Persistence of Phase Coexistence in Disaturated Phosphatidylcholine Monolayers at High Surface Pressures

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ABSTRACT Prior reports that the coexistence of the liquid-expanded (LE) and liquid-condensed (LC) phases in phospholipid monolayers terminates in a critical point have been compromised by experimental difficulties with Langmuir troughs at high surface pressures and temperatures. The studies reported here used the continuous interface of a captive bubble to minimize these problems during measurements of the phase behavior for monolayers containing the phosphatidylcholines with the four different possible combinations of palmitoyl and/or myristoyl acyl residues. Isothermal compression produced surface pressure-area curves for dipalmitoyl phosphatidylcholine (DPPC) that were indistinguishable from previously published data obtained with Langmuir troughs. During isobaric heating, a steep increase in molecular area corresponding to the main LC-LE phase transition persisted for all four compounds to 45 mN/m, at which collapse of the LE phase first occurred. No other discontinuities to suggest other phase transitions were apparent. Isobars for DPPC at higher pressures were complicated by collapse of the monolayer, but continued to show evidence up to 65 mN/m for at least the onset of the LC-LE transition. The persistence of the main phase transition to high surface pressures suggests that a critical point for these monolayers of disaturated phospholipids is either nonexistent or inaccessible at an air-water interface.

INTRODUCTION

Biologists, chemists, and physicists have studied the behavior of phospholipid monolayers. In addition to serving as models for understanding the behavior of cellular membranes and pulmonary surfactant, the two-dimensional interfacial films provide simple accessible systems for studying phase transitions (Nagle and Bonner, 1976; Knobler and Desai, 1992). Perhaps the greatest attention has focused on the main transition between the liquid-expanded (LE) and liquid-condensed (LC) phases. In the LE phase, molecular density is sufficiently high to allow van der Waals interactions, but low enough that the aliphatic tails have random tilt and bond orientation. The driving parameter for the transition from the LE to the LC phase is the ordering of the tails into an all-*trans* state with a uniform tilt relative to the interface (Möhwald, 1995). A plateau in the surface pressure-molecular area (π-$\bar{A}$) isotherm is the best-known manifestation of this transition. Microscopic methods that distinguish the two phases confirm that this plateau represents the region of phase coexistence (Lösche et al., 1983).

The studies reported here concern the behavior of this coexistence region at surface pressures approaching and above the collapse pressure for an LE film. In series of experiments conducted at increasing temperatures, the coexistence plateau not only shifts to higher pressures, but also becomes shorter in length along the molecular area axis, suggesting that under the appropriate conditions, the molecular areas of the two phases would become identical at a critical point (Phillips and Chapman, 1968; Träuble et al., 1974; Albrecht et al., 1978). Conclusive demonstration of the critical point has been hampered by experimental problems. Best estimates of the critical pressure suggest that it occurs very close to or above the level at which LE phospholipids collapse (Albrecht et al., 1978). The critical point would also occur at conditions for which maintenance of temperature, humidity, and confinement of the compressed film on a standard Langmuir trough is difficult. Small air bubbles immersed in an aqueous subphase provide an alternate experimental approach. An axisymmetric bubble can be used as a surface balance by spreading a film at its air-liquid interface and then manipulating its volume (see Note 1 at end of text). Published equations allow determination of the interfacial area and surface tension of the bubble from its shape (Malcolm and Elliott, 1980; Schoel et al., 1994). The relatively small size of the interface makes the regulation of temperature considerably easier than for a standard Langmuir trough. The continuous air-liquid interface of a bubble eliminates any possibility of escape by the monolayer around or along confining barriers (Schürch et al., 1989, 1992, 1994; Putz et al., 1994, 1998; Schoel et al., 1994). In the studies reported here, we have used a captive bubble as a surface balance to seek confirmation at high surface pressures for the termination of the LE-LC coexistence region at a critical point. Our experiments consider four disaturated phosphatidylcholines, emphasizing dipalmitoyl phosphatidylcholine (DPPC) in particular because of its importance for the function of pulmonary surfactant and the extensive information available from previously published studies.

Received for publication 22 March 1999 and in final form 6 July 1999.

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0006-3495/99/12/3134/10 $2.00

MATERIALS AND METHODS

Materials

DPPC, 1-palmitoyl-2-myristoyl phosphatidylcholine (PMPC), 1-myristoyl-2-palmitoyl phosphatidylcholine (MPPC), and dimyristoyl phosphatidylcholine (DMPC) were obtained from Avanti Polar Lipids (Alabaster, AL) and used without further characterization or purification. Agarose was obtained from Sigma (St. Louis, MO) and purified by extraction (Bligh and Dyer, 1959). Water was distilled and then filtered through Macropure, Ultrapure DI, and Organic Free Cartridges from Barnstead Thermolyne Corp. (Dubuque, IA). The following reagents were purchased commercially and used without further purification or analysis: high purity chloroform and methanol (Burdick and Jackson, Muskegon, MI); GibcoBRL brand N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid (HEPES) (Life Technologies, Grand Island, NY); $CaCl_2 \cdot 2H_2O$ (J.T. Baker Inc., Phillipsburg, NJ); and NaCl (Mallinckrodt Specialty Chemicals Co., Paris, KY).

