On defining total lung capacity in the mouse

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Submitted 9 October 2003; accepted in final form 18 December 2003

Soutiere, Shawn E., and Wayne Mitzner. On defining total lung capacity in the mouse. J Appl Physiol 96: 1658–1664, 2004; 10.1152/japplphysiol.01098.2003.—Maximal lung volume or total lung capacity in experimental animals is dependent on the pressure to which the lungs are inflated. Although 25–30 cmH2O are nominally used for such inflations, mouse pressure-volume (P-V) curves show little flattening on inflation to those pressures. In the present study, we examined P-V relations and mean alveolar chord length in three strains (C3H/HeJ, A/J, and C57BL/6J) at multiple inflation pressures. Mice were anesthetized, and their lungs were degassed in vivo by absorption of 100% O2. P-V curves were then recorded in situ with increasing peak inflation pressure in 10-cmH2O increments up to 90 cmH2O. Lungs were quickly frozen at selected pressures for morphometric analysis. The inflation limbs never showed the appearance of a plateau, with lung volume increasing 40–60% as inflation pressure was increased from 30 to 60 cmH2O. In contrast, parallel flat deflation limbs were always observed, regardless of the inflation pressure, indicating that the presence of a flat deflation curve cannot be used to justify measurement of total lung capacity in mice. Alveolar size increased monotonically with increasing pressure in all strains, and there was no evidence of irreversible lung damage from these inflations to high pressures. These results suggest that the mouse lung never reaches a maximal volume, even up to nonphysiological pressures >80 cmH2O.

THE CHARACTERISTIC FORM of the respiratory system quasi-static pressure-volume (P-V) relation is consistent across humans and most laboratory species (1, 18, 25). Bachofen and collaborators (1) studied three species, the cat, monkey, and dog, and noted no remarkable interspecies differences in P-V characteristics in these species. The P-V curves are normally characterized by a sigmoidal inflation limb, which is presumed to be limited by an elastic limit that occurs at high distension of lung tissue (11, 13, 20, 26). The deflation limb also shows a sigmoid shape, being relatively flat during the initial deflation from high pressures. These results suggest that the mouse lung never reaches a maximal volume, even up to nonphysiological pressures >80 cmH2O.

The mouse P-V curve superficially appears to follow this pattern. However, closer inspection of the curves from mice shows that, although the inflation limb starts to plateau >10 cmH2O, as transpulmonary pressure (Ptp) increases >20 cmH2O, an inflection occurs whereupon the volume begins to increase further (15, 31, 34). Thus, at Ptp that would normally be used to define TLC, i.e., 25–30 cmH2O, the mouse inflation limb is now in a range of increasing lung compliance. This fact makes it clear that the definition of total lung capacity (TLC) in the mouse cannot be determined similarly to what is commonly done in other animal models. This use of the term TLC derives from humans, where TLC is defined as the lung volume at the end of a maximal voluntary inspiration. Experimental animals clearly cannot be instructed to do such a maneuver, and “maximal” lung volumes are thus generally defined at user-selected inflation pressures. Because in the present study we do not want to define a fixed maximal inflation pressure, we use the term TLC in a vernacular sense simply to indicate maximal lung volume (15). For better or worse, this has become a fairly ubiquitous usage. Because of the increasing use of the mouse as a model species in pulmonary physiology and pathophysiology, our experiments were designed first to determine at what pressures the mouse lung begins to show a limit to inflation; second, how alveolar size changes at high Ptp; and third, how this phenomenon varies in different mouse strains. Our results suggest that the mouse lung continually expands even up to nonphysiological pressures over 80 cmH2O and that the strain differences in alveolar size previously observed at normal inflation pressures (28a) are maintained even at the substantially increased alveolar dimensions at these high pressures.

MATERIALS AND METHODS

Animals. Males from three standard inbred strains (C3H/HeJ, A/J, and C57BL/6J), 6–8 wk of age (Jackson Laboratories, Bar Harbor, ME), were used in this study. Animals were housed in an animal facility and provided food and water ad libitum. The protocol was approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University.

