Effects of collagenase and elastase on the mechanical properties of lung tissue strips

HUICHIN YUAN,1 STEFANIDA KONONOV,1 FRANCISCO S. A. CAVALCANTE,1 KENNETH R. LUTCHEN,1 EDWARD P. INGENITO,2 AND BEŁA SUKI1

1Department of Biomedical Engineering, Boston University, and 2Brigham and Women’s Hospital, Boston, Massachusetts 02215

Received 9 November 1999; accepted in final form 3 February 2000

Yuan, Huichin, Stefanida Kononov, Francisco S. A. Cavalcante, Kenneth R. Lutchen, Edward P. Ingenito, and Béla Suki. Effects of collagenase and elastase on the mechanical properties of lung tissue strips. J Appl Physiol 89: 3–14, 2000.—The dynamic stiffness (H), damping coefficient (G), and harmonic distortion (kₜ) characterizing tissue nonlinearity of lung parenchymal strips from guinea pigs were assessed before and after treatment with elastase or collagenase between 0.1 and 3.74 Hz. After digestion, data were obtained both at the same mean length and at the same mean force of the strip as before digestion. At the same mean length, G and H decreased by ~33% after elastase and by ~47% after collagenase treatment. At the same mean force, G and H increased by ~7% after elastase and by ~25% after collagenase treatment. The kₜ increased more after collagenase (40%) than after elastase (20%) treatment. These findings suggest that, after digestion, the fraction of intact fibers increases before and after treatment with elastase or collagenase. The kₜ increased more after collagenase (40%) than after elastase (20%) treatment. These findings suggest that, after digestion, the fraction of intact fibers decreases, which, at the same mean length, leads to a decrease in moduli. At the same mean force, collagen fibers operate at a higher portion of their stress-strain curve, which results in an increase in moduli. Also, G and H were coupled so that hysteresivity (G/H) did not change after treatments. However, kₜ was decoupled from elasticity and was sensitive to stretching of collagen, which may be of value in detecting structural alterations in the connective tissue of the lung.

stiffness; damping; tissue resistance; nonlinearity; network elements in the parenchyma are responsible for the linear viscoelastic and nonlinear behavior of lung tissue?

The lung tissue has a number of important structural components, such as the connective tissue network, interstitial cells, and the surface lining layer. For example, the surface lining layer is a significant stress-bearing element in the lung tissue. Liquid filling of the lung abolishes the gas-liquid interface and thus eliminates a significant portion of the hysteresis of the isolated air-filled lung (1, 5), which supports the notion that surface lining is an important contributor to tissue resistance. Schurch et al. (31) measured the surface tension-area curve of pulmonary surfactant extracts. They found that, after a few consecutive cycles, the hysteresis of the surface film during small-amplitude cycling became negligible. This suggests that, during tidal breathing, the contributions of surface film to lung hysteresis are small. The surface film, however, may have an indirect influence on lung hysteresis through the geometric alterations of the alveoli due to reduced surface forces at low lung volumes (33). Similarly, in a recent study (41), we found that the dynamic properties of parenchymal tissue strips from guinea pigs measured before and after loss of cell viability were statistically identical. Because of the absence of cross-bridge cycling, the average energy dissipation in nonviable samples was only ~10% smaller than in viable samples. Therefore, from these studies, one may conclude that lung parenchymal mechanics are most likely dominated by the extracellular matrix. The next question is then how alterations in the extracellular matrix that occur in various interstitial lung diseases, such as emphysema or fibrosis, affect the elastic and hysteretic properties of lung parenchymal tissues.

The primary aim of this study was to gain more insight into the origin of tissue elastic and hysteretic properties. To achieve this goal, we investigated the linear and nonlinear mechanical contributions of the constituents of the connective tissue, namely, the collagen-elastin fiber network, to the overall mechanical properties of lung tissue strips under the effects of
various biochemical interventions mimicking interstitial lung diseases.

METHODS

Sample Preparation

Lung parenchymal strips were obtained from healthy male guinea pigs weighing 400–450 g and killed by intraperitoneal injection of pentobarbital sodium. The fresh lungs were removed from the thoracic cavity, placed in oxygenated Krebs-Ringer organ bath perfusate (in mM) 5 KCl, 137 NaCl, 2 CaCl$_2$, 1 MgSO$_4$, 1 NaH$_2$PO$_4$, and 24 NaHCO$_3$ on ice, and studied within 1 h. The pH was controlled with bubbling 5% CO$_2$–95% O$_2$. Tissue strips of 4.5 × 4.5 × 10 mm in dimensions were prepared, and then each end of the tissue strip was fixed by cyanoacrylate glue to small metal clips attached to straight steel wires. The assembly was placed in a vertical glass tissue bath (Wilbur Scientific, Boston, MA), with the upper wire attached to a force transducer and the lower wire to the lever arm of a displacement generator.

Experimental Setup

The apparatus is described in detail in Ref. 41. Briefly, a servo-controlled lever arm (model 300H, Cambridge Technologies) provided the desired elongations. The force developed by the tissue strip was measured by a force transducer (model 400A, Cambridge Technologies). Calibrations were performed between 0 and 2 g force by using standard weights. The transducer system was tested as in Ref. 41 and was shown to be linear and accurate to within 1% over the force range (0–2 g) of interest with a hysteresis at least 10 times smaller than that of the tissue strip. The servo-controlled lever arm was driven by a displacement signal generated by a computer. The signal was sent out from the digital/analog port of a data-acquisition board (DT2812, Data Emmanuel, Cambridge, MA) and then smoothed with a low-pass filter (8-pole, R858L8EX, Frequency Devices, Haverhill, MA) with a cutoff frequency of 15 Hz. Both displacement and force signals were low-pass filtered at 15 Hz and then sampled at 50 Hz.