Methods

Captive bubble apparatus

These studies used a pressure-driven captive bubble device with minimal modifications from an apparatus described previously (Putz et al., 1998; Fig. 1). This instrument measures the shape of an axisymmetric air bubble floating in a liquid subphase against a 1% agarose ceiling. The chamber for the instrument consists of an optical cuvette partially filled with agarose, the surface of which is molded with a slight indentation to maintain the bubble centered. After the cuvette is filled with subphase, sealed, and inverted, an appropriate volume of air (80–90 μl for our experiments) is injected to form the bubble. Changes in hydrostatic pressure applied to the subphase manipulate the volume of the bubble and consequently its surface area. The height and width of the bubble, 4 mm and 6 mm, respectively, for an 80-μl bubble in water, provide the basis for calculating its volume, surface area, and surface tension. A computer-controlled syringe drive (Harvard Apparatus, Cambridge, MA) manipulates an air-filled syringe to vary the hydrostatic pressure applied to the subphase over a possible range from 0.4 to 3.5 atmospheres. A CCD camera (TM-7EX, PULNiX Inc., Sunnyvale, CA, obtained from Motion Analysis, Eugene, OR) monitors the profile of the bubble in the vertical axis to ensure that the bubble has the axisymmetric shape for which the equations are applicable. A separate camera aligned in the horizontal axis measures the height and width of the bubble. Temperature is monitored and regulated using a temperature controller (YSI Inc., Yellow Springs, OH or Cole-Palmer, Vernon Hills, IL) with a thermistor probe (YSI) and heating pads (Minco, Minneapolis, MN) applied externally along the sides of the chamber. Temperature is maintained within ±0.1°C of the target value. Variation of ±0.6°C produced detectable discontinuities in isotherms during the heating cycle. Hydrostatic pressure is measured with a diaphragmatic pressure transducer (Validyne, Northridge, CA). All experiments reported here used a subphase containing 10 mM HEPES pH 7.0, 1.5 mM $CaCl_2$, and 150 mM NaCl (HSC) unless noted otherwise.

Computer interface

The bubble was manipulated and measured using programs constructed with the graphical user interface LabVIEW (National Instruments, Austin, TX). Images of the bubble were captured by framegrabber (PCI-1408, National Instruments) and analyzed in real time using the image-analysis program IMAQ (National Instruments). Calibration factors for converting pixels to absolute dimensions were obtained by imaging ball bearings of known diameter (Putz et al., 1994). The volume, surface area, and surface tension were calculated from the height and width of the bubble using previously published equations (Schoel et al., 1994). Surface pressures were calculated from the difference in surface tension between published values for water at the appropriate temperatures (Washburn, 1926–1930) and the measurements for the interfacial monolayers. The program routinely averaged results from two to four frames to provide a recorded set of values approximately every 0.25 s. The applied hydrostatic pressure and temperature were measured via an input/output board (PCI-1200, National Instruments). The syringe pump was manipulated using the same board to control characteristics of the bubble specified for particular experiments.

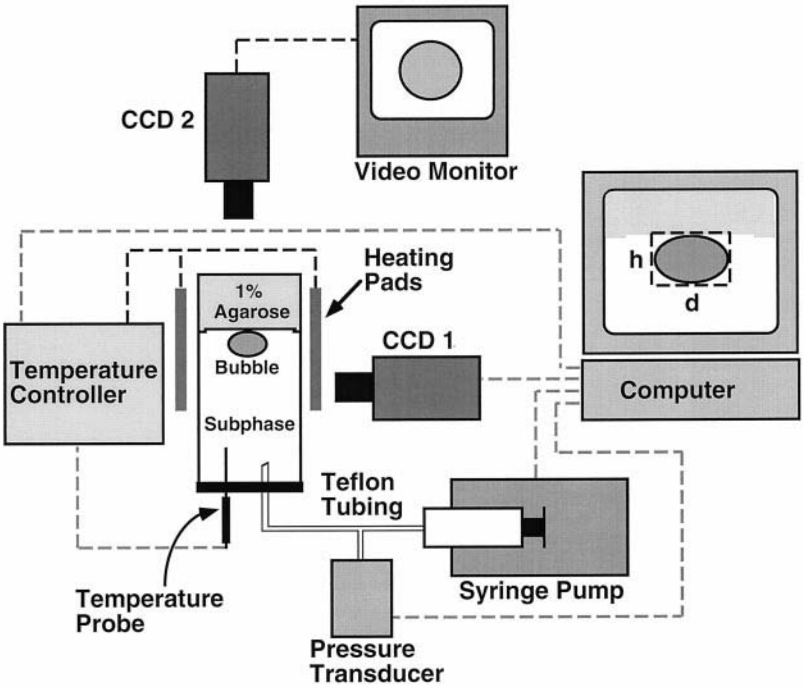

FIGURE 1 Schematic diagram of the captive bubble apparatus. An air bubble floats below a ceiling of 1% agarose in HSC or water. CCD cameras 1 and 2 record the profiles of the bubble in the horizontal and vertical axes, respectively. A computer calculates surface pressure, surface area, and volume of the bubble from measurements of its height (h) and diameter (d) obtained from a captured image of its profile, and manipulates the bubble according to the constraints of the particular experiment by varying hydrostatic pressure via a computer-controlled syringe pump. Temperature is measured and regulated by a temperature controller with a thermistor probe and thermofoil heating pads. Materials and Methods provides a more complete description.

Spreading the films

Our experiments studied films of phosphatidylcholines spread on the surface of the captive bubble. Spreading solvent for most experiments was chloroform/methanol in a 1:2 (v/v) mixture, although a 1:1 combination worked equally well. Following formation of an 80–90-μl bubble in a subphase of ~2.5 ml, 0.05–0.08 μl phosphatidylcholine at appropriate concentration was injected through a 0.5-μl syringe and needle (SGE, Australia) that touched the air-liquid interface to produce a monolayer with initial surface pressure (π) below 11 mN/m and molecular area ($\bar{A}$) >70 $Å^2$/molecule. Spreading solvent was removed by extensive replacement of the subphase. The bubbles were first compressed to a surface pressure of 40–65 mN/m, after which an outflow valve was opened and adjusted so that HSC infused at a constant rate maintained constant surface pressure. The total volume of buffer flushed through the subphase exceeded 20 ml.

Isothermal compressions and expansions

After the spreading and washing of the phosphatidylcholine monolayer, surface pressure was reduced, the chamber was opened to atmospheric pressure, and the temperature was adjusted to the desired value. After closing the outlet valve, hydrostatic pressure was first lowered to reduce surface pressure to zero. Isothermal measurements of surface pressure and area were then recorded during compression at a constant rate of <2.5 $Å^2$/molecule/min. Expansion curves, when measured, used the same rate.

