Changes in Sedimentation of Surfactant in Ventilated Excised Rat Lungs

PHYSICAL ALTERATIONS IN SURFACTANT ASSOCIATED WITH THE DEVELOPMENT AND REVERSAL OF ATELECTASIS

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ABSTRACT We ventilated excised rat lungs at a constant tidal volume (CTV); they developed areas of atelectasis which could be reversed by a large inflation (CTV + I) or prevented by the addition of positive end-expiratory pressure to the CTV. To explore the possibility that these modes of ventilation led to changes in surfactant, we lavaged the lungs and centrifuged the returns at 500 *g*; we measured the amount of disaturated phosphatidylcholine (DSPC) in the resultant pellet and supernatant fluid as a marker for surfactant. We found 16.9±1.5 (mean±SE), 38.0±2.4, 18.3±1.6, and 21.7±2.3% of the total lavage DSPC, in the pellet from freshly excised, CTV, CTV + I, and positive end-expiratory pressure to the CTV lungs, respectively. The total amount of lavage DSPC was the same in all groups.

The ultrastructure of acellular material pelleted by sequential centrifugation of lavage returns at 500, 1,000, and 60,000 *g* was examined. We found mostly tubular myelin in the 500-*g* and 1,000-*g* pellets, but no tubular myelin in the 60,000-*g* pellet.

Air inflation pressure-volume measurements from the degassed state revealed that the opening pressure and recoil pressures up to 75% of total lung capacity were significantly higher in the CTV than in the CTV + I lungs. There were no differences between these groups in air deflation or in saline inflation and deflation pressure-volume measurements. Our findings suggest that CTV leads to increases in the tubular myelin form of surfactant and that this leads to increased surface tension in alveoli which results in alveolar collapse.

INTRODUCTION

Ventilation in the resting tidal volume range or below, without an occasional deep breath, leads, in both animals and man, to a fall in pulmonary compliance, to the development of areas of atelectasis, and hypoxemia (1–4). These changes may be almost completely reversed by a deep inflation. It is thought that the fall in compliance and the atelectasis are a result of an increase in alveolar surface tension (5) and that the hypoxemia is caused by continued perfusion of collapsed alveolar areas (4).

Our present study was stimulated by the observation that the 500-*g* pellet produced by centrifuging lavage returns from lungs ventilated at low volumes contained a greater percentage of the total lavage phospholipids than did the pellet of lavage returns from lungs ventilated at high volumes. This suggested to us that the mode of ventilation might lead to a physical alteration in pulmonary surfactant. We therefore examined the effect of different modes of ventilation on (*a*) the sedimentation behavior in a centrifugal field, of disaturated phosphatidylcholine (DSPC),[1] as a marker for pulmonary surfactant, (*b*) the recoil of the lung due to surface and tissue forces, and (*c*) the development of atelectasis. Finally, we observed the ultrastructural appearance of the material sedimented by centrifuging

This work was presented in part at the Annual Meeting of the American Federation of Clinical Research, 29 April–1 May 1978, San Francisco, Calif.

Received for publication 9 October 1978 and in revised form 2 April 1979.

[1] *Abbreviations used in this paper*: CTV(+I), constant tidal volume ventilation (plus a large terminal inflation to 40 cm H_2O transpulmonary pressure); DSPC, disaturated phosphatidylcholine; PEEP, positive end-expiratory pressure; P-V, pressure-volume; TLC, total lung capacity.

J. Clin. Invest. © *The American Society for Clinical Investigation, Inc.* · *0021-9738/79/08/0600/09* $1.00
Volume 64 August 1979 600–608

lung lavage returns sequentially at 500, 1,000, and 60,000 *g*.

METHODS

Preparation of excised rat lungs. Specific pathogen-free male hooded Long Evans rats, weighing 200–250 g (Charles River Breeding Laboratories, Wilmington, Mass.), were killed by exsanguination after 50 mg/kg body wt Nembutal anesthesia (Abbott Laboratories, North Chicago, Ill.) i.p. The lungs and trachea up to the second tracheal ring were excised, cleaned of extraneous tissue, and then weighed. Lungs were discarded if there was grossly visible hemorrhage of consolidation, or if the lung weight to body weight ratio deviated by >2 SD from a mean value of 0.00523±0.00046 obtained in our laboratory from measurements on 50 normal-appearing lungs.