Protocol

Before the protocol was started, the system was aligned by adjusting the horizontal position of the actuator so that the hysteresis of the strip displayed on an oscilloscope during sinusoidal oscillations was minimized. This is an important step because any friction between the steel wire and the wall of the glass container can significantly contribute to the measured hysteresis of the tissue strip. The experiments were performed at room temperature. The strip was preconditioned by first performing single slow stretch to 2 g of mean force. The mean force was then reset to a desired value, and the strip was oscillated sinusoidally at 1 Hz for ~5 min to avoid the transients due to stretching the strip to 2 g of force

Proteolytic enzymes were used to elucidate the effects of the connective tissue components on the mechanical properties of lung parenchyma. Collagen or elastin fibers were digested with collagenase (1 mg, Sigma Chemical) or pancreatic elastase (5 μl, Sigma Chemical). During digestion treatment, the strip was placed in a chamber filled with PBS solution (37°C, pH 7.4) to which the appropriate enzyme solution was added.

A total of 16 lung parenchymal strips was studied before and after enzyme treatment: eight strips were treated with elastase for 60 min, and eight strips were treated with collagenase for 30 min. The dynamic properties of the strips were measured at a mean force of 1 g with strain amplitudes of 5, 10, and 15%. After enzyme treatment, the tissue strips lose part of their elastic recoil. As a consequence, when the strips are stretched to the same distending force, the corresponding length will be larger than it was before the treatment. Thus the operating point on which the oscillations are superimposed can be chosen as either the same static length or the same static force of the strip as before treatment. To better characterize the alterations in the mechanical properties, we repeated the oscillatory measurements both at the same mean length and at the same mean force as in control. Additionally, in three strips from both the elastase- and the collagenase-treated groups, the quasi-static stress-strain curve was measured, and, in one strip from both groups, the time response of the mechanical moduli was measured every 15 or 30 min for up to 3 h.

Measurement Approach

The quasi-static stress-strain curve was measured by using a triangular wave in strain with a period of 200 s. For the dynamic measurements, instead of the traditional sinusoidal oscillation approach, we used a broad-band pseudorandom displacement input signal, which allows a rapid assessment of the dynamic mechanical properties of the tissue strips. The frequency composition of the displacement signal was chosen so that the influence due to nonlinearities on the estimated apparent complex moduli is minimized and so that higher order nonlinearities can be identified for characterizing tissue nonlinearities. The signal followed the composition proposed by Victor and Shapley (39) and contained six input frequencies, a flat power spectrum, and a set of random phases (see Table 1). The length of the sequence was 4,096 points, and the sampling rate was 60 Hz, which corresponds to a time period of 68 s. For each dynamic force-displacement measurement, three cycles were delivered, and only the last two cycles were collected to avoid the effects of transients on the dynamic moduli. This kind of sparse frequency composition of the input allows a rapid assessment of the impedance of modulus spectrum of the system simultaneously at many frequencies, and the estimated apparent spectrum is very smooth as if it were measured with separate sinusoidal of some equivalent amplitude (36).

Data Analysis

Characterization of strip mechanics. The length input and force output signals were normalized to obtain strain $\varepsilon$ and stress $T$ as

$$
\varepsilon(t) = \frac{|l(t) - l_o|}{l_o}; \ T(t) = F(t)/A_o \tag{1}
$$

where $l$ is length, $l_o$ is reference length of the strip, $F$ is force, $A_o$ is cross-sectional area of the strip corresponding to $l_o$, and $t$ is time. The mechanical properties of the strips are charac-

Table 1. Frequency components and phase angles of the input signal

<table>
<thead>
<tr>
<th>Harmonic Component</th>
<th>7</th>
<th>15</th>
<th>31</th>
<th>63</th>
<th>127</th>
<th>255</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency, Hz</td>
<td>0.10</td>
<td>0.22</td>
<td>0.45</td>
<td>0.92</td>
<td>1.86</td>
<td>3.74</td>
</tr>
<tr>
<td>Phase angle, rad</td>
<td>1.03</td>
<td>−1.84</td>
<td>1.66</td>
<td>−0.24</td>
<td>−0.92</td>
<td>0.71</td>
</tr>
</tbody>
</table>
characterized by the complex modulus $G^*$ defined in the frequency domain as

$$G^*(\omega) = T(\omega)/\epsilon(\omega) = G'(\omega) + jG''(\omega)$$

where $j$ is the imaginary unit, $\omega$ is the circular frequency, $G'$ is the storage modulus or the component of the stress that is in phase with strain, and $G''$ is the loss modulus or the component of the stress that is in phase with strain rate. The data records of $T(t)$ and $\epsilon(t)$ were first transformed to $T(\omega)$ and $\epsilon(\omega)$, respectively, which were then divided into four blocks (4,096 points/cycle) with an overlap percentage of 25%. The complex spectra for each cycle were obtained by taking the fast Fourier transforms of the blocks. The $G^*$ was then estimated in the frequency domain by taking the ratio of the cross-power spectrum of $T$ and $\epsilon$ and the autopower spectrum of $\epsilon$. The $G^*$ was also corrected for the frequency response of the measuring apparatus, which was mainly due to asynchronous sampling of the displacement and force channels. The hysteretic properties of the tissue were characterized by the tissue hysteresivity, $\eta$, introduced by Fredberg and Stemovnic (9), which is defined as the ratio of dissipated to stored energy over a force-length cycle and is simply $G'G''$. This allows the calculation of $\eta$ as a function of frequency.