Isobaric expansions

After the spreading and washing steps, the bubble was adjusted at 25°C to achieve a surface pressure of 25 mN/m. Repeated measurements of compression isotherms determined an average molecular area at that pressure (e.g., 50 $Å^2$/molecule for DPPC). Molecular areas deviating from that value by >0.5 $Å^2$/molecule were concluded to reflect errors in spreading the monolayer or determining the concentration (Albrecht, 1989), and the films were rejected. For acceptable monolayers, hydrostatic pressure was lowered to reduce surface pressure to zero. The monolayer was then compressed to the desired surface pressure at a rate < 5.0 $Å^2$/molecule/min. π-$\bar{A}$ isotherms were monitored to ensure that a well-defined plateau indicated an uncontaminated film. Isobaric measurements of molecular area and temperature were then performed by computer manipulation of the syringe pump to maintain the desired surface pressure while ramping the voltage applied to the heating pads at a constant rate.

RESULTS

Experiments on the captive bubble differ in important respects from studies with a Langmuir trough. The controlled variable, for instance, is the hydrostatic pressure applied to the subphase. The volume, surface area, and surface pressure are all dependent measurements. With our apparatus, volume changed during isothermal compressions at a nearly constant rate (Fig. 2). Surface area, however, did not. At higher surface pressures, the bubble tended to preserve its surface area despite decreases in its volume. Consequently, the compression of monolayers in our experiments slowed substantially at higher surface pressures (Fig. 2). The compression rates reported here represent maximum values. Noise in the measured surface pressure was greater than for most data obtained with a Wilhelmy plate, which to some extent reflects the limits of our standard CCD camera. Noise also varied as a function of surface pressure because of different susceptibilities to errors in the measurements. At 0 mN/m, where the height (h) to diameter (d) ratio was above 0.5, an error of ±1 pixel in h or d resulted in an error of ±0.9 or 0.2 mN/m, respectively. At a surface pressure near 70 mN/m, where $h/d < 0.2$, the same error in h or d led to a difference of only ±0.07 or 0.0003 mN/m, respectively (Figs. 2 and 3). The balance between surface tension and buoyancy determines the shape of the bubble, and when high surface tensions dominate, the relatively spherical shape becomes less responsive to changes in surface tension (Schoel et al., 1994). Small differences in the measured dimensions then produce larger fluctuations in the calculated surface tension.

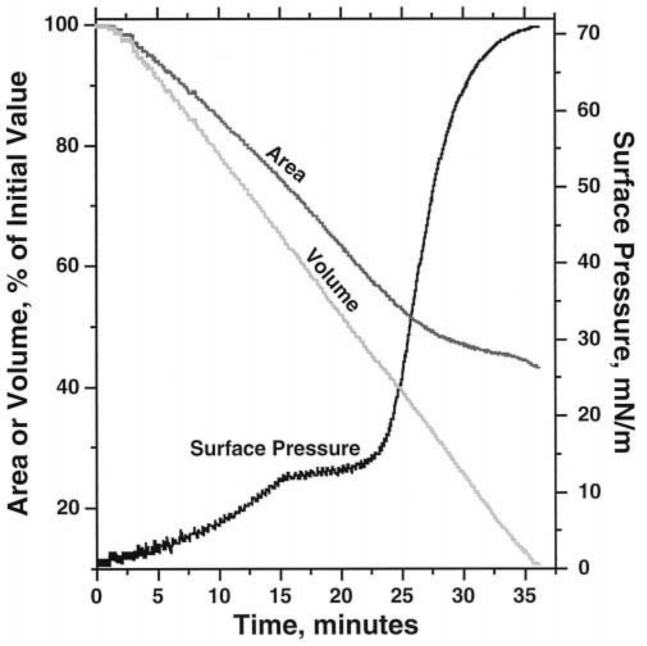

FIGURE 2 Variation of measured characteristics during compression of an air bubble coated with an interfacial monolayer of DPPC. Infusion from an air-filled syringe at a constant rate of 0.5 ml/min increased hydrostatic pressure applied to the subphase surrounding the bubble. The temperature was 25°C.

Experiments on the captive bubble also required a different method for removing spreading solvent. Initial experiments in which solvent simply evaporated into the airspace enclosed within the bubble produced isotherms for DPPC with little evidence of the coexistence plateau. Exchange of large volumes through the subphase to take advantage of the small solubility of chloroform in water improved the curves, but a premature rise from the plateau persisted as long as the washing occurred with the monolayer close to the point of lift-off. Compression of the films to a significant surface pressure in an attempt to exclude any solvent from the film produced the best results (Fig. 3). The molecular area of the films varied between experiments more than the surface pressure (Fig. 3), as anticipated from the error expected in preparing and applying the spread solutions (Albrecht, 1989). In general, however, the isotherms for DPPC on the captive bubble closely duplicated previously reported careful measurements (Albrecht et al., 1978) obtained with a Langmuir trough (Fig. 3).