Ventilation of excised lungs. The lungs were placed in a humidified chamber kept at 37°C and ventilated with a Harvard constant-volume respirator (Harvard Apparatus Co., Inc., Millis, Mass.) delivering humidified air at 37°C. We used the nomogram of Kleinman and Radford (6) to determine the tidal volume. The lungs were divided into three different groups. One group (constant tidal volume [CTV]) was ventilated at the nomogram-derived tidal volume 80 times/min for 2 h. Another group (CTV + I) was similarly ventilated but at the end of 2 h the lungs were slowly inflated once to a transpulmonary pressure of 40 cm H_2O; at that point the entire lung appeared reexpanded. The P_{tp} was then lowered to zero. The third group of lungs was ventilated as those in the CTV group except that positive end expiratory pressure (PEEP) of 10 cm H_2O was used throughout the 2 h.

Endobronchial lavage. Lungs were lavaged five times through the trachea using 5 ml of ice-cold 0.15 M NaCl/g of initial lung weight each time. The returns, collected by gravity, were pooled. Bloody or blood-tinged returns were discarded.

Sedimentation of surfactant. The pooled lavage returns from each lung were centrifuged at 500 *g* for 20 min at 4°C. Tubes with gross evidence of blood in the pellet were discarded. The supernatant fluid was separated from the pellet by gently pouring it off. The tube was washed gently with a few milliliters of saline and the washings were added to the supernatant fluid. DSPC was used as a marker for surfactant and the amount of DSPC in the pellet was expressed as a percentage of the total amount of DSPC in the lavage returns.

Measurement of phospholipid and DSPC. Lipids were extracted by the method of Bligh and Dyer (7). An internal standard of [^{14}C]dipalmitoyl lecithin was added before extraction to enable correction for losses in this and subsequent steps. The lipid extracts were dried under nitrogen and brought to a known volume in chloroform-methanol (2:1 vol/vol). A sample was taken for measurement of phosphorus (8) and thus the phospholipid content was estimated. Another sample was taken and DSPC was isolated using the osmium tetroxide method (9) and measured by phosphorus determination.

Cell content. We used a Neubauer chamber to count the number of cells in the pellets obtained by centrifuging lavage returns from both CTV and CTV + I lungs.

Determination of isopycnic density. The isopycnic density of material in the lavage returns was determined by centrifuging the returns to sedimentation equilibrium in a continuous density gradient of sodium bromide. The gradient was formed from two solutions; one of density 1.174 g/ml, obtained by adding the required salts (10) to the lavage returns, and the other of density 1.008 g/ml. The resulting gradient was linear and ranged in density from 1.05 to 1.12 g/ml. The gradients were centrifuged at 60,000 *g* for 17 h with a Beckman SW 25.1 rotor in a Beckman L-2 preparative ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 4°C. A visible band of material was observed and 1 ml was aspirated from the center of the band and the density measured using calibrated specific gravity bottles.

Light microscopy and volume changes. After ventilation, the presence or absence of grossly visible liver-like areas in

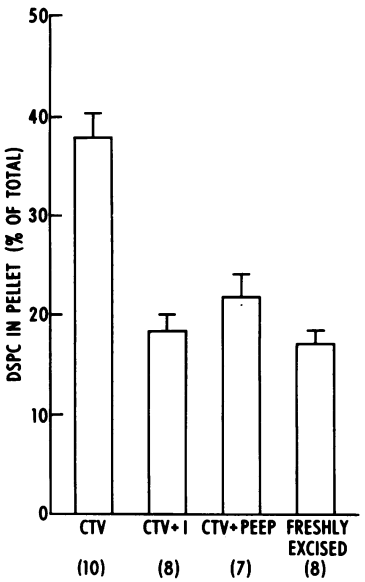


FIGURE 1 Effect of different modes of ventilation on the sedimentation of surfactant. Lavage returns from freshly excised rat lungs or lungs ventilated in vitro for 2 h at 37°C were centrifuged at 500 *g* for 20 min at 4°C. The amount of DSPC in the pellet was expressed as a percentage of the total DSPC in the lavage. The bars denote mean±SE. The figure in parentheses indicate the number of experiments. (CTV group vs. each of other groups $P < 0.001$.)

