Contribution of collagen matrix to passive left ventricular mechanics in isolated rat hearts

DEIDRE A. MACKENNA, JEFFREY H. OMENS, ANDREW D. MCCulloch, AND JAMES W. COVell

Departments of Medicine (Cardiology) and Applied Mechanics and Engineering Sciences (Bioengineering), University of California, San Diego, La Jolla, California 92093

MacKenna, Deidre A., Jeffrey H. Omens, Andrew D. McCulloch, and James W. Covell. Contribution of collagen matrix to passive left ventricular mechanics in isolated rat hearts. Am. J. Physiol. 266 (Heart Circ. Physiol. 35): H1007–H1018, 1994.—Although it makes up only 2–6% of left ventricular dry weight, collagen is thought to be the major structural protein determining passive ventricular stiffness. However, the relationship between structure of the extracellular matrix and passive mechanics is not understood. Hence, to deplete the collagen matrix, 16 rat hearts were perfused with bacterial collagenase for 60 min. Quantitative morphology using picrosirius red revealed a 36% decrease in collagen area fraction predominantly in the medium-sized fibers. Scanning electron microscopy revealed damage to the endomysial struts. Passive pressure-volume curves showed increases in left ventricular volume at all pressures (from 0.203 ± 0.061 to 0.265 ± 0.061 ml at 5 mmHg, P < 0.0001). Strain during loading, calculated from lengths obtained from a triplet of piezoelectric crystals, was unchanged with collagen depletion. However, remodeling strain computed from the collagenase-treated state referred to the Krebs solution-treated state at the same ventricular pressure showed both circumferential (0.145 ± 0.166 to 0.170 ± 0.158) and longitudinal (0.070 ± 0.120 to 0.068 ± 0.069) stretching. Sarcomere lengths increased at all depths (5.2% at midwall). Thus alterations in the extracellular matrix lead to increased ventricular volume and sarcomere lengths without altering ventricular compliance.

collagenase; diastolic function; remodeling; extracellular matrix; residual stress; picrosirius red

THE ULTRASTRUCTURE of the extracellular collagen matrix in the myocardium has been studied in detail (5, 32), and substantial information is available on the biochemistry and molecular biology of cardiac collagen (12, 28). Although it comprises only 2–6% of left ventricular (LV) dry weight in normal mammalian hearts (37), the extracellular collagen matrix is generally thought to be the major structural component contributing to the passive stiffness of the resting myocardium (35). Various pathophysiological states alter the extracellular collagen matrix as well as myocyte function (36, 40). A greater understanding of the role of collagen in myocardial mechanics may provide insight into functional alterations in these disease states.

The objective of this study was to acutely degrade the extracellular collagen matrix and measure changes in passive LV mechanics. Two-dimensional (2D) finite strain analysis has been used previously to quantify the local stretch and shear that occur during ventricular pressure loading (26). These scale-invariant measures represent local deformation during filling and can be used in continuum mechanics models to estimate passive material properties of intact myocardium (17, 30).

To deplete the collagen extracellular matrix, isolated arrested rat hearts were perfused with a low concentration of crude bacterial collagenase (29). The damage was assessed by using hydroxyproline assays, light microscopy, and scanning electron microscopy (SEM). To characterize passive ventricular mechanics, we measured local 2D strain as well as the global pressure-volume relationships. The mechanical measurements were made at least three times for each heart: before perfusion, after treatment with a noncollagenase-containing perfusate to control for the irreversible effects of perfusion alone, and finally after treatment with the collagenase solution. In this way, it was possible to measure directly the remodeling of the tissue as the collagen matrix was degraded.

Collagenase treatment disrupted the small and medium collagen fibrils and increased LV volume at all pressures but did not change the pressure-strain relationship. Furthermore, the plastic remodeling revealed stretching in the direction of the muscle, which was partially explained by increased sarcomere length. Therefore fibrillar collagen may be more important for maintaining LV geometry by constraining sarcomere length than by contributing to passive ventricular stiffness.

METHODS

Experimental preparation. Twenty-four Sprague-Dawley rats (350–450 g body wt) were anesthetized with pentobarbital sodium (100 mg/kg). The animals were ventilated with air, and the heart was exposed with a median sternotomy. The ascending aorta was clamped, and 2–3 ml of hypothermic hyperkalemic cardioplegic buffer were injected directly into the LV through the apex to arrest the heart. Care was taken to avoid overdistending the LV. The solution contained (in mM) 60 KCl, 118 NaCl, 27.2 NaHCO3, 1.0 KH2PO4, 1.2 MgSO4, and 11.1 glucose. 2,3-Butanedione monoxime (30 mM) and heparin (10,000 U/l) were also added to the solution to delay ischemic contracture and prevent coagulation, respectively. The heart was quickly excised, rinsed, and weighed. A 2.8-mm-diameter cannula with side perforations was secured in the aorta. Perfusion was visually verified as the blood was briefly flushed from the coronary vessels. The heart was immersed in excess perfusate during coronary perfusion. A purse-string suture around the mitral annulus was used to secure a stainless steel cannula with a small balloon attached that was inserted into the LV. The cannula was connected to a volume infusion pump (Harvard Apparatus) and a Statham P23 ID pressure transducer zeroed at the level of the heart (Fig. 1). The compliance of the balloon did not contribute to the measured cavity pressure at all LV volumes, which were always < 0.5 ml (30). A drainage tube was positioned in the LV apex to evacuate