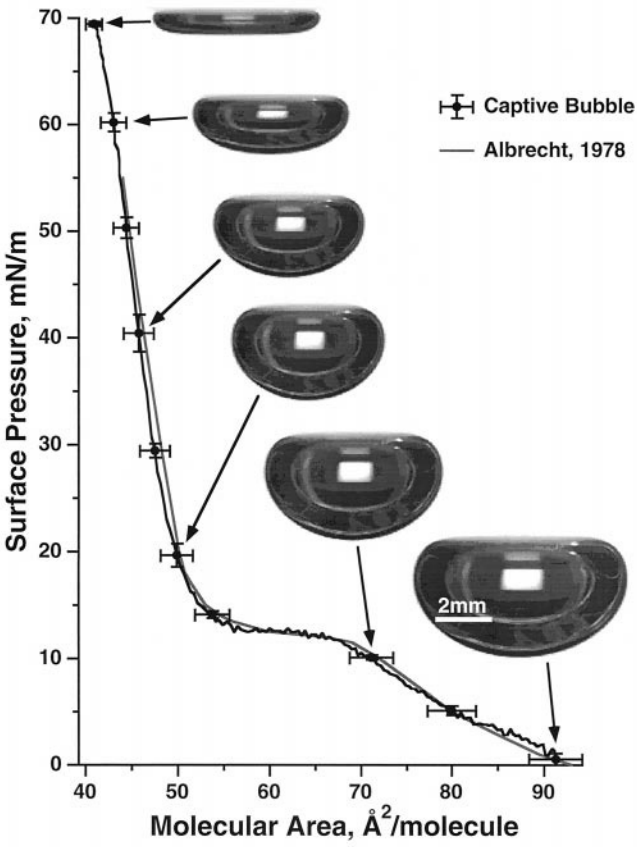

FIGURE 3 Surface pressure (π) − molecular area ($\bar{A}$) isotherms for monolayer of DPPC. Films were compressed at 2.5 Å^2/molecule/min on a captive bubble at 25°C. The black line gives the mean of three experiments obtained by averaging π and $\bar{A}$ at specific increments in volume of the bubble. Error bars give means ± SD for each coordinate. The gray line was transcribed from a previously published graph for α-DPPC compressed on a Langmuir trough at 25°C (Albrecht et al., 1978). Inserted images show representative profiles used to calculate the characteristics of the bubble for one of the three experiments.

Experiments on the captive bubble differ from studies with Langmuir troughs in that pressure in the bulk phases varies during the experiment. To determine if hydrostatic pressure affected transition surface pressures, films of DPPC were spread to different initial molecular areas. Expansion or compression at 28°C to 1 mN/m then produced monolayers at the same surface pressure but different bulk phase pressures. During subsequent compression, the surface pressure of the main phase transition differed by less than 1 mN/m over the range of bulk phase pressures from 0.7 to 2.8 atmospheres.

The bubble was also used to measure expansion isobars (Fig. 4). Samples were heated at a nearly constant rate while the computer manipulated the syringe to maintain surface pressure constant to within ±0.4 mN/m. For isobars at and below 50 mN/m, temperature was increased by 11°C/h (Fig. 4, *A* and *B*). At pressures below 45 mN/m, the curves for DPPC consisted of three linear segments (Fig. 4 *A*). The initial rise above ambient temperature produced a linear expansion of the LC phase that averaged 0.10 ± 0.02 Å^2/molecule/°C. Within a span of 1 to 2°C, expansion rates suddenly increased, marking the onset of the LC-LE phase transition. With increasing pressure, the slope of this second segment decreased from 7.5 Å^2/molecule/°C at 10 mN/m to 2.2 Å^2/molecule/°C at 45 mN/m. The length of the transition, given as the difference between the initial and final molecular areas for the steeply ascending segment, also decreased, falling from 19.8 to 7.4 Å^2/molecule between 10 and 45 mN/m. At the upper end of the main transition, the slope decreased as abruptly as it had increased at the onset. Rates of expansion in this third linear region for the LE phase, averaging 0.28 ± 0.03 Å^2/molecule/°C, were slightly greater than for the initial segment before the transition. The initial and final segments showed no evidence of any discontinuity at surface pressures $\leq$45 mN/m (Fig. 4 *A*).

We also performed limited studies with three other phosphatidylcholines to confirm that findings for DPPC occurred with other compounds (Fig. 4 *B*). PMPC, MPPC, and DMPC at 45 mN/m all produced isobars with characteristics similar to curves for DPPC. Molecular areas for PMPC and MPPC initially increased at 0.09 ± 0.01 Å^2/molecule/°C. At 27°C for PMPC and 32°C for MPPC, expansion increased by an order of magnitude to 1.3 ± 0.2 Å^2/molecule/°C. The restriction of our apparatus to temperatures above ambient values prevented access for DMPC to the initial relatively flat part of the isobar. The coexistence region appeared to begin at approximately the minimum temperature of 20°C, during which the film expanded at a rate of 0.77 Å^2/molecule/°C. For all four compounds, the coexistence segment extended over an average difference in molecular areas of 7.8 ± 0.9 Å^2/molecule. The slopes then decreased to an average of 0.23 ± 0.05 Å^2/molecule/°C in the LE phase. The transitions for all three compounds were less discrete than for DPPC, and this difference could reflect different levels of purity (S. Tristram-Nagle, Y. Isaacson, Y. Lyatskaya, Y. Liu, K. Brummond, J. Kataras, and J. F. Nagle, submitted for publication). The isobars at 45 mN/m provide the same evidence for these three additional phospholipids as for DPPC that the main phase transition, but no other transition, persisted at least to this pressure.

The isobars for DPPC first showed evidence for collapse of the monolayer at 45 mN/m. The film completed the main transition at that pressure and began its subsequent expansion before area suddenly decreased (Fig. 4 *A*). Isobars at 45 mN/m showed similar behavior for the other three phospholipids (Fig. 4 *B*). We interpreted this decline in terms of a loss of molecules from the interface rather than a true decrease in molecular area. Collapse for all four phospholipids at 45 mN/m began at an average temperature of 49 ± 1°C and an average molecular area of 57 ± 1 Å^2/molecule. If during collapse the molecules that remain at the interface have a constant molecular area, then the films lost an average of $(4.4 \pm 0.9) \cdot 10^{10}$ molecules, or 0.03%, per second. At 50 mN/m, the onset of collapse for DPPC, indicated by decreasing area, occurred slightly before the main transition began (Fig. 4 *A*). The curve did begin an ascent at 42°C, but this segment was interrupted early in the