TABLE I
Effect of Mode of Ventilation on Volume and Phospholipid Content of Lavage Returns

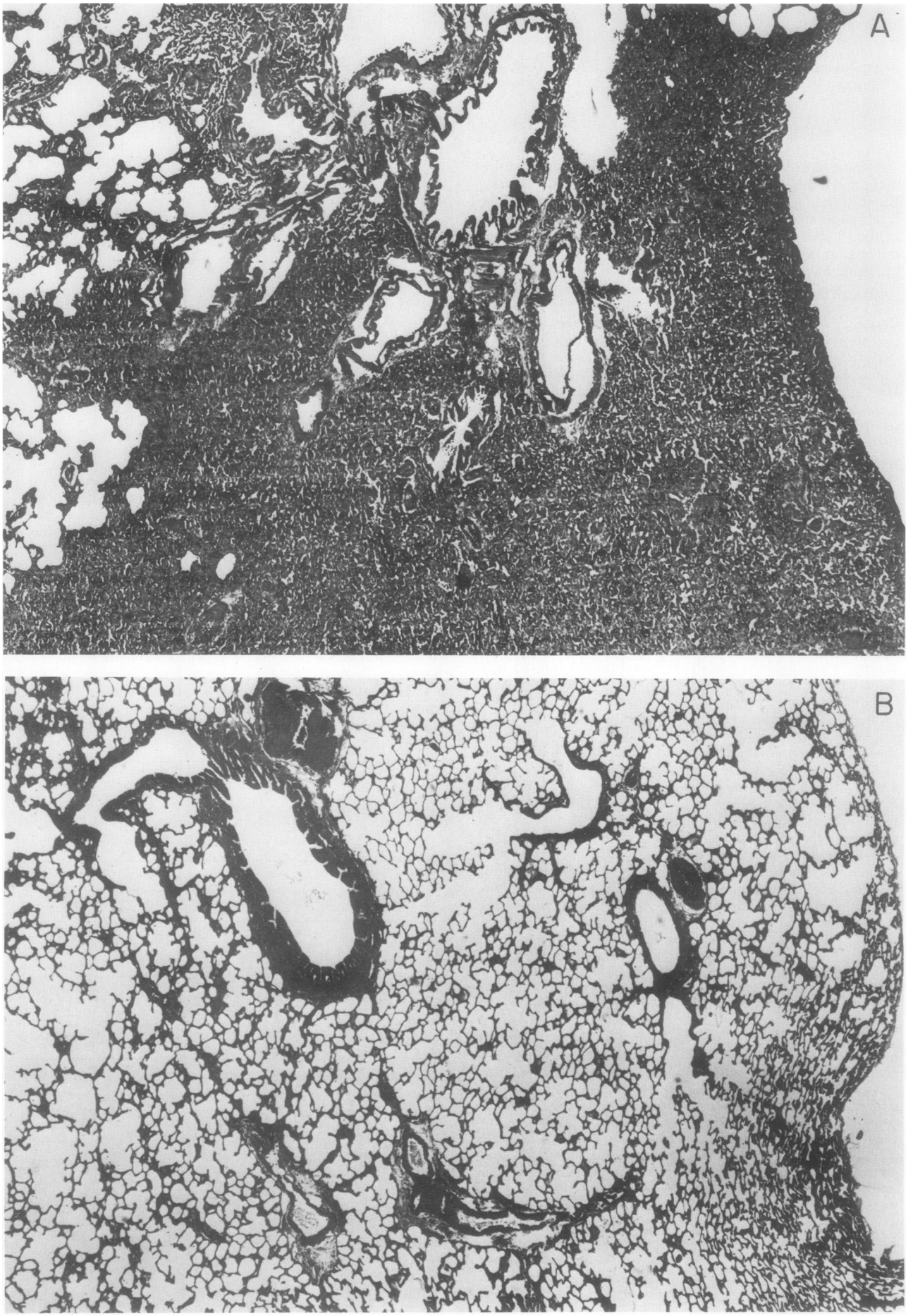
Ventilation	Lavage returns		
	Volume	DSPC*	DSPC/phospholipid‡
	%	*μg/g lung*	%
CTV (10)	85.2±1.0	946±64	45.3±1.1
CTV + I (8)	86.6±0.9	980±64	47.1±1.7
CTV + PEEP (7)	84.9±1.0	985±53	46.7±2.5
Freshly excised lungs (8)	89.9±1.5§	886±44	47.3±0.5

Lungs were lavaged either immediately after sacrifice (freshly excised) or after 2 h of in-vitro ventilation at 37°C. Mean±SE are given. The figures in parentheses indicate the number of animals.

* No significant difference between the groups.

‡ No significant difference between the groups.

§ $P < 0.025$ compared to CTV and CTV + PEEP groups. Other groups not significantly different from each other.


A
B

the lungs was noted. Tissue blocks were taken from the liver-like areas of a few CTV lungs and from anatomically corresponding areas of a few CTV + I lungs, and paraffin sections stained with hematoxylin and eosin were prepared for light microscopy. The volume of other lungs was measured by water displacement (11) before and after they were degassed in a vacuum jar.

