turn, may be related to the apparent microviscosity, ζ, of the medium surrounding the probe by the Perrin equation (Weber, 1953). A full discussion of the assumptions involved in this treatment and of the relative sense in which the term "apparent microviscosity" should be understood has been given (Lentz et al., 1976a). In this earlier discussion, it was noted that the great practical utility of employing microviscosity changes for detecting phase transitions was that the natural logarithm of viscosity was expected to vary linearly with the reciprocal absolute temperature (Eyring and Jhon, 1969). In this study, we have taken further advantage of this property of the microviscosity and have presented our data as plots of the activation energy (Eyring and Jhon, 1969) of the microviscosity vs. temperature, where the activation energy is given by:

$$\Delta H^{\ddagger} = R\partial \ln \eta / \partial \ln (1/T) \quad (1)$$

Above the main phase transition, $\Delta H^{\ddagger}$ should be constant, but as the vesicles are cooled through their liquid-crystal to gel transition, $\Delta H^{\ddagger}$ should peak sharply, as does the excess heat capacity in a calorimetric experiment. Below the main phase transition, the motion of DPH becomes so anisotropic that the assumptions involved in the calculation of microviscosity break down (Kawato et al., 1977). Nonetheless, plots of $\Delta H^{\ddagger}$ vs. temperature remained empirically useful in defining the position of the pretransition, since the natural logarithm of the microviscosity varied very little with temperature below the main transition, except in the range of the pretransition (Lentz et al., 1976a). Values of the fluorescence lifetime for DPH in vesicle dispersions at various temperatures, needed in the derivation of apparent microviscosity, were estimated from a limiting low-temperature value of 11.4 ns as previously described (Lentz et al., 1976a). Derivitization of apparent microviscosity, other calculations, plotting, and statistical analysis were carried out using an IBM-370 computer in conjunction with a Cal Comp plotter at the University of North Carolina Computation Center.

Temperature-Jump Measurements. In order to produce a rapid change in temperature in the fluorimeter sample chamber, the bath and sample chamber were first equilibrated at the starting temperature of the temperature-jump experiment. The bath circulating pump was then switched off and the water was siphoned from the bath chamber. Water at the desired final temperature of the jump was added to the bath chamber and the circulating pump was switched back on. Using this procedure, with continuous stirring in the cuvette, the temperature of a vesicle dispersion could be jumped through the pretransition region in 2–3 min, depending on the magnitude of the jump. The anisotropy of DPH fluorescence was recorded for some time after the initial temperature jump and plotted according to eq 2 in order to obtain the rate constant for return of the vesicle suspension to equilibrium.

$$\ln \{[r(t) - r_2]/[r_1 - r_2]\} = -kt \quad (2)$$

Here, $r(t)$ is the DPH fluorescence anisotropy at time t, r_2 and r_1 are the anisotropies at the final and initial temperatures, respectively, and k is the first-order rate constant for relaxation of the vesicle suspension from state 1 to 2.

Calorimetry. The excess heat capacity was measured using a differential scanning calorimeter based on the heat leak principle. The general procedure has been described previously (Suurkuusk et al., 1976). In these experiments, approximately 0.7 mL of the lipid dispersion was scanned at a rate of 15 °C h^{-1}. The reported melting temperature, T_m, is that at which the heat capacity was a maximum.

Results

Calorimetry. The apparent heat capacity of a dispersion of DPPC multilamellar vesicles in the absence and presence of DPH is shown in Figure 1. Differences in the T_m values were observed for samples free of and containing DPH. These results, and those obtained for different lipids, are summarized in Table I. In addition, the observed thermodynamic param-

TABLE I: Phase Transition Parameters for Phosphatidylcholine Dispersions.