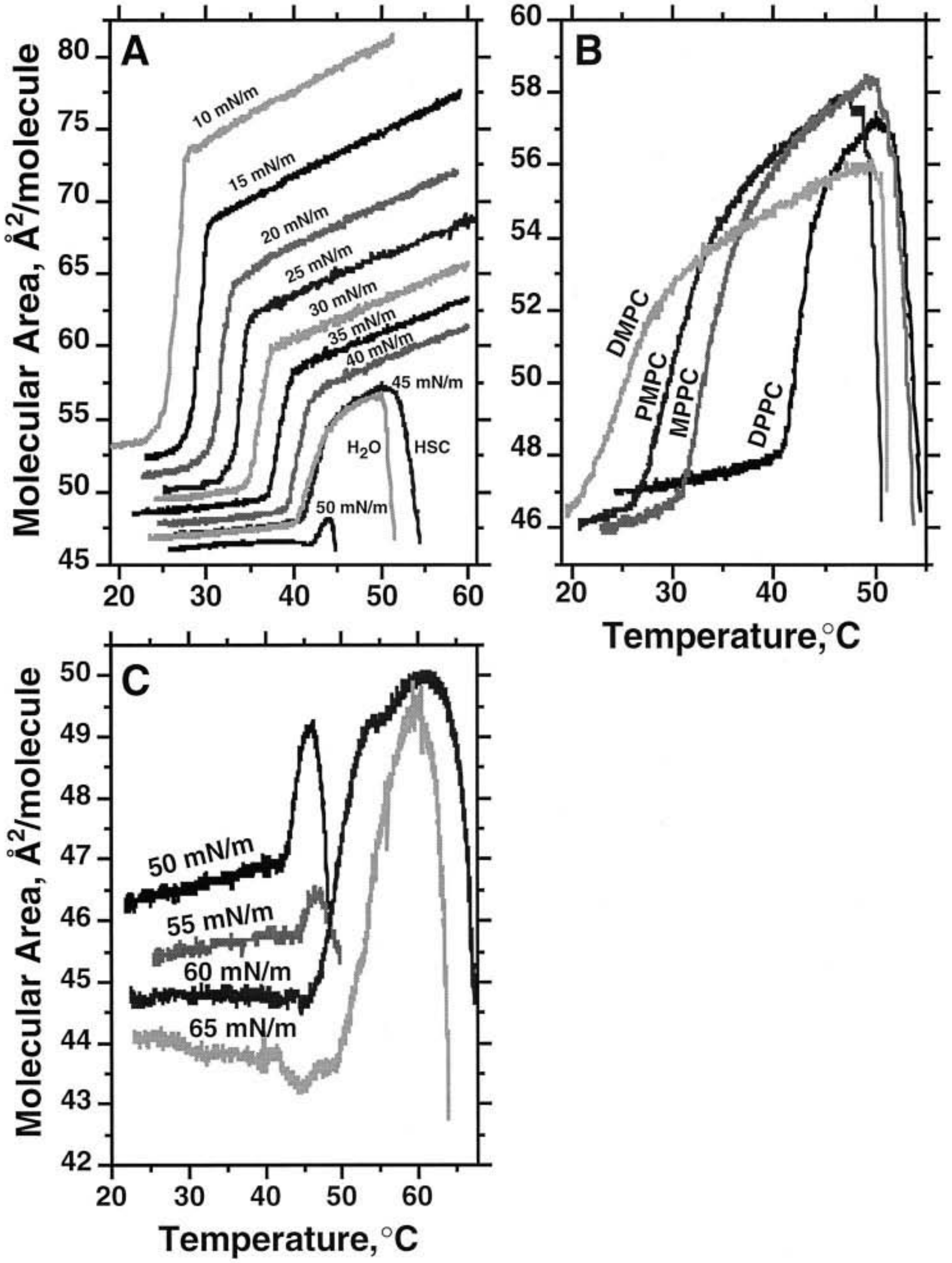

FIGURE 4 Expansion isobars for phospholipid monolayers at various surface pressures. Phospholipids (*A*, DPPC; *B*, DPPC, PMPC, MPPC, and DMPC; *C*, DPPC) were spread on the surface of a captive bubble, compressed at room temperature to the indicated surface pressure, and then heated while adjusting the volume of the bubble to maintain constant surface pressure. All experiments used a subphase of HSC with the exception of the gray curve for DPPC at 45 mN/m (*A*) which used water. Temperature increased at either 11°/h (*A* and *B*) or 2°C/min (*C*).

transition by the sharp drop in area beginning at 44°C (Fig. 4 *A*). These results indicate that the main transition for DPPC persisted at least to 50 mN/m, but that collapse complicated efforts to follow it to higher pressures. At 50 mN/m and above, simultaneous molecular expansion and collapse prevented calculation of the rate for either process.

Subsequent experiments heated monolayers of DPPC at the faster rate of 2°C/min used previously by others (Albrecht et al., 1978) to emphasize the expansion of the film caused by phase behavior in preference to the decrease in area caused by collapse. Because of collapse, calculations of molecular area were not reliable, and changes in surface area were measured in units of percent of initial area. The first segment of the isobar before the onset of the main transition at 50 mN/m was again linear, with a slope of 0.10 ± 0.02%/°C (Fig. 4 *C*). The slope of this segment dropped to 0.07 ± 0.02%/°C at 55 mN/m, 0 ± 0.007%/°C at 60 mN/m, and −0.10 ± 0.05%/°C at 65 mN/m. The isobaric areas, however, began to increase at temperatures predicted by the onset of the coexistence region at lower pressures. Coexistence at 50, 55, 60, and 65 mN/m began at 42°, 44°, 45°, and 49°C, respectively, with increases in slope to 2.5 ± 0.6, 1.0 ± 0.6, 1.6 ± 0.1, and 1.5 ± 0.1%/°C. The steep segments at these pressures indicated that the main transition persisted at least to 65 mN/m.

The length of ascent in the coexistence region varied in an unexpected manner. The increase in surface pressure above 45 mN/m did not produce a progressively earlier termination of the main transition. Although collapse occurred earlier at 55 mN/m than at 50 mN/m, after an expansion of only 1.6 ± 0.1% as opposed to 4.5 ± 0.3% initial area, it occurred substantially later at 60 mN/m. The isobar actually completed the main transition after an expansion of 9.5 ± 0.2% initial area at that pressure, and had begun the post-transitional expansion at a rate of 0.2 ± 0.1%/°C before the area began its abrupt decline at 60°C. At 65 mN/m, the steep ascent again persisted through the greater length of 13 ± 1% initial area, but collapsed at 60°C before establishing any subsequent linear expansion that would define the end of the transition. The longer coexistence regions in the isobars above 50 mN/m indicated that higher surface pressures, further above the collapse pressure, created more stable films rather than earlier collapse.