Pressure-volume (P-V) measurements. Air P-V measurements were made using previously described equipment (12) in a system that, without the lung, had a compliance of 0.003 ml/cm H_2O. Lungs were degassed and inflation was begun. Initially, 0.5-ml volumes of air were introduced to a total of 3 ml, and then 1.0-ml volumes were introduced until a pressure of or slightly exceeding 35 cm H_2O was reached. The pressure was recorded 2 min after each addition of air. The volumes were expressed as a percentage of the total volume present at 35 cm H_2O, which we designated total lung capacity (TLC), and a P-V curve was plotted for each lung. The recoil pressures at specified volumes were read off the curve and these values then grouped for CTV and CTV + I lungs. Air deflation P-V measurements were made as previously described (12).

Saline P-V measurements were made as described by Mead et al. (13); the volume of saline in the lung at 10 cm H_2O was taken as TLC. When saline was used to distend the degassed rat lungs, we noted air bubbles in the saline withdrawn during the subsequent deflation maneuver. We also noted relatively high inflation pressures and a large degree of hysteresis in the P-V diagram (not shown). These findings suggested an air-liquid interface was still present. During the second inflation-deflation cycle however, no air bubbles were seen and the hysteresis was minimal; we thus used the P-V values from this cycle.

These difficulties with the rat and our need to compare the initial curves of CTV and CTV + I lungs, led us to perform studies with rabbits, expecting the larger size of the airways would allow more complete removal of air before initial inflation with saline. Excised rabbit lungs were ventilated at 40 times/min in the CTV or CTV + I modes. This resulted in gross changes similar to those in the rat lungs ventilated at CTV and, as in the rats, these changes were reversed by a terminal inflation (CTV + I). We did not detect any evidence of retained air during the inflation-deflation maneuvers and hence we used the values obtained in the first cycle.

Ultrastructural studies. Pellets formed by centrifuging the lung lavage returns were fixed and prepared for electron microscope analysis as previously described in detail for lung tissue (14).

Statistical analysis. For all parameters measured the values for individual animals were averaged per experimental group and the standard error of the group mean was calculated. The significance of differences between groups was calculated by an unpaired t test anaylsis (15).

RESULTS

Mode of ventilation and sedimentation of phospholipids. Compared to the freshly excised lung, ventilation in the CTV mode for 2 h more than doubled the percentage of lavage DSPC which sedimented at 500 g (Fig. 1). A single inflation (CTV + I) returned the percent of DSPC pelleted to the level found in freshly excised lungs, whereas the addition of PEEP to the CTV mode of ventilation almost completely prevented the rise in the percent of DSPC pelleted. Since these modes of ventilation did not change the total amount of DSPC in the lavage returns (Table I), there is both a relative and an absolute increase in the amount of DSPC pelleted at 500 g. These modes of ventilation did not alter the volume of lavage returns or the ratio of DSPC to total phospholipids; however, slightly more saline was recovered from freshly excised than from ventilated lungs (Table I). There were no differences in the number of cells per gram of lung pelleted at 500 g between the CTV lungs ($3.99 \pm 0.19 \times 10^6$, mean±SE) and the CTV + I lungs ($4.32 \pm 0.08 \times 10^6$, $P > 0.1$).

Isopycnic density. The isopycnic density was 1.0790±0.0019 g/ml (mean±SE, $n = 8$) and 1.0816 ±0.0027 g/ml ($n = 7, P > 0.4$) for the band in the lavage returns from CTV and CTV + I lungs, respectively.

Effect of the mode of ventilation on lung volume, atelectasis, and P-V relations. Lungs ventilated at CTV for 2 h contained significantly less air than lungs ventilated for 2 h in the CTV + I mode. The predegassing lung volumes (milliliters per gram lung) were 1.34±0.04 ($n = 5$) and 1.95±0.11 ($n = 5, P < 0.001$) for CTV and CTV + I lungs, respectively; postdegassing volumes were 1.04±0.04 and 1.08±0.08, $P > 0.5$ for the same groups of lungs.

All lungs ventilated in the CTV mode for 2 h developed areas which were liver-like in gross appearance. Microscopically, these areas had extensive alveolar atelectasis (Fig. 2A). A single inflation to a transpulmonary pressure of 40 cm H_2O reversed the gross and also the microscopic evidence of atelectasis (Fig. 2B). CTV + PEEP lungs did not develop areas of macroscopic atelectasis.

The pressure required to inflate the degassed lung to 5% of TLC was significantly greater in the CTV lungs than in the CTV + I lungs (Fig. 3). The recoil pressures at all other lung volumes up to 75% of TLC were also significantly greater in the CTV lungs. There were no statistically significant differences in elastic recoil noted during deflation in air-filled lungs (not shown) or during saline inflation and deflation of the rat lung (not shown). The P-V curves obtained by saline inflation of rabbit lungs from a degassed state were also not different between the two groups (Fig. 4).