Quasi-static P-V curves. Each animal was weighed and anesthetized with pentobarbital sodium intraperitoneally at a dose of 75 mg/kg body wt. After anesthesia, a tracheostomy was performed, and the lungs were connected to a ventilator. The animal was ventilated with 100% O2 for 10 min before the lungs were sealed off with a stopcock for 5 min to degas the lungs. Quasi-static P-V curves were immediately performed in situ with a system detailed in previous studies (22). Briefly, the system consists of a water-filled syringe pumping water in and out of a vertical air-filled column. The vertical water column serves as a leak-free piston whose position can be precisely controlled. Air from the top of the column is connected to the tracheal cannula. Air volume changes were determined by measuring the displacement of the syringe plunger with a linear displacement transformer (model 244–000, Transtek, Ellington, CT) after correcting for gas compression in the system. Airway pressure (Paw) and volume were recorded by PowerLab digital data-acquisition system (ADInstruments, Castle Hill, Australia). The initial inflation rate was low (0.009 ml/s) to ensure that initial lung recruitment from the degassed state did not result in excessive pressures. Once a pressure of 30 cmH2O was reached, the flow rate was increased to

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0.035 ml/s for the remaining inflation and deflation maneuvers. The lower limit of deflation was −10 cmH₂O. The upper limit of inflation was increased in 10-cmH₂O increments until a desired pressure was reached or the lung ruptured. We assessed the presence of irreversible damage at the very high inflation pressure by interposing an inflation cycle to 30 cmH₂O between two pressure cycles to 60 cmH₂O.

**Histology.** A total of 30 mice, 18 A/J, 6 C3, and 6 B6, were used for morphometric analysis of the lung at multiple inflation and deflation pressures. Each animal was prepared exactly as for the quasi-static P-V curve. Following degassing of the lung, all extrathoracic tissue was cut away, leaving an isolated thorax with the diaphragm intact and the trachea cannulated. P-V maneuvers were then initiated, and, at preselected points along the inflation or deflation limb, the trachea was clamped. The isolated thorax was quickly transferred (<5 s) to 500 ml of 2-methylbutane (−180°C) precooled with liquid nitrogen (29). The specimens were then processed for freeze substitution (30). They were transferred to absolute acetone precooled to −70°C. After at least 48 h and one additional acetone change, the thoraxes were quickly (~30 s) sliced into 5-mm disks by using a band saw (blade thickness, 0.7 mm) maintained in a cold room, keeping them frozen solid at all times. The disks were returned to fresh −70°C acetone for an additional 24 h. They were then put into a refrigerator-freezer at −20°C for 2 h and then into a refrigerator at 4°C for 2 h. Sections of left lungs were then carefully separated from the rest of the tissue disk, washed in room-temperature acetone, and embedded in glycol methacrylate (Polysciences, Warrington, PA). Two-micrometer-thick sections were cut with a microtome and stained with methylene blue.

From each left lung, 12–18 nonoverlapping 640 × 480 μm fields were sampled, deliberately avoiding the large airways and blood vessels. Each of these regions was photographed for digital analysis, and conventional morphometric methods were used to determine air space chord length, as previously described (6, 12, 17, 18, 28a, 33). Briefly, a grid with parallel lines spaced at 35 μm was overlaid onto each histological image, and the length of each chord, defined by the distance between intercepts with lung tissue, was measured. As in previous studies (17, 28a), chords <8 μm and >250 μm were excluded. Mean alveolar chord length (Lₐₐ) was calculated as the mean of the pooled chords from each animal.

**Statistical analysis.** Statistical differences in Lₐₐ between strains were analyzed by one-way ANOVA with the Bonferroni correction for multiple pairwise comparisons by using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA). Intrastrain morphometric and lung volume parameters from the multiple fixation pressures were compared with t-tests. For all statistics, a 95% confidence level was considered statistically significant. Chord length frequency distributions were generated by pooling all of the chords from each strain and grouping the chord lengths into 7-μm bins.

