Rapid Compression Transforms Interfacial Monolayers of Pulmonary Surfactant

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ABSTRACT Films of pulmonary surfactant in the lung are metastable at surface pressures well above the equilibrium spreading pressure of 45 mN/m but commonly collapse at that pressure when compressed in vitro. The studies reported here determined the effect of compression rate on the ability of monolayers containing extracted calf surfactant at 37°C to maintain very high surface pressures on the continuous interface of a captive bubble. Increasing the rate from 2 Å^2/phospholipid/min (i.e., 3% of (initial area at 40 mN/m)/min) to 23%/s produced only transient increases to 48 mN/m. Above a threshold rate of 32%/s, however, surface pressures reached >68 mN/m. After the rapid compression, static films maintained surface pressures within ±1 mN/m both at these maximum values and at lower pressures following expansion at <5%/min to ≥45 mN/m. Experiments with dimyristoyl phosphatidylcholine at 37°C produced similar results. These findings indicate that compression at rates comparable to values in the lungs can transform at least some phospholipid monolayers from a form that collapses readily at the equilibrium spreading pressure to one that is metastable for prolonged periods at higher pressures. Our results also suggest that transformation of surfactant films can occur without refinement of their composition.

INTRODUCTION

Films of pulmonary surfactant in the lung achieve surface pressures that are exceptional for both their magnitude and their stability. A thin film of surfactant coats the air-liquid interface of the alveoli, and when compressed during exhalation by the decreasing alveolar surface area, surface pressure approaches 70 mN/m (Horie and Hildebrandt, 1971; Schürch, 1982). The compressed films are metastable at these pressures. In excised lungs held at constant volume, the slow rate of increase in airway pressure suggests that surface pressure falls from values of 68 mN/m at a rate of approximately 0.1 mN/m min^{-1} (Horie and Hildebrandt, 1971). Direct measurements using fluorocarbon droplets deposited on the surfactant film in situ provide comparable values (Schürch, 1982). This metastability of static surfactant films at surface pressures well above equilibrium values in the absence of dynamic compression is arguably more remarkable than the magnitude of the surface pressure itself.

Replication of such high stable surface pressures in vitro with well-defined monolayers containing the complete set of surfactant constituents has proven difficult. Spread films compressed on a Langmuir trough at physiological temperatures generally collapse when they reach the equilibrium surface pressure, with constituents leaving the interface to form a bulk phase and restore the equilibrium density. This observation suggests that the surfactant film in the lung must undergo some process of transformation into a different structure capable of maintaining high surface pressures. Most models contend that structural transformation reflects a change in composition. Of the multiple components present in pulmonary surfactant, only the most prevalent constituent, dipalmitoyl phosphatidylcholine (DPPC), can achieve and maintain high surface pressures in films containing a single compound at physiological temperatures on a Langmuir trough. The transformation in structural stability of the surfactant film is generally thought to reflect a change from the complete composition of the freshly secreted material to one that is greatly enriched in DPPC. Processes that can produce transformation include compression through an interval of collapse (Hildebran et al., 1979), repeated compression in the presence of excess material beyond the content of the monolayer (Keough, 1984), and formation of the film by adsorption rather than spreading (Schürch et al., 1995).

The studies reported here determined whether rapid compression could also achieve transformation. Compression that exceeds the rate at which a film can relax, either by rearrangement of constituents within the film or by collapse from the interface, must elevate surface pressure. Numerous insoluble monolayers, including films of pulmonary surfactant, display this effect (Rabinovitch et al., 1960; Sims and Zografi, 1971; Boonman et al., 1987; Kato, 1990; Rapp and Gruler, 1990; Kato et al., 1991; Angelova et al., 1996; Kwok et al., 1996; Kampf et al., 1999). Films, however, that achieve high pressures in this manner are generally unstable, and surface pressure decays toward equilibrium as soon as active compression ceases. We show here that compression at speeds inaccessible on the Langmuir trough but in the range of rates that occur in the lung converts monolayers of pulmonary surfactant and of at least one fluid phospholipid from a form that collapses readily at 45 mN/m to one

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that is metastable at higher surface pressures. The characteristics of the transformed films and of the process by which conversion occurs challenge current models of the stable surfactant film.

MATERIALS AND METHODS

Materials

Our experiments used extracts of lung surfactant obtained from calves (calf lung surfactant extract (CLSE)). CLSE is obtained by lavaging calf lungs, removing cells from the recovered fluid by low-speed centrifugation, spinning at higher speed to collect the large surfactant particles, and extracting the pelleted material into chloroform (Notter et al., 1983). Extraction removes the glycoprotein SP-A recovered in the large surfactant particles along with contaminating serum proteins but retains all other constituents of native surfactant. CLSE was among the first surfactants successfully used to treat premature babies (Kwong et al., 1985) and has been characterized extensively since (e.g., Kendig et al., 1989; Kahn et al., 1995). CLSE used in these studies, obtained from ONY (Amherst, NY), represented material combined from multiple lungs and was used without further purification or characterization.

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: dipalmitoyl phosphatidylcholine (DPPC) and dimyristoyl phosphatidylcholine (DMPC) (Avanti Polar Lipids, Alabaster, AL); high-purity chloroform and methanol (Burdick and Jackson, Muskegon, MI); GibcoBRL Ultra Pure brand Hepes, purity >99.7%, heavy metals <20 ppm (Life Technologies, Grand Island, NY); $CaCl_2 \cdot 2H_2O$ (J.T. Baker Inc., Phillipsburg, NJ); NaCl (Mallinckrodt Specialty Chemicals Co., Paris, KY).

