Influence of Hydration on the Formation and Stability of the Critical Bilayer State

Kazuhiro Fukada[†] and Norman L. Gershfeld*

National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Building 6, Room 139, Bethesda, Maryland 20892-2755

Received: March 12, 1997; In Final Form: June 18, 1997[⊗]

Phospholipid dispersions that undergo a multilamellar vesicle (MLV) to unilamellar vesicle (ULV) transformation at a critical temperature T^* form a single bilayer spontaneously and rapidly at the air−water interface. The "surface bilayer" rapidly covers the entire air−water surface, yielding a surface tension ∼70 mN/m. This indicates that the outermost layer of the surface bilayer is its hydration layer. At 100% relative humidity (R.H.) the surface bilayer is stable indefinitely but becomes unstable, forming a monolayer, when the R.H. is reduced. The rate of breakdown to monolayer varies inversely with the R.H. The rapid formation of the surface bilayer is a manifestation of the superposition of surface forces on the MLV−ULV transformation that occurs simultaneously within the bulk dispersion at T^*. Surface forces act primarily to accelerate the MLV−ULV transformation.

I. Introduction

The spontaneous formation of a bilayer that spans the entire air−water surface of aqueous phospholipid dispersions and at a critical temperature T^* that depends on the lipid composition has been demonstrated by radiotracer[1,2] and permeability[3] studies. Parallel observations of the heat capacity[4] and mechanical properties[5] of the dispersed phase of phospholipid bilayers indicate that molecular and topological events commensurate with a transformation to unilamellar vesicles occur within the bulk phase at the same temperature that the "surface bilayer" forms. Thus, phospholipid dispersions exhibit a heat capacity anomaly with energetics comparable to that for the transformation of multilamellar vesicles (MLV's) to unilamellar vesicles (ULV's) against the opposing interbilayer van der Waals force.[4] Moreover, the response of these dispersions to a shear stress indicates that bilayer weakening occurs in a narrow temperature interval around T^*.[5] Because the unilamellar state forms at a temperature singularity T^* and exhibits a heat capacity anomaly and a slowing of the rate of the MLV−ULV transformation with temperature[4] as $(T^* - T)^{-1}$, which are properties associated with critical states,[6] the unilamellar structure that forms at T^* is considered to be a critical state. At temperatures above and below T^* the bulk dispersion consists only of MLV's. These are general properties of phospholipids, having also been observed with multicomponent mixtures from a wide variety of cell membranes. For each of these complex mixtures T^* equals the physiological temperature of the cell from which the lipids are extracted.[5]

To augment our understanding of the properties of this unique state, we have examined the influence of relative humidity on the rate of formation and stability of the surface bilayer. The impetus for this study is a paradox that arose from the initial observations of the surface bilayer,[1,2] namely, a discrepancy between the measured and theoretical value for the surface tension of a bilayer at the air−water interface. The basic issues involved are as follows. A bilayer that forms at the air−water surface would necessarily have an upper surface consisting primarily of hydrated polar groups (Figure 1). In principle, such a structure will be stable provided water in the vapor phase is in equilibrium with the hydration layer of the bilayer, i.e., at the vapor pressure of water at T^*. Since the surface tension of a flat bilayer in suspension (vesicles with large radius of curvature) is near zero,[7] at the air−water surface with hydrated polar groups exposed to air the surface tension of the surface bilayer will be determined primarily by the hydration layer and therefore should be similar to that of a water surface with a surface tension of ∼70 mN/m (Figure 1). It is therefore paradoxical that the reported value for the surface tension of the dispersion at T^* is ∼20 mN/m,[1,2] a value consistent with a condensed lipid monolayer whose upper surface is effectively that of a fluid hydrocarbon (Figure 1).

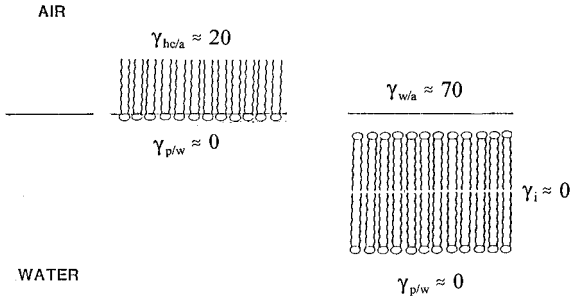

Figure 1. Models illustrating probable orientation of lipid molecules in a monolayer and in a bilayer at the air−water surface. The surface tensions listed are for the contributions of each of the interfaces to the measured surface tension. The following quantities are defined for the monolayer: hc/a, hydrocarbon chain−air; p/w, polar group−water. The following quantities are defined for the bilayer: w/a, hydration layer−air; i, interface between opposed hydrocarbon chains. The water layer above the surface bilayer is its hydration layer. Surface pressure is $\pi = \gamma_f - \gamma_w$ where f and w refer to the surface tension of the film-covered and water surface, respectively.