*Viscoelastic modeling.* The special design of the input signal allows a robust estimation of the apparent linear transfer function of the system at the input frequencies in the absence of very strong nonlinearities. Therefore, to evaluate the dynamic properties of the tissue strip, we first fit a linear viscoelastic model to the complex modulus spectra at the six input frequencies. Many viscoelastic tissue models have been proposed in the literature (3, 10–14, 19, 22, 28, 29, 35). We chose the constant-phase model, which originated from the power law type of stress relaxation of a rubber balloon (13)

$$T(t) \propto t^{-\beta}$$

where $\beta$ is the relaxation exponent. The Fourier transform of Eq. 3 was later applied to lung impedance spectra in the frequency domain by Hantos et al. (11, 12). The tissue impedance $Z(\omega)$ is described by

$$Z(\omega) = G^*(\omega)/j\omega = \frac{G}{\omega^\alpha} - j\frac{H}{\omega^\alpha}$$

with

$$\alpha = \frac{2}{\pi} \tan^{-1}\left(\frac{H}{G}\right) = 1 - \beta$$

where the parameters $G$ and $H$ are the tissue damping and elastance coefficients, respectively. Note that $\alpha$ is not an independent parameter in the model, and it governs the frequency dependence of the real and imaginary parts of $Z(\omega)$. With only two parameters ($G$ and $H$), Hantos and co-workers showed that this model can fit the tissue impedance in cat and dog lungs better than other viscoelastic models (11, 12). Additionally, a mathematical framework and a possible molecular basis of Eqs. 3 and 4 has also been offered (35). In our previous study (41), we also observed that the lung tissue behaved as if it had a purely viscous component $R$, which was also added to the complex modulus $G^*(\omega)$ such that

$$G^*(\omega) = H\omega^\alpha + j(G\omega^\beta + R\omega)$$

Note that the first term is the $G'$, which increases slowly and quasi-logarithmically with $\omega$ where the exponent $\beta$ is a small number between 0.04 and 0.1 (3, 11, 35). The second term is the $G''$, which also increases quasi-logarithmically and with the same exponent as the $G'$ because $R_0$ is negligible at low frequencies. The tissue $\eta$ in this model is the ratio of the imaginary and real parts of $G^*$ and is a function of frequency. However, because the term $R_0$ is small compared with $Go^\beta$ at low frequencies and to remain consistent with previous analyses of whole lung mechanics (11, 18, 35), we define $\eta$ as the ratio $G'H$ except when we examine the frequency dependence of $\eta$ directly from $G'$ and $G''$. Using a global optimization algorithm (6), we estimated the model parameters by minimizing the following root-mean-square error (RMSE)

$$\text{RMSE} = \sqrt{\frac{1}{N} \sum_{i=1}^{N} (G^*(\omega)_\text{data} - G^*(\omega)_\text{model})^2}$$

where $\omega_i$ refers to the input frequencies and $N = 6$.

*Harmonic distortion.* When applying a broad-band input, the degree of system nonlinearity can be characterized by how the moduli depend on the amplitude of the strain input. Another way of characterizing nonlinearity is via the so-called extended harmonic distortion index $k_\delta$, which quantifies the amount of both harmonic distortion and cross talk in the output signal (43). The coefficient $k_\delta$ is defined as

$$k_\delta = \frac{P_\text{TOT}}{P_{\text{NI}}} \times 100\%$$

where $P_{\text{TOT}}$ is the total power in the output and $P_{\text{NI}}$ is the output power due to system nonlinearities only, i.e., the power at noninput frequencies. The advantage of using $k_\delta$ is that it can be calculated from a single spectrum, whereas the traditional method of characterizing nonlinearity requires the measurement of the moduli at several distinct amplitudes.

**RESULTS**

The quasi-static stress-strain curve of two tissue strips before and after digestion with either elastase or collagenase is shown in Fig. 1. There is a noticeable
intersample variability in the stress-strain curves of the control samples similar to that found by Maksym and Bates (20). The effect of both treatments is to shift the stress-strain curves to the right, resulting in a softer tissue. Thus for any given strain before and after treatment, the slope of the curve and hence the incremental elastic modulus ($Y$) of the tissue must be smaller after digestion. However, this may not be the case when the slopes are studied at the same distending stress because of the nonlinear nature of the curves. This phenomenon is further examined in terms of the dynamic moduli of the tissue (see below). In general, collagenase always produced a larger shift in the stress-strain curve than did elastase.