Time-course of changes in sedimentation of DSPC and development of atelectasis. Within 15 min after the onset of CTV the percentage of DSPC sedimenting

FIGURE 2 (A) Photomicrograph of section from liver-like areas of CTV rat lung (×25). Note the extensive atelectasis. (B) Photomicrograph of section from CTV + I rat lung (×25). Note that the atelectasis has been reversed.

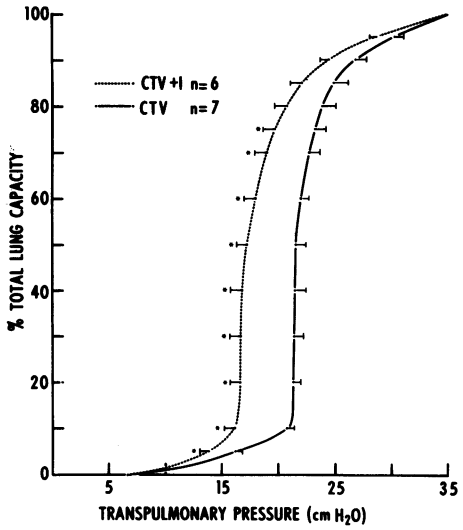


FIGURE 3 Air inflation P-V curves for CTV and CTV + I lungs. The lungs were degassed and inflated in 0.5-ml increments up to a total of 3 ml, and then in 1.0-ml increments until a transpulmonary pressure of 35 cm H_2O was exceeded. The pressure at each volume was noted after 2 min. Curves were plotted for individual lungs and the pressure values at predetermined volumes were noted and then grouped. The values shown are mean±SE. (*) Statistically significant differences, $P < 0.05$ at 5 and 75% of TLC, $P < 0.025$ at 60 and 70% of TLC, $P < 0.01$ at 50% of TLC, $P < 0.005$ at 10, 20, 30, and 40% of TLC.

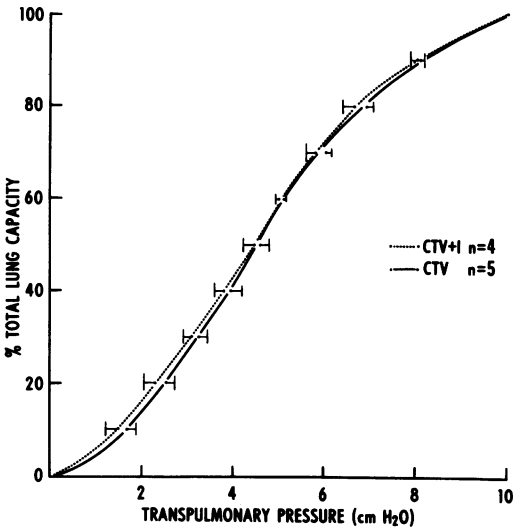


FIGURE 4 Saline inflation curves for CTV and CTV + I rabbit lungs. The curves shown represent the first inflation from the degassed state. The pressures were noted 1 min after each increment of saline was added, individual curves were plotted and the pressures at predetermined volumes were determined and grouped. The values shown are mean±SE. The two curves are not significantly different.

at 500 *g* increased ≅1.5 times (Table II). Thereafter, the rate of increase was less but at 1 h the percent DSPC sedimented was significantly higher than at 15 min. This increase in sedimentation preceded grossly visible atelectasis (Table II).

Ultrastructure of sedimented materials. The pellet formed by centrifuging lavage returns from CTV lungs at 500 *g* for 20 min contained macrophages and acellular material (Fig. 5A). The latter appeared mainly as tubular myelin (Fig. 5B) which is a well-recognized component of the acellular lining layer of alveoli (16, 17). The pellet formed when the supernatant fluid from the 500-*g* centrifugation was centrifuged at 1,000-*g* for 20 min was similar in appearance (not shown) to the 500-*g* pellet. When the 1,000-*g* supernatant material was centrifuged at 60,000 *g* for 60 min the pellet formed contained amorphous particles and was free of cells and of tubular myelin (Fig. 5C). The ultrastructure of the pellets obtained from CTV + I lungs were similar in appearance to corresponding pellets from the CTV +I lungs.