Another aspect of the phenomenon concerns the rate of surface bilayer formation as determined by radiotracers compared with the time required for the surface tension to reach its equilibrium value. Radiotracers indicate that surface bilayer formation is completed essentially within several minutes[2] while the corresponding surface tension measurements require as much as 24 h before equilibrium is reached.[2]

The radiotracer and surface tension studies have now been reconciled by experiments that focus on the conditions for

* To whom correspondence should be addressed. Fax: 301-402-0009. E-mail: ngo@cu.nih.gov.
[†] Current address: Department of Chemistry, Tokyo Metropolitan University,
[⊗] Abstract published in *Advance ACS Abstracts,* September 1, 1997.

S1089-5647(97)00928-0

maintaining an equilibrium hydration layer for the surface bilayer. In this study we show that when 100% relative humidity is maintained at T^*, the surface tension of the surface bilayer is, as predicted, essentially that of a water surface, and it is attained very rapidly. However, if less than the equilibrium water vapor pressure is present, the surface bilayer disassembles, forming a lipid monolayer with a rate that varies inversely with the relative humidity. The slow rates of monolayer formation observed in the inital studies are attributable to relative humidities that approach but do not equal 100%. A concluding discussion will examine the significance of this result for the mechanism and thermodynamics of assembly of the surface bilayer and the critical unilamellar state.

II. Methods

A. Materials. Dimyristoylphosphatidylcholine (DMPC) was obtained from Avanti Polar Lipids, Inc., Alabama. The sample purity was given as >99%. Impurities on the order of 0.5 mol % are sufficient to modify the surface tension−temperature relation.[8] Since conventional analytical methods (e.g., thin-layer chromatography) are usually insensitive at this level of lipid impurity, we have estimated the purity of this sample from the premelting heat capacity anomaly at $T < T_m$ [9] using a microcalorimeter designed to measure isothermal heat capacities.[10] The purity of the DMPC as measured by this instrument is ~99.2 mol %. Although this level of impurity is likely to give surface tension−temperature relations slightly different from our previous results with highly purified DMPC,[1,2] it is sufficiently pure to be used for illustrating the influence of hydration on formation of the surface bilayer. This allows us to avoid the lengthy purification procedure necessary for producing pure DMPC.[8] All experiments are performed in the absence of light, since we have observed that 1−2 days' exposure to fluorescent lighting will cause some contamination to appear in surface films (unpublished).

B. Film Formation and Measurement of Surface Tension.. Surface tensions were obtained by the Wilhelmy plate method.[11] Plates made of quartz, Pt, and filter paper each with similar dimensions were employed. Identical surface tensions were obtained with each. We have used the Pt plate for the majority of the experiments. The plate is suspended from a Cahn electrobalance (Cerritos, CA) by a thin wire and kept in a fixed position. The measurement consists of raising the entire constant temperature chamber and cell containing the lipid by a laboratory jack until the water surface just touches the Wilhelmy plate. This initial reading is taken as the surface tension of water at the temperature of the measurement. The surface tension of water was measured between 13 and 36 °C and agreed (±0.2 mN/m) with literature values.[12] The rate of change of surface tension with time was obtained by a strip chart recording of the Cahn electrobalance output. The data are recorded in units of surface tension γ, but we report them as surface pressure defined by $\pi = \gamma_w - \gamma_f$ where subscripts w and f refer to the water and film-covered surfaces, respectively.

Since lipid dispersions were utilized in the initial publications,[1,2] we have used the equilibrium spreading pressure method[11] in which lipid is confined to the air−water surface to compare with our initial studies and to establish that our results are independent of the method used to form the films. The principal aim of the equilibrium spreading pressure experiment is to obtain the properties of the bulk lipid from measurements of the equilibrium surface film.[13] However, formation of surface films in equilibrium with the bulk phospholipid can be problematic primarily because of the difficulty of establishing the equilibrium state of the bulk lipid in water.[14] Examples of the sensitivity of surface tension to metastable bulk DMPC preparations have been reported;[15−17] the metastability effects are particularly obvious at temperatures below the gel−liquid crystal transition temperature T_m. These have been shown to be largely due to incomplete hydration of the bulk phase lipid or to the use of organic solvents in the preparation of the dispersions. In our studies all bulk preparations are completely hydrated, and the dispersions are prepared without the use of organic solvents.