Figure 2 shows the data and model fits of $G'$ and $G''$ and $\eta$ as a function of frequency in a typical strip, in control and after treatment with elastase when the length of the tissue was set to the value before digestion (same mean length). In control, both $G'$ and $G''$ increased steadily and approximately linearly with the logarithm of frequency up to $\sim 1$ Hz, after which $G''$ increased faster, most likely due to the effect of the Newtonian resistance of the tissue. Also, both $G'$ and $G''$ showed a slight negative strain amplitude dependence. The ranges of $G'$ and $G''$ in control match those obtained in our previous study (41). The observed dynamic behavior in parenchymal strips from guinea pigs is consistent with data found in other species with the use of a sinusoidal approach (24, 28) and in whole animal studies (11, 12, 18, 29). After elastase treatment, the mean force in the tissue strip decreased by $\sim 20\%$. Correspondingly, both $G'$ and $G''$ shifted down by 40\% in a parallel fashion. The frequency dependence of $G'$ and $G''$ displayed a similar behavior as in control, except that the small strain amplitude dependence almost completely disappeared. Interestingly, $\eta$, calculated as $G''/G'$ as a function of frequency, showed virtually no dependence on frequency (except a small increase above 2 Hz), strain amplitude, and the conditions of the strip (Fig. 2C). The mean value of $\eta$ was $\sim 0.1$, which is in good agreement with other reported data (11, 26, 35, 41). The linear viscoelastic model provided good quality fits to the data. However, the RMSE values obtained from fitting the model to the data depended slightly on the condition. Compared with control, they decreased by $\sim 10\%$ at the same mean length and increased by 50–80\% at the same mean force (data not shown).

We evaluated the tissue properties before and after elastase and collagenase treatment by examining the parameters $G$, $H$, $R$, $\eta$, and $k_d$. The population means of the parameters obtained at strain amplitudes of 5, 10, and 15\% before and after elastase treatment are shown in Fig. 3 and before and after collagenase treatment in Fig. 4. In general, $G$ and $H$ decreased and $R$ and $k_d$ increased with increasing strain amplitude and were statistically significant in many cases. At the same mean length, $G$ and $H$ decreased after elastase treatment by 30–35\% ($P < 0.02$), and they decreased even more significantly with collagenase treatment by 45–50\% ($P < 0.0004$). Although $R$ did not change after elastase treatment, it decreased statistically significantly in the collagenase-treated condition ($P < 0.0001$) independent of the strain amplitude. The effect of treatment (collagenase or elastase) on both $G$ and $H$ was highly significant, but there was no difference in the reduction in $G$ due to elastase or collagenase, whereas $H$ was statistically significantly smaller after collagenase treatment ($P < 0.01$). This resulted in a slight (but not significant) increase in $\eta$ after collagenase treatment so that the difference in $\eta$ after collagenase and elastase became significant ($P < 0.05$). Nevertheless, $\eta$ did not change compared with control after either treatment. The $k_d$ increased after both treatments but reached a statistically significant level ($P < 0.001$) only after collagenase treatment. These changes in the parameters were accompanied by significant decreases in the static operating stress in the strip ($\sim 20\%$ and $\sim 30\%$ decreases after elastase and collagenase treatment, respectively, compared with control).

**Fig. 2.** Storage modulus ($G'$; A), loss modulus ($G''$; B), and hysteresivity ($\eta$; C) as a function of frequency for a typical strip. Solid and open symbols, data before and after elastase treatment, respectively. Circles and triangles, data at strain amplitudes of 5 and 15\%, respectively. Solid lines are model fits.
At the same mean force, both G and H increased statistically significantly after collagenase treatment by 21% ($P < 0.0002$) and 29% ($P < 0.0001$), respectively, whereas G and H increased only slightly after elastase treatment by 6 and 8%, respectively. The value of $h$ showed a 10% increase after collagenase treatment and a 17% reduction after elastase treatment, which was statistically significant ($P < 0.05$). The values of $R$ increased statistically significantly after collagenase treatment ($P < 0.01$) but not after elastase treatment. The $k_d$, and hence the degree of tissue nonlinearity, was more sensitive to collagenase treatment. The $k_d$ increased significantly by 40% ($P < 0.0001$) after the strips were treated with collagenase, whereas the corresponding percent increase in elastase-treated strips was only 20% ($P < 0.008$).

The time responses of the mechanical parameters normalized with respect to their control values in two strips after elastase and collagenase treatment are shown in Fig. 5. The digestion of collagen fibers had a markedly stronger and faster effect on the parameters than did the digestion of elastin fibers. Both G and H decreased by ~60% after 1 h of collagenase treatment. The elastase caused a similar but a slower response in the strip. Both G and H decreased by 50% after 3 h of treatment. The values of $R$ and $h$ fluctuated and did not show systematic change after either enzyme treatment. The $k_d$ increased by ~10% after 1 h following elastase and collagenase treatment.

**DISCUSSION**

In the present study, we focused on the mechanical role of the extracellular matrix, i.e., the elastin-collagen fiber network, by measuring the mechanics of tissue strips in an organ bath, which eliminates the influence of air-liquid interface. The mechanical roles of elastin and collagen fibers were probed by examining the mechanical
properties of tissue strips obtained before and after treatments with elastase and collagenase. Our main findings are as follows: 1) because the $G'$ and $G''$ were very sensitive to both collagenase and elastase treatment, the elastin-collagen fiber network dominates the macroscopic elastic and dissipative properties of the tissue strip; 2) because $h$ was nearly insensitive to either treatment, $h$ appears to be independent of the number of intact fibers; and 3) because the operating stress and strain amplitudes we used in the study cover approximately the range of normal breathing around functional residual capacity, collagen fibers also contribute to tissue elasticity during normal breathing. Before interpreting these results, we first discuss some important considerations regarding the manner in which the enzymatic digestion takes place.