0363-6135/94 $3.00 Copyright © 1994 the American Physiological Society H1007
Local and global ventricular mechanics: group II. Another 10 hearts were used to study 2D midwall strain as well as ventricular pressure-volume relations (group II). These hearts were also perfused with Krebs and collagenase solutions. To determine 2D midwall strain in the plane parallel to the epicardium, three miniature piezoelectric crystals (0.6–0.8 mm diam) were implanted in the lateral free wall (30). The crystals formed an approximately equilateral triangle with sides 3–6 mm long. By using a Triton sonomicrometer system (system 6 modified for 0.1-mm calibration and 0.5-mm minimum crystal separation), the ultrasonic transit times between each of the crystal pairs were converted into segment lengths, and these data together with the LV pressure and volume were acquired at 25 Hz as the heart was passively loaded. Data were recorded for four different times; pressure and volume data were obtained immediately after the hearts were isolated just as in group I. The crystals were then implanted in the midwall and, and the pressure, volume, and segment length data were again recorded. Data were then obtained after the heart was perfused with the Krebs solution for 15 min. The hearts were then perfused with the collagenase solution for 60 min total. Every 15 min, perfusion was stopped, and the heart was loaded through 10 cycles replicating the previous protocol; however, this was performed while the heart was still submerged in the perfusate so as not to disturb the piezoelectric crystal wires. After 60 min of collagenase perfusion, the heart was removed from the bath, the ventricle was preconditioned, and pressure, volume, and segment lengths were again acquired. The heart was kept moist with perfusate during the data acquisition periods.

2D midwall strains referred to the zero-LV pressure state were computed from the segment lengths with respect to circumferential and longitudinal axes. Each of the three length signals obtained during the inflation portion of the loading cycle was fitted by least squares as a cubic function of LV volume (30). Finite strain components were calculated directly from lengths interpolated at 0.05-mL increments of LV volume by using the quadratic form that defines the symmetric Green’s strain tensor $E_{ij}$ (16)

$$\frac{ds^2 - dS^2}{dS^2} = 2E_{ij}dX_i dX_j , \quad i,j = 1...2$$

where $dS$ is the length in the reference state, $ds$ is the corresponding length in the deformed state. In this case, $X_1$ is the circumferential coordinate $\Theta$, and $X_2$ is the longitudinal coordinate $Z$. Hence from this homogeneous strain analysis we obtained the circumferential and longitudinal extensional strain components and the torsional shear strain component over the range of LV filling pressures.

In addition to the strain due to loading, we defined the remodeling strain as the change in configuration from one treatment state to another at matched LV pressure loads. Because there were three time points where length signals were acquired, there were three possible combinations of states defining different remodeling strains. For statistical comparisons, we defined remodeling strain in each of the perfusion-treated states referred to the same untreated state before perfusion i.e., strain after Krebs treatment referred to the unperfused state, and strain after collagenase treatment referred to the unperfused state. Third, to examine the deformation of the tissue independent of changes due only to perfusion treatment, another remodeling strain was defined by the configuration of the crystals after collagenase treatment referred to the Krebs-treated state.

Biochemistry and ultrastructural evaluation. After the mechanical tests, the hearts were prepared for biochemical and histological evaluation. The heart was weighed again to estimate the amount of edema. The right ventricle was removed.
and frozen for biochemical analysis. The severed right coronary artery branches were occluded, and the remaining tissue was perfusion fixed and stored overnight in glutaraldehyde (2.5% in phosphate buffer). Crystal location and triangle orientation were measured on the fixed LV. An equatorial cross-sectional ring that contained the crystal triangle was used to measure collagen area fraction, sarcomere length, and myofiber angle. In four hearts, the remaining tissue was used for ultrastructural examination with SEM.

The right ventricles of 14 collagenase-treated hearts were analyzed for collagen content by using a hydroxyproline assay. Fourteen additional sex- and weight-matched nonperfused control rats were killed, and the hydroxyproline contents of the right ventricles were used as control values. The samples were dried and weighed to determine tissue dry weight, hydrolyzed in 6 N HCl at 110°C for 24 h, and analyzed for hydroxyproline concentration as described by Bergman and Loxley (1). Collagen concentration was estimated assuming that hydroxyproline composes 14% of the collagen molecules. No correction was made for the contribution of elastin to the hydroxyproline concentration. (All results are presented as μg collagen/mg dry heart wt.)