In our studies milligram quantities of DMPC are supported in the air−water surface within a small (1 cm^2) Pt grid shaped as a basket. The lipid is confined to a small region of the surface of the measuring cell, thereby providing a relatively large source of hydrated lipid from which the surface films may spread. Some dispersion preparations are also examined for comparison with the equilibrium-spreading pressure studies. DMPC films that form at $T < T_m$ are gaseous with surface pressures $\pi < 0.1$ mN/m.[18] This is below the sensitivity of our present instrumentation and therefore will appear as zero. Since T_m for DMPC is 23.8 °C, we chose to start each experiment between 22 and 23 °C so that even in the presence of lipid the surface tension of water could be obtained at this temperature. This procedure provides an internal calibration of the system. Equilibration of DMPC at 23 °C for 4 h did not yield any significant surface pressures. Other studies[15−17] with DMPC at 23 °C report a small increase in surface pressure at this temperature after about 2 h but not at lower temperatures (e.g., 22 °C). Moreover, these reports indicate that after 2−5 days the surface pressure sometimes increases inexplicably to substantial levels. We have not observed these effects, and the discrepancy with our studies may be due to our use of complete darkness when measuring surface pressures to avoid any possibility of photoinduced decomposition.

The experimental protocol consists of equilibrating the lipid on the water surface at 22−23 °C for a minimum of 30 min to hydrate the lipid and to equilibrate the vapor space. Extended equilibration times, up to 4 h, did not alter the experimental results. After completion of the equilibration the entire system is heated to the experimental temperature. To heat the constant temperature chamber 10 °C above the inital temperature generally takes less than 3 min (see below).

The rate of monolayer formation was obtained by measuring π as a function of time t, using the following relation[19,20]

$$d\pi/dt = K(\pi_f - \pi_t) \quad (1)$$

where the subscripts f and t are for the final (time-independent) and instantaneous values of π, respectively. K, the rate constant, is obtained from the integration of eq 1 as

$$K = \frac{1}{t} \ln \frac{\pi_f}{\pi_f - \pi_t} \quad (2)$$

C. Constant Temperature and 100% Relative Humidity Chamber. The concept that a stable bilayer will form at the air−water surface only with equilibrium water vapor, i.e., 100% relative humidity, pointed to the surface tension measurements as the possible source of the paradox. Radiotracer measurements are typically at 100% relative humidity because they are made in a closed system with a small vapor space.[2] However, 100% relative humidity for surface tension measurements is generally difficult to attain because complete enclosure of the vapor space is usually compromised by external controls for manipulating the measuring device. In the present studies a compact anodized square aluminum box (9 cm × 9 cm × 5 cm), with a flat cover consisting of two equal sections that may

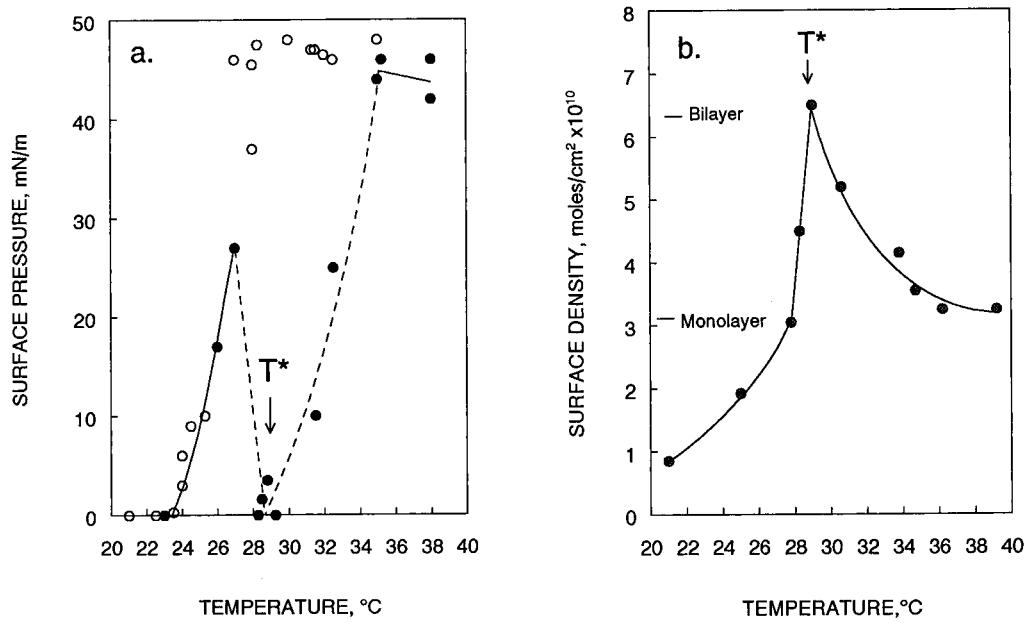

Figure 2. (a) Surface pressure−temperature phase diagram for DMPC, showing the influence of relative humidity. Surface-confined lipid is equilibrated at 23 °C, then heated rapidly to the experimental temperature *T*. ● is for 100% relative humidity. ○ is for ∼90% relative humidity. (b) Film density−temperature relation obtained using dispersions of ^{3}H-DMPC, redrawn from Tajima and Gershfeld,[2] at 100% relative humidity. Monolayer density is 55 Å^2/molecule.

