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Surface properties of rat pulmonary surfactant studied with the captive bubble method: adsorption, hysteresis, stability

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Surface tension-area relations from pulmonary surfactant were obtained with a new apparatus that contains a leak free captive bubble of controllable size. Rat pulmonary surfactant was studied at phospholipid concentrations of 50, 200 and 400 μg/ml. At the highest concentration, adsorption was rapid, reaching surface tensions below 30 mN/m within 1 s, while at the lowest concentration, approximately 3 min were required. Upon a first quasi static or dynamic compression, stable surface tensions below 1 mN/m could be obtained by a film area reduction of approximately 50%. After three to four cycles the surface tension-area relations became stationary, and the tension fell from 25–30 to approximately 1 mN/m for a film area reduction of less than 20%. Hysteresis became negligible, provided the films were not collapsed by further area reduction. Under these conditions, the films could be cycled for more than 20 min without any noticeable loss in surface activity. After only three to four consecutive cycles, surfactant films exhibited the low surface tensions, collapse rates and compressibilities characteristic of alveolar surfaces in situ. Remarkably, surface tension and area are interrelated in the captive bubble which may promote low and stable surface tensions. If the surface tension of the captive bubble suddenly increases ('click') because of mechanical vibration or unstable surfactant, the bubble shape changes from flat to more spherical. The associated isovolumetric decrease in surface area prevents the surface tension from rising as much as it would have in a constant-area situation. This feedback mechanism may also have a favorable effect in stabilizing alveolar surface tension at low lung volumes.

Introduction

The area enclosed by cyclic, isothermal pressure-volume or surface tension-area plots represents the irreversible heat loss which must be provided by additional work in the cycle. Such added work for pressure-volume or surface tension-area relations of lungs is called 'hysteresis' and is provided by the respiratory muscles.

The pressure-volume hysteresis of the air-filled lung and its recoil force have tissue and surface components. It is well known that liquid filling of the lung abolishes the gas-liquid interface and, with it, most of the hysteresis of the isolated air-filled lung [1–3]. It has been difficult to isolate the tissue contribution to pressure-volume relations from the contributions due to the surfactant film because of the complex interrelationship between surface tension and lung structure. Nevertheless, most of the pressure-volume hysteresis is thought to be caused by the surface tension-area relationship of the alveolar surfactant film [4,5]. Since Clements [6] introduced the Langmuir-Wilhemly surface balance to study pulmonary surfactant extracts, surface tension-area relations obtained in that apparatus have been thought to represent the in situ behavior of the alveolar film. The study reported here examines this assumption, using a recently described in vitro technique for studying surface tension of alveolar lavage material [7].

Several important aspects of in situ pressure-volume relations are not reflected in the behavior of alveolar

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surfactant films investigated in the Langmuir-Wilhelmy balance. In a series of fundamental studies on excised lungs, Bachofen, Hildebrandt and co-workers [1,2] found that neither tissue hysteresis nor surface hysteresis were notably affected by ventilation rates, for cycling periods from 70 to 2000 s/cycle. Hildebrandt [8] found that pressure-volume loop size remained invariant for frequencies from 0.6 to 120 cycles/min. Bachofen [9] found in human subjects that the work of breathing was only slightly affected by the breathing frequency, for frequencies from 10 to 50 breaths/min. In contrast, shape and area of surface tension-area relations varied with frequency in a study of the dynamic behavior of surfactant films conducted in a Langmuir-Wilhelmy balance [10]. In these experiments the hysteresis area at 1 cycle/min was four times that at 11 cycles/min.

In an attempt to overcome difficulties with Langmuir balances, some investigators have been using small air bubbles formed on subsurface tubes in cuvettes containing as little as 20 μl of fluid [11,12]. Such bubbles can be pulsated, therefore allowing surface films to be expanded and compressed, and lowest surface tension to be measured. It is also possible to record surfactant adsorption under dynamic conditions by following the surface tension at maximum bubble size at each pulsation. Surface tension-area relations could also be obtained, however, this would be quite difficult because of the small bubble size, approximately 0.5 mm in diameter. Although this development was an improvement over the surface balance method, there still remain problems with surface leaks up the plastic tube on which the bubbles are formed. When surfactant films reach a surface tension below the surface free energy of the plastic, the film tends to creep along the plastic-air surface. This phenomenon was absent from Pattle's original bubble method [13] which made it more suitable for the measurement of film stability than the more modern apparatus just described. A refinement of Pattle's method, using bubbles of 0.5-3 cm in diameter has now been developed to produce adsorption records, surface tension-area relations and leak-free film stability measurements under quasi-static and dynamic conditions [7]. The apparatus contains a leak proof bubble of controllable size in order to determine more reliable surface tension-area relationships.

We reported recently [7] tests of the new captive bubble apparatus, using solvent-spread films of pure DPPC, and adsorbed films from lipid extract of bovine pulmonary surfactant and from purified rabbit surfactant. After only one to two compressions, the rabbit surfactant films exhibited the low surface tension, collapse rates and compressibilities characteristic of the alveolar surface in situ, and approaching the behavior of spread DPPC films. The bubble 'clicking' phenomenon described earlier by Pattle was also reproduced, but only with the bovine extract, which did not perform as well as the rabbit surfactant in surface tests.