Methods

Captive bubble apparatus

These studies used a pressure-driven captive bubble device (Putz et al., 1994a, 1998) described previously (Crane et al., 1999). The instrument measures the shape of an axisymmetric air bubble floating below an agarose ceiling during changes in hydrostatic pressure applied to the liquid subphase (Schürch et al., 1989). The height and width of the bubble provide the basis for calculating its volume, surface area, and surface tension (Malcolm and Elliott, 1980; Schoel et al., 1994). If a surfactant film is formed at the bubble's interface, the system can then be used as a surface balance, although surface area, which normally is the independent variable, is dependent on the hydrostatic pressure applied to the subphase. Applied hydrostatic pressure provided either by a computer-controlled syringe drive (Harvard Apparatus, Cambridge, MA) or by compressed nitrogen varied over a possible range from 0.4 to 3.5 atm. CCD cameras monitored either the profile of the bubble in the vertical axis to ensure axisymmetric shape or in the horizontal axis to measure its height and width. Accurate analysis of the interfacial characteristics requires an axisymmetric bubble. The correct shape was confirmed before all experiments and intermittently during slow manipulations but not during rapid changes. Temperature was monitored with a thermistor probe (YSI, Yellow Springs, OH) and controlled by a temperature regulator (either YSI or Cole-Palmer) via heating pads (Minco, Minneapolis, MN) applied along the sides of the chamber. All experiments used a subphase containing 10 mM Hepes, pH 7.0, 150 mM NaCl, and 1.5 mM $CaCl_2$ (HSC).

Computer interface

The bubble was manipulated and measured by computer 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). 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). The algorithm used requires only the two measured values and so is faster than the axisymmetric drop shape analysis of Neumann and co-workers (Rotenberg et al., 1983), which requires several measurements to define the bubble's profile. The simpler calculations allowed determination of the interfacial characteristics in real time, although the basis of determining very low surface tensions, when the ratio of height/diameter (h/d) is less than 0.097, is based on extrapolation of surface tension to zero when $h/d = 0$ (Schoel et al., 1994) rather than solution of the Young-LaPlace equation (Rotenberg et al., 1983). Surface pressures were calculated from the difference between previously published surface tensions for water at the appropriate temperatures (Washburn, 1926–1930) and the measured surface tension. The program routinely averaged results from two to four frames to provide a recorded set of values at a rate of ~4 Hz. For rates of compression that required more frequent analysis, images were first recorded on a video cassette recorder and then analyzed individually, using the camera speed of 30 frames per second to provide elapsed time. By using an input/output board (PCI-1200, National Instruments) to control the syringe pump, the computer could manipulate applied hydrostatic pressure. This approach allowed regulation of surface pressure, surface area, or volume, either to maintain one of these variables constant or to change it in a predetermined manner.

Spreading of the films

Our experiments studied films spread on the surface of the captive bubble. Following formation of an 80–100-μl bubble, 0.05–0.08 μl of CLSE or DMPC in chloroform:methanol (1:1, v:v) at appropriate concentrations were injected through a 0.5-μl syringe and needle (Scientific Glass Engineering, Ringwood, Victoria, Australia) that touched the air-liquid interface to achieve an initial surface pressure below 42 mN/m. To remove the spreading solvent, bubbles were then compressed to a surface pressure of 40–42 mN/m. With further HSC infusing, an outlet valve was opened and adjusted to maintain a constant surface pressure while allowing at least 20 ml of buffer to flow through the subphase. This protocol for spreading the monolayer and removing the solvent was developed in studies with DPPC (Crane et al., 1999). Compression of such monolayers produced surface pressure/surface area isotherms exactly comparable to curves obtained on standard Langmuir troughs (Crane et al., 1999). The compression isotherm for DPPC at 37°C, and in particular the slope of the segment above 50 mN/m, provided a sensitive criterion for indicating the presence of contaminating substances.

Manipulation of the bubble

Routine compression and expansion of the bubble used the computer-controlled syringe drive to manipulate the hydrostatic pressure applied to the subphase. Compressions beyond the maximum rates possible with the syringe drive instead used pressure from a tank of compressed nitrogen. Application of 2.0 atm pressure across the adjustable opening of a needle valve provided variable rates of compression.

RESULTS

In these studies, we achieved a wide range of compression rates by using a captive bubble as a surface balance. The

proponents of this method have pointed out the significant advantage over more traditional methods of compressing films that the continuous interface of the bubble eliminates any possibility of escape along confining barriers (Schürch et al., 1989; Putz et al., 1994b). The bubble also provides better regulation of humidity and temperature and a wider range of speeds. At 37°C, compression of CLSE monolayers at <5 Å^2/phospholipid/min (5% initial area/min), a rate that is well within the range accessible on Langmuir troughs, increased surface pressure monotonously to a plateau at 45 mN/m (Fig. 1). Further compression produced minimal further increase in surface pressure. Hysteresis during reexpansion was perhaps less than commonly observed on troughs, but monolayers of CLSE on the captive bubble generally produced behavior comparable to that on more conventional surface balances, with collapse following compression above equilibrium densities, and reinsertion of collapsed constituents during subsequent expansion.

For faster compressions, we used a different method to manipulate the size of the bubble. Rather than using a syringe-drive to vary hydrostatic pressure, we instead applied a jump in hydrostatic pressure from a source of compressed gas across a needle valve of variable resistance. The change introduced two new issues. First, if the faster compression exceeded the rate of heat exchange and therefore was adiabatic rather than isothermal, it would produce a significant increase in temperature. Any such heating of the gas phase, however, should be evident from measurements of PV (pressure times volume), which to the extent that the bubble behaves like an ideal gas would reflect nT (moles times temperature). PV actually dropped following compression, presumably because of an increase in dissolved gas at the higher pressure. Although our results could not exclude a transient increase in temperature, they did suggest that none occurred. The second issue concerning the two methods of manipulating the bubble was the difference in patterns of compression. In contrast to experiments with the syringe pump (Crane et al., 1999), the rate of change for both volume and area in the pressure-jump experiments varied over the course of the compression during their asymptotic approach to final values (Fig. 2). The rates reported here represented the change in area during the first 15% of compression. Hysteresis between the initial rapid compression and slow expansion back to 45 mN/m suggested a loss of material from the interface so that the molecular content of the film became unknown. We therefore expressed areas as the percent of their initial values at 45 mN/m, designated A_0, rather than area per molecule.