be slid apart, served as a constant temperature high-humidity chamber for surface tensions. A 2 mm diameter hole was drilled in the center of the split cover to allow the thin wire support of the Wilhelmy plate to pass through. The interior volume is maintained at constant temperature by Peltier devices positioned on all five exterior surfaces of the box, including each half of the cover; the bottom surface is covered by insulation. Solutions for surface tension measurement are contained in a small Pyrex dish (4.5 cm i.d., 3.5 cm height) that is placed in the center of the constant temperature box. The dish is immersed in a pool of water, which serves to humidify the interior and provide for efficient heat transfer from the metal box to the Pyrex dish. Constant temperature (±0.1 °C) is maintained with a thermistor set in the pool of water surrounding the Pyrex dish and incorporated into a feedback circuit for controlling the power suppy to the Peltier devices. Rapid temperature changes of 10 °C could be achieved within several minutes.

Three levels of relative humidity are used in this study. (a) For 100% relative humidity: the metal box is filled with water to the same level as that of the solution in the Pyrex dish; wet filter paper lining the interior walls of the box is immersed in the surrounding water. In addition, after the Wilhelmy plate is immersed in the surface, the glass dish is covered with wet filter paper, leaving sufficient area for the supporting wire of the plate to move freely. We have measured the temperature of the bulk water and of the water surface in the glass dish with thermistors placed 5 mm below and above the surface. With appropriate care in keeping the filter paper wet all temperatures agree within 0.1 °C, indicating that evaporation was minimal and that the vapor space was at 100% relative humidity. (b) The second level of relative humidity is achieved when wet filter paper is not used to maintain high humidities. Temperature differences between the water surface and the air space above it are about 1 °C, indicating some evaporation is occurring. We estimate the relative humidity to be ∼90%, the approximate decrease in vapor pressure due to cooling. (c) The third level of relative humidity was achieved by opening the chamber to the room air, typically 70−80%. Although these are relatively crude distinctions of relative humidity, they proved to be sufficiently reproducible to demonstrate the sensitivity of the surface bilayer to changes in relative humidity.

III. Results

A. Influence of Relative Humidity on the Surface Tension of the "Surface Bilayer". Figure 2a shows the surface pressure of fully hydrated DMPC crystals confined to the air−water surface as a function of temperature. Figure 2b gives the surface density of DMPC as a function of temperature previously obtained by use of radiotracers;[1,2] the data is reproduced here to facilitate our discussion. Of particular significance is the film density, which varies between a condensed monolayer and a bilayer between 26 and 34 °C.

Each surface pressure in Figure 2a was obtained by equilibrating the lipid at 23 °C and then heating rapidly (<3 min) to the experimental temperature. The open points represent films that were formed under conditions in which the relative humidity is ∼90% (see Methods section). These data, in general, agree with previous results obtained with dispersions of highly purified DMPC[1,2] except that they are displaced by approximately 0.5 °C to lower temperatures. As noted previously (see Methods section), this result is typical for DMPC with a contamination of about 0.5 mol %.[8] For films that form at temperatures below T_m (23.8 °C) the surface pressure is very near zero. This is to be expected for the very low concentration, gaslike films that form at $T < T_m$.[18] The surface density of the monolayer is approximately one-fifth to one-third that of a condensed monolayer (Figure 2b). Under this relative humidity (<100%) π increases with temperature and reaches a maximum at *T**, the high values signifying the presence of monolayers in the surface.

The filled points in Figure 2a represent separate runs under the condition of 100% relative humidity. At temperatures below 26 and above 34 °C the surface pressure is not dependent on relative humidity. However, between these temperatures there is a dramatic decrease in the surface pressure, with a minimum of $\pi \approx 0$ (i.e., $\gamma_f \approx 70$ mN/m) at 28.5−29.1 °C near the critical temperature *T** of 29 °C. As Figure 2b indicates, the surface density exceeds that for a condensed monolayer in this tem-

TABLE 1: Influence of Temperature and Relative Humidity (R.H.) on *K*, the Rate of Monolayer Formation[c]

T, °C	*K*, min^{-1}	R.H., %
24−26	0.1 ± 0.05	70−100
>34	0.1 ± 0.05	70−100
26−34	0.1 ± 0.05[a]	∼90
26−34	0.003 ± 0.001[b]	∼90
*T** (28.5−29.1)	0	100