The tissue ring from the center of the LV was embedded in paraffin, and 10-μm sections were cut parallel to the epicardium from the center of the LV free wall. Ten to fifteen sections were obtained at 200-μm intervals through the entire thickness of the wall and stained for 90 min with picrosirius red 0.1% Sirius Red F3BA in saturated picric acid), which is specific for collagen when viewed with polarized light microscopy (20). Picrosirius red provides high-contrast images by enhancing the birefringence of the fibrillar collagen, and different colors are associated with different fiber sizes (10). A low-power bright-field view (about ×10 magnification) was acquired with an image analysis system (Data Translation DT-2255 with Apple Macintosh IICi host) and printed as a black-and-white half-tone image. A standard grid of five equally spaced points was drawn on the center of the micrograph, and the microscope stage was positioned using the location of local anatomic markers. Regions near large vessels were excluded. The tissue was examined with polarized light under higher power (×40 objective) and the section rotated on a circular stage until the autobirefringence of the muscle was extinguished (~45° from the polarizing axis). Color images (24-bit; JVC color charge-coupled device camera) of the bright-field and polarized views were acquired for each location. The color images were recoded into hue (dominant wavelength), saturation (color purity), and brightness images (14). The area of birefringent collagen was determined by thresholding the brightness channel of the polarized image, and the total area of tissue in each view was determined from thresholding the saturation channel of the bright-field image. The associated colors of the birefringent collagen were determined from the corresponding pixels on the hue channel. The total collagen area fraction was calculated as the ratio of the birefringent collagen area to tissue area in each view. Collagen area fraction was measured in five views from each of five sections from five collagenase-treated and five control hearts where four of the controls were Krebs treated and one was a sex- and age-matched nonperfused control heart.

By use of the same sectioning and staining protocol, the transmural distribution of sarcomere length was measured in eight collagenase-treated and eight control hearts, where the controls were four Krebs-treated and four sex- and weight-matched nonperfused controls. The lengths of 15 groups of 10 adjacent sarcomeres were measured in digitally acquired images from the center of sections using either bright-field or polarization microscopy under high power (×100 objective) for 10–12 sections in each heart. The system was calibrated by using an objective micrometer with 10-μm spacing that yielded a magnification factor of 8.02 pixels/μm. Muscle fiber angle was measured with respect to the circumferential axis in eight collagenase-treated hearts by using a microscope eyepiece calibrated in 1° increments.

For SEM, the glutaraldehyde-fixed tissue was postfixed in 2% OsO₄ and then dehydrated in a series of graded ethanol. The tissue was then critical point dried with liquid CO₂ as the intermediate fluid. The slices were mounted on stubs so that the equatorial plane could be viewed with the microscope, and the samples were coated with 180–200 nm of gold. The specimens were photographed with a Cambridge 360 scanning electron microscope at a variety of magnifications (×100–5,000) to examine the amount and organization of the collagen ultrastructure. Regions of samples with preparation artifact were avoided. The observer (D. A. MacKenna) was not blinded to the source of the tissue.

Statistical analysis. LV volume and strain measurements were analyzed with a two-factor repeated-measures analysis of variance (ANOVA) with LV pressure and perfusion treatment (i.e., no perfusion, 15 min Krebs, and 60 min collagenase) both as repeated factors. A nested ANOVA design was used for collagen area fraction to evaluate the effect of treatment (Krebs vs. collagenase treatment) and variation between individual hearts. Myocyte sarcomere length data were analyzed by analysis of covariance with transmural location as the covariate and treatment (Krebs vs. collagenase) as the independent factor. Statistical significance was set at the 95% confidence level (P = 0.05). To determine the effects of specific treatments, post hoc contrasts were made between the means of the data, and Bonferroni corrections were applied to account for multiple comparisons.

RESULTS

The mean body weight was 388 ± 34 (SD) g for the six group I rats and 404 ± 30 g for the 10 group II rats with mean heart-to-body weight ratios of 3.51 ± 0.24 and 3.21 ± 0.11 g/kg, respectively. The eight Krebs-perfused external control rats had a mean body weight of 402 ± 46 g. The data acquisition times and volume of perfusate were similar for both groups I and II (Table 1). After perfusion, the 16 group I and II hearts exhibited a mean 23.5 ± 20.1% increase in heart weight. In three of the eight Krebs-perfused external control hearts, the preperfusion heart weights were not recorded. However, the remaining five hearts increased 25.0 ± 17.5% during the

<table>
<thead>
<tr>
<th>Table 1. Data acquisition times and perfusion flow rates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>Cumulative time after arrest, min</td>
</tr>
<tr>
<td>Before perfusion</td>
</tr>
<tr>
<td>Crystal implant</td>
</tr>
<tr>
<td>60 min collagenase</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 and 10 hearts in groups I and II, respectively. Values before perfusion were measured immediately after hearts were excised and balloon was inserted into ventricle. Perfusion flow rate was estimated from fluid that overflowed from containing dish. Krebs and collagenase perfusions were performed for 15 and 60 min total, respectively.
course of the 60 min of perfusion. This was not statistically different from the group I and II hearts (P > 0.7 by Student’s t test).