**RESULTS**

Repeated P-V curves with increasing limits of inflation. Figure 1A is a representative plot of the first inflation and deflation from the degassed condition and the second complete curve to 30 cmH₂O. It is clear from both inflation limbs that, at 30 cmH₂O, there is no flattening or any indication that a

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**Figure 1.** Original tracings of respiratory system pressure-volume (P-V) relation-ship from a single A/J mouse. A: initial inflation from the degassed condition to 30 cmH₂O, deflation to residual volume, and a second inflation and deflation cycle. B: inflation and deflation to 60 cmH₂O, twice the pressure used previously to define total lung capacity. C: mechanical integrity of the lung following inflation to nonphysiological pressure. It depicts an inflation and deflation cycle to 30 cmH₂O before (solid line) and following (dashed line) the inflation to 60 cmH₂O. D: all sequential P-V maneuvers performed on this lung are depicted. Rupture of the lung occurred at the start of deflation from 80 cmH₂O. Vₜₖₒ lung volume at 30 cmH₂O.
maximal lung volume has been reached. Figure 1B overlays the subsequent inflation and deflation limbs to a maximal pressure cycle of 60 cm H2O onto the curves in Fig. 1A. The inflation limb for the curve to 60 cm H2O is seen to superimpose on the 30-cmH2O subsequent inflation limb, reaching the same volume at 30 cm H2O. With a further increase in inflation volume to 60 cm H2O, there is a continual and substantial increase in lung volume, resulting, in this example, in an additional 40% increase in lung volume by 60 cm H2O. With inflation >30 cm H2O, there is now clear indication of lung stiffening as the P-V curve bends toward the pressure axis. The deflation limb from 60 cm H2O is almost perfectly parallel with the one from the inflation to 30 cm H2O, despite this inflation to double the pressure. Figure 1C superimposes a P-V curve to a peak pressure of 30 cm H2O done after the curve to a peak of 60 cm H2O. The inflation and deflation limbs nearly superimpose on the first 30-cmH2O curve done before the one to 60 cm H2O. Figure 1D superimposes all P-V cycles conducted in this mouse, done with 10-cmH2O increments in peak pressure up to rupture in this animal at 80 cm H2O. All deflation limbs are parallel, regardless of the magnitude of inflation pressure. Lung volume at pressures >40 cm H2O is also shown to continue to increase almost linearly up to lung rupture.

Table 1 shows lung volume and morphometric data for the three strains studied. For analysis, we only report data up to a peak pressure of 60 cm H2O, a pressure that caused no gross damage or leak in any of the mice. Lung volumes at 30 cm H2O (V30) were very similar to those previously reported (29). V30 for the C3 animals (1.45 ml) were 52 and 35% larger than for B6 (0.95 ml) and A/J (1.07 ml), respectively. All three strains showed a statistically significant and substantial increase in lung volume at 60 cm H2O (V60), compared with V30. The largest relative increase in V60 was in the B6 strain (78%), whereas the lung volume increase with the same Paw increase was 33% in the C3 and 33% in the A/J strains.

**Morphometry of lungs quick-frozen at 30 and 60 cm H2O.** Consistent with previous morphometric studies on these strains (8a), there is significant interstrain variation in Lmax. Low-power (×4) photomicrographs of lungs frozen at 60-cmH2O Paw, illustrating the qualitative interstrain differences in lung architecture, appear in Fig. 2, and the quantitative results for Lma are shown in Table 1. Relative to both the C3 and A/J mice, the B6 demonstrates a significantly (P < 0.01) smaller Lma at 30 and 60 cm H2O. The Lma of the C3 and A/J are not statistically distinguishable at a Paw of 30 cm H2O. There were no statistically significant increases in Lma going from 30- to 60-cmH2O Paw in any strain. However, the 17.0% increase in Lma from 30 to 60 cm H2O in B6 mice approached standard confidence levels (P = 0.061). Furthermore, as described next, there were significant increases in Lma at lower lung volumes in the A/J strain.