The foregoing discussion is important because surface tension and area in the lung are interrelated and operate within a narrow (physiologic) range. Morphometric data and direct measurements of surface tension in excised lungs have demonstrated that alveolar surface tension on inflation is higher than on deflation, while at 40% total lung capacity (TLC), the surface area is larger on deflation than on inflation [5]. Although hysteresis can be produced in surface balances by pulmonary surfactant films, it has not been possible to adequately quantify surface tension-area hysteresis because of film leakage. In this study we report surface tension-area relations from pulmonary surfactant with a leak-free captive bubble tensiometer using area excursions that are physiologically relevant.

Materials and Methods

The captive bubble method has been described recently [7]. Briefly, bubble size and thus the surface tension of any insoluble film at the bubble surface is altered by changing the pressure within the closed chamber (Fig. 1). The present version consists of a thermostated (37°C) 5 ml glass syringe (Unimetrics, gastight, Shorewood, IL) mounted between the stage and nosepiece holder of a microscope stand. The sample chamber is filled with an aqueous medium, either a salt solution or a surfactant suspension. After placing a bubble of atmospheric air, 2–3 mm in diameter, at the center of the agarose ceiling, the chamber pressure is reduced to 0.1–0.02 atm by withdrawing the syringe plunger with the focusing drive of the microscope stand, causing the bubble to double its original diameter to 4–6 mm. This represents the starting point for adsorption measurements and the beginning of continuous video recording [7]. During the adsorption period the chamber contents are stirred by a magnetic stirrer inside the chamber. After adsorption, the pressure in the sample chamber is increased stepwise or continuously by pushing in the shaft of the syringe plunger. As the pressure increases the bubble volume and surface area decrease, compressing the adsorbed surfactant monolayer at the air-water interface. The bubble progressively flattens, indicating a lower surface tension (Fig. 2). The surfactant film may also be formed by spreading the surfactant at the interface with a syringe, e.g. spreading a solution of phospholipid in organic lipid solvent.

From the video picture of the bubble, the bubble surface tension is determined by using the fourth degree polynomial approximation of Malcolm and Elliott [14], for sessile or captive bubbles having a 180° contact angle. To determine the bubble surface area we digi-

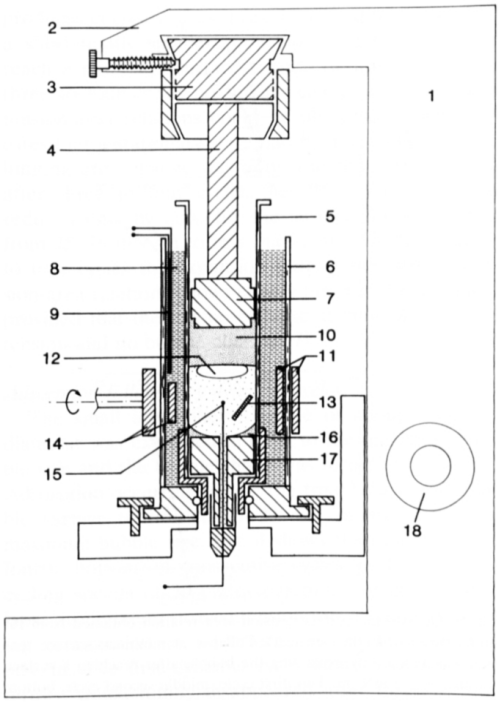

Fig. 1. The captive bubble surface tensiometer. 1, 2, Microscope stand (Nikon). 3, Chuck for clamping syringe plunger onto microscope stand. 4, Plunger. 5, Glass cylinder (gastight syringe). 6, Plastic chamber with glass windows. 7, Teflon piston. 8, Water. 9, Heating element. 10, 1% agarose gel. 11, Magnets, position adjustable. 12, Air bubble. 13, Stir bar. 14, Magnetic stir bars. 15, Temperature probe. 16, 1% agarose meniscus or stainless steel funnel to prevent sticking of air bubbles. 17, Teflon plug. 18, Focusing knob. Procedure: A bubble of atmospheric air, 2 mm in diameter is formed below a slightly concave 1% agarose ceiling. The chamber, a 5 ml syringe (Unimetrics, gastight, Shorewood, IL) is closed pressure-tight and mounted between the stage and nosepiece holder of a microscope stand. The focusing mechanism of the microscope stand is used to drive the syringe piston. The pressure in the chamber is reduced to expand the bubble to maximum size, 6–8 mm in diameter. The bubble area and therefore the surfactant film area decreases when pressure to the chamber is applied which decreases the bubble size.

tized the photographs and calculated the area with computer programs based on the formulas of Rotenberg et al. [15] which can be applied for bubbles that are not too flat at the apex [7]. These formulas can also be used with bubble contour data to calculate the surface tension, surface area, volume and contact angle of bubbles having higher surface tensions and other contact angles. Application of the Rotenberg formulas to our data resulted in calculated surface tensions identical to those obtained with the Malcolm and Elliott [14] method. The measurements of chamber pressure is not needed for these calculations. In this study we have used the approximation of Malcolm and Elliott [14] to calculate surface tension. This procedure requires only measurements of bubble height and diameter. The area was calculated by multiple regression analysis of the height and diameter measurements using a formula derived from bubble contour data and rotational areas.