In the 10 hearts used for strain analysis, the mean apex-to-base distance was 14.0 ± 1.0 mm, and the circumferential location of the crystal triangle was 25 ± 35° counterclockwise from the estimated center of the LV free wall as determined by extending a line from the midpoint of the septum through the center of the LV. The mean wall thickness measured from the fixed tissue in the unloaded state was 3.5 ± 0.5 mm, and the outer diameter was 12.0 ± 0.5 mm.

Right ventricular hydroxyproline content was 24 ± 6 μg collagen/mg tissue dry wt in collagenase-treated hearts vs. 27 ± 3 μg collagen/mg tissue dry wt in sex- and weight-matched controls. This 12% change in collagen did not achieve statistical significance (P = 0.16). However, collagen area fraction measured using quantitative morphometry showed a significant 36% decrease in birefringent fibrillar collagen stained with picrosirius red (2.1 ± 1.2 and 3.3 ± 1.8% for collagenase- and Krebs-treated hearts, respectively; P < 0.05). The 256 colors/hues were lumped into five 32-hue categories with median colors defined as red, orange, yellow, green, and teal (Fig. 2). Visual verification of the composite signal revealed that the remaining signal (blue-magenta; hue range 135–231) was extracellular background noise (vessel lumen, sectioning artifact, and so forth). The area fraction of orange and yellow fibers decreased significantly with collagenase treatment, indicating a decrease in medium-sized fibers (Fig. 3). The red, green, and teal fibers (large and small) tended to decrease in area fraction, but the difference was not statistically different. Nested ANOVA showed that the variation between individual samples was statistically significant (P < 0.001, P < 0.001, and P < 0.005, for red, green, and teal fibers, respectively), which may decrease the power of the measurement. In contrast, the background pixels were not statistically different either between treatments or hearts, indicating that the method was consistent for all hearts. Qualitative evaluation of the scanning electron micrographs of the collagenase-treated tissue revealed damage to all portions of the collagen extracellular matrix. However, the damage was most dramatic in the smaller endomysial weave and struts (Fig. 4). This agrees well with the 24% decrease in green and teal fibers by the picrosirius-polarization method. In all samples, there were regions where normal collagen ultrastructure was evident; however, the overall appearance indicated a loss of collagen more pronounced in these smaller fibers. The larger perimysial tendons were evident, but their structural integrity could not be evaluated with this method.

The pressure-volume curves for the eight hearts perfused only with Krebs solution for 1 h demonstrated a shift to the left with Krebs perfusion (mean LV volume decreased from 0.277 ± 0.054 to 0.250 ± 0.045 ml at 10 mmHg, P < 0.02 for whole curve). In contrast, the curves of the collagenase-perfused hearts shifted to the right after each of the four 15-min intervals as shown in Fig. 5 for one heart. The effect of perfusion treatment on the LV volume for the six group I hearts was statistically significant (P < 0.0001). Table 2 summarizes the results of the post hoc contrasts between the mean LV volumes after the different perfusion treatments. The mean LV volume after collagenase perfusion for longer than 15 min was significantly greater than before perfusion or after 15 min Krebs perfusion. LV volume after 15 min of perfusion with Krebs was not different from that before perfusion. Statistical analysis of the pressure-volume data for the 16 hearts included in both groups I and II (Fig. 6) led to the same conclusions. Post hoc contrasts showed that the volume after 60 min collagenase treatment was significantly different from either 15 min Krebs treatment or before any treatment but that volume after 15 min Krebs treatment was not significantly different from that before treatment (P > 0.9).
Typical 2D midwall strains in one heart computed from the segment lengths during passive inflation are shown in Fig. 7 as a function of ventricular volume at three different times (before perfusion, after 15 min Krebs treatment, and after 60 min collagenase treatment). In all hearts, circumferential and longitudinal strains were positive and torsional shear strain was negative. The mean results for the 10 group II hearts are given in Table 3, and the strains after Krebs and collagenase treatment are shown in Fig. 8. Repeated-measures ANOVA did not show significant differences in any of the mean strain components between perfusion treatments ($P > 0.3$ for each strain component).