Faster rates of compression produced results that changed considerably over a narrow range of speeds (Fig. 3). At $23 \pm 2\%$ A_0/s, results were similar to those at $<5\%$ A_0/min despite the almost 300-fold faster compression. Surface pressure again followed a plateau and increased minimally above the value of 45 mN/m established at slower rates. At $32 \pm 1\%$ A_0/s, however, surface pressure reached 67.1 ±

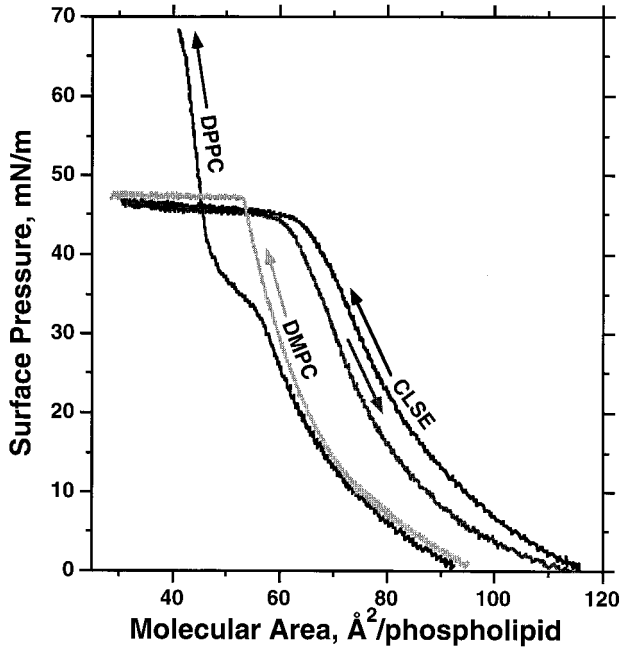

FIGURE 1 Surface pressure/molecular area isotherms for CLSE, DMPC, and DPPC. Monolayers were compressed at 37°C on a captive bubble at a rate of <5 Å^2/phospholipid/min. Arrows indicate traces during compression for all three films and expansion for CLSE only.

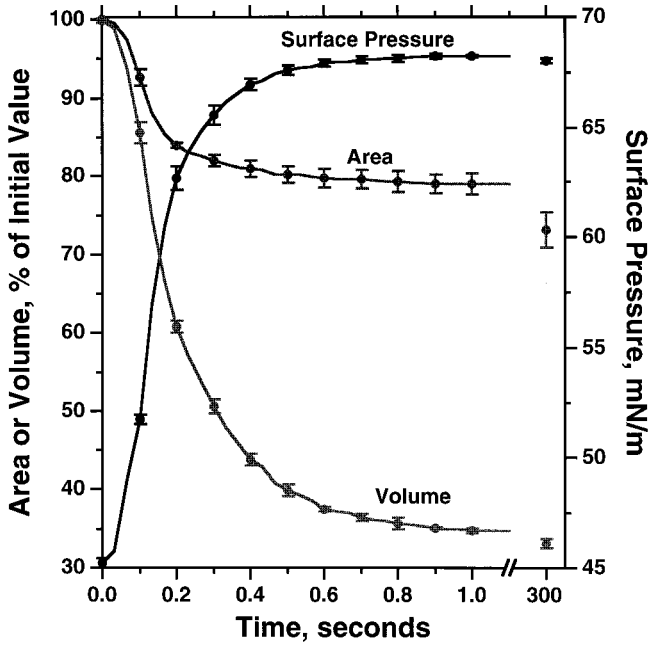

FIGURE 2 Variation of volume, area, and surface pressure for monolayers of CLSE during rapid compression. CLSE was compressed at <5 Å^2/phospholipid/min to 45 mN/m and then rapidly by a sudden increase in the hydrostatic pressure applied to the subphase. Area and volume are expressed as the percent of initial values. Error bars give ±SD at selected points for the mean of three experiments. Temperature = 37°C.

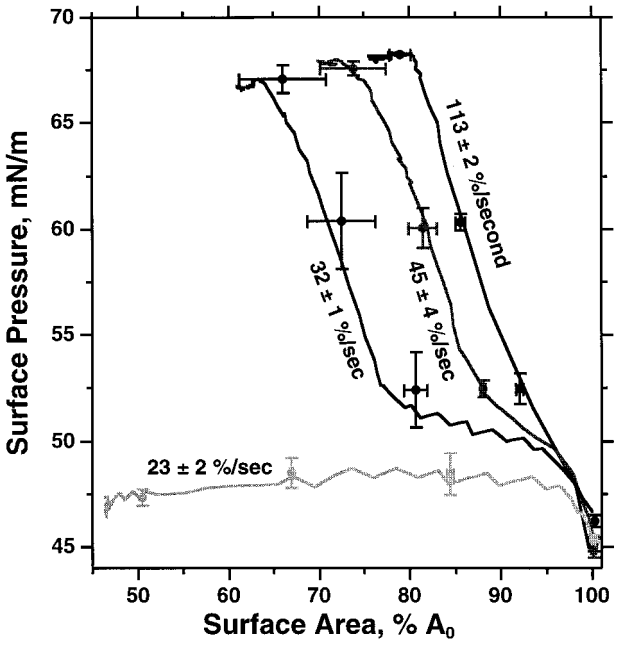

FIGURE 3 Compression of CLSE monolayers at different rates. Films were compressed initially at a rate of <5 Å^2/phospholipid/min to a surface pressure of 45 mN/m and then subsequently at the different rates indicated. Areas are expressed as percent of initial values at 45 mN/m (% A_0). Solid curves give data from a single experiment representative of three replicates. Symbols give mean ± SD for data averaged at selected volumes for the three experiments. Stated rates reflect the initial 15% of compression. Temperature = 37°C.