		main transition			pretransition		
lipid	method	T_m	ΔH (kcal/mol)	$\Delta T_{1/2}$	T_m	ΔH (kcal/mol)	reference
DMPC	differential	23.9[a] ± 0.1	5.0 ± 0.2	0.2	14.1 ± 0.3	1.0 ± 0.5	this work
DPPC	scanning	41.3[a] ± 0.1	8.6 ± 0.3	0.4	35.2 ± 0.3	1.5 ± 0.5	
DSPC	calorimetry	54.5[a] ± 0.1	10.2 ± 0.4	0.6	48.5 ± 0.3	1.7 ± 0.5	
DMPC + DPH	differential	23.4 ± 0.1	5.0 ± 0.2	0.2	13.0 ± 0.3	1.0 ± 0.5	this work
DPPC + DPH	scanning	40.8[a] ± 0.1	8.6 ± 0.3	0.4	33.5 ± 0.3	1.5 ± 0.5	
DSPC + DPH	calorimetry	53.7 ± 0.1	10.4 ± 0.4	0.6	46.4 ± 0.3	1.7 ± 0.5	
DMPC	DPH	23.8 ± 0.25[c]		1.6	10.0[d] 12.4[e]		this work
DPPC	fluorescence	40.6 ± 0.25[b]		1.6[a]	22.9,[d] 29.8,[h] 31.9[e]		
DSPC	depolarization	53.7 ± 0.5[c]		1.9	43.2,[d] 45.6[e]		
DMPC	differential	23.9	5.4	0.3[l]	14.2[g]	1.0	Mabrey and Sturtevant
DPPC	scanning	41.4	8.7	0.3[l]	35.3[g]	1.8	(1976, 1977)
DSPC	calorimetry	54.9	10.6	0.5[l]	51.5[g]	1.8	
DPPC	Tempo partitioning	41.5		3.6[i]	30.7[f,j]		Gaffney and Chen (1977)
DMPC	*cis*-parinaric	23.0			10[g]		Sklar et al. (1977)
DPPC	fluorescence	42.0		1-2	32[g]		
DSPC	intensity	54.0			49[g]		
DMPC	turbidity	24.3			10.0[j] 15.8,[k]		Tsong and Kanehisa
DPPC	at 546 nm	41.1			23.5,[j] 32.2[k]		(1977)

[a] Heating scan, 15 °C/h. [b] Average of ten heating and cooling scans at different rates. [c] Average of four heating and cooling scans at different rates. [d] Cooling scan, 18 °C/h. [e] Heating scan, 18 °C/h. [f] Cooling scan, 6-7 °C/h. [g] Heating scan, 30 °C/h. [h] Zero-scan rate. [i] Estimated from figures. [j] Cooling scan, 9 °C/h. [k] Heating scan, 9 °C/h. [l] Calculated, $\Delta T_{1/2} \simeq 6.9\ T_m^2/N\Delta H$.

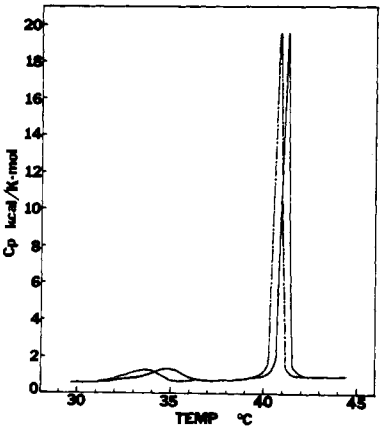

FIGURE 1: Calorimetric scans of DPPC dispersions: pure DPPC at about 10 μmol/mL (—); DPPC containing 1 DPH per 500 lipid molecules (- - -). Scan rate = +15 °C/h.

eters are compared in Table I with similar results obtained by recent calorimetric and other techniques.

Fluorescence Depolarization. Figure 2 displays the microviscosity activation energy vs. temperature plots for DMPC, DPPC, and DSPC multilamellar vesicles. The T_m values, determined from the peak in the activation energy plots, are also recorded in Table I. Excellent agreement is seen between the two sets of calorimetric results. However, less satisfying agreement is apparent between the two fluorimetric results (DPH fluorescence depolarization and *cis*-parinaric acid fluorescence intensity) and the calorimetric results.

Evaluation of Hysteresis. Figure 2 contains the activation energy vs. temperature plots for scans made both in heating and cooling directions. The hysteresis in the pretransition is quite evident for all three phosphatidylcholine species. Conversely, no hysteresis was observed for the main phase transition in any of the phosphatides examined.