In 8 of the 10 group II hearts, the crystal signals did not require significant adjustments to the gain or trigger levels; hence it was possible to calculate remodeling strains from one time to another (before perfusion, 15 min Krebs treatment, and 60 min collagenase treatment) at matching LV pressures. To determine whether these strains were similar to the shift in the pressure-volume relationship, the absolute change in LV volume was calculated in the same eight hearts. To examine the effect of collagen depletion statistically, strain and volume change were calculated at both perfusion-treated states (Krebs and collagenase) referred to the preperfusion state. However, to isolate the effects of the collagen degradation, independent of the influence of perfusion, only the changes that occurred during the 60-min collagenase period were examined where remodeling was defined as the final postcollagenase state referred to that after Krebs perfusion.

Compared with the volume before any treatment, 60 min collagenase treatment increased LV volume by an average of 0.055 ± 0.039 ml (including 0.032 ± 0.030 ml at 0 mmHg LV pressure). This volume shift was signifi-

![Graph showing pressure-volume curves](image)

**Fig. 5.** Typical pressure-volume curves for a group I heart after 6 treatments. After perfusion with Krebs solution for 15 min, curve shifted slightly to left, but difference was not statistically significant for average of 6 hearts. In contrast, treatment with collagenase caused curve to shift progressively right.

| Table 2. Statistical probabilities for pressure-volume data as a function of treatment in group I hearts |
|----------------------------------------|--------|--------|--------|--------|--------|
| Before Perfusion | 15 min Krebs | 15 min Collagenase | Collagenase 30 min | Collagenase 45 min |
| 15 min Krebs | NS | Collagenase | Collagenase | Collagenase |
| 15 min collagenase | NS | NS | NS | NS |
| 30 min collagenase | $P < 0.05$ | $P < 0.05$ | NS | NS |
| 45 min collagenase | $P < 0.01$ | $P < 0.01$ | NS | NS |
| 60 min collagenase | $P < 0.01$ | $P < 0.01$ | $P < 0.05$ | NS | NS |

$n = 6$ group I hearts. Data were analyzed by 2-way analysis of variance with both perfusion protocol and volume as repeated measures. Post hoc contrasts were made by comparing means and correcting for multiple comparisons by using a Bonferroni correction factor. NS, not significant.

![Scanning electron micrographs](image)

**Fig. 4.** Scanning electron micrographs of normal (A) and collagenase-perfused (B) rat hearts. Note substantially decreased number of endomysial collagen mesh and intermyocyte struts in collagenase-perfused tissue. Larger perimysial collagen fibers are still present although difficult to quantify. Original magnification of both micrographs, ×5,000.
In the eight group II hearts evaluated, myofiber angle had a linear transmural variation ($r^2 = 0.940 \pm 0.045$) that varied from $-56 \pm 12^\circ$ on the epicardium to $45 \pm 23^\circ$ on the endocardium (estimated by linear regression through all the measurements). At the midwall, i.e., the location of the strain measurement, the mean muscle fiber angle was $-4 \pm 12^\circ$; hence, the midwall circumferential strain component was considered to be the fiber strain and the longitudinal strain the cross-fiber strain. Similarly, the sarcomere length extension, which is in the direction of the muscle fiber, corresponded to circumferential stretching.

Remodeling strain was calculated in the collagenase-treated state referred to the Krebs-treated state at matching LV pressure (Fig. 9B). All three components of this remodeling strain were independent of LV pressure, indicating that collagenase perfusion changed the local shape of the ventricle but not the elastic properties of the myocardium. Table 4 summarizes the statistical comparisons of the Krebs and collagenase remodeling strain referred to the preperfusion state. The circumferential and longitudinal remodeling strain components were all positive and significantly larger with collagenase perfusion but were independent of LV pressure. The torsional shear remodeling strain was not significantly affected either by collagenase treatment or pressure. Linear regression of the remodeling strain components using a no-intercept model revealed significant correlation for circumferential and longitudinal remodeling vs. volumetric strain (volume after collagenase treatment/volume after Krebs treatment - 1) at all LV pressures ($P < 0.05$ for all pressures). The shear remodeling did not correlate with volumetric strain ($P > 0.3$ for all pressures).

Mean sarcomere length was longer in eight collagenase-treated hearts than in four Krebs-treated and four sex- and weight-matched controls (Fig. 10). The transmural gradient decreased 22% with collagenase treatment ($-3.8 \times 10^{-5} \pm 1.1 \times 10^{-5}$ and $-4.9 \times 10^{-5} \pm 9.7 \times 10^{-6} \mu$m sarcomere length/μm wall thickness for collagenase treated and controls, respectively). Analysis of covariance revealed a significant effect of both treatment ($P < 0.0001$) and position ($P < 0.0001$) with no significant interaction ($P > 0.4$).
Table 3. Mean strain during loading referred to zero LV pressure