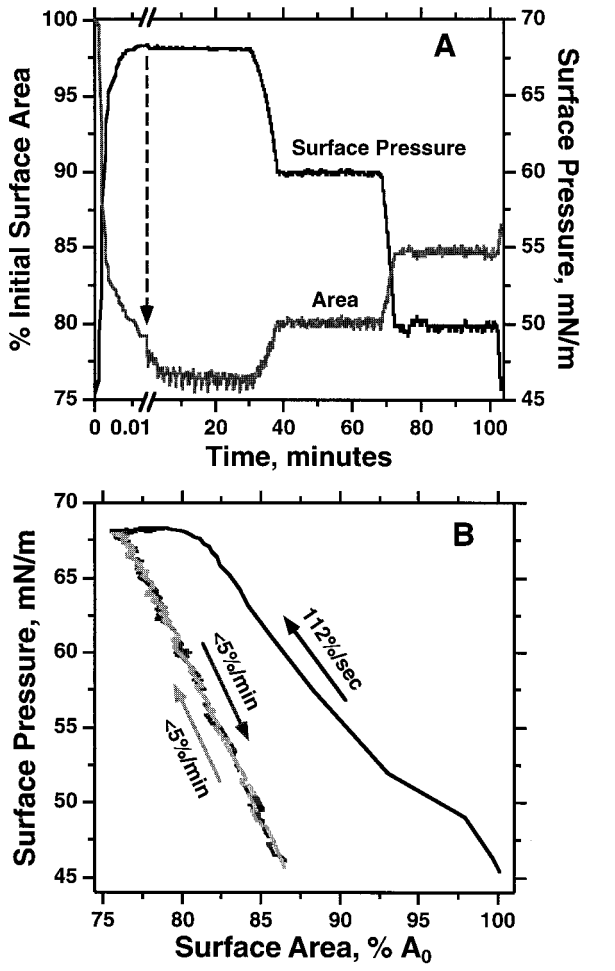

FIGURE 4 Stability of CLSE film following rapid compression. A CLSE monolayer was compressed from an initial surface pressure of 45 mN/m at a rate $>100\%$ A_0/s. The resulting film was monitored for 30 min initially at the maximum surface pressure and then following expansion at $<5\%$ A_0/min to 60 and 50 mN/m. Volume was measured and maintained constant after achieving each surface pressure. The film was then recompressed continuously at $<5\%$ A_0/min. (*A*) Surface pressure (*black line*) and surface area (*gray line*) plotted as functions of time during the initial compression and expansion to 45 mN/m. The vertical dashed line indicates the area at which the film first achieved maximum surface pressure. (*B*) Surface pressure plotted versus surface area for the same experiment, including the final slow recompression not shown in (*A*). Arrows indicate the direction and rate of change in area for the different segments of the experiment. Temperature = 37°C. Data shown are representative of three experiments.

0.6 mN/m. Increasing the speed of compression further to 45 ± 4% and 113 ± 2% A_0/s had little effect on the maximum surface pressures (67.6 ± 0.3 and 68.2 ± 0.1 mN/m, respectively), but the reduction in area required to achieve these pressures decreased. In increasing the rate from 32% to 45% to 113% A_0/s, the change in area required to reach 67 mN/m fell from 34 ± 5% to 24 ± 2% to 19 ± 1%, respectively. The variation resulted from different responses to the initial change in area. The isotherms at the three different rates $\geq 2\%$ A_0/s all fit the general pattern of an initial increase in surface pressure, with values rising above those for slower compressions when area had changed by only 3%, followed by a more horizontal segment and then a steep linear ascent (Fig. 3). In all three cases, the linear portion began at roughly the same surface pressure (52 ± 1 mN/m) and had roughly the same slope (1.27 ± 0.05 mN/m/% A_0). Faster compression primarily affected the reduction in area required before surface pressure began to rise above 52 mN/m.

The rapidly compressed films also lost area at high surface pressures. The bubble reached minimum volume (not shown) and maximum surface pressure simultaneously, but surface area continued to change (Fig. 4, *A* and *B*). For compression at 113% A_0/s, for instance, area decreased by 4 ± 2% A_0 after compression of the bubble ceased.

The rapidly compressed films that achieved high surface pressure were impressively stable (Fig. 4). Bubbles held at constant volume after completion of the rapid compression maintained surface pressure and surface area constant within 2% for periods of at least 30 min (Fig. 4 *A*). This stability was characteristic of the films not only at the maximum pressures but also after expansion to lower surface pressures. After slow expansion to 60 and 50 mN/m, the rapidly compressed films again maintained surface pres-

sure and area essentially constant for 30 min (Fig. 4 *A*). Subsequent to rapid compression, slow expansion and recompression at ≤5% A_0/min followed the same curve and could be repeated without loss in area as long as surface pressure remained in the range of 45–68 mN/m (Fig. 4 *B*). The absence of hysteresis provides further evidence that collapse of the film was minimal over the duration of these experiments and for the full range of surface pressures above equilibrium spreading values.