**Volumes and morphometry of lungs quick-frozen at multiple inflation pressures.** In the A/J strain, morphometric analysis was performed at four additional points on the P-V relationship (two inflation and two deflation). At the lowest inflation point (7 cm H2O), considerable heterogeneity in air space size across microscopic fields from the same lung was observed. Figure 3 illustrates a typical example. This image shows a normal-looking alveolated region on the left with large unseptated spaces on the right. In addition, there were also some regions of local atelectasis in this lung (not shown). At 15 cm H2O, the gross heterogeneous appearance of fields from a single animal

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**Table 1. Lung volume and morphometric parameters for standard inbred strains of mice**

<table>
<thead>
<tr>
<th>Strain</th>
<th>P Fixation, cmH2O</th>
<th>Lung Volume, ml</th>
<th>Mean Alveolar Chord Length, μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3H/HeJ</td>
<td>30</td>
<td>1.45±0.10^d</td>
<td>63.1±5.0^e</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1.92±0.18^ae</td>
<td>69.1±1.3^e</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>30</td>
<td>0.95±0.06</td>
<td>44.7±2.9</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1.69±0.14^d</td>
<td>52.3±2.4</td>
</tr>
<tr>
<td>A/J</td>
<td>15</td>
<td>0.61±0.03</td>
<td>48.1±0.4^d</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1.07±0.08^d</td>
<td>60.1±1.7^d</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1.42±0.13^d</td>
<td>63.7±1.0^d</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.91±0.06</td>
<td>58.5±0.1^e</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.70±0.05</td>
<td>51.5±1.3</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 3 for all pressures in each strain. P Fixation, airway pressure at which the lung was frozen; i, frozen on inflation; d, frozen on deflation following inflation to 30 cm H2O. Symbols indicate statistically significant (P < 0.05) differences as follows: *7 vs. 15 cmH2O on inflation or deflation; ^15 vs. 30 cmH2O; ^30 vs. 60 cmH2O, ^A/J vs. B6; ^C3 vs. B6, ^C3 vs. A/J.

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**Fig. 2. Photomicrographs of representative microscopic fields from the air-inflated lungs of C3H/HeJ (C3; A), A/J (B), and C57BL/6J (B6; C) animals that were quick-frozen at an airway pressure of 60 cmH2O.** This side-by-side comparison qualitatively illustrates the quantitative, C3 largest, intermediate A/J, and B6 smallest, differences found in mean alveolar chord length (Lma) among these strains of mice.
is significantly reduced. With inflation from 7 to 15 cmH\(_2\)O, the mean \(L_{\text{ma}}\) increased significantly \(-50\%\) from 31.9 \(\pm\) 3.3 (SE) to 48.1 \(\pm\) 0.4 \(\mu\)m (Table 1). Figure 4 shows the frequency and cumulative distributions of chord lengths at the different pressures. For clarity, only the inflation data are shown. Figure 4 shows that the increase in \(L_{\text{ma}}\) with inflation results from a loss of the population of very small chords. For example, at 7 cmH\(_2\)O, chords \(<50\ \mu\)m comprise \(-85\%\) of the total chords. By the time 60 cmH\(_2\)O is reached, only 50% of the chords are \(<50\ \mu\)m.

**Correlation of \(L_{\text{ma}}\) with lung volume.** On average, there is a monotonic relation between inflation pressure and \(L_{\text{ma}}\). However, the \(L_{\text{ma}}\) is significantly smaller at equivalent pressures on inflation compared with that on deflation following inflation to 30 cmH\(_2\)O. Lung volume is also larger on inflation, so, to examine the relation between lung volume and \(L_{\text{ma}}\), we have plotted lung volume against \(L_{\text{ma}}\) in Fig. 5. Figure 5 shows that the relation between alveolar size and lung volume is independent of whether the lung is inflating or deflating. Thus the P-V hysteresis seems to correspond to the average air space dimension in this strain, such that \(L_{\text{ma}}\) is closely linked to lung volume and not inflation pressure.