<table>
<thead>
<tr>
<th></th>
<th>Before Perfusion</th>
<th>15 min Krebs</th>
<th>60 min Collagenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_{50}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 mmHg</td>
<td>0.065 ± 0.043</td>
<td>0.058 ± 0.036</td>
<td>0.088 ± 0.062</td>
</tr>
<tr>
<td>20 mmHg</td>
<td>0.114 ± 0.061</td>
<td>0.091 ± 0.055</td>
<td>0.131 ± 0.087</td>
</tr>
<tr>
<td>$E_{25}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 mmHg</td>
<td>0.045 ± 0.031</td>
<td>0.050 ± 0.018</td>
<td>0.053 ± 0.042</td>
</tr>
<tr>
<td>20 mmHg</td>
<td>0.079 ± 0.060</td>
<td>0.089 ± 0.041</td>
<td>0.079 ± 0.064</td>
</tr>
<tr>
<td>$E_{90}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 mmHg</td>
<td>-0.028 ± 0.019</td>
<td>-0.026 ± 0.013</td>
<td>-0.028 ± 0.021</td>
</tr>
<tr>
<td>20 mmHg</td>
<td>-0.047 ± 0.031</td>
<td>-0.039 ± 0.019</td>
<td>-0.032 ± 0.026</td>
</tr>
</tbody>
</table>

Values are means ± SD. Circumferential ($E_{50}$) and longitudinal strains ($E_{25}$) are positive and increase with pressure but do not change with perfusion state. Shear strain ($E_{90}$) is negative, indicating a left-handed torsion.

**DISCUSSION**

This study was designed to provide insight into the relationship between the structure of the cardiac extracellular matrix and the mechanical properties of the resting myocardium. Based primarily on ultrastructural observations of normal and diseased tissue, it has been proposed that the cardiac extracellular matrix prevents overstretching of myocytes during filling by increasing passive stiffness at higher pressures (35) and facilitates capillary alignment and patency during systolic contraction (5). However, few have developed methods to intervene with the normal collagen ultrastructure in healthy hearts and to make measurements to test these ideas (7, 29). In this study, we used bacterial collagenase to degrade the collagen network in the isolated arrested rat heart and measure changes in both global filling and local deformation of the LV myocardium. With collagen depletion, the LV enlarged at all pressures, but the passive material stiffness, as indicated by the pressure-strain relation, did not change. Damage induced by the bacterial collagenase primarily affected the endomysial and medium-sized fibers and led to increased unloaded LV volume and sarcomere length. Thus the unexpected result of the present study is that it is possible to damage the cardiac extracellular matrix significantly without altering either the slope of the pressure-volume relationship or the local in-plane tissue compliance.

**Collagen degradation.** Although the decreases in hydroxyproline concentration were small, the ultrastructural organization of the fibrillar collagen was substantially altered as observed by light and electron microscopy. Caulfield and Wolkowicz (7, 8) and Matsubara et al. (25) have used the sulphydryl agents 5,5'-dithiobis(2-nitrobenzoic acid) and oxidized glutathione (GSSG) to activate endogenous collagenases for both acute and chronic dissolution of collagen. Their results were similar to the present findings, where the changes in the extracellular collagen matrix were primarily apparent in the endomysial struts and weave.

Although collagen fibers are the easiest components of the extracellular matrix to view with SEM, there are several other components that compose the cardiac interstitium. Glicosaminoglycans and other glycoproteins are closely associated with collagen (28). These molecules are highly hydrophilic and help to maintain water content. Preliminary studies using a purified form of bacterial collagenase (PN C-0773, Sigma Chemical) and the protocol for the group I hearts did not always lead to changes in the pressure-volume curves or noticeable differences in SEM. This purified bacterial collagenase was filtered and recycled for several experiments. Only after perfusion through two or three hearts were changes in passive mechanics observed. Examination of the protein content of the perfused effluent showed that after successive recycling, other large molecules were present in the perfusate that may have aided in collagen degradation. We found that it was necessary to use a collagenase that contained a very low concentration of nonspecific protease (PN C-9891, Sigma Chemical) to obtain repeatable results. This indicates that the glycosaminoglycans or other structural proteins probably protect the cleavage sites required by the collagenase enzymes. The low levels of protease did not have adverse

---

![Fig. 8. Mean ± SE strain components at 5 left ventricular (LV) pressures, shown after 15 min Krebs perfusion and 60 min collagenase perfusion. A: circumferential strain component. B: longitudinal strain component. C: torsional shear strain component. Changes were not statistically significant for any component, but small increases were observed in circumferential strain.](image-url)
Fig. 9. Mean ± SD increase in volume (A) and remodeling strains (B) for 8 rat hearts between collagenase- and Krebs-treated states. These changes in configuration were independent of pressure and reflect uniform shift of pressure-volume curves. Remodeling strain shows stretching in both circumferential and longitudinal directions without significant torsion.