The extracellular matrix can be altered biochemically by various enzyme treatments. The extent of biochemical and structural change in the fiber matrix depends largely on the specificity of these enzymes. Modification of the physical characteristics of the tissue by the enzymes depends on a number of factors, such as the molecular structure of the target and its affinity for the enzyme, penetration of the enzyme, and perhaps the total amount of tissue to be digested. Pancreatic elastase is a highly active elastin-decomposing enzyme and degrades amino acids at sites of the elastin molecule (23, 27). Collagenase attacks and cleaves amino acids at specific binding sites of the collagen molecule (17, 32). From electron micrographs, Karlinsky et al. (15) observed disruption of collagen fibers in parenchymal tissue treated with collagenase and of elastic fibers treated with elastase. They have also shown that the collagenase was not active against elastin and that elastase had no detectable activity against collagen. In the present study, no attempt was made to confirm the specificity and extent of degradation of elastin and collagen produced by elastase and collagenase treatment. Nevertheless, from our results it appears that the enzyme digestion is appropriate to study the mechanical contributions of the specific

Fig. 4. Means ± SD of $G$ (A), $H$ (B), $R$ (C), $\eta$ (D), and $k_d$ (E) measured at 3 different strain amplitudes before (Ctr) and after collagenase treatment taken at the same ML or same MF as in control. *Statistically significant difference ($P < 0.05$) between the value of a parameter (at a given strain amplitude and condition ML or MF) and its corresponding value in control.
structural constituents, such as elastin or collagen, in the peripheral part of the parenchyma.

**Tissue Elastic Behavior**

Our data suggest that both elastin and collagen contribute to tissue elasticity in the strain range applied, which roughly corresponds to functional residual capacity during normal breathing. This somewhat contradicts the notion of independent functionality of elastin and collagen fibers. Karlinsky et al. (15) reported that the compliance of elastase-treated lungs decreased only at low and medium lung volumes, whereas compliance of collagenase-treated lungs decreased only at high lung volume. Their data suggest that elastin provides great extensibility at low volumes, whereas collagen limits lung extensibility at high volumes. However, data obtained from experiments in tissue strips and lungs are not directly comparable because of the differences in boundary conditions, protocols, lack of gas-liquid interface, and so on. Also, the parenchymal strip undergoes uniaxial deformation, whereas lungs undergo a more uniform expansion during breathing. Although one can compare the quasi-static stress-strain curves before and after treatment, we were primarily interested in the dynamic contribution of the fibers to the macroscopic behavior of the tissues. Thus we measured the dynamic moduli and nonlinearity of the samples before and after digestion around two operating points on the stress-strain curve: at the same mean length and at the same mean stress as in control. These two specific operating points were chosen to better understand the separate roles of lung volume and transpulmonary pressure in lung mechanics.

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**Fig. 5. Time dependence of the mechanical moduli** $G$ (A), $H$ (B), $R$ (C), $\eta$ (D), and $k_d$ (E) normalized by their value immediately before digestion was started ($G_c$, $H_c$, $R_c$, $\eta_c$, and $k_d,c$, respectively). ●, Data obtained during elastase digestion; ■, data obtained during collagenase digestion.
At the same mean length or strain level, tissue elasticity as characterized by H substantially decreased after either elastase or collagenase treatment. This appears to be in agreement with the notion that the extracellular elastin-collagen fiber network is the principal contributor to tissue elasticity. The collagen fibers are nonlinear and behave as a nearly perfect spring with large stiffness and can be extended in length by only a few percent (34). The elastin fibers are linear and viscoelastic with a stiffness of at least two orders of magnitude lower than that of the collagen fibers (10). It is worth noting that the H of the tissue strip is far less than that of collagen or elastin fibers alone. This is most likely a network effect: the total stiffness of a large network of interconnected springs can be less than the spring constant of individual springs. After digestion, the number of collagen or elastin fibers is reduced in the fiber network. When this network is stretched to the same mean length as before digestion, due to the reduction in the number of springs, the static stress developed by the network will be less than before digestion. Thus the incremental modulus or H of the system will also decrease. The fact that digesting both elastin and collagen results in a large decrease in H is also noteworthy. Mercer and Crapo (23) found that the spatial distribution of collagen is fairly uniform in the tissue. If we take a composite network of soft and very stiff springs, such as that proposed by Wilson and Bachofen (40) in which the stiff springs are spatially uniformly distributed and stretch the network, it is conceivable that most of the elastic response will primarily be due to the stiff springs. Alternatively, if the soft springs are partially eliminated (e.g., by digestion of elastin), the H of the system should not change appreciably. Yet Fig. 1 shows a large change in the quasi-static stress-strain curve, and Fig. 5 shows that there is a significant decrease in H as a function of time during elastin digestion. Given that elastin has a Young’s modulus (Y) at least two orders of magnitude smaller than collagen, it is surprising that, after elastin digestion, H decreases fairly rapidly by 40% in 1 h. This indicates that the actual topology of the network must play an important role in its macroscopic behavior.