Comparison of the film at 45 mN/m before and after rapid compression provided the most direct demonstration of the effects of that maneuver (Fig. 4 *B*). After compression at >100% A_0/s and slow reexpansion to 45 mN/m, the film occupied an area, designated A_1, that was 17 ± 4% less than A_0. The compressibility, $-1/A \times dA/d\pi$, during compression at <5% A_0/min had fallen from the initial value of 264 ± 100 m/N to 5.3 ± 0.1 m/N. The value for monolayers of DPPC under these conditions was 3.9 ± 0.3 m/N. The rate of collapse, $-1/A \times dA/dt$, decreased from at least 6 h^{-1} at 47 mN/m before transformation to roughly 1×10^{-3} h^{-1} at 50 mN/m after the rapid compression.

Hysteresis developed for the transformed film if expansion lowered surface pressure below 45 mN/m (Fig. 5). Surface pressure decreased continuously during expansion at <5% A_0/min until reaching ~40 mN/m but then remained between 39 and 41 mN/m during further expansion to A_0 (Fig. 5 *A*). When subsequently recompressed at the same slow rate to 45 mN/m, the film reached 95 ± 3% A_0, indicating that of the 17% difference between A_0 and A_1, 5% represented material excluded from the interface that was unable to reinsert into the film. The remaining 12% resulted from some combination of reduction in molecular area and exclusion of components into a pool that respreads readily between 40 and 45 mN/m.

The extent to which the surface was expanded beyond A_1 determined subsequent behavior (Fig. 5 *B*). When recompressed at <5% A_0/min, two of three films expanded to 108.0 ± 1.0% A_1 and all films expanded to ≥104.2 ± 0.0% A_1 again reached surface pressures above 65 mN/m. The recompression isotherms consisted of a relatively horizontal segment beginning at 45 mN/m in which surface pressure changed little while area was reduced below A_1, followed by a steep ascent (Fig. 5 *B*). Similar to the initial isotherms during rapid compression, the steep ascending segments of these slow recompressions began between 50 and 55 mN/m. The slopes of the steeply rising portions of the curves (2.0 ± 0.3 mN/m/% A_1) were equivalent to values from the initial reexpansion (1.8 ± 0.1 mN/m/% A_1). The extent to which these steep parallel segments were shifted to lower areas exceeded the overexpansion of the film beyond A_1. After expansion to 102.1 ± 0.1% A_1, the parallel segment during recompression was shifted relative to the isotherm during the slow expansion by 5 ± 2% of A_1. For the curves that did reach high surface pressure following expansion to 108% of A_1, the parallel segment was shifted to smaller area by 32 ± 9% of A_1. Expansion to 115.7 ± 0.6% of A_1 prevented the film from ever reaching surface pressure >48 mN/m.

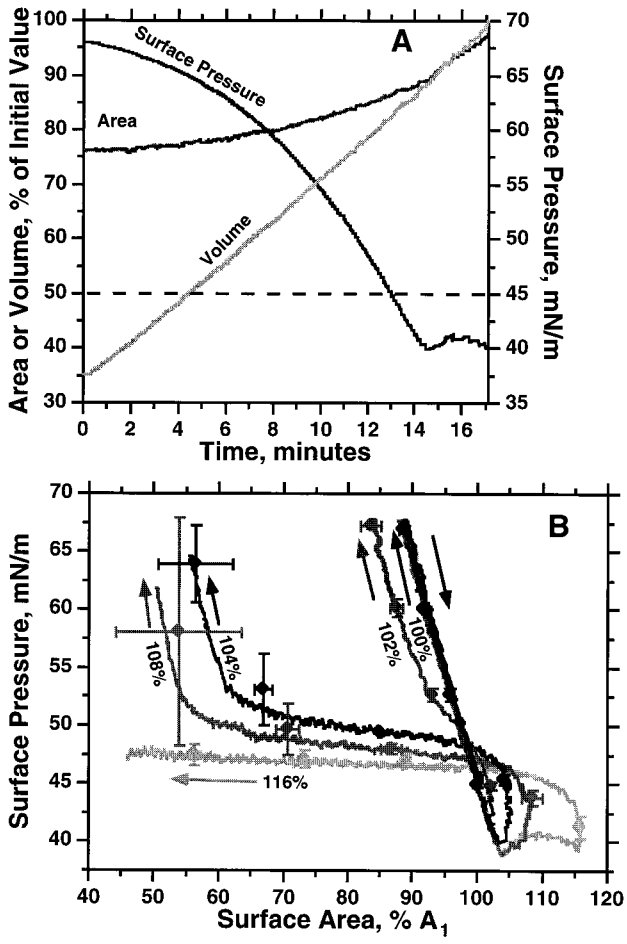

FIGURE 5 Expansion of rapidly compressed CLSE to surface pressures below 45 mN/m. CLSE spread to form initial monolayers with surface pressure <40 mN/m was compressed at <5% initial area/min to 45 mN/m and then at >100%/s to high surface pressures. (*A*) Variation of surface pressure, area, and volume with time during subsequent expansion at <5% A_0/min to the original volume at 45 mN/m before rapid compression. Dashed horizontal line indicates 45 mN/m. Surface area (*dark gray curve*) and volume (*light gray line*) are expressed as percent of initial values. (*B*) Variation of surface pressure with area during expansion at <5%/min to different maximum areas and during subsequent recompression. After expansion and then 10 min with the bubble held at constant maximum area, the films were recompressed at <5% A_0/min. Surface area is normalized to the area (A_1) at which surface pressure reached 45 mN/m during expansion. Each curve is representative of three experiments and is labeled according to maximum area. Symbols give mean area ± SD averaged for the three experiments at selected surface pressures. Temperature = 37°C.