Effects on the myocytes when viewed with either SEM or light microscopy (i.e., no contraction bands or cell rupture). Furthermore, because this product plus added trypsin is used for myocyte isolation (23), we conclude that damage to the myocytes is unlikely and feel confident that it has not altered the results. The concentration of collagenase used in this study (10 mg/l) was established through a series of sequential dilution experiments. When the concentration was an order of magnitude higher, the myocardium was macroscopically disrupted after 30–45 min of perfusion. Because the objective of this study was to achieve small but repeatable changes in the ventricular mechanics, the milder concentration was selected.

Experimental design: potential complications of edema. The prolonged crystalloid perfusion led to a

20–25% increase in heart weight either with or without the addition of collagenase. Because the edema was similar for both treatments, it is unlikely that the changes observed in either the structure or mechanics were due to edema. However, it is well known that crystalloid perfusion leads to changes in water content in most preparations (24, 38). In the heart, which is 80% water, a 4% increase in water content, measured from the wet-to-dry weight ratio, leads to a 25% increase in heart weight. Other preparations where isolated hearts were perfused with Krebs solution for extended periods have shown variable changes in water content that corresponded to an 11–57% increase in heart weight (13, 24).

A large accumulation of fluid in the interstitium could potentially damage the extracellular matrix, but this was probably not the case in the current study. In addition to the precautions mentioned, the similarly edematous Krebs-perfused hearts were used as the controls for the histology; hence, if edema had caused the damage, no difference would have been observed. Caulfield and Wolkowicz (7) have perfused isolated rat hearts for up to 3 h and did not observe differences from normal tissue. Although the change in heart weight was not reported, it is likely to have been similar to this and other studies using Krebs as a perfusate (2, 39). Also, the control values measured in this study for collagen area fraction are very similar to control values of other groups using picrosirius red (4, 31) or trichrome (19) to quantify changes in collagen.

Furthermore, fluid may have entered the myocytes and caused intracellular damage. Although this was not directly examined in the current study, sarcomere length was quantified in four edematous Krebs-perfused hearts and four nonperfused hearts as the control values, and these were not different from each other. Additionally, in both groups of the perfused hearts (Krebs and collagenase), sarcomere length was examined at 150 locations in each heart, and contraction

Table 4. Statistical probabilities for remodeling volume and strain using a 2-way repeated-measures analysis of variance

<table>
<thead>
<tr>
<th>Remodeling</th>
<th>Treatment</th>
<th>Pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV volume</td>
<td>$P &lt; 0.005$</td>
<td>$P &gt; 0.5$</td>
</tr>
<tr>
<td>$E_{θθ}$</td>
<td>$P &lt; 0.05$</td>
<td>$P &gt; 0.5$</td>
</tr>
<tr>
<td>$E_{zz}$</td>
<td>$P &lt; 0.05$</td>
<td>$P &gt; 0.9$</td>
</tr>
<tr>
<td>$E_{θz}$</td>
<td>$P &gt; 0.3$</td>
<td>$P &gt; 0.1$</td>
</tr>
</tbody>
</table>

Values are given for 8 of 10 group II hearts; 2 group II hearts were excluded because of difficulty with crystal signal on 1 channel. All data were calculated with experimental treatment state (Krebs or collagenase) referred to unperfused state at matching left ventricular (LV) pressure. All data are independent of LV pressure.
bands were not observed, further indicating that intracellular damage was minimal.

Finally, to assess the influence of the increase in LV mass on the findings, the equatorial region, where strain was measured, was modeled as a cylinder, and the 2D remodeling strain and changes in cavity volume were used to approximate the relative volume of the myocardium in different states. With it assumed that the crystals remained at the midwall, 90% of the increase in heart weight can be predicted by remodeling that occurs during the initial 15 min of Krebs perfusion. Preliminary studies in our laboratory to reduce the increases in heart weight observed with Krebs perfusion revealed that the majority of the heart weight increase occurs in the first 10–15 min of perfusion. Furthermore, use of various osmolarities of mannitol or dextran did not alleviate the edema. However, it was found that submerging the tissue in fluid tended to reduce the edema, but the mechanism for this is not understood. These findings regarding the rapid onset of edema in this preparation emphasize the need for the initial Krebs perfusion to act as the internal control state. It also indicates that the remodeling strains accurately express changes in local tissue shape due to collagen depletion and not edema.

**Histological and mechanical analysis.** The picrosirius red-polarization method has been used by many investigators to examine fibrillar collagen in the myocardium (4, 11, 31) and other tissues (20). The total collagen area fraction results from the control hearts agree closely with control values from other studies using picrosirius red (4, 31). Initially the different birefringence colors were thought to correlate with the different types of collagen (21), but more recently they have been shown to correlate with the different-sized fibers, where red are large and green are small fibers (10). Unfortunately, the quantitative fibril diameter-color correspondence is unknown because birefringence color may also be affected by section thickness (22) and surrounding medium (10), both of which were held constant for the current comparison. Furthermore, the nature of optical anisotropy is such that the resulting retardation colors repeat periodically as the difference between the fast and slow refractive indexes increases. Hence determining the order of the retardation, which could predict the relationship between the retardation and color spectrum, is difficult (34). Although the picrosirius red-polarization method has been used extensively, few studies have used quantitative image analysis (31), and none has segmented the color images to examine fibril size distribution. This new application allows examination not only of the overall changes in collagen but also of which fiber sizes were affected.