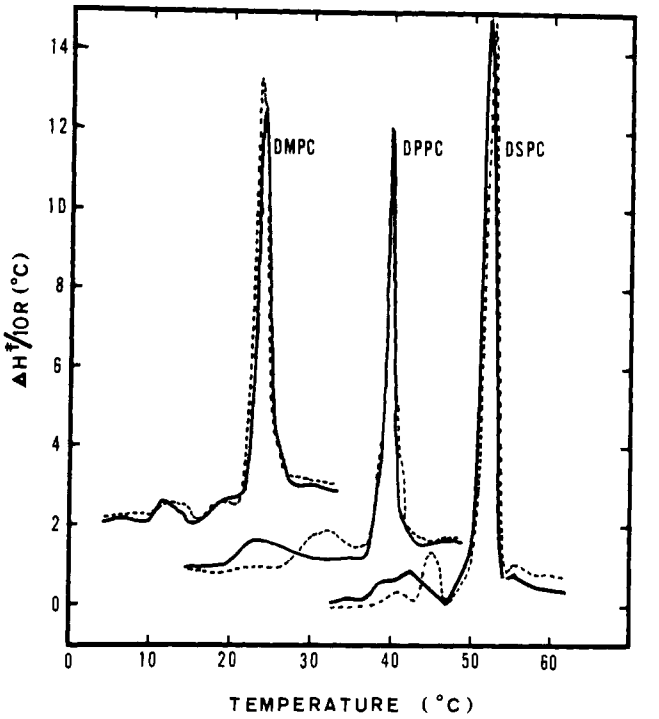

FIGURE 2: Fluorimetric scans of phosphatidylcholine dispersions. Activation energy of the microviscosity vs. sample temperature recorded at the midpoint of the cuvette. Scan rate = ±18 °C/h. Sample concentrations were about 0.5 μmol/mL. The data for DPPC and DMPC are displaced upward by 1 and 2 ordinate units, respectively. Cooling scans (—); heating scans (---).

In order to determine the precise origin of the hysteresis in the pretransition, microviscosity activation energy scans were

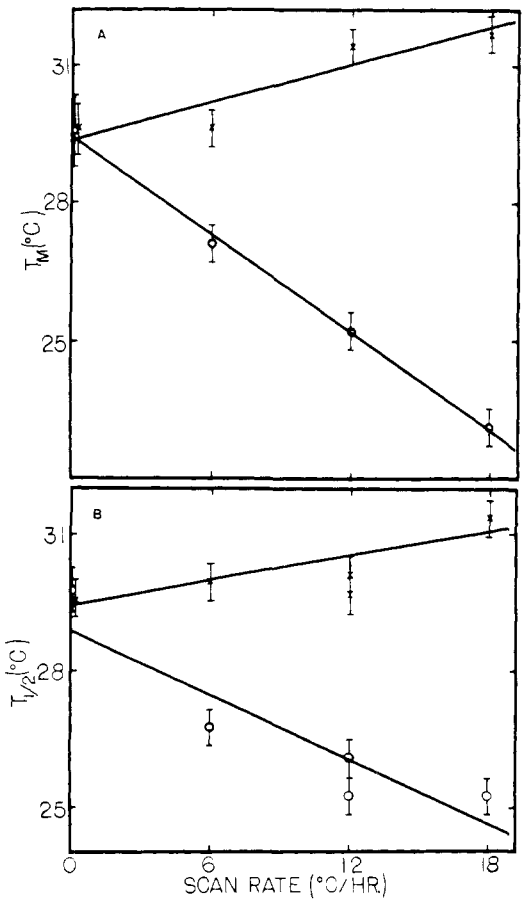

FIGURE 3: Temperature of the pretransition in DPPC multilamellar vesicles as a function of fluorimetric scan rate. (A) Peak temperature of the pretransition range, T_m. (B) Mean temperature of the pretransition range, $T_{1/2}$, in plots of microviscosity activation energy vs. temperature. The lines drawn through the data are least-square fits.

recorded at different scanning rates, and the resultant pretransition characteristic temperatures were plotted vs. scan rate, as shown in Figure 3. The shape of the activation energy scans in the pretransition region was often ill defined. The peak activation energy (T_m) was often quite far removed from the mean temperature of the transition range ($T_{1/2}$). Such behavior would be expected if the scanning rate were comparable to the rate of the transition. The variations with scan rate of T_m and $T_{1/2}$ shown in Figure 3 are also consistent with this hypothesis. Analysis of the scan rate dependence of T_m and $T_{1/2}$ according to a two-state model leads to a simple expression for the first-order rate constant of the transition:

$$k = \ln 2/b \qquad (3)$$

where b is the change in T_m or $T_{1/2}$ for an increment in scan rate (see Figure 3). Values of k are given in Table II.