Natural surfactant was extracted from the lungs of adult Fischer 344 rats (male and female) using three aliquots of lavageate for each lung adjusted to the total lung volume. The lavageate was a buffered salt solution composed of 140 mM NaCl and 10 mM Hepes at pH 6.9. The lavageates from three lungs were pooled and centrifuged at $500 \times g$ to remove cells, and then at $60000 \times g$ at 4°C for 1 h to bring down a cell free pellet. This pellet was suspended in 1.5 ml of 140 mM NaCl + 10 mM Hepes + 2.5 mM $CaCl_2$, pH 6.9. An

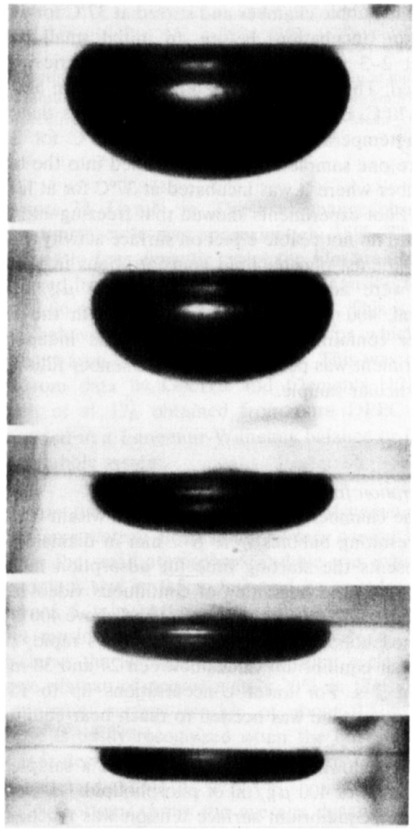

Fig. 2. Bubble shape at five different surface tensions, from top to bottom: 25, 16, 4, 2, 1 mN/m. The surfactant was rat pulmonary surfactant.

aliquot of 0.5 ml of this sample was used to determine the total phospholipid content [16]. The sample phospholipid concentration was then adjusted to 800 μg/ml by adding the above mentioned buffered salt solution containing 2.5 mM $CaCl_2$. To test the surface activity 1.5 ml of the adjusted sample was filled into the bubble chamber. A bubble of atmospheric air 2–3 mm in diameter was placed at the center of the agarose ceiling, and the temperature was raised to 37 ± 1°C within 10 min. Adsorption and a first quasi static compression was performed as described below. Surface activity was considered acceptable if (1) adsorption to 25–28 mN/m occurred within 2 s and (2) minimum surface tension upon the first quasi static compression following adsorption was at or below 5 mN/m.

We obtained a total of four samples from 12 rats. One sample was rejected because the minimum surface tension was above 5 mN/m. The remaining three samples were pooled, then divided into four aliquots. A fresh sample of 1.5 ml from one aliquot was filled into the bubble chamber and stirred at 37°C for at least 30 min (incubation) before an initial small bubble, about 2–3 mm in diameter, of atmospheric air was formed. The rest of the aliquots were frozen and kept at −70°C. Before the test, one aliquot was thawed at room temperature and then kept at 3°C for 3–4 h before one sample of 1.5 ml was filled into the bubble chamber where it was incubated at 37°C for at least 15 min. Pilot experiments showed that freezing and thawing had no noticeable effect on surface activity. For the experiments, phospholipid concentrations in the samples were adjusted to 50 μg/ml, 100 μg/ml, 200 μg/ml, 400 μg/ml and 800 μg/ml with the Hepes buffer containing 2.5 mM $CaCl_2$. One independent experiment was performed using a chamber filling from a particular sample.

Results

Adsorption followed by quasi-static cycles

The chamber pressure was reduced within 0.5 s and the resulting bubbles were 6–7 mm in diameter. This represents the starting time for adsorption measurements and the beginning of continuous video recording. For sample concentrations at and above 400 μg/ml of phospholipids, adsorption was always rapid, reaching near equilibrium values between 24 and 30 mN/m within 2 s. For lower concentrations up to 15 min adsorption time was needed to reach near equilibrium values.

The following studies were done at a sample concentration of 400 μg/ml of phospholipid.