Our experiments used a subphase that contained electrolyte and buffer to allow direct comparison of our current findings to our prior studies (Discher et al., 1996, 1999), which were designed to replicate the conditions encountered by a film of pulmonary surfactant in the lung. Selected experiments with DPPC on the captive bubble using water for the subphase showed no significant differences from our results with buffered electrolyte (Fig. 4 *A*).

The well-defined changes between the different segments of the isobars provided clear-cut points for the beginning and end of the main transition. These fit well with comparable values for the beginning and end of the plateau regions in isotherms performed over a range of temperatures (Fig. 5). Expansion isotherms provided plateaus that were more horizontal than during compression, and that agreed particularly well with the expansion isobars (Fig. 5). Both isobaric and isothermal data indicated that molecular areas at the ends of the main transition became more similar at higher surface pressures and temperatures, but that the differences appeared to decrease asymptotically and did not approach zero (Fig. 6).

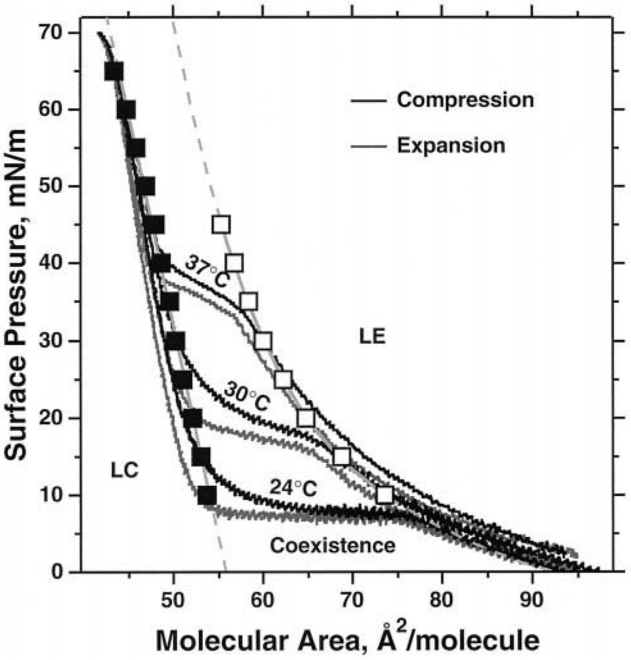

FIGURE 5 π-$\bar{A}$ data for DPPC from both isothermal and isobaric experiments. Black and gray lines without symbols indicate isothermal data obtained during compression and expansion, respectively, at 2.5 Å^2/molecule/min for the indicated temperatures. Open and filled squares indicate the onset and completion, respectively, of the main transition in expansion isobars. Light gray lines estimate possible phase boundaries, and are obtained from the least-squares fit of these areas to a linear relationship for the LC phase and to a fourth-order polynomial for the LE phase, with the dashed extensions indicating extrapolation of these equations to surface pressures beyond the actual measurements.

DISCUSSION

The captive bubble produces isotherms for monolayers of DPPC that are comparable to prior results obtained on standard Langmuir troughs, showing a prominent plateau at the expected molecular areas that tilts above the horizontal and that shortens with increasing temperature (Figs. 3 and 5). Removal of spreading solvent from the monolayers proves to be a surmountable problem handled by multiple exchanges of the subphase while the film is held at fixed elevated surface pressures. Isobars produced during heating at a constant surface pressure demonstrate the main phase transition particularly well (Fig. 4). The slope of the coexistence segment deviates from the vertical step expected for a truly first-order transition, in agreement with the nonhorizontal plateau in the isotherms. Prior reports have attributed this deviation either to low-level contamination (Pallas and Pethica, 1985; Hifeda and Rayfield, 1992) or molecular clustering (Israelachvili, 1994; Ruckenstein and Li, 1995). Others have noted (Pallas and Pethica, 1985) that the LC end of the coexistence plateau in isotherms is more clearly defined during expansion than compression (Fig. 4). We (data not shown) and others (Albrecht et al., 1978) have similarly found that expansion isobars caused by heating have coexistence segments that are more vertical than those during compression produced by cooling.

Our data provide evidence only for the LC-LE main transition. The isobars for the four phospholipids before and after the coexistence segment are linear without evidence of a change in slope to support the presence of other transitions. Our results are therefore unable to support the presence of two second-order transitions suggested previously. We find no evidence for either the solid-to-LC transition in the segment at areas below the main transition (Albrecht et al., 1978; Cadenhead et al., 1980), or for the anisotropic-to-isotropic liquid transition at areas above the coexistence region (Albrecht et al., 1978). A change in shear viscosity provided the most convincing evidence for the liquid transition in previous reports (Albrecht et al., 1978), and we have made no attempt to repeat those measurements. Our results, therefore, although providing no evidence of these additional transitions, also are unable to disprove their existence.

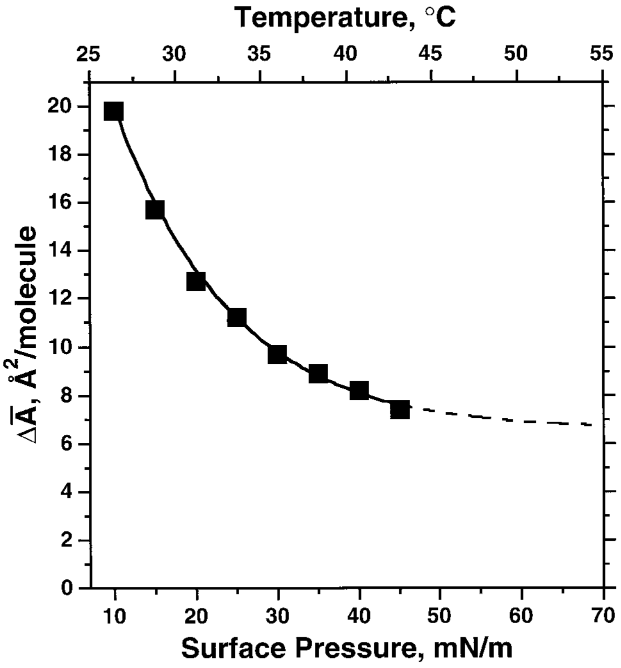

FIGURE 6 Change in molecular areas across the main phase transition for DPPC at different surface pressures and temperatures. $\Delta\bar{A}$ is the difference between the molecular areas at the top and bottom of the steep ascent in expansion isobars. The curve gives the best fit of the equation $y = a + b \cdot e^{-c \cdot x}$ to the data, with the dashed line indicating the extrapolation beyond the measured values.