Significant changes were noted histologically for both sarcomere length and collagen area fraction. To properly interpret any changes in sarcomere length, it is important that all tissue is fixed at the same reference volume or pressure. In this study, the tissue was all fixed at 0 mmHg LV pressure, the same reference state as the strain during loading. Furthermore, to reduce the effects of transmural position on the interpretation, an analysis of covariance was used to analyze the data because it treats position as a continuous variable. Additional potential problems may have occurred with fixation or cellular injury. Perfusion fixation with glutaraldehyde, ethanol dehydration, and paraffin embedding are all known to cause tissue shrinkage (18). However, presumably this would have been similar for both histological groups, and the relative differences would remain. Additionally, these data are similar to those measured in rat hearts that have been immersion fixed with glutaraldehyde (33). Finally, contraction bands were not present over large regions of tissue, indicating that the fixation was relatively uniform and the myocytes were not injured from the edema, collagenase treatment, or tissue fixation.

Piezoelectric crystals have been used extensively to measure uniaxial segment length changes as well as 2D strain analysis (15) but have only recently been adapted to rat hearts (30). Although measurement of 2D finite strain has advantages over uniaxial strain measurements, there are some limitations when this method is applied to small animals. Although the crystals were small (0.6–0.8 mm), they still occupied about one-quarter of the ventricular wall thickness. Furthermore, verification of the exact placement of the crystals in the midwall was difficult. Another problem was the tissue damage that may occur around the crystal during perfusion. Accumulation of fluid around the insertion site may have introduced changes in the orientation or shape of the crystals during the experiment. However, in 8 of the 10 hearts, we found that 60 min perfusion with collagenase did not significantly alter the magnitude and shape of the received ultrasound pulse, and changes in segment length were consistent. In the remaining two hearts, adjustments in the signal amplification and triggering were needed to track one of the crystal signals. In these hearts the remaining two segment lengths increased as well. Moreover, the circumferential and longitudinal remodeling strain components showed significant correlation with relative changes in ventricular volume at all LV pressures. Consequently, these consistent correlations, together with the fact that the effect on the pressure-volume curves from the instrumented hearts (group II) was very similar to that from the noninstrumented hearts (group I), indicate that local tissue damage has not significantly affected the measurements.

**Mechanical changes.** Changes in passive LV mechanics were associated with changes in the ultrastructure of the collagen network. Perfusion of the LV with collagenase caused an increase in LV volume at all pressures. This shift was uniform over the range of LV pressures; hence the LV stiffness or slope of the pressure-volume curve did not change. This is consistent with the finding that strain referred to the zero-pressure state did not change. In contrast, treatment with Krebs solution alone for 1 h led to a slight stiffening effect that changed the slope of the pressure-volume curve as well as the LV volume. This stiffening, presumably due to edema, may have masked small changes in slope due to collagen degradation. It is possible that this edema, which would have increased LV stiffness and decreased ventricular
volume, may have balanced decreases in stiffness due to collagen degradation. It may also have reduced the increase in LV volume after collagenase perfusion.

Preconditioning after perfusion may also influence the pressure-volume curves. In this preparation, both perfusion and possibly submersion of the heart in perfusate led to an accumulation of fluid in the myocardium that necessitated preconditioning after each period of perfusion to ensure that all residual fluid had been evacuated from the ventricle through the vent. Additionally, ischemic contracture, which may also influence the pressure-volume relationship, was blocked with 2,3-butanedione monoxime, an agent shown to interfere with the Ca^{2+}-troponin interaction (27). Similar to the effect of edema, inadequate preconditioning or contracture would have masked the observed results by decreasing LV volume after perfusion.

Matubara et al. (25) observed changes in ventricular pressure-volume curves similar to those found in this study. At 4 wk after two intravenous infusions of GSSG, they observed a shift to the right in the pressure-volume curves as well as a 30% decrease in collagen area fraction. The GSSG apparently activated native collagenases that altered the ultrastructure most noticeably at the level of the small fibers. However, local deformations were not examined, and control data were obtained from age- and sex-matched controls rather than from the same hearts; thus changes in local shape were not assessed.

Local deformations were measured to study the effects of collagen degradation on the passive elastic stiffness as represented in the pressure-strain relationships. Borg et al. (3) estimated an elastic modulus in rat and hamster hearts at various ages by using