**DISCUSSION**

**Unique characteristics of the mouse P-V curve.** Leith (15) was the first to note the “double-humped” P-V inflation curve of the mouse lung. He speculated that perhaps the maximal lung volume or TLC might not be defined by pressures in experimental laboratory settings. An inflation limb with a double inflection is sometimes seen in other species, including human, and a theoretical model was proposed with different opening pressures for alveoli and the smallest airways (25). Unfortunately, this previous model was not tested with either physiological or histological experiments, but it does indicate that two populations of opening pressures could theoretically explain this observation. There have been no subsequent studies that attempted to address this potentially important observation in mice (or other species), although there have been recent studies where this peculiar inflation limb has been published without apparent notice or comment (31, 34). The classic explanation for the inflation limb of the P-V curve is that the initial lower corner (convexity toward the pressure axis) reflects opening of closed alveoli and airways. At increasing pressures, everything is open, and the upper corner (concavity toward the pressure axis) represents a transition to where a limit to distension of the lung parenchyma is caused by structural constraints (8, 20).

Repeated P-V curves with increasing limits of inflation. In this study, we have clearly shown that, once the second inflation knee occurs, the lung keeps getting bigger, in an almost linear manner, with increasing pressure. This continues up to the point of rupture at what would normally be considered quite unphysiological pressures. The nature of this steadily increasing inflation limb, however, makes it unclear how to define maximal lung volume or TLC in the mouse.
Such definition is important in comparisons between different mouse strains or within a strain before and after some experimental intervention. If the inflation limb is seen to progressively flatten as high pressures are reached, one may comfortably assume that one is near to a maximal lung volume. In many species, this appears to be the situation at a pressure of \( \sim 30 \text{ cmH}_2\text{O} \), and this pressure was used by Tankersley et al. (31) to compare lung volumes in different mouse strains. While their results clearly documented different P-V relations and different lung volumes in the different strains, part of these observed differences may relate to the fact that the second knee of the curve occurs at different inflation pressures in the different strains. Indeed, when the three strains previously studied were inflated to 60 cmH\(_2\)O, the large differences in TLC previously reported were greatly minimized, with the B6 lungs surpassing that of the A/J in size. This observation that B6 lungs are substantially more compliant over this range of high pressures may be indicative of a fundamental ultrastructural difference whose impact at lower pressures or in disease models is uncertain.

When P-V curves are generated, it is often an implicit assumption that the flat part of the curve at the start of the deflation represents TLC. Indeed, inflation limbs are rarely even reported. However, as clearly shown in the present study, one can achieve nearly flat, parallel deflation limbs, regardless of the inflation pressure or lung volume. Thus one clearly cannot use the upper flat part of the deflation limb as an index of maximal lung volume. This point was emphasized some years ago by Leith (16), but not many subsequent investigators paid attention to his counsel. These observations regarding the interpretation of the flat deflation curve have ramifications for those investigators who attempt to fit the deflation curves with an exponential (3, 10, 24) or sigmoidal function (23, 32). Clearly, in the mouse, one could obtain quite different parameters of the fit, depending on what inflation pressure is selected.

In the present study, to minimize physical lung trauma, we inflated the lungs inside the thorax, but we did not measure the pressure in the pleural space. Previous work (4, 14, 15, 31) has indicated that the compliance of the relaxed mouse chest wall is very large, and, therefore, pressure in the pleural space of the relaxed thorax of the mouse at inflation pressures up to 30 cmH\(_2\)O would be trivial. Tankersley et al. (31) showed that P-V curves done with and without chest wall intact were almost superimposable, and Lai and Chou (14) actually estimated pleural pressure from esophageal pressure, showing it to be \(<3 \text{ cmH}_2\text{O} \) at a Paw of 30 cmH\(_2\)O. We do not know whether this remains true at the highest inflation pressures used in this study. If the thoracic wall did begin to reach some elastic constraints, this would decrease the effective Ptp below the maximal values used. However, this would not affect the interpretation or qualitative conclusions based on the data.