Temperature-Jump Study. Treatment of the temperature-jump experiments is illustrated by the two plots of Figure 4. In these plots, we have recorded $\ln [r(t) - r_2]/[r_1 - r_2]$ as a function of the time following the onset of the temperature jump (see eq 2). Following a short time required for temperature equilibration in the sample, this quantity varied linearly with time, as predicted by eq 2. The characteristic times derived from the slopes of linear regions for four separate temperature-jump experiments are given in Table II. The temperature-jump data are, therefore, consistent with a model of the pretransition of dipalmitoylphosphatidylcholine as a two-state transition following first-order kinetics, with a characteristic time of 5 ± 2 min.

TABLE II: Characteristic Times for the Pretransition in DPPC Multilamellar Vesicles.

method	$t_{1/2}$ (heating) (min)	$t_{1/2}$ (cooling) (min)
temp jump (19 ↔ 38 °C)	3.6 ± 0.9	4.9 ± 0.3
temp jump (27 ↔ 33 °C)	4.1 ± 0.7	7.7 ± 3.5
T_m (peak) vs. scan rate	8.0 ± 3	30.0 ± 3.5
T_m (mean) vs. scan rate	14.0 ± 3	17.0 ± 3.5

Discussion

We have found that the apparent characteristic temperatures for the pretransition in three different phosphatidylcholine, multilamellar vesicle systems depend on the method used to monitor the transition. The T_m values were observed to vary with the rate and direction of scanning in a series of fluorimetric experiments. In addition, for roughly constant scan rates, calorimetrically and fluorimetrically determined T_m values were observed to differ.

The dependence on scan rate of the pretransition characteristic temperatures has been shown to result primarily from the slow rate of this transition. Direct temperature-jump experiments with DPPC multilamellar vesicles indicated a "relaxation time" of 5 ± 2 min for this transition (Table II). This value is in rough agreement with the value derived from analysis of the variation of apparent T_m with heating rate but is smaller than the value obtained from a similar analysis of cooling scans. This discrepancy could be due to the simplistic model assumed in order to derive a rate constant from the scan rate data. It is also possible that a super cooling phenomenon occurs during cooling scans and is not detectable during temperature-jump experiments. This possibility is consistent with the observation of greater hysteresis effects in cooling dilatometric scans of the DPPC pretransition region than in heating scans (J. F. Nagle, private communication). Whatever the reason for the difference between these two estimates, it is clear that the pretransition is slow, with characteristic times on the order of several minutes.

The pretransition of glycerophosphatide dispersions is known to be associated with the glycerophosphorylcholine head group and, for this reason, has been assigned to a conformational change in the head-group region of the bilayer (Ladbrooke and Chapman, 1969). Vaughan and Keough (1974) have shown that any modification of the choline head group leads to the immediate disappearance of the pretransition. Aside from these correlations of the transition with the presence of the choline head group, no direct data exist to confirm that this transition is actually associated with a conformational change in the phospholipid head-group region. By contrast, we have noted in a previous publication (Lentz et al., 1976a) that the sensitivity of DPH fluorescence depolarization to the pretransition argued in favor of some change in the hydrophobic portion of the bilayer being associated with the transition. Such a change in the hydrophobic region may or may not be associated with a conformational change in the head group. Rand et al. (1975) have presented low- and high-angle X-ray diffraction and monolayer data that they interpreted as indicating the existence of an L_β phase (extended chains perpendicular to the bilayer surface) between about 35° and 42°. Janiak et al. (1976) interpreted similar X-ray data, in somewhat more detail, and concluded that the bilayer assumed a rippled conformation, with tilted acyl chains ($P_{\beta'}$ phase) in

this temperature range. A subsequent freeze-fracture electron microscopy study provided data interpreted as being consistent with the $P_{\beta'}$ proposal (Luna and McConnell, 1977). Both of these proposals require that substantial changes in individual molecular positions be coordinated over fairly long distances. Such a long-range reorganization of an ordered matrix might be expected to be slow and thus consistent with our observations of characteristic times for the transition in the range of 3–11 min. In addition, both sets of X-ray data provide evidence for a change in acyl-chain packing in the region of the pretransition, in agreement with our fluorescence results. Whatever the detailed nature of the conformational change associated with the pretransition, our enthalpy data (Table I) suggest that it does not vary significantly with increasing chain length of the phosphatidylcholine.