[a] Initial rapid process. [b] Secondary slow process. [c] Hydrated DMPC is confined to the air−water surface, equilibrated for a minimum of 30 min at 23 °C and then heated rapidly to the experimental temperature *T*.

perature range, reaching the bilayer density at *T**. We have maintained these conditions at *T** for as long as 6−8 h, with the surface pressure remaining at zero. This is far longer than is necessary to observe film-spreading at other temperatures, e.g., for $T < 26$ °C or $T > 34$ °C, when changes in surface tension are noted almost immediately upon reaching the experimental temperature. The very low surface pressure signifies that the surface tension remains at the value for water. Thus, at *T** and 100% relative humidity where the surface bilayer forms,[2] the surface tension is the value predicted for the hydrated surface of a bilayer (Figure 1). Between 26 °C and *T** and between *T** and 35 °C, at 100% relative humidity, the $\pi-T$ relation in Figure 2 is represented by dashed lines to indicate that the values of π are somewhat variable and scatter along these lines.

B. Influence of Relative Humidity on the Rates of Monolayer and Surface Bilayer Formation. Rate constants for monolayer formation *K*, from eq 2 (see Methods section), under various conditions of relative humidity and temperature are tabulated in Table 1. Below 26 and above 34 °C the rate of monolayer formation can be described by a single process that is independent of the relative humidity. The magnitude of the rate constant for this process is $K = 0.1 \pm 0.05$ min^{-1}. This value encompasses all the data for temperatures below 26 and above 34 °C, and for the full range of relative humidities, 70−100%. Thus, when the temperature of the hydrated lipid is raised from 23 °C to temperatures below 26 °C or very rapidly to temperatures above 34 °C, there is an instantaneous increase in surface pressure as soon as the experimental temperature is reached. The stable, final value of π is reached within 30 min for the lower temperatures and within several hours for $T > 34$ °C. Similar behavior has been observed in the region of T_m (23.8 °C) where DMPC forms gaslike films; a rapid change in surface pressure is observed immediately after crystals are introduced on the surface at T_m, and the equilibrium value is reached shortly thereafter.[18] Since these relatively rapid rates pertain to the formation of gaslike and condensed monolayer states, we attribute this value of *K* to the spreading of a monolayer from multilamellar liquid crystals of DMPC. This process does not depend on the relative humidity.

The major influence of relative humidity on the rate of film formation is found between 26 and 34 °C, and particularly at 29 °C, where the surface bilayer forms. For relative humidities of ∼90% upon heating the lipid rapidly from 23 °C to temperatures within this 8 °C range there is an initial rapid increase in π that lasts several minutes, and this is followed by a second, much slower process in which the surface pressure gradually rises until the equilibrium value is reached. Estimates of $K = 0.1$ min^{-1} for the initial rapid process are typical for spreading of a monolayer from the multilamellar liquid crystal (see above and Table 1). The rate constant for the secondary slow increase in π is $K = 0.003 \pm 0.001$ min^{-1}, 1−2 orders of magnitude smaller than the value for the initial spreading process. Constant values of surface pressure are attained only after 20−40 h, giving rates comparable to those reported with dispersions at these temperatures.[2,16] The disparate rates observed for the two stages of monolayer spreading indicate that two distinct process are involved: a rapid initial stage that is typical of monolayers spreading from multilamellar liquid crystals, and a second much slower monolayer spreading process that is found only in the temperature region where surface bilayers form. The initial rapid process—monolayer-spreading from multilamellar liquid crystals—is independent of the relative humidity. We shall show below that the second slow process of monolayer formation is associated with the stability of the surface bilayer and is strongly dependent on the relative humidity. The final, stable values of π for relative humidity of ∼90% are given in Figure 2a.

With the relative humidity at 100%, heating the lipid rapidly from 23 to 29 °C, *T**, the surface pressure remains at zero (see Figure 2a), which is the expected value for the surface bilayer, even after 6−8 h. If the monolayer had formed during this period, it would have been detected almost immediately upon reaching *T**. Therefore, the surface bilayer that appears at *T** (Figure 2b) must have formed within the time required to reach this temperature, i.e., 2−3 min. Indeed, under these conditions, the radiotracer studies[2] show a comparable rapid rate for the formation of the surface bilayer at *T**.