After equilibrium surface tension was reached, the first quasi-static cycle was performed by increasing the pressure in the sample chamber stepwise and by waiting at each step until the bubble shape no longer changed noticeably within 10–20 s, indicating that the film surface tension changed less than 0.5 mN/m (the sensitivity of the method) in that period. Between 10 and 15 steps were taken for the compression part of the cycle. The compression part was frequently characterized by a region during which film compressibility was relatively large, that is, the surface tension changed little for a given bubble area change (Fig. 3). Below a tension of 15 mN/m compressibility rapidly decreased, indicating increasing film stiffness. Starting at 25 mN/m, the area reduction necessary to produce minimum surface tensions below 2 mN/m, upon this first compression, was between 30 and 80%, with an average of about 50%. After reaching a minimum surface tension below 2 mN/m which could be easily estimated from the height of these flat bubbles, the bubble was slowly expanded for 1–2 min at tensions above approximately 26 mN/m. Transient surface tension under these quasi-static conditions never exceeded 30 mN/m and decreased in approximately 2 s to 25–26 mN/m after each expansion step at tensions above 25 mN/m. After reaching the original maximum bubble size, a second cycle was started, followed by two more quasi-static cycles. Fig. 3 shows that continuous cycling

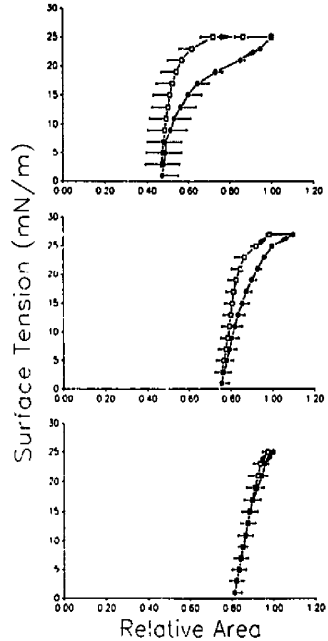

Fig. 3. Quasi-static surface tension-area relation obtained from rat pulmonary surfactant (see text). Collapse at minimum surface tension was avoided by expanding the bubble after reaching a surface tension of 1–2 mN/m. Top, first cycle, middle second cycle, bottom fourth cycle. Note: decreasing area reduction necessary to reach near zero tension upon consecutive cycling, and reduced hysteresis. Filled circles, compression. Mean ± 1 S.E., eight independent experiments.

produces decreasing compressibility and hysteresis and a substantially smaller reduction in area needed to reach a minimum surface tension of 1 mN/m After three to four cycles no further change in the surface tension-area relations could be observed, which indicates that a stationary state had been established. The limiting area change was approximately 20%, that is, after three to four cycles the film area had to be reduced only by 20% for the surface tension to fall from 25–26 mN/m to less than 1 mN/m. After three to four cycles the expansion part of the surface tension-area relation follows closely the compression part, provided that there is no collapse at minimum surface tension and no bubble clicking [7].

Adsorption followed by dynamic cycles

The small atmospheric bubble of about 2 mm in diameter was expanded in 0.5 s to the maximum bubble size and the bubble shape was recorded for 5 min. Adsorption occurring during the first 2 s lowered bubble surface tension to 25–28 mN/m after attaining maximum bubble size. Fig. 4 shows the first (top) and fourth (bottom) of consecutive cycles, performed with cycling speeds of 30 cycles per min. Again, film collapse at minimum surface tension was avoided by starting reexpansion at a surface tension between 1–2 mN/m. The first dynamic cycle shows a narrow hysteresis, which totally disappeared after three to four cycles. The transient maximum surface tension for these

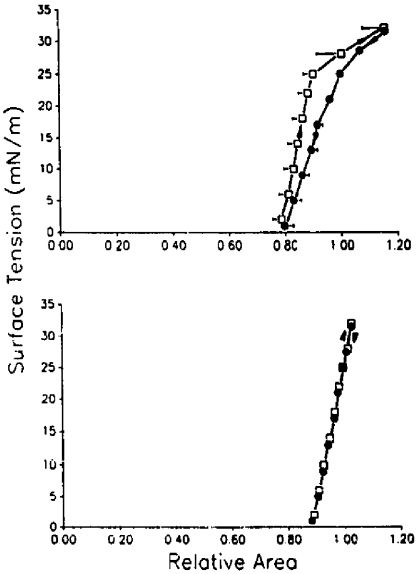

Fig. 4. First (top) and fourth (bottom) surface tension-area relations (see text) produced by dynamic cycles (30 cycles/min). Filled circles, compression. Mean ± 1 S.E., seven independent experiments.

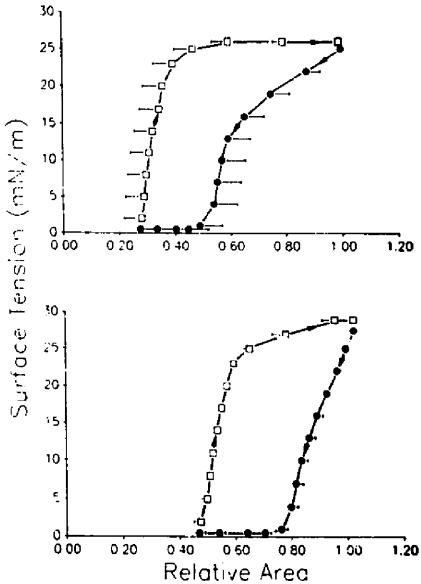

Fig. 5. Quasi-static (top) first cycle with collapse plateau at minimum surface tension and dynamic (bottom) cycles (30 cycles/min), after ten consecutive cycles. Filled circles, compression. Mean ± 1 S.E., four independent experiments.

cycles was 32–33 mN/m. The area change observed for the fourth cycle was approximately 15%. That is, after three to four dynamic cycles the film area had to be reduced by only 15% for the surface tension to fall from 25–26 mN/m to less than 1 mN/m. This is close to the behavior of pure DPPC films for which the equivalent area change is about 10%. This was calculated from data by Goerke and Clements [17] and Schürch et al. [7], obtained from pure DPPC films compressed in a Langmuir-Wilhelmy balance or in the captive bubble system.