DISCUSSION

There are several possible explanations for the increased sedimentation of DSPC in the CTV lungs. Since pulmonary macrophages contain DSPC (18), increased numbers of macrophages on the alveolar surface might explain the differences; however, the number of macrophages was the same in the 500-*g* pellets of lavages from CTV and CTV + I lungs. Also, the ratio of DSPC to total phospholipid was not lower in the lavage of CTV lungs as might be expected if there were increased numbers of macrophages. It is possible that the mode of ventilation affected phagocytosis of surfactant by macrophages and that phagocytosed surfactant was responsible for the increased amount of DSPC in the 500-*g* pellet. We think this is unlikely

TABLE II

Time-Course of Development of Macroscopic Atelectasis and Changes in Sedimentation of DSPC

Ventilation	Lungs with areas of atelectasis	Pellet DSPC/total DSPC
min	%	
0 (8)	0	16.9±1.5
15 (7)	0	28.6±1.8*
30 (7)	57	31.2±2.6
60 (8)	100	35.6±1.6‡
120 (10)	100	38.0±2.4

Excised lungs were ventilated for the times indicated at constant nomogram tidal volume at 37°C. Mean±SE are given where applicable. The figures in parentheses indicate the number of animals.

* $P < 0.001$ vs. 0-min group.

‡ $P < 0.025$ vs. 15-min group.

because it would require that the phagocytosed surfactant be immediately released from the cells in response to a single large inflation. The presence of atelectasis in the CTV lungs raised the possibility that lavage fluid was distributed differently in these lungs than in the CTV + I lungs leading to sampling differences. We do not think this occurred because all areas of the lungs appeared completely inflated with the first of five lavages.

We also considered the possibility that the mode of ventilation increased the amount of surfactant present in a physical state which sedimented at 500 *g*. In this regard, if one considers factors governing particle sedimentation in a centrifugal field (19) it becomes apparent that the volume and the density of the sedimenting particle determine the velocity of the sedimentation if the density of the medium and centrifugal force remain constant. The similarity in isopycnic density between the bands in lavage material from CTV and CTV + I lungs suggests that differences in density do not explain the differences in the amount (absolute and relative) of DSPC pelleted.

These considerations led us to an ultrastructural examination of the pelleted material. It showed that the acellular component of the 500-*g* pellet was mainly tubular myelin. This is of particular interest because tubular myelin seems to be a constituent of the in vivo alveolar lining layer as evidenced by its presence in lungs fixed by vascular perfusion (17) and in lungs examined by freeze-etching methods (16). It is rich in phospholipids and, although it is surface active (20) its spreading and surface tension lowering properties are impaired compared to surfactant in the endobronchial lavage fluid (21). The 60,000-*g* pellet by contrast contains smaller aggregates similar in appearance to other components of the hypophase of the duplex alveolar lining layer.

Based on our studies on the behavior of DSPC in a centrifugal field and on the ultrastructural appearance of the acellular pelleted material we suggest that the CTV mode of ventilation results in the build-up of tubular myelin in the lining layer and that this is reflected in the increased pelleting of DSPC. To understand how this may occur we need to consider the behavior of partially soluble molecules at an air-liquid interface. This area has been investigated and clearly discussed by Clements and Tierney and their colleagues (5, 22, 23). In essence, a fall in alveolar surface area during expiration leads to compression of molecules in the surface lining film which is thought to cause a loss of surfactant molecules from the film. Ries and Kimball (24) have provided information on the anatomical form one might expect surfactant to take when squeezed from the lining film during compression. Their electron micrographs of fatty-acid monolayers brought to a collapsed state by severe compression led them to suggest that during collapse the monolayer folds over to form layers two molecules thick. This is particularly pertinent to our morphological findings because Weibel et al. (25) found the lamellae of tubular myelin to have a 42-Å periodicity. This is consistent with lamellae of figures formed by repeating bimolecular leaflets of phospholipids in water as viewed in the electron microscope (26).