The main transition for the four disaturated phosphatidylcholines studied persists at least to the pressure at which collapse begins (Fig. 4 *B*). The precipitous decline in area at 45 mN/m that indicates rapid collapse begins when the temperature reaches 50°C. The delay of collapse after the transition to the more expanded phase suggests a collapse pressure that varies with temperature. The persistence, however, of the main transition to this point suggests that prior isotherms interpreted as indicating the termination of the coexistence region at a critical point may in fact have resulted from other phenomena, such as the progressive tilt of the coexistence plateau with increasing temperature or escape from confinement.

Collapse complicates interpretation of results above 45 mN/m. Area during the isobaric heating responds to the two competing processes of thermal expansion and loss of molecules by collapse. Values of molecular area are no longer accurate because the number of molecules within the monolayer becomes unknown. The area falls when the rate of collapse exceeds the rate of expansion. More rapid heating increases the rate of thermal expansion and allows the changes caused by the main transition to remain evident further through the transition. The maximum area achieved is then determined by the relative rates of the two processes. The variation of the maximum area for DPPC indicates a previously reported but somewhat surprising dependence of collapse rate on surface pressure. Prior investigators have shown on Langmuir troughs that collapse of DPPC initially accelerates when surface pressure increases above the collapse pressure, but then slows as surface pressure approaches 70 mN/m (Goerke and Gonzales, 1981). Our results confirm these findings with a different experimental system. The maximum area achieved by the isobar falls during the initial elevation in surface pressure from 45 to 55 mN/m, suggesting an increasing rate of collapse. Going to 60 and 65 mN/m, however, causes the maximum area to increase, suggesting a slower collapse despite the greater elevation above collapse pressure.

The isobars for DPPC above 45 mN/m all show evidence of the main transition. An approximate upper limit for the isobaric studies occurs at 65 mN/m. At that surface pressure, the upper end of the main transition occurs at or above 60°C. The surface tension of water falls with increasing temperature, and at 60°C, it reaches 66 mN/m. With further heating or at higher surface pressures, the system therefore reaches interfacial tensions approaching zero, and becomes intrinsically unstable. The experiments then suggest that the main transition for DPPC monolayers on an aqueous subphase persists to the maximum possible surface pressure.

Behavior under equilibrium conditions can be extrapolated to predict conditions at which a critical point would occur. When a first-order transition terminates at a critical point, both the molecular entropies and the molecular areas for the two phases become equal. For molecular entropies, the two-dimensional Clapeyron equation indicates that

$$\frac{d\pi_{\mathrm{M}}}{dT} = \frac{\Delta\bar{S}}{\Delta\bar{A}} = \frac{\lambda}{T \cdot \Delta\bar{A}}$$

where π_{M} is the surface pressure of the main transition, $\Delta\bar{S}$ and $\Delta\bar{A}$ are the differences in molecular entropy and area between the two phases, and λ is the latent heat (Phillips and Chapman, 1968). When $\Delta\bar{S}$ becomes zero at the termination of a first-order transition, λ must also vanish. The latent heats calculated from the Clapeyron equation can be fit to a linear relationship with measured temperature or surface pressure (Fig. 7). If such a linear relationship persisted, latent heat would vanish at a critical point with $T_{\mathrm{c}} = 57$°C, $\pi_{\mathrm{c}} = 77$ mN/m. This surface pressure lies well outside the conditions accessible to a monolayer on an aqueous subphase.

Molecular areas for the two phases similarly suggest that they should remain distinct. The difference in molecular areas across the main transition falls with increasing temperature and pressure, but asymptotically rather than linearly. Exponential curves that fit the data well would never reach values of $\bar{A}$ below 6 Å^2/molecule (Fig. 6). The measurements of area then agree with the latent heats that the critical point would be highly inaccessible, or possibly nonexistent. This result and the shape of the coexistence region fit at least in general terms with a prior theoretical calculation. A rigorously solvable model in which the acyl chains are considered as a two-dimensional lattice (Nagle, 1973) predicts a wedge-shaped coexistence region that ter-

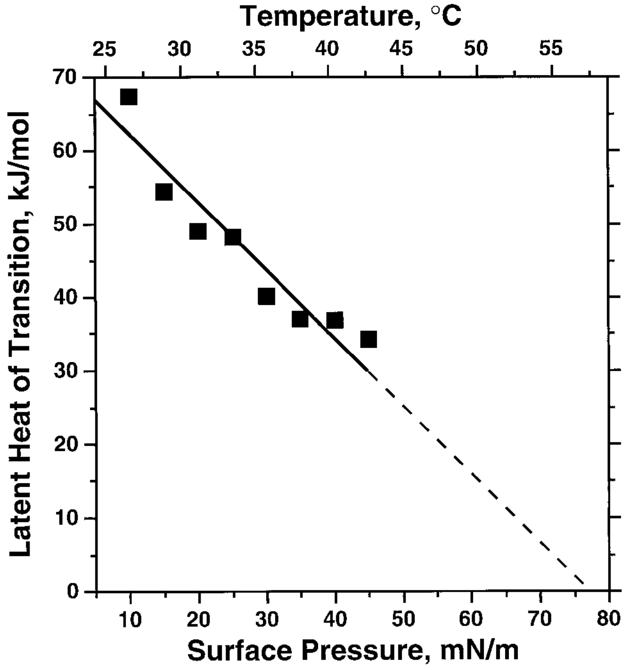

FIGURE 7 Calculated latent heat of the main transition for DPPC. Temperatures and molecular areas at the beginning and end of the main transition were obtained from expansion isobars and used to calculate latent heats as functions of temperature and surface pressure. The straight line gives the least-squares fit to the data, with the dashed line indicating the extrapolation to zero latent heat.

minates only at a critical point with T_c = 530°C and π_c = 293 mN/m (Nagle, 1986).