Adsorption followed by quasi-static and dynamic cycles with collapse plateau at minimum surface tension

Again the small atmospheric bubble was expanded to maximum size in 0.5 s, followed by an adsorption period of 5 min. After 2 s adsorption the bubble surface tension was between 25 and 28 mN/m. Fig. 5 (top) shows the average quasi-static first cycle with a collapse plateau of approximately 20% in film area at the minimum surface tension of about 0.5 mN/m. Collapse is easily recognized when the bubble height no longer decreases and the bubble starts to shrink in its diameter.

Fig. 5 (bottom) shows the average dynamic (30 cycles/min) hysteresis loop obtained after approximately ten consecutive dynamic cycles. The points represent the mean ± 1 S.E. for four independent experiments.

As can be seen from the small standard errors, the loops tend to reach a stationary state with a constant collapse plateau. The area change needed for the surface tension to fall from about 30 mN/m to less than 1 mN/m was approximately 25%.

Stability at minimum surface tension

Monolayer metastability for the surfactant preparations varied little with the compression-expansion history (Fig. 6). Films which produced minimum surface tensions upon a quasi-static first compression of approximately 60% or less were quite stable at minimum surface tension. After stopping compression surface tension rose from below 1 mN/m to 2–3 mN/m over a 30 min period and the corresponding decrease in bubble area was 3% after one min and 12% after 30 min. Stability after four to ten consecutive quasi-static cycles tended to improve slightly: the surface tension rose to approximately 1.5 mN/m over a 30 min period. The related decrease in bubble area was 1% after 1 min and 10% after 30 min. After 10 min of dynamic cycling from 25 mN/m to minimum surface tension, a minimum of 0.5 mN/m was achieved. Cycling was stopped at this low value and surface tension rose to less than 1 mN/m over the subsequent 10 min period. The corresponding area change was approximately 5%.

Surfactant activity in relation to sample concentration

As stated above, for sample concentrations below 200 μg/ml of phospholipids, the time for the surface tension to reach near equilibrium (while stirring) was up to 15 min (Fig. 7). We observed a substantial effect

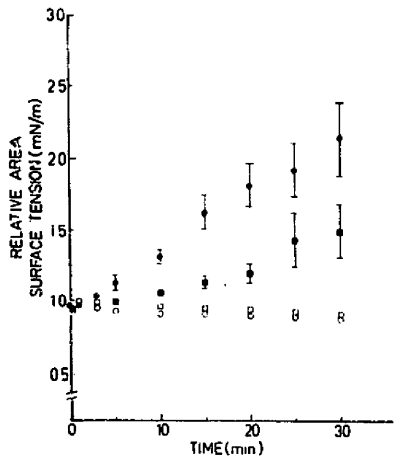

Fig. 6. Surface tension vs. time and corresponding surface area vs. time relations following the first (○) and the fourth quasi-static compression (■). Open symbols represent the corresponding area vs. time relations. Mean ± 1 S.E., $n = 4$.

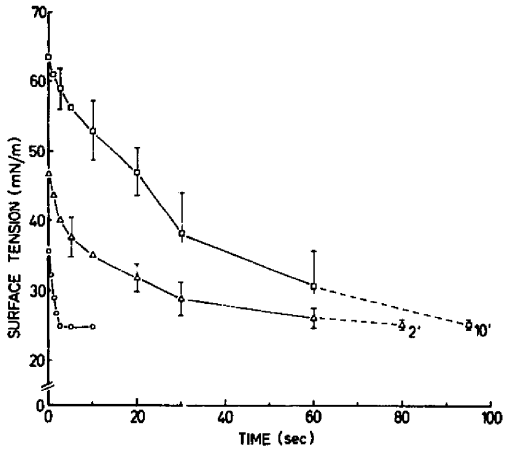

Fig. 7. Adsorption vs. time relations for sample concentrations of 400 (○), 100 (△), and 50 μg/ml (□) of phospholipid. The small atmospheric bubble (see text) was increased ten-fold in its surface area in 0.5 s to maximum bubble size. This was the starting point for the adsorption measurements. Mean ± 1 S.E., $n = 4$.

of reduced sample concentration on consecutive hysteresis loops, that is, hysteresis loops with collapse plateaus at minimum surface tension. At sample concentrations at and below about 100 μg/ml of phospholipid, the surface tension at maximum bubble area increased with cycling time, reaching maximum surface tensions of 50 mN/m after five to ten cycles. Minimum surface tension was similarly affected: frequently after only five to ten cycles the minimum surface tension was above 20 mN/m even for film area reductions of more than 80%.