To better understand tissue elasticity, we can study how various network models that have been proposed in the literature would respond to digestion. These models include fiber recruitment type of models, such as those proposed by LaBan (16), Romero et al. (30), or Maksym et al. (20, 21), the line element model of the alveolar duct introduced by Wilson and Bachofen (40), or other geometric models (7, 21). In the finite element model of the alveolar duct and alveoli of Denny and Schroter (7), the elastin and collagen fibers run parallel in the alveolar walls. In the alveolar duct model of Wilson and Bachofen (40), the parenchyma is conceptualized as a hexagonal mesh of line elements. The H of each line element represents the resultant elasticity of both collagen and elastin fibers. In both models, elastin and collagen would be stretched simultaneously as the degree of deformation increases. However, the contribution of the stiff collagen fibers becomes significant only at high strains. This also means that the network should not be very sensitive to collagen digestion at low strains. Our data are not consistent with this interpretation. First, the tissue is extremely sensitive to collagen digestion at all strains (see quasi-static stress-strain curve in Fig. 1). Second, if collagen and elastin are indeed mechanically in parallel, then the total elastic response would be dominated by the much stiffer collagen. Thus disruption of elastin fibers induced by enzymes could not result in a substantial decrease in the elasticity of the tissue. Indeed, in the model of Denny and Schroter (7), it was necessary to eliminate over 95% of elastin to obtain an appreciable effect on the stress-strain curve. The recruitment models of Maksym and Bates (20) and Romero et al. (30) predict that the elastic behavior of lung tissue is mostly determined by the stretched soft elastin fibers, but with increasing strain the stiff collagen fibers are recruited. Also, as they begin to contribute to the mechanics, the stress-strain curve becomes more and more nonlinear. This idea is very attractive. Depending on the model configuration and the initial prestress, these kinds of models may be able to account for the fact that digesting either elastin or collagen can result in a large change in the stress-strain curve.

At the same prestress level, H estimated from the oscillatory data remained approximately the same after treatment with elastase, whereas it increased significantly after treatment with collagenase. Moreno et al. (26) found that H in elastase-treated strips remained unchanged at a fixed low prestress level, which is in agreement with our results. However, they also found that, after elastase treatment, H increased by up to several fold in magnitude at a fixed high prestress level. Because of disruption of elastin fibers, the tissue strips had to be stretched to a larger length to reach the same prestress as in control. As a result, recruitment of intact collagen fibers under a larger degree of extension most likely dominated tissue elasticity at the macroscopic level. From this analysis, one would expect a decrease in tissue elasticity when the tissue strip was treated with collagenase. Surprisingly, however, at the same prestress, H also increased after collagenase treatment.

We can better understand the difference between incremental tissue elasticity at the same mean length and at the same mean stress by developing a simple continuum model of the fiber network. Let us consider a system composed of a spatial distribution of elastic springs in parallel with each characterized by its incremental spring constant $k = k(x, y; \varepsilon)$. Here $x$ and $y$ are the coordinates along the cross-sectional area ($A$) of the strip, and $\varepsilon$ is the strain in the $z$ direction. We also assume that the incremental modulus $Y$ of the strip explicitly depends on $\varepsilon$, because the tissue displays an exponential stress-strain curve (34). If the density of springs, i.e., the number of springs per unit $A$ is $\rho(x, y)$, then $Y$ is given by
can be written as a stress-strain curve, digestion. Due to the exponential nonlinearity of the material, the concentration, and so on. After digestion, the fibers are stretched more and their mean stress before and after digestion, then the strain will increase after digestion. This is reflected in the particular stress relaxation behavior or the frequency dependence of the complex modulus. When a step in strain is applied to the tissue, the stress follows a slowly decaying power law over many time decades (3, 29, 35). This type of behavior is equivalent to a frequency dependence of the modulus that is consistent with our constant-phase model in Eq. 4. Indeed, the constant-phase model fits the data reasonably well under all of the conditions we studied. More importantly, the exponent \( \beta \) of the power law in Eq. 4, which is related to the \( \eta \) of the material, changed very little following alterations of the extracellular matrix constituents. In other words, parameter \( G \) behaves very similarly to parameter \( H \) under all conditions: at the same mean length, the \( G \) decreased significantly after elastase and collagenase treatment, whereas, at the same prestress, it remained the same after treated with elastase and increased significantly after collagenase treatment. This resulted in a small decrease in \( \eta \) after elastin digestion. The elastin digestion has an effect on the elastin fibers, which are viscoelastic. On the other hand, collagen is almost purely elastic. Thus, if the contribution of elastin fibers to the macroscopic viscoelasticity decreases, one would expect that the tissue would become more elastic. Nevertheless, changes in \( \eta \) were small compared with changes in \( G \) and \( H \). This can be visualized by plotting the relative change in \( G \) against the relative change in \( H \) for the various conditions. Figure 6 shows that, indeed, changes in the dissipative properties always follow changes in the elastic properties of the fiber network. In Fig. 6, we also show data from our previous study using methacholine challenge (41). The data suggest that, irrespective of the manner in which we alter tissue elasticity (e.g., challenge of interstitial cells, passive length change, digestion of elastin or collagen), \( G \) always follows it. This, in accord with Fredberg and Stamenovic (9), reflects the very fundamental property of lung tissue as a composite matter.