One mechanism by which rapid compression might transform a monolayer of CLSE is refinement of its composition. Experiments were performed with monolayers containing a single fluid phospholipid to determine whether the rapid compression could transform a film for which compositional refinement was impossible. Previous isobaric expansions on the captive bubble showed that DMPC at 45 mN/m

is in the fluid liquid-expanded state at temperatures above ~22°C (Crane et al., 1999). Compression of DMPC at <5 Å^2/molecule/min (<5% A_0/min) and 37°C produced the isotherm expected for fluid films, with an initial smooth increase in surface pressure followed by a sharply defined plateau beginning at 47.4 ± 0.2 mN/m (Fig. 1). Compression to 54% A_0 failed to elevate surface pressure above 48.0 ± 0.4 mN/m (Fig. 1). Rapid compression at 105 ± 9% A_0/s instead increased surface pressure to 68.0 ± 0.1 mN/m with a decrease in area to only 78 ± 2% of A_0 (Fig. 6). Unlike CLSE, surface area as well as surface pressure stopped changing when compression of the bubble ceased (Fig. 6). Like CLSE, rapidly compressed films of DMPC were metastable. Surface pressure decreased by only 0.2 ± 0.3 mN/m from its maximum value over 10 min with the bubble held at constant volume and 0.1 mN/m during one experiment over 30 min. The rapidly compressed films of DMPC were also metastable at lower surface pressures, with expansion to 45 mN/m and recompression to 65 mN/m at rates <5% A_0/min producing identical isotherms without hysteresis. The area A_1 of the film at 45 mN/m following rapid compression was 8 ± 2% less than A_0. When area was appropriately normalized, the isotherms for the slow compression of the film before and after transformation continued along the same curve (Fig. 7). Just before collapse from the interface at 47.5 mN/m, compressibility of the initial film (4.6 ± 0.5 m/N) was similar to that of the transformed film (5.5 ± 0.2 m/N) (Fig. 7). The isotherm for the transformed film during slow expansion and recompression was linear, with no plateau or change in slope to suggest a discrete phase transition.

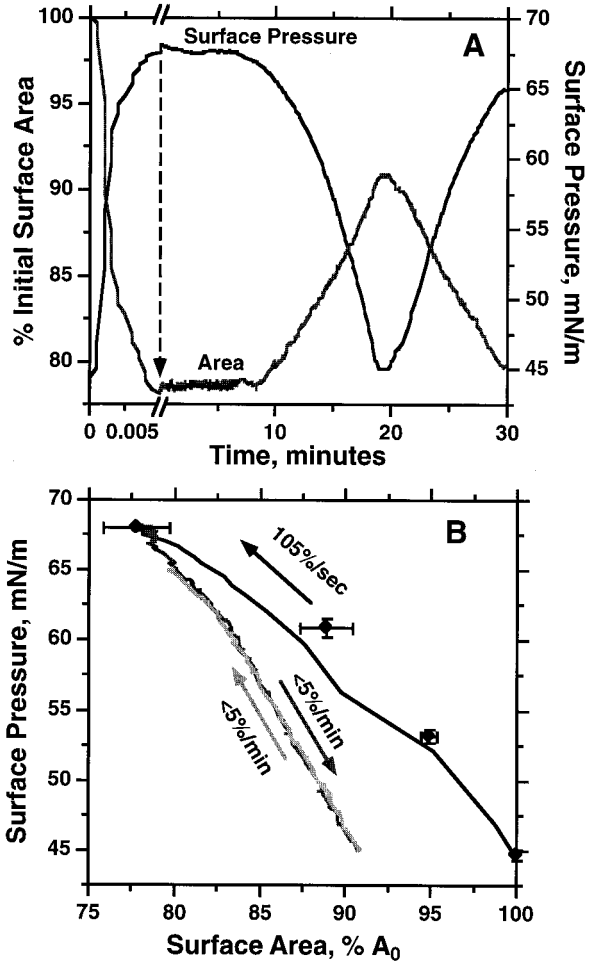

FIGURE 6 Effect of rapid compression on DMPC monolayers. DMPC was spread to an initial surface pressure <40 mN/m, compressed at <5% initial area/min to 45 mN/m, and then at 105%/s. The bubble was maintained at constant minimum volume for 10 min and then expanded continuously at <5% initial area/min to 45 mN/m. After a brief interval at that surface pressure, the bubble was recompressed at the same rate to 65 mN/m. (*A*) Surface pressure and surface area plotted versus time. The dashed vertical line indicates the area at which surface pressure achieved its maximum value. (*B*) Surface pressure plotted versus surface area. Curves give the data from an individual experiment representative of three replicates. Symbols in *B* give mean ± SD averaged for the three experiments at selected volumes during the rapid compression. Temperature = 37°C.

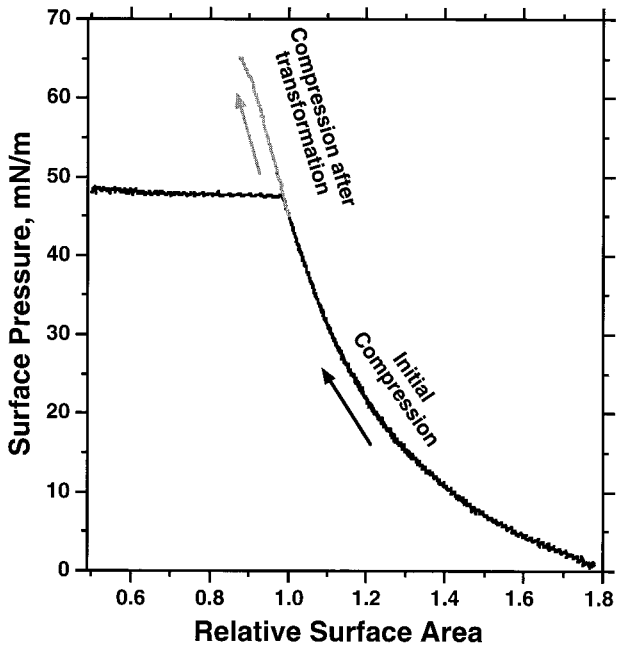

FIGURE 7 Comparison of DMPC monolayers before and after rapid compression. DMPC monolayers with an initial surface pressure <40 mN/m were either expanded to 0 mN/m or immediately compressed at a rate of <5% initial area/min to 45 mN/m. In the former case, the film was compressed at a rate of <5 Å^2/phospholipid/min, as shown in black. In the latter case, rapid compression at 105%/s to >67 mN/m and subsequent slow expansion back to 45 mN/m was performed. A recompression at <5% initial area/min to 65 mN/m is shown in gray. Surface area is expressed relative to A_0 for the untransformed monolayer and relative to A_1 for the film after rapid compression. Temperature = 37°C.