Since the surface bilayer forms readily and is stable at *T** and 100% relative humidity, we can examine the influence of relative humidity on its stability (Table 2). In this experiment DMPC is equilibrated at 23 °C and then heated rapidly to *T** to form the surface bilayer. The relative humidity in the chamber is then reduced by replacing the water pool in which the Pyrex dish is immersed with 80 wt % LiCl solution, giving a chemical activity of ∼0.12 and relative humidity of its equilibrium vapor of ∼10%.[21] The exchange of water by the salt solution is achieved with narrow bore polyethylene tubing attached to a syringe needle and inserted through a small hole in the wall of the chamber. The exchange occurs without changing the internal ambient temperature of the chamber. In this arrangement a water vapor pressure gradient will form between the LiCl solution and the aqueous dispersion. By assuming a linear gradient, we estimate that the relative humidity falls to 95−99% at approximately 50 μm (roughly the mean free path of a water molecule at this temperature) above the film surface.

Approximately 1 h after injecting the LiCl solution the surface pressure begins to increase. This delay is likely the time to establish the water vapor pressure gradient between the LiCl solution and the film surface. The increase in surface pressure is described by a single rate constant $K = 0.002$ min^{-1}. This rate cannot be attributed to monolayer-spreading from the surface-confined multilamellar liquid crystal because that process is 1−2 orders of magnitude faster and is independent of the relative humidity. The slow rate of spreading can only be due to the breakdown of the surface bilayer to monolayer when the relative humidity is decreased.

Thus, the stability of the surface bilayer is extremely sensitive to relative humidity. A dramatic example of this sensitivity is seen if the cover of the enclosure is partially opened, allowing the relative humidity to drop from 100% and to approach that of the room, 70−80%. Within seconds after opening the chamber there is a rapid increase in π of 10−15 m Nm^{-1}. The surface pressure continues to increase, reaching a final value that is identical with that for the films obtained with <100% relative humidity. This final value is reached within less than an hour, with a rate constant $K = 0.3 \pm 0.1$ min^{-1}, 2−3 times faster than the rate observed for spreading of monolayers from

TABLE 2: Influence of Relative Humidity (R.H.) on Stability of Surface Bilayer at *T, 29 °C**

R.H., %	K, min^{-1}
100	0
95−99[a]	0.002 ± 0.001
70−80	0.3 ± 0.1

[a] 80 wt % LiCl solution substituted for water pool. See text for details.

the multilamellar liquid crystals under the same conditions of relative humidity (Table 1). This striking effect has been observed for films formed at other temperatures between 26 and 34 °C and 100% relative humidity where film densities exceed those of condensed monolayers (see Figure 2b).

Although both temperature and relative humidity change when the enclosure cover is opened, it is unlikely that the rapid change in surface pressure is due to a change in temperature, since the surface temperature is reduced only about 1 °C below the bulk temperature. Moreover, π is strongly temperature-dependent for $T < T^*$ (Figure 2a); a significant lowering of the surface temperature would show greatly reduced values of π and not the rapid increase that is observed.

Two mechanisms of monolayer formation have now been identified: one involves the spreading from multilamellar liquid crystals, with $K = 0.1$ min^{-1} and relatively insensitive to temperature and relative humidity (Table 1). The second mechanism entails breakdown of the surface bilayer with a rate that depends on the relative humidity and occurs only in the region of *T** (Table 2). To test which of these mechanisms of monolayer spreading applies to the radiotracer studies,[2] we have measured K for 0.2 mg/mL dispersions of DMPC at selected temperatures within this 8 °C range around *T** and ∼90% relative humidity. For this experiment the dispersion is heated from 23 °C to the experimental temperature. In each instance we observe only a slow increase in π with $K = 0.003$ min^{-1} that is characteristic of surface bilayer breakdown. The initial rapid increase in π seen with the lipid confined to the surface at this relative humidity is not observed. This slow process of bilayer breakdown appears to be rate determining for the spreading of monolayers in this temperature interval with relative humidities of ∼90%. This relative humidity is typical for temperature-controlled enclosures[15,16] and in our previous studies with DMPC is the likely source of the disparity in rates between the radiotracer and the surface tension measurements.

Once formed, the surface bilayer at *T** and 100% relative humidity is very stable as exemplified by its response to heating. It is possible to heat the structure to temperatures as high as 35 °C and still retain its structure with $\pi \approx 0$ for several hours (the period of observation). Yet a small change to 95−99% relative humidity (replacing water with concentrated LiCl solutions) is sufficient to destabilize the structure and trigger its breakdown to monolayer. It should be noted that when DMPC is heated rapidly from 23 to 35 °C without pausing at *T**, only monolayer formation is observed (see Figure 2a).

We have also tried to determine whether surface bilayer breakdown is reversible by monitoring π when 100% relative humidity is reestablished. In several experiments a reversal to reduced surface pressures has been observed when π is low (<5−10 mN/m). However, once the high equilibrium surface pressures are attained, the process does not appear to reverse when 100% relative humidity is restored.