The origin of this peculiar hysteretic behavior of lung tissue is still speculative. Fredberg et al. (8) proposed that cross-bridge cycling within the contractile cells in the parenchyma plays an important role in tissue \( \eta \). Although it has been demonstrated that cross-bridge cycling may affect tissue hysteresis during active cell contraction induced by various types of agonists (8), it does not seem to be important in baseline conditions.

\[
Y(\varepsilon) = \int_A \rho(x, y)k(x, y; \varepsilon)dx\,dy \quad (8)
\]

For simplicity, we assume that the system is homogeneous, i.e., \( k \) and \( \rho \) are the same everywhere inside the tissue, so that Eq. 8 reduces to

\[
Y(\varepsilon) = A\rho_0 k(\varepsilon) \quad (9)
\]

where \( \rho_0 \) denotes the equilibrium density of fibers. Suppose now that the effect of digestion on the tissue is to uniformly reduce the density of fibers in the tissue. We express this mathematically by explicitly making \( \rho \) a function of time

\[
\rho(t) = f(t)\rho_0 \quad (10)
\]

where \( f \) is the fraction of remaining fibers after a digestion period of \( t \) and depends on the fiber type (e.g., elastin or collagen), the actual enzyme and its specificity, the concentration, and so on. After digestion, Eq. 9 can be written as

\[
Y(\varepsilon, t) = Af(t)\rho_0 k(\varepsilon) \quad (11)
\]

Equation 11 is now in a form suitable to discuss our results. If we compare the incremental moduli at the same mean length before and after digestion, then \( \varepsilon \) is the same in Eq. 11 for the control and the digested strip. However, because \( f(t) < 1 \), \( Y \) after digestion must be smaller than in control. If we compare \( \bar{Y} \) at the same mean stress before and after digestion, then the strain \( \varepsilon_1 \) before digestion is smaller than the strain \( \varepsilon_2 \) after digestion. Due to the exponential nonlinearity of the stress-strain curve, \( k(\varepsilon_1) < k(\varepsilon_2) \). Thus, after digestion, the fibers are stretched more and their \( k \) is higher. However, we also have a decreased fiber density. Therefore, if the increase in \( k \) due to an increase in strain from \( \varepsilon_1 \) to \( \varepsilon_2 \) is larger than the decrease in \( f(t) \), the macroscopic \( \bar{Y} \) will increase after digestion. This is the case for collagenase digestion (Fig. 4). Alternatively, when the increase in \( k \) is balanced by a decrease in fiber density, then the macroscopic incremental \( \bar{Y} \) can take similar values before and after digestion just like in our data following elastase treatment (Fig. 3). Thus the competing effects of decreasing fiber density and stretching the remaining fibers will determine the incremental moduli in any given condition.

**Tissue Hysteretic Behavior**

The viscoelastic properties of lung tissue are reflected in the particular stress relaxation behavior or the frequency dependence of the complex modulus. When a step in strain is applied to the tissue, the stress follows a slowly decaying power law over many time decades (3, 29, 35). This type of behavior is equivalent to a frequency dependence of the modulus that is consistent with our constant-phase model in Eq. 4. Indeed, the constant-phase model fits the data reasonably well under all of the conditions we studied. More importantly, the exponent \( \beta \) of the power law in Eq. 4, which is related to the \( \eta \) of the material, changed very little following alterations of the extracellular matrix constituents. In other words, parameter \( G \) behaves very similarly to parameter \( H \) under all conditions: at the same mean length, the \( G \) decreased significantly after elastase and collagenase treatment, whereas, at the same prestress, it remained the same after treated with elastase and increased significantly after collagenase treatment. This resulted in a small decrease in \( \eta \) after elastin digestion. The elastin digestion has an effect on the elastin fibers, which are viscoelastic. On the other hand, collagen is almost purely elastic. Thus, if the contribution of elastin fibers to the macroscopic viscoelasticity decreases, one would expect that the tissue would become more elastic. Nevertheless, changes in \( \eta \) were small compared with changes in \( G \) and \( H \). This can be visualized by plotting the relative change in \( G \) against the relative change in \( H \) for the various conditions. Figure 6 shows that, indeed, changes in the dissipative properties always follow changes in the elastic properties of the fiber network. In Fig. 6, we also show data from our previous study using methacholine challenge (41). The data suggest that, irrespective of the manner in which we alter tissue elasticity (e.g., challenge of interstitial cells, passive length change, digestion of elastin or collagen), \( G \) always follows it. This, in accord with Fredberg and Stamenovic (9), reflects the very fundamental property of lung tissue as a composite matter.

The origin of this peculiar hysteretic behavior of lung tissue is still speculative. Fredberg et al. (8) proposed that cross-bridge cycling within the contractile cells in the parenchyma plays an important role in tissue \( \eta \). Although it has been demonstrated that cross-bridge cycling may affect tissue hysteresis during active cell contraction induced by various types of agonists (8), it does not seem to be important in baseline conditions.
because we found that cross-bridge cycling contributes at most 10% to total tissue hysteresis (41). An alternative explanation is the fiber-fiber interaction mechanism described by Mijailovich et al. (25). In this model, the fibers are in close contact, and the load between the fibers is transferred by a stick-and-slip motion. The friction generated between the fibers is considered as the primary source of tissue hysteresis. This model provides predictions that are in reasonable agreement with the measured H and η. Although it is still uncertain whether physical contacts exist between fibers in the lung parenchyma, Brown et al. (4) provided some experimental evidence using electron microscopy that, in certain connective tissue structures such as ligamentum propatagiale, elastin and collagen fibers are in close contact, and hence they may be mechanically connected in parallel. Suki et al. (35) suggested that a mechanistic basis for the power law stress-relaxation behavior is a consequence of the so-called “reptation” motion of the collagen-elastin fibers, whereby the fibers rearrange through a series of highly constrained “wormlike” displacements or undulations under the influence of external stresses. In this picture, the distribution of fiber diameter and length is an important factor in determining the power law type of stress relaxation. Our data suggest that tissue η and hence the stress relaxation exponent were marginally affected by fiber digestion. Thus the reptation model would contradict our data if the enzymes induced fragmentation of the fibers and hence alterations in fiber concentration and distribution of fiber width and length. However, Morris and Stone (27) showed in electron microscopic images that elastase induced small holes within the elastin fibers without removing parts of the fibers. Whereas a collagen molecule is cleaved by collagenase at a specific site leading to the production of two fragments, considering that a collagen fiber consists of a large number of cross-linked tropocollagen molecules, fragmentation of parts of these molecules may not lead to complete cleavage of an entire collagen fiber. Thus, during digestion, the fibers would be damaged and losing elasticity, but the actual mechanism of fiber motion may not be significantly altered. Therefore, our data do not exclude, neither do they support, fiber reptation as the mechanism for tissue η. If fiber fragmentation does occur after digestion, then it is likely that the cross linking between fibers and the ground substance may play an important role in lung tissue resistance. Stromberg and Wiederhielm (34) argued that the stress relaxation could be considered a consequence of delayed alignments of the fibers with macroscopic stress. This delay would result from the resistance against the movement of the fibers within the dense, viscous ground substance.