DISCUSSION

The behavior of extracted calf surfactant in a monolayer on the surface of a shrinking captive bubble depends greatly on the rate of compression. Although the symmetry of compression differs between the captive bubble and standard Langmuir troughs, the two devices produce similar behavior when the speed of compression is the same. At 37°C, films of CLSE on the bubble collapse immediately when they reach the equilibrium spreading pressure of ~45 mN/m, just as they do on standard troughs. More rapid compression, however, converts the film to a form that is metastable over a broad range of higher pressures. We consider first the process of transformation, then the nature of the transformed film, and finally the physiological implications of our results.

Transformation of the film to the more stable form requires compression above a narrowly defined threshold rate. We favor the explanation that the change in area must exceed the rate at which constituents can collapse from the interface, that the resulting decrease in molecular area causes surface pressure to rise, and that the extreme compression at very high surface pressures transforms the film. This scenario fits with prior observations on single-component monolayers containing saturated phospholipids in which elevation of surface pressures above equilibrium spreading values initially accelerated collapse, but rates of relaxation then slowed when pressures reached very high values (Goerke and Gonzales, 1981). Rapid compression on the captive bubble, however, exposes the film in our experiments to effects other than high surface pressure that could also contribute to transformation. The horizontal plateau in the compression isotherm above 67 mN/m almost certainly results from collapse of the monolayer, and formation of a three-dimensional structure could be essential for transformation. The initial rapid change in area could by itself be sufficient to produce a stable film even before an appreciable rise in surface pressure occurs. The isotherms above and below the threshold rate deviate after only a 3% change in area, and this early distinction between films that will prove to have different stabilities at the end of compression favors an early transformation. Determination of which factor–the high surface pressure, formation of collapsed structures, or the rapid pulse of compression–causes the transformation awaits development of the technical capability to stop the rapid compression reproducibly at specific points along the same isotherm.

Increasing the rate produces a characteristic change in the shape of the isotherms. When the rate of compression first exceeds the threshold required to achieve high surface pressures, the isotherm still follows an initial fairly flat plateau before surface pressure begins to rise steeply. The curves are reminiscent of behavior reported by others previously in which surfactant films on Langmuir toughs required compression through an extensive plateau before isotherms curved upward (Hildebran et al., 1979; Keough, 1984). A central question concerning the function of pulmonary surfactant is the nature of the structural change during this plateau that enables the film to withstand high surface pressures. Our results show that progressively increasing the compression speed first shortens and then eliminates the initial plateau, thereby transforming the film with a smaller change in area. One model that fits with these different data is that the initial collapse of material in some way hinders and slows subsequent collapse, thereby decreasing the rate of compression required to reduce molecular area and cause surface pressure to rise.

The effect of excess material beyond the contents of a monolayer has received increased recognition recently because of evidence that adsorbed surfactant forms multilayers both in vitro (Oosterlaken-Dijksterhuis et al., 1991; Schürch et al., 1995; Yu and Possmayer, 1996) and in vivo (Hills, 1988; Schürch and Bachofen, 1995). This finding has led to speculation that the thickness of surfactant films contributes to their stability. The collapsed material in our experiments raises the possibility that transformation during rapid compression occurs by formation of a multilayer. The change in area, however, that is required for transformation is quite small. The rapidly compressed film occupies on average 17% less than the initial monolayer, and as little as 13% less for individual experiments (Fig. 4 *B*). A trilayer formed from the average excluded material would cover at most 8% of the interface. Even a bilayer, which would be energetically unfavorable, would occupy only 20%. Any multilayers formed during rapid compression must then represent localized structures, separated by stretches of monolayer. Although such multilayers might slow collapse during the initial rapid compression, the intervening monolayer would be incapable of sustaining high surface pressures if it remained in the initial state. Formation of multilayers therefore cannot alone explain the conversion of surfactant to a more stable structure.

Transformation during rapid compression also seems unlikely to reflect a major change in the film's composition. Most investigators have concluded that the stability of the surfactant film observed in vivo requires a refined composition. During standard experiments on the Langmuir trough, static monolayers containing single components sustain high surface pressures only in the liquid-condensed phase (Smith and Berg, 1980). DPPC is the only constituent of pulmonary surfactant that forms the condensed phase in single-component films at physiological temperatures (King, 1984). Consequently, the standard view has been that the functional film of surfactant in the lung must be markedly enriched in DPPC relative to the complete mixture. The squeeze-out model proposes that exclusion of other constituents above the equilibrium spreading pressure produces the necessary enrichment (Watkins, 1968; Clements, 1977; Bangham et al., 1979). More recent studies have suggested that selective adsorption of DPPC during formation of the

film might instead change the composition before the onset of compression (Schürch et al., 1995). In our experiments, however, the reduction in area is inadequate to produce a substantial change in the content of DPPC. Extracted calf surfactant contains 33% DPPC (Kahn et al., 1995). Because the molecular area of the other phospholipids would be greater than for DPPC, the 17% change in area resulting from compression at >100% A_0/s indicates that thin films of surfactant can sustain physiological stability when containing less than 40% DPPC.