The fact that we kept the thorax intact may also be related to the lack of obvious or irreversible damage observed when lungs are inflated to such high pressures. Our observations that we could superimpose P-V curves done to 30 cmH\(_2\)O (Fig. 1C), with an intervening one done to 60 cmH\(_2\)O suggest little acute functional damage.

**Morphological results and visual examination.** There have been a number of morphological investigations in other species of the structural changes accompanying the P-V relationship (5, 7, 21). One major problem with all such studies is how to fix the lungs to preserve what the lung looks like in vivo. The three most common fixation methods are inflation with a liquid fixative, air inflation with vascular perfusion (9), or quick-freezing (29). Each of these techniques has its own specific limitations. For the objectives of the present study, we selected quick-freezing as the one with the least problems. Inflation by tracheal instillation with liquid fixative would of necessity change the surface properties of the normal in vivo lung. Although vascular perfusion provides excellent images of air-filled lungs, this method would not work at the high inflation pressures that we were studying here. To obtain adequate uniform perfusion, one would need to have the perfusion pressure at least as high as the Paw, and, even if uniform perfusion could be obtained, perfusion pressures of such magnitude would surely lead to an edematous lung. Freezing is not ideal, because the lungs do not freeze instantaneously, and there can be some distortions associated with a slow freezing process (29). This may possibly be the reason why we observed such heterogeneity in alveolar size at low-inflation pressures. However, such heterogeneity has also been observed in other morphological studies of pressure volume hysteresis that applied vascular perfusion for fixation (9). We, therefore, believe that the heterogeneity in alveolar size observed may reflect the situation present before fixation. In the mouse, the potential problems associated with slow freezing have been minimized, first because of the small mass of the mouse lung, and second because of the further reduction of tissue mass by
removal of extrathoracic tissue, while still maintaining the lung within the thorax.

With regard to this freezing method, it is worth noting that the alveolar size obtained with this freezing method shows differences from those obtained in a recent study using fixation with tracheal instillation of Zenker’s solution (28a). Soutiere et al. (28a) measured alveolar size in the same strains at 7- and 14-cmH2O instillation pressure, and their results showed Lma to be 53.9, 46.6, and 41.7 μm in the C3, A/I, and B6 mice, respectively. In the present study, we only have Lma in the C3 and B6 strains at pressures of 30 and 60 cmH2O, and these values are slightly larger than with Zenker’s fixative (Table 1). For the A/I strain, the range of Lma, with lungs frozen at pressures from 7 to 60 cmH2O overlaps the value found previously. Perhaps the most important difference, however, is the fact that the frozen lung shows an increase in Lma in going from 7 to 15 cmH2O, whereas the lungs fixed with liquid instillation showed no increase in Lma. At the present time, we do not have enough information to be able to explain this difference. It may possibly be related to the heterogeneity that we found in the lungs frozen at 7 cmH2O. However, it is important to emphasize that it is not proper to compare lungs fixed at the same pressure inflated with fluid or air. Not only will the lung volumes likely be different, but also, as we have shown in the present work, the previous inability to increase alveolar size with increased fixation pressure may reflect the fact that fixative itself may be working quickly enough to limit lung expansion. As a final comment on these general problems with lung fixation, we note a very recent paper that demonstrates the potential to measure alveolar size in situ without fixation. Sera et al. (27) presented a method using micro-computed tomography with radiodense staining to visualize structures as small as alveoli in rat lungs in situ. Although their objective was not to quantify alveolar size changes, their qualitative images are of sufficient resolution to show substantial alveolar enlargement with a lung volume increase from FRC to TLC.