IV. Discussion

At *T** and 100% relative humidity a single bilayer forms spontaneously and rapidly in the air−water surface with a surface tension that is expected for a bilayer with one surface exposed to air (Figure 1). The structure is stable indefinitely as long as these conditions are maintained. However, the stability of the surface bilayer is exquisitely sensitive to the chemical potential of the water vapor, with a small reduction to 90−99% relative humidity, causing its transformation to a monolayer.

These results further emphasize the uniqueness of the properties of DMPC in the temperature interval of 26−34 °C. The formation of a bilayer in the air−water surface reflects phenomena that occur simultaneously within the bulk lipid dispersions. Thus, a heat capacity anomaly[4] and a weakening of the bilayer structure[5] appear in a small temperature region coincident with *T**. These properties are manifestations of molecular interactions within the bilayer, which are necessarily associated with the MLV−ULV transformation. The single bilayer structure will form wherever the lipid is present, in the bulk dispersion or at a surface, even with relative humidities <100%. The formation of a stable surface bilayer at 100% relative humidity indicates that the air−water surface and bulk water become indistinguishable at *T** with respect to this process. The free energy change in forming the bilayer in the surface is ($\pi \times$ film area) $= 0$. The principal distinction between the unilamellar state in the surface and in the bulk of the dispersion lies in their relative rates of formation. The surface bilayer forms rapidly, within a matter of minutes, while in bulk dispersions the MLV−ULV transformation may take many days.[4] These differences in rate are primarily due to the presence of surface forces. A model for how surface forces may act to accelerate the formation of the surface bilayer at *T** is considered in the following.

Multilamellar vesicles (MLV's) in the air−water surface have a portion of the outermost bilayer of each particle exposed to air. For the portion of the bilayer exposed to air the surface tension is ∼70 mN/m, while for the portion still immersed in water the surface tension is zero.[7] Consequently, the outermost bilayer of the MLV will be subjected to a Laplace pressure difference across the line of contact of the vesicle with the air−water surface, which will act to shear the outermost bilayer along the line of contact. If this shearing force exceeds the shear threshold of the bilayer, it will be severed and spreading of the bilayer across the surface is now possible. This shearing force will appear only across the outermost bilayer of the MLV because all interior ones have only water on both surfaces. Although the Laplace pressure is relatively insensitive to temperature, bilayer weakening in DMPC dispersions has been observed in the temperature interval near *T** (26−34 °C).[5] Thus, this model appears to be a realistic possibility for explaining why the surface bilayer appears rapidly at this temperature. The slow rate of the MLV−ULV transformation in bulk dispersions is likely due in part to the absence of the Laplace pressure-induced shear and in part to the high activation energy associated with the opening of external bilayers to release those in the interior of the MLV's.

The films that form at 100% relative humidity and at temperatures approaching *T** provide further insights into the phenomenon associated with the formation of the critical unilamellar state. The radiotracer studies[2] (Figure 2b) show that the film density for DMPC increases monotonically from a low value (near zero) at $T_m = 23.5$ °C to the surface bilayer density at $T^* = 29$ °C, and for temperatures that exceed *T** the surface density decreases continuously from that of a bilayer to the value of a condensed monolayer at 34 °C. Thus, at 26−29 and 29−34 °C, the critical region, the concentration of the surface film varies between that of a monolayer and a bilayer.

These are stable states as reflected in both the radiotracer and surface tension measurements (parts a and b of Figure 2) at these temperatures. A possible explanation for these results may lie within a theory of bilayer assembly in which detergents stabilize flat bilayer fragments as intermediate states for the assembly of unilamellar vesicles.[22] In a similar vein the surface film densities that fall between monolayer and bilayer represent an equilibrium between monolayer (analogous to detergent) and flat bilayer fragments. Since the phenomenon of bilayer weakening occurs only in the critical temperature region, with the bilayer at its weakest at T^*,[5] a temperature-dependent response to the shear imparted by the Laplace pressures in the surface might therefore yield an increasing number of bilayer fragments (or an increased fragment size) and a reduced monolayer concentration as T^* is approached. In support of this model we have found that at each temperature within the critical region of 26−34 °C when the relative humidity is reduced to 70−80% by opening the chamber to the room, there is an instantaneous and rapid increase in surface pressure, signifying presence of bilayer-like structures, which ultimately reaches the equilibrium monolayer value indicated in Figure 2.