Tissue Nonlinearities

Lung tissue displays two distinct types of nonlinearities: nonlinear elastic properties characterized by the quasi-static stress-strain curve (Refs. 20, 33; Fig. 1) and dynamic nonlinearities characterized by the dependence of the dynamic moduli on strain amplitude (Refs. 24, 41; Figs. 3 and 4). Of particular interest is the fact that the nature of static and dynamic nonlinearities is opposite: the incremental k along the quasi-static stress-strain curve is an increasing function of stress and strain, whereas the dynamic nonlinearity is a decreasing function of strain amplitude around a fixed operating point. The quasi-static nonlinearity is likely related to the recruitment-like behavior of collagen fibers (20, 21, 30) during stretching due to the highly nonlinear stress-strain behavior of collagen itself (34). The origin of the dynamic nonlinearity is still not clear. It is likely that the nonlinearly elastic collagen, the linearly viscoelastic elastin, and the viscous ground substance all contribute to this phenomenon. Several models have been suggested to account for this type of nonlinearities. For example, various cascade combinations of linear viscoelastic and nonlinear elastic subsystems were applied to both whole lung pressure-volume (19, 37) and tissue strip stress-strain data (22, 42). Although these models can fit the data well, they are phenomenological in nature. Recently, Bates (2) derived a nonlinear model of lung tissue rheology in which the fibers in the tissue are modeled as rigid rods. When a step in strain is applied to the tissue, the fibers rearrange with an instantaneous elastic response, and, with time, the original random orientation of the fibers is restored via diffusion, which gives rise to stress relaxation. The total stress response of this model is nearly separable in instantaneous stress and time-dependent relaxation, and hence the model provides a possible mechanistic basis of Fung’s (10) quasi-linear viscoelastic theory. Although this model is able to account for the nonlinearities in the instantaneous elastic response of the tissue during a stress relaxation.
experiment, it fails to account for the dynamic nonlinearities.

Our present findings suggest that dynamic nonlinearities increase after a change in the structure of the extracellular matrix. At the same mean stress, the $k_d$ increased considerably after collagenase treatment and slightly after elastin digestion. In our previous study, we postulated that increases in tissue dynamic nonlinearities following methacholine challenge were a consequence of stiffening of the fiber network induced by cell contraction (41). If this were true, any increase or decrease in $H$ would be followed by an appropriate increase or decrease in $k_d$. In other words, one might expect a similar coupling between elasticity and nonlinearity as the coupling between $G$ and $H$ shown in Fig. 6. However, whereas at the same mean length $H$ decreased after either digestion, we never found a decrease in $k_d$ that seemed to violate this coupling. Indeed, plotting the relative change in $k_d$ as a function of the relative change in $H$ in Fig. 7 demonstrates that there is no single linear relationship between these variables. Fig. 7 also shows our previous data of $k_d$ and $H$ after methacholine challenge of the tissue strips. A striking feature of the data is that, when the $H$ of the tissue is changed via a passive change in mean stress or an active cell contraction, the data follow a well-defined trajectory with a positive slope. However, when the fiber network is biochemically and hence structurally altered, the data follow an apparently orthogonal trajectory. Recall that macroscopic elasticity is determined by the number of fibers and their incremental $H$ (see Eq. 11). It may be that the elasticity at the same mean length is mostly determined by the number of intact fibers and not by the nonlinear stress-strain curve of the collagen. After digestion, the number of intact fibers decreases. Thus the corresponding $H$ decreases too, and the points on the graph must move to the left. On the other hand, because the remaining collagen fibers operate at a slightly higher mean length, the system becomes more nonlinear and $k_d$ moves north. This decoupling of elasticity and nonlinearity may prove very useful in detecting biochemical and/or structural alterations in the connective tissue matrix due to the presence of interstitial diseases.

In summary, our findings suggest that, after digestion 1) the fraction of intact fibers decreases; 2) at the same mean length, a decrease in the number of intact fibers leads to a decrease in dynamic moduli; 3) at the same mean force, the collagen fibers become more stretched than at the same mean length, and hence they operate at a higher portion of the nonlinear stress-strain curve, which results in an increase in the dynamic moduli; and 4) whereas damping and elasticity appeared to be always coupled, dynamic nonlinearity was decoupled from elasticity after digestion and was sensitive to the stretching of collagen. This latter fact may find important implication in detecting structural alterations in the connective tissue of the lung.

This study was supported by National Heart, Lung, and Blood Institute Grants HL-59215–01A1 and HL-62269.

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