Volumes and morphometry of lungs quick-frozen at multiple inflation pressures. The heterogeneous appearance of air-inflated lungs at low-inflation pressures has been previously observed by Gil and Weibel (9) during air inflation to 7 mmHg and fixation by vascular perfusion. At 7-cmH2O inflation, we also observed a quite heterogeneous appearance of lung tissue (Fig. 3). Such heterogeneity may limit the utility of a single variable like traditional mean linear intercept or the Lma. However, it does raise the important question as to how the lung goes from such structural heterogeneity to a more homogeneous appearance at higher pressures. That is, do the smaller units enlarge, as they seem to in rats (21, 27), or do the larger units unfold to increase surface area, as several studies would indicate (2, 6, 9, 28)? The answer to this question is beyond the scope of this paper, but, because, in many species, there is good evidence for the latter mechanism, we might have expected this to be the situation in mouse lungs as well. Our data in mice, however, do not support this. The chord length frequency distributions (Fig. 4) show an unambiguous rightward shift with increasing Paw, and results in Fig. 5 clearly show a monotonic relation between Lma and lung volume. The lack of a significant increase from 30 to 60 cmH2O may have resulted from several different causes. First, we may just need a larger sample size to detect the decreasing increments at higher pressures. Second, there may be increased fixation shrinkage at 60 cmH2O that would decrease the measured difference in Lma. Although such shrinkage from processing frozen lungs is normally small (<10%), it is known to be slightly greater at higher pressures (19). Although the extent of this has never been quantified at the pressures we have studied, if the effect extended to 60 cmH2O, then this would have reduced the measured difference in Lma at the two highest pressures. Third, there actually might not be a significant change in Lma over this pressure range. We should also note that the alveolar size should be more properly correlated with lung volume rather than pressure. The lung P-V curve links these two variables, but, as alveolar size and lung volume are structural variables, it is reasonable to expect some linkage between them. Thus part of the reason for finding no statistically significant change in Lma from 30 to 60 cmH2O may be simply that the volume change is too small to allow us to detect an Lma change, given the limited sample size. Changes in Lma are much smaller than lung volume changes, varying as the cube root of lung volume, so noise levels take on a greater significance with this linear measurement.

One additional mechanism about which we can only guess at this time is how can the heterogeneity observed at 7 cmH2O be resolved with an increase in mean alveolar size as inflation pressure increases? We suggest that the end result occurs because of the net effect of two simultaneous processes. Clearly there must be alveolar unfolding with lung inflation in the large air spaces shown in Fig. 3, right. With increasing lung volumes, if alveolar septa unfold, then mean chord length through this region with large air spaces on Fig. 3, right, should decrease. However, alveoli in already unfolded regions seem to be able to enlarge, and this effect must be the dominant one for the whole distribution. Evidence to support this hypothesis is not easily obtainable with histology, but, as noted, the application of micro-computed tomography (27) has the potential to resolve this long-standing controversy.

Finally, we may speculate on the implications of our observations. One of the reasons for measuring TLC is that it presumably tells us about the size and physical properties of the lung. If there is a maximal volume, it should reflect some elastic limit of the connective tissue structure. In mice, however, our findings show that this is never even approximated at the maximal pressure normally used for P-V curves. Indeed, in some strains at 30 cmH2O, the inflation limb still shows convex curvature toward the pressure axis! Unfortunately, at the present time, we have few clues regarding the causes of this double-humped inflation limb. Until some structural basis for this is better understood, it will be very difficult to interpret differences or changes in the TLC within and across strains. In the past, most investigators have simply compared the volume at a specified pressure. While this still may be acceptable, one can easily envision slight shifts in the position of the inflation limb knees that may lead to results and conclusions entirely unrelated to structural constraints on lung or alveolar size.

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J Appl Physiol • VOL 96 • MAY 2004 • www.jap.org