Prolonged cycling following initial adsorption

Again the small atmospheric bubble of about 2 mm in diameter was expanded in approximately 0.5 s to the maximum bubble size. The phospholipid concentration was 400 μg/ml. Adsorption following expansion lowered the surface tension to 25–28 mN/m at maximum size. Following a first quasi-static cycle, the bubble was pulsed continuously at a frequency of 20 cycle/min between a surface tension of 20–23 mN/m and a minimum tension below 1 mN/m. Cycling was continued for a total of 20 min without interruption. Fig. 8 (top) demonstrates the compression part of the quasi-static first cycle (●) and compression part of the second dynamic cycle (□). Fig. 8 (bottom) shows the compression parts after 10 min (●) and after 20 min (□) continuous cycling. Surface tension-area relations of expansion followed exactly those of compression. Further, there was no change in these relations during the entire cycling time of 20 min, indicating no loss of surface active material during prolonged cycling.

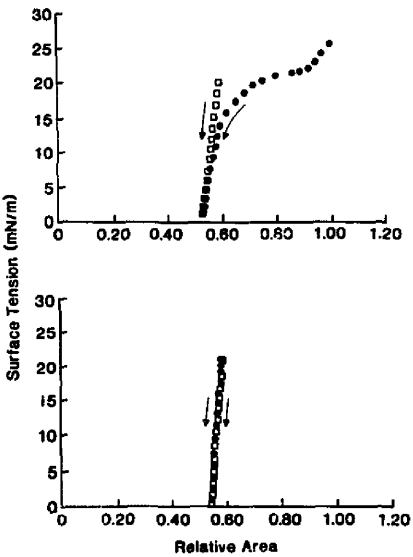

Fig. 8. Quasi-static compression followed by continuous dynamic (20 cpm) cycling between surface tensions of 23 and 1 mN/m for 20 min. Top: First quasi-static compression (•) followed by first dynamic compression (□) Bottom: Dynamic compression after 10 min (•) and 20 min (□) continuous cycling.

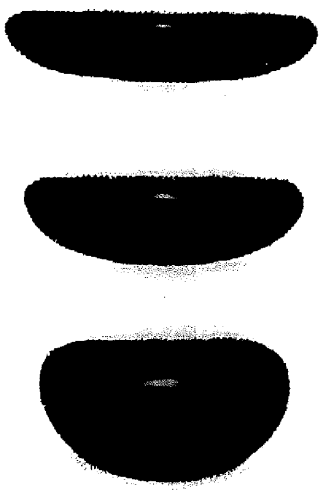

Fig. 9. Example of bubble clicks: a sudden increase in surface tension from 1.0 (top) to 1.9 (middle) to 7.9 mN/m (bottom). The corresponding areas were 0.22, 0.20, 0.18 cm^2. The clicks were caused by mechanical vibration. The increase in surface tension is accompanied by a decrease in surface area, while the bubble volume remains constant.

Interrelation between surface tensions and area

Fig. 9 demonstrates a self-regulatory mechanism to minimize the free surface energy of the captive bubble. An accidental increase in surface tension ('click') reduces the surface area by a change of shape of the bubble which in turn mitigates the initial step increase.

Discussion

It is well-known since the early work of von Neergaard [18] and Radford [19] that excised, gas-free lungs when inflated with air exhibit larger pressure-volume hysteresis than when-inflated with fluid. Similarly, large surface tension-area hysteresis loops were demonstrated by Clements in his pioneering studies of pulmonary surfactant, using the Langmuir-Wilhelmy balance [6]. Later, Bachofen, Hildebrandt, and coworkers showed that for repeated pressure-volume loops, hysteresis depends on the lung volume at which reinflation starts, becoming wider the lower the starting volumes [1]. Very little hysteresis of pressure-volume and related surface tension-area curves was demonstrated in more recent work, where alveolar surface tension was measured in situ with a microdroplet spreading method [5]. The results obtained in these later experiments conducted either in situ by pressure-volume recordings or by alveolar micropuncture compared with surface tension-area loops obtained with the Langmuir-Wilhelmy balance demonstrate the following quantitative inconsistencies: (a) the size of hysteresis or the energy dissipation per cycle is relatively large only in excised lungs that are inflated from volumes considerably lower than 40% TLC or from about zero transpulmonary pressure. In healthy, normally breathing lungs, the pressure-volume losses are relatively small [1]. On the other hand as pointed out above, surface tension-area hysteresis is quite large in Langmuir-Wilhelmy loops [20]; (b) the rate and magnitude of stress relaxation and creep are substantially greater in vitro [8,21]; (c) the temperature dependence of physical properties is slight in whole lung [22] but appreciable in the surface balance [23]; (d) in lungs, surface tension falls rapidly to near zero values after only about 25–30% decrease in surface area [5,7] compared to 50–80% compression required in the surface balance or in the pulsating bubble surfactometer. Finally, (e) re-expansion of the lining film in situ produces considerably less hysteresis than would be predicted from surface balance studies of extracts [1,23].

The factors that determine shape and area of continuous dynamic surface tension-area loops obtained in vitro, assuming no artifacts due to surface leakage are as follows:

1. Maximum surface tension depends on the balance between the rate of adsorption of surface active material and the rate of expansion of surface area. If

the rate of film formation is equal to or faster than the rate of area expansion, then the maximum surface tension for normal pulmonary surfactant preparations at total phospholipid concentrations at and above 400 μg/ml (this work) will approach the equilibrium surface tension between 24 and 28 mN/m at maximum area [17]. If film formation is slower, then surface tension at maximum area will be greater than the equilibrium tension.

2. Minimum surface tension depends on a number of factors, including surface compression rate, the extent of film compression, on film surfactant content and on film adsorption rate because for dynamic cycles the time allowed for adsorption is restricted. These last two factors depend in turn on the quality of the surfactant preparations, and on the efficiency of the enrichment process toward pure dipalmitoylphosphatidylcholine (DPPC) during film compression [26,27]. Hysteresis also depends on enrichment with DPPC. Nohara et al. [28] showed that surfactant preparations enriched with DPPC are less compressible, that is to say, these films reach near zero surface tension after less compression than do films formed from non-enriched surfactant. Consequently, hysteresis loops produced in surface balance studies show a long horizontal collapse plateau at minimum surface tension since film areas are compressed to a fixed residual area, e.g. 50% or less of the original area. If these collapse plateaus are relatively large in continuous, stationary hysteresis loops, then film formation from surfactant stores either from the collapsed phase associated with the interface or from the subphase reservoir compensates the material that leaves the film proper at minimum surface tension.

3. From the mechanisms described above it is clear that hysteresis depends not only on maximum and minimum surface tension but also on a number of interrelated factors whose influence is difficult to analyze in Langmuir-Wilhelmy studies. One of the most intractable problems has been film leakage past the restraining walls in Langmuir troughs [17]. Early investigators recognized this and used continuous Teflon ribbons in rectangular troughs [24] or rhombic frames [25]. While this procedure eliminated the leakage that occurs between the barrier and trough walls in conventional balances, leakage along the wall-liquid interfaces, that is, spreading of film material along these interfaces was not eliminated. Few studies have considered this important artifact.

The captive bubble device

For lungs held at 37°C and deflated to 40% TLC the surface tension measured in individual alveoli is close to zero and extremely stable [29]. Remarkably, a minimum compression rate is not required for the alveolar surface tension to fall to near zero values, i.e., minimum surface tensions can be obtained under quasi-static conditions. In contrast, a similar stable behavior of lung surfactant films at 37°C cannot be reproduced either with the Langmuir-Wilhelmy balance or with the pulsating bubble surfactometer [12]. Although additional and as yet unknown factors may be involved, the in situ-in vitro differences in film stability are usually explained by film leaks as mentioned above [17]. The conventional Langmuir-Wilhelmy balance has a large perimeter to surface area ratio, offering the opportunity for monolayer material to creep up or down over the surfaces of the restraining walls and barrier. Film leakage seems not to be as important for rigid, low surface tension films of materials like DPPC at room temperature. However, for the more fluid films such as those formed by native lung surface active material at 37°C, the problem of film containment during the initial compressions is nearly insoluble [17]. In another surface balance design, the pulsating bubble surfactometer [12] monolayer material can still exit the surface via inlet tubes connected to the bubbles and possibly along the inner walls of the chamber. To our knowledge low tensions are routinely attainable with this latter apparatus only when high cycling rates (20 cycles/min) are used [30]. Thus, for quasi-static measures of the lowest surface tension attainable, for studies of monolayer stability and of mechanisms involved in creating film hysteresis, we feel the captive bubble approach has advantages compared to previously published methods. Near zero surface tensions can be obtained upon the first quasi-static or dynamic compression following adsorption. However, the calculations take more time, and larger samples (about 1 ml) compared to the pulsating bubble surfactometer (20 μl) are needed. This last disadvantage is partially compensated for, because lower surfactant concentrations, about 400 μg/ml of phospholipid, give reproducible hysteresis loops.

The captive bubble method should be suitable for studies on the role of surfactant proteins in surfactant film formation because the reduction in film area necessary to produce near zero surface tension following adsorption can be obtained. This information in combination with film compressibility data might help to clarify the function of surfactant proteins not only in adsorption but also in film purification or surface sorting.

Metastability of lung surfactant monolayers

Monolayers of lung surface active material and of some saturated phospholipids can reach surface tensions below 25 mN/m when compressed. This is a metastable state, however, and with time the surface tension of such monolayers held at constant area tends to rise back up to this equilibrium value [17]. Nevertheless, normal respiration seems to take place in this

region of low surface tension, so the rapidity with which this rise occurs is an important criterion for adequate surface activity. The rapid surface leaks present in more conventional surface balances at these low surface tensions at 37°C compete with slower collapse processes inherent in all these films, rendering the measurement of the latter impossible in such systems.

In the captive bubble system, however, films of lung surface active material have proven more stable in that they relax far more slowly to the limiting value of 25 mN/m [7]. We are given further confidence that this rise of surface tension is measuring a natural process by the fact that it occurs with a time course similar to that seen in excised lungs at 37°C held at constant volume [29] and in other related lung measurements [31,32].