Apart from discrepancies introduced by differences in scan rates, our results reveal slight differences between the calorimetric and fluorimetric T_m values for the main transition and especially for the pretransition in phosphatidylcholine dispersions. The main transition discrepancies are revealed by the data in Table I, in that the calorimetric values are between 0.1 and 0.8 °C higher than the fluorimetric values. By contrast, for phosphatidylcholine dispersions containing 1 DPH per 500 lipid molecules, the calorimetric and fluorimetric T_m values shown in Table I are essentially identical. It thus appears that the fluorescence probe perturbs the lipid matrix in such a way as to lower the main transition slightly. It is interesting that the DPH-induced depression of T_m varies directly with the length of the phosphatidylcholine acyl side chain. Since the enthalpy of the transition increases with acyl chain length, while the transition temperature remains relatively constant (on the absolute scale), the colligative property rules for ideal liquid solution–ideal solid solution phase equilibria would predict an inverse dependence of T_m depression on chain length (Moore, 1962; Seltz, 1934). We conclude that the mixing of DPH with phosphatidylcholine lamellar phases must be nonideal, even at very low mole fractions of DPH. It is possible that the observed correlation of T_m depression with acyl chain length is related to the fact that the partial molar volume changes associated with the main transition increase with increasing chain length (Nagle and Wilkinson, 1978). The presence of a volume-filling impurity, such as DPH, should stabilize the less dense phase relative to the more dense phase, i.e., the L_α phase relative to the $P_{\beta'}$ or L_β phase. This added stabilization of the fluid phase would result in a lowering of the phase transition temperature, and the magnitude of the shift in T_m would increase with increased difference in the density of the two phases.

The discrepancies between the calorimetric and fluorimetric results for the pretransition are even more dramatic. The data in Table I reveal that the fluorimetrically detected pretransition T_m for DPPC is about 1.5 °C lower than that detected calorimetrically in a sample containing DPH. It is not clear whether this difference is within the considerable error range involved in defining the characteristic temperature for the pretransition. If the difference were significant, it might suggest that DPH partitions more favorably into the high-temperature than the low-temperature phase bordering the pretransition. Additional studies will be required to establish if this may be the case. In any event, our data make it clear that DPH and other membrane probes (see Table I) somewhat perturb the bilayer structural changes they are meant to detect.