Both monolayer and surface bilayer form spontaneously and are stable, although under different conditions of relative humidity at T^*. We are therefore confronted with the dilemma of deciding which is the equilibrium state. Since the breakdown of the surface bilayer to monolayer when the relative humidity falls below 100% does not appear to be reversible when π reaches its maximum value, we assume that the monolayer is the equilibrium state. The surface bilayer that forms is therefore metastable with respect to the monolayer. The structure is stable only at 100% relative humidity and acts as a skin to prevent formation of the equilibrium monolayer at T^*. Its formation in the surface is a consequence of the MLV−ULV transformation in the bulk dispersion, which is completely independent of the surface phenomenon. Indeed, surface bilayer formation occurs rapidly even when the relative humidity is less than 100%.

The monolayer that forms in equilibrium with bulk phospholipid at T^* has a unique significance. The $\pi-T$ and $\pi-X_i$ relations for dispersions of binary phospholipid mixtures[23] indicates that at T^*

$$d\pi/dT = d\pi/dX_i = 0$$

These results lead to the following deductions for the equilibrium monolayer at T^*:

$$X_i\,(\text{monolayer}) = X_i\,(\text{bilayer})$$

$$s_i\,(\text{monolayer}) = s_i\,(\text{bilayer})$$

$$h_i\,(\text{monolayer}) = h_i\,(\text{bilayer})$$

where s_i, h_i, and X_i are the partial molar entropy, enthalpy, and mole fraction of the ith component, respectively. These relations signify that the equilibrium monolayer that forms at T^* is energetically identical with the critical bilayer state.

Acknowledgment. We thank Mr. Gary Melvin for design and construction of the constant temperature chamber. We also thank Drs. Schneider and Cammenga for providing a preprint of their recent studies.

References and Notes

(1) Gershfeld, N. L; Tajima, K. *Nature (London)* **1979**, *279*, 708.
(2) Tajima, K.; Gershfeld, N. L. *Biophys. J.* **1985**, *47*, 203.
(3) Ginsberg, L.; Gershfeld, N. L. *Biophys. J.* **1985**, *47*, 211.
(4) Gershfeld, N. L.; Mudd, C. P.; Tajima, K.; Berger, R. L. *Biophys. J.* **1993**, *65*, 1174.
(5) Gershfeld, N. L.; Ginsberg, L. *J. Membr. Biol.* **1997**, *156*, 279.
(6) Sengers, J. V. In *Supercritical Fluids*; Kiran, E., Levelt Sengers, J. M. H., Eds.; Kluwer Academic: Netherlands, 1994; pp 231−271.
(7) Gruen, D. W. R.; Wolfe, J. *Biochim. Biophys. Acta* **1982**, *688*, 572.
(8) Tajima, K.; Gershfeld, N. L. *J. Colloid Interface Sci.* **1981**, *81*, 283.
(9) Sturtevant, J. M. In *Physical Methods of Organic Chemistry*; Weissberger, A., Ed.; Interscience: New York, 1945; Vol. I, p 350ff.
(10) Mudd, C. P.; Gershfeld, N. L.; Berger, R. L.; Tajima, K. J. *Biochem. Biophys. Meth*ods **1993**, *26*, 149. Jin, A.; Mudd, C. P.; Gershfeld, N. L. *Biophys. J.* **1996**, *70*, A112.
(11) Gaines, G. L., Jr. *Insoluble Monolayers at Liquid-Gas Interfaces*; Interscience: New York, 1966.
(12) Weast, R. C. *Handbook of Chemistry and Physics*; Chemical Rubber Co.: Cleveland, 1967.
(13) Gershfeld, N. L. *Annu. Rev. Phys. Chem.* **1976**, *27*, 349.
(14) Lasic, D. D. *J. Colloid Interface Sci.* **1990**, *140*, 349.
(15) Barnes, G. T.; Lawrie, G. A.; Battersby, B. J.; Sarge, S. M.; Cammenga, H. K.; Schneider, P. B. *Thin Solid Films* **1992**, *242*, 201.
(16) Lawrie, G. A.; Schneider, P. B.; Battersby, B. J.; Barnes, G. T.; Cammenga, H. K. *Chem. Phys. Lipids* **1996**, *79*, 1.
(17) Schneider, P. B.; Cammenga, H. K. *Thin Solid Films* **1996**, *284*−*285*, 27.
(18) Gershfeld, N. L.; Tajima, K. *J. Colloid Interface Sci.* **1977**, *59*, 597.
(19) Cary, A.; Rideal, E. K. *Proc. R. Soc. London* **1925**, *109A*, 301.
(20) Motomura, K. *J. Colloid Interface Sci.* **1967**, *23*, 313.
(21) Robinson, R. A. *Electrolyte Solutions*, 2nd ed.; Academic Press: New York, 1959.
(22) Lasic, D. D. *Biochem. J.* **1989**, *258*, 1.
(23) Gershfeld, N. L. *J. Phys. Chem.* **1989**, *93*, 5256.