Our results with DMPC challenge the more fundamental observation that films reaching the equilibrium spreading pressure in the liquid-expanded phase must be unstable at higher densities. Rapid compression produced the same transformation for DMPC as for CLSE of a film that collapsed just above 45 mN/m to one that was metastable over the broad range of higher pressures (Fig. 6). Preliminary experiments with palmitoyl-oleoyl phosphatidylcholine and palmitoyl-palmitoleoyl phosphatidylcholine at 37°C and with DPPC at 65°C suggest that our results extend to other compounds above their gel-to-liquid crystal transition temperatures. We assume that the similar behavior of DMPC and CLSE reflect the same phenomenon, and that refinement of the surfactant films plays little or no role in the formation of a metastable film.

The most remarkable aspect of our results is the persistence of high stability during expansion of the film to lower surface pressures. The slow rates of collapse and low compressibilities that define the transformed state are not restricted to the high pressures >60 mN/m at which they have been documented previously (Goerke and Gonzales, 1981). They also persist when surface pressure is reduced to 45 mN/m. Further expansion does restore the original instability, but the process is complicated. Although the transformed film may at some point revert to its initial state, material excluded from the interface during the rapid compression also can reinsert into the monolayer when surface pressure falls below the equilibrium spreading value. The ability of collapsed surfactant to respread into the interface is well documented (e.g., Wang et al., 1995), and such a process best explains the roughly constant surface pressure at 40 mN/m when the film approaches A_0 during slow expansion (Fig. 5). Restoration of the original unstable behavior could then reflect the presence of reinserted material still in the initial unstable state, the reversal of a structural transformation, or some combination of the two. Following expansion to pressures between 40 and 45 mN/m, the isotherm during recompression follows a roughly isobaric plateau for a length that depends on the extent of expansion beyond A_1, but surface pressure then rises with the same compressibility as the original transformed film. Although expansion to below 45 mN/m allows material to reinsert into the interface, restoring stability with slow recompression does require a reduction in area below that of the transformed film that is greater than that occupied by the material that reinserts. Our results are most easily explained in terms of differential stability, with material reinserting into the transformed film to form focal fluid structures that not only collapse readily above 45 mN/m but also promote the collapse of some surrounding film.

The persistence of the high stability at lower surface pressures complicates evaluation of various models for the transformed structure. Expansion of the rapidly compressed film to 45 mN/m restores the initial thermodynamic conditions but not the initial state. The film must therefore be kinetically trapped in the more dense configuration that is capable of sustaining high surface pressures, with a relaxation time to the initial structure that is long relative to the duration of our experiments. Consequently, several possible explanations for transformation are difficult to assess. Hypothetical structures that could conceivably be reached only by a pulse to high surface pressure, normally inaccessible because of collapse, and that might have the appropriate stability include the liquid-condensed phase, a crystalline phase analogous to the sub-gel of bilayers (Katsaras et al., 1995), and the anisotropic fluid phase distinguished by Albrecht and co-workers by its greater viscosity (Albrecht et al., 1978). All should produce characteristic changes at discrete pressures in either molecular area or compressibility if expansion and return to the initial state occurred under equilibrium conditions. A rapid rise in viscosity analogous to that of a supercooled liquid (Angell, 1995), but produced by a quench in pressure rather than temperature (Liu and Nagel, 1998), could trap the system as an amorphous solid and prevent relaxation to the bulk phase, and might again have characteristic behavior during expansion. The kinetic entrapment, however, means that the absence of any discontinuities in the isotherms could simply reflect an expansion too fast to observe them. The featureless linear traces for transformed CLSE and DMPC above 45 mN/m, as well as the comparison of the compressibilities of DMPC before and after transformation, provide no evidence concerning these models. The structure of the transformed film remains an enigma.

The most certain physiological implication of our results concerns the relationship between stability of a film and its composition. Previous studies have interpreted films with similar stabilities and compressibilities as having similar compositions (Hildebran et al., 1979; Schürch et al., 1989). Surfactant compressibilities and collapse rates similar to DPPC were thought to have undergone a compositional change and become substantially enriched in that compound. Our results with DMPC demonstrate conclusively that films at physiological temperature with physical characteristics similar to DPPC can have different compositions. Our studies show that surfactant films can form structures that are similar to DPPC in stability without undergoing a major compositional change, and contradict the general understanding that the film must lose most material other

than DPPC to become stable above equilibrium spreading pressures.

The physiological relevance of the rapid compression that transforms the films is less certain. The threshold rate required in these studies does lie within the range of compressions expected in normal breathing. Subsequent experiments in our laboratory conducted as preliminary work for other studies have also achieved transformation with rates as low as 1%/s, presumably reflecting improved elimination of contaminants during the complicated experimental procedure. Furthermore, our results indicate that once formed, a stable film might persist through multiple cycles of slow tidal breathing as long as the surface area remains within certain bounds. Previous studies, however, have suggested that surfactant films can be stable during initial compressions that are quasi-static. Degassed excised lungs can show normal compliance during their first quasi-static deflation (Bermel et al., 1984). Adsorbed films in vitro can be quite stable during a first compression that consists of small discrete steps (Schürch et al., 1995). We speculate that the presence of a multilayer formed during adsorption might lower the threshold rate of compression required to achieve transformation. If the multilayer slowed collapse, then the rate of compression required to reduce molecular area and increase surface pressure would also be lower, perhaps resulting in transformation during a slower quasi-static compression. This model leads to specific hypotheses that remain untested.

In summary, compression above a threshold rate at 37°C of monolayers containing extracted calf surfactant transforms the films from a structure that collapses immediately at 45 mN/m to one that is metastable over a broad range of higher surface pressures. The small decrease in area required to achieve the greater stability and the similar behavior of rapidly compressed DMPC monolayers suggest that the transformation reflects an alteration other than simply a change in composition.

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