Limited experiments with other disaturated phosphatidylcholines suggest phase behavior qualitatively similar to that of DPPC (Fig. 4 *B*). PMPC, MPPC, and DMPC in bilayers have gel-to-liquid crystal transitions at lower temperatures than DPPC (Davis et al., 1981). If the LC-LE phase transition in monolayers also occurred at lower temperatures, any critical point might be shifted and therefore more accessible to conditions achievable for phospholipid monolayers. The main phase transitions for PMPC, MPPC, and DMPC do occur at lower temperatures than for DPPC. Isobars, however, at 45 mN/m for all four compounds indicate that the main transitions persist to the collapse pressure, and $\bar{A}$ shows no signs of decreasing for compounds that melt at lower temperatures.

The discrepancy between our results and those that previously suggested the termination of the main phase transition is direct. We have used the classical measurements of molecular area rather than the more recently developed microscopic methods that allow direct observation of phase behavior in monolayers (see Note 2). The elimination by the captive bubble of experimental problems with the Langmuir trough provides a possible explanation for the different results. Temperature and humidity are more easily controlled within the bubble. The continuous interface eliminates escape along or around confining barriers. The enclosed interface of the bubble should prevent subsequent contamination by airborne factors that can alter results on the Langmuir trough (Hifeda and Rayfield, 1992; Klopfer and Vanderlick, 1996). We therefore speculate that our different results reflect the experimental advantages of the captive bubble.

There is, however, a theoretical ambiguity inherent in the use of the captive bubble as a surface balance, although it seems unlikely to explain our different results. With the captive bubble, the critical temperature and surface pressure could in theory depend on the bulk phase pressure. This possibility originates from the Gibbs phase rule, which in its most general form states that for a system with c components, p phases, and n intensive variables, the number of degrees of freedom f is determined by $f = c - p + n$.

Although temperature and pressure generally provide the intensive variables sufficient to describe bulk phase systems, an interfacial film also requires surface pressure, and n increases from 2 to 3 (Crisp, 1949; Gaines, 1966). The restriction of experiments on Langmuir troughs to atmospheric pressure determines one degree of freedom, and the phase rule reduces to the standard form with $n = 2$. The captive bubble, however, has no such restriction, and the additional degree of freedom means that surface pressures, including critical surface pressures, could vary with hydrostatic pressure. The surface pressure of the LE-LC transition for DPPC, however, varies at most by 1 mN/m over the range of bulk phase pressures available with our apparatus. The supra-atmospheric pressures in our experiments seem unlikely to explain the apparent absence of the critical point suggested by previous experiments on Langmuir troughs.

Our measurements raise the possibility that the phase diagram for phosphatidylcholine monolayers might be simpler than previously proposed. Conversion of one phase to the other at a critical point must be possible by continuous changes in conditions without at any point being able to distinguish which phase is present, and so the symmetries cannot be distinct (Landau and Lifshitz, 1958). Under commonly measured conditions, the LC and LE phases are anisotropic and isotropic, respectively (Moy et al., 1986), and so they do not meet this constraint. Two additional features of the phase diagram could still permit the coexistence region to terminate. First, under conditions closer to the critical point, one or both of the LC and LE phases could convert to phases with a common symmetry. Second, the coexistence region could terminate at a tricritical point. The first-order transition would then continue at higher surface pressures as a second-order transition for which the molecular area and entropy would vary continuously, but symmetry would change at a distinct point. The existence of two expanded phases, anisotropic fluid and isotropic fluid, and of the tricritical point have both been reported previously (Albrecht et al., 1978). Our results suggest a simpler phase diagram. We find no evidence for a transition between different fluid phases, nor of the critical point that implies that such a transition should exist. Just as the liquid-solid transition in three-dimensional materials persists indefinitely (Pippard, 1960), so might the LE-LC transition for phospholipid monolayers.

In summary, our results show that the captive bubble used as a surface balance for studying monolayers produces results comparable to those from standard Langmuir troughs, but with possible advantages for control of temperature, humidity, and confinement of the film. Isobars obtained with monolayers of four disaturated phosphatidylcholines demonstrate only the main transition between the LE and LC phases. Coexistence of the phases persists in all cases at least to the onset of collapse. For DPPC, measurements complicated by ongoing collapse suggest that no critical point exists at conditions that can be achieved by monolayers at an air-water interface.

NOTES

1. Relationships expressing surface tension and area apply equally to axisymmetric droplets and bubbles (Malcolm and Elliott, 1980). Other investigators have previously used droplets as surface balances (Kwok et al., 1996).
2. Another investigator has in fact advised us that fluorescence microscopy on DPPC monolayers at 40°C failed to detect the phase separation predicted by our results (M. Lösche, personal communication). We and this investigator agree, however, that experimental difficulties such as convection with heating of the subphase and the limits of microscopic resolution, particularly in light of the smaller size of LC domains with increasing temperature, make this observation inconclusive.

S.H. gratefully acknowledges the guidance of Dr. Jon Goerke in the initial construction of a functional captive bubble apparatus. Dr. David Grainger, Dr. Viola Vogel, and William Schief have discussed work in progress. Drs. David Grainger, Mathias Lösche, John Nagle, and Christian Salesse provided helpful criticism of the manuscript before submission. Walter Anyan performed initial studies that established this method in one of our laboratories (S.H.). Ethan Smith completed the experiments after the initial acceptance of the manuscript that documented the invariance of surface pressure with hydrostatic pressure.

Funding was provided by the following organizations: the Department of Anaesthesia and General Intensive Care Medicine, The Leopold-Franzens University of Innsbruck (G.P.); the Whitaker Foundation (S.H.); the American Lung Association of Oregon (S.H.); and the National Institutes of Health (Grants HL 03502 and 60914 to S.H.). Page charges were deferred in part by the friends and family of Vern McKee.

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