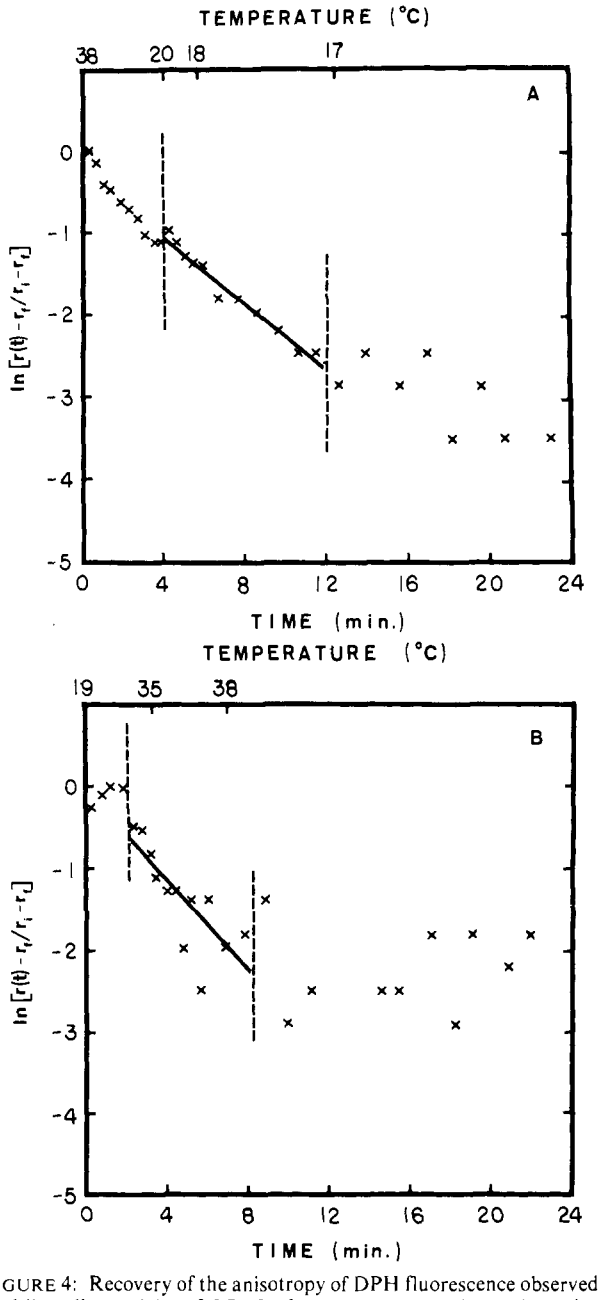

FIGURE 4: Recovery of the anisotropy of DPH fluorescence observed in multilamellar vesicles of DPPC after a temperature jump through the temperature range of the pretransition. The time following the jump is recorded on one abscissa, while the temperature recorded at the midpoint of the sample cuvette is recorded on the other abscissa. The quantity plotted on the ordinate is described in the text. Characteristic times for the pretransition were obtained from a least squares linear fit of the data within the region indicated by verticle bars; (A) cooling temperature jump; (B) heating temperature jump.

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Binding of Recrystallized and Chromatographically Purified 8-Anilino-1-naphthalenesulfonate to *Escherichia coli lac* Repressor†

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ABSTRACT: 8-Anilino-1-naphthalenesulfonate (Ans), recrystallized from water as the magnesium salt, contains a fluorescent impurity representing 0.3% of the absorbance at 351 nm. This impurity can be removed by Sephadex LH-20 chromatography. The chromatographic and spectral properties of this impurity suggest that it is bis(Ans), a dimer of Ans. This bis(Ans) impurity makes a significant contribution to the fluorescence increment observed when *lac* repressor is added to recrystallized Ans. This occurs because bis(Ans) binds much more tightly to this protein than does Ans. The dissociation constant divided by the number of binding sites per subunit is 3.1×10^{-6} M for bis(Ans); the corresponding value for Ans is $> 1 \times 10^{-4}$ M. Because of their differing absorption spectra, the impact of this bis(Ans) impurity is especially large with excitation wavelengths above 400 nm. Users of recrystallized Ans should consider the potential consequences of this impurity whenever working with a protein to which Ans binds weakly.

The fluorescent compound 8-anilino-1-naphthalenesulfonate has been commonly used as a probe of protein structure (Brand & Gohlke, 1972). This probe binds noncovalently to a variety of proteins, typically with a large decrease in the wavelength of maximum fluorescence emission and a large increase in the fluorescence quantum yield. These changes are favored by binding sites that are nonpolar (Stryer, 1965) and act rigidly within a nanosecond time scale (Gafni et al., 1977).

We have used Ans[1] to probe structural changes within the *lac* repressor of *Escherichia coli* (Worah et al., 1978). During the course of this study, we came to realize that Ans, purified by recrystallization of the magnesium salt from water, contains an impurity that contributes significantly to the fluorescence enhancement observed in the presence of *lac* repressor. We report a method for removing this impurity from recrystallized Ans that has allowed us to directly evaluate the effects of this impurity by comparing recrystallized and purified Ans. This impurity has been isolated and tentatively identified as bis(Ans), a dimer of Ans first synthesized by Rosen & Weber (1969).

We will discuss the various factors which enable this bis(Ans) impurity to make a prominent contribution to the fluorescence increment observed when a protein is added to recrystallized Ans. This study serves as a caution to the many investigators using Ans to consider the potential consequences of this bis(Ans) impurity.

Klungsøyr (1971) reported removing some darkly colored impurities from Ans by passage through a Sephadex G-25

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[1] Abbreviations used: Ans, 8-anilino-1-naphthalenesulfonate; bis(Ans), bis[4,4′-(8-anilino-1-naphthalenesulfonate)]; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

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