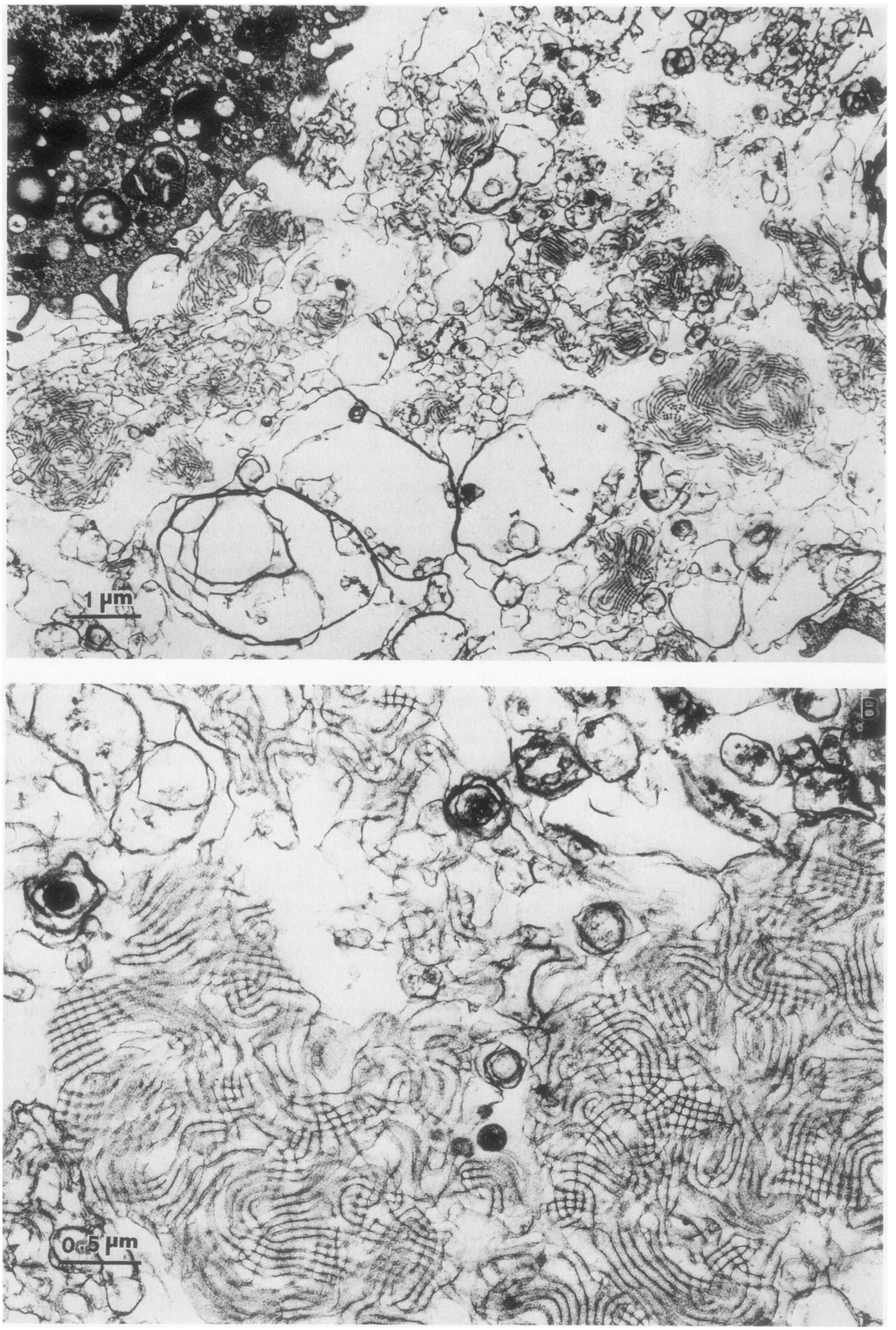
We interpret the higher opening pressures and the higher recoil pressures in air inflated CTV lungs compared to the air inflated CTV + I lungs, in conjunction with the absence of differences between these groups during saline inflation, to indicate a higher surface tension in the CTV lungs. This view is consistent with current concepts of lung elastic recoil including the proposed relationship between surface tension and opening pressure (5, 13, 27, 28). After inflation to TLC the deflation P-V relations are the same in the two groups; this indicates that the recoil pressures due to surface forces have returned to control values in the CTV lungs after inflation. Further evidence of an increase in surface tension in the CTV lungs is the development of gross and microscopic atelectasis with the resultant measured volume loss in these lungs.

We thus conclude that CTV ventilation leads to increases in sedimentation of surfactant without change in density, increases in the tubular myelin form of surfactant, increased surface tension in the alveoli and atelectasis. All are reversed with a single large inflation. Our experiments do not allow us to directly link the presence of increased amounts of DSPC as tubular myelin with the development of alveolar collapse. However, evidence exists indicating that the aggregation of surfactant into tubular myelin results in the formation of surfactant with diminished spreading and surface tension lowering ability (21). This could lead to increased alveolar surface tension and alveolar collapse.

The precise manner in which a deep breath might lead to disaggregation of tubular myelin is unclear. It may occur because, as the alveolar surface area increases, the lining film becomes thinner and the tubular myelin is brought to the air surface where conditions favor spreading (29, 30). These events would result in a return of lung recoil and alveolar stability toward normal, in a reversal of atelectasis and in a decreased sedimentation of surfactant. The mechanism of action of PEEP may be to increase end-expiratory alveolar surface area, decreasing the surface film compression and thereby decreasing formation of tubular myelin.