Since surface tension and area (A) are interrelated for the captive bubble, we have calculated the change in surface tension due to the change in area with time. From Fig. 3 bottom, we obtained the average film compressibility for the fourth cycle, at a surface tension of 5 mN/m:

$$\frac{1}{A}\frac{dA}{d\gamma} = 0.06\ \mathrm{m(mN)^{-1}}$$

Using this value and the surface area changes after 1 and 30 min, we obtained surface tension changes of approximately 0.15 and 2.0 mN/m, respectively. Therefore, assuming constant surface area, the surface tension would have risen to 3.5 mN/m in a 30 min period not to 1.5 mN/m as measured with the captive bubble. However, this estimate depends strongly on the value obtained for the film compressibility. From the standard errors of the mean, it is obvious that film compressibility could easily approach the value of pure DPPC films, 0.005 m $(\mathrm{mN})^{-1}$. Using this value, the surface tension increase at constant area would be approximately 23 mN/m in 30 min, 2 mN/m in 1 min. Thus, in 30 min the surface tension would have risen to a value close to 25 mN/m, the equilibrium surface tension.

Clements [26] suggested that the extraordinary in situ stability of the alveolar lining layer at small surface areas (i.e. at functional residual capacity) was due to a squeezing out of components other than DPPC from the alveolar film during the preceding lung deflation. This is in line with the demonstration of the behavior of mixed films [33]. Others have added DPPC to lung lipid extracts to improve the ability of films of these extracts to reach low minimum surface tensions [28]. Experiments with a pulsating bubble surfactometer showed a decrease in surface tension at minimum radius when bubbles created in suspensions of surface active materials were pulsated for a period of 2 min or more [30]. Therefore, an enrichment with DPPC of the surface active material in the surface monolayer by breathing movements might play a role in vivo. Our results support the observations of Possmayer et al. [30]. Repeated cycling of surfactant films probably enriches these films in DPPC, enhancing their stability at low surface tensions. After only three to four cycles, the film area reduction needed to decrease surface tension from 25–30 mN/m to less than 1 mN/m, is approximately 15%, a value very close to that obtained from pure DPPC films, which require about 10% film reduction in film area for the above surface tension range.

The smaller area change observed for dynamic cycles (15%) compared to that of quasi static cycles (20%) for the surface tension change from 25 to less than 1 mN/m is probably because there was less squeeze-out, and a slightly higher transient surface tension at maximum bubble area which might promote a more efficient surfactant adsorption for such cycles. Thus the low monolayer compressibility, characteristic of low tension DPPC films [17], is reflected in the decreasing percent area reduction necessary to produce low surface tension films after repeated monolayer cycling (Fig. 3). This lung surfactant monolayer property, demonstrated with the captive bubble apparatus, agrees well with other measurements made in lungs [5,34].

Repeated cycling and hysteresis

Following the first quasi static cycles, the bubble was pulsed continuously at 20 cycles/min for a total of 20 min without interruption between values below 25 mN/m (20–23 mN/m) and 1–2 mN/m. After four consecutive cycles, the compression part of these cycles exactly followed the expansion part. Further, there was no change in these relations during the entire cycling time of 20 min, indicating no net loss of surface active material during prolonged cycling. After repeated cycling between surface tensions of 25–30 mN/m and collapse at minimum surface tension (collapse plateau) our results indicate that film material is displaced from the film and is no longer available for film formation upon bubble expansion. This is particularly evident for relatively low sample concentrations, below approximately 100 μg/ml of phospholipids. At higher concentrations, surface tension-area hysteresis loops that include collapse plateaus at minimum surface tensions approach a stationary shape after five to ten dynamic (30 cycles/min) cycles. Therefore, for relatively high sample concentrations, the subphase reservoir may contain sufficient phospholipid-protein complexes for efficient respreading and new film formation, or the collapsed material may enter the film again in the presence of sufficient surfactant associated proteins. The exact mechanisms for these findings need to be

clarified in future studies. However, for relatively low surfactant concentrations, respreading of collapsed material apparently does not occur. This observation might be relevant for replacement therapies with artificial surfactants in cases of the respiratory distress syndrome of the newborn (RDS). Maintaining a certain level of positive end expiratory pressure might be beneficial from the point of view of film stability, because film collapse at minimum surface tension would be avoided as surface tensions might stay above the minimum at end expiration. If collapse is avoided, our experiments have shown that surfactant films can be cycled between 1–2 and 22 mN/m for 20 min without any loss of surface activity even at relatively low sample concentrations.

Interrelation between surface tension and area

This interrelation, illustrated by bubble clicking, may also play a role in stabilizing the peripheral air spaces of the lung. There is ample evidence that in the lung increases in surface tension result in a decrease of free alveolar surface area by a subtle interplay between tissue and surface forces in order to minimize the total free energy of the system [35,36]. Hence, a local disturbance of surface tension will not destabilize the respiratory surface, but by the ensuing change in shape and surface area of the microstructures the increase in surface tension will be toned down and a new stable equilibrium re-established.

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