ACKNOWLEDGMENTS

This work was supported in part by Pulmonary Academic Award HL00374; National Institutes of Health grants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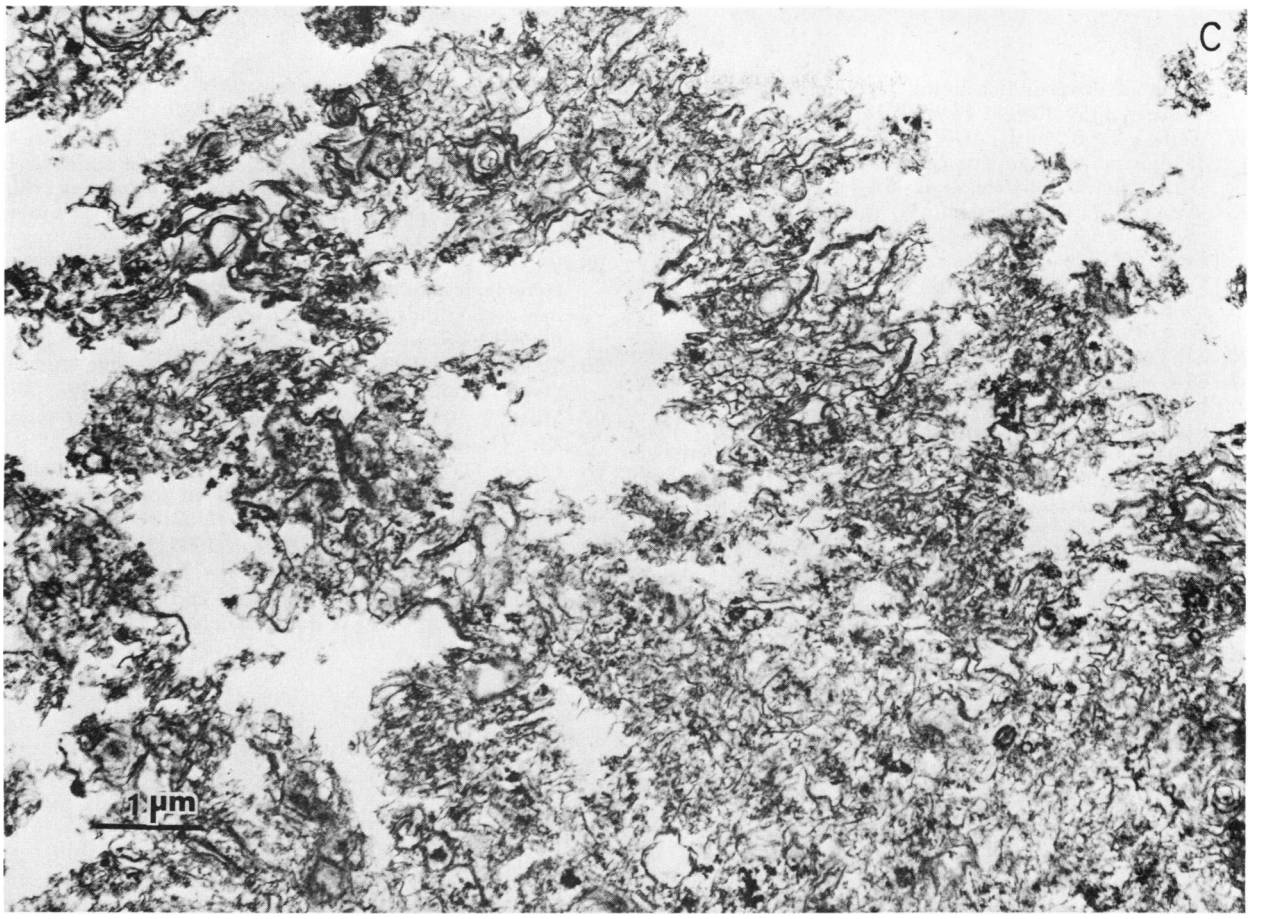

FIGURE 5 (A) Electron micrograph of pellets. 500 *g* pellet at ×13,500 showing pulmonary alveolar macrophage and tubular myelin. (B) 500 *g* pellet at ×30,000 showing tubular myelin. (C) 60,000 *g* pellet (from supernatant fluid obtained after 1,000 *g* centrifugation) at ×13,500 showing amorphous material free of tubular myelin.

HL20366, HL22333, and HL07283; Veterans Administration Research funds; the Veterans Administration Research Electron Microscopy Laboratory; and the American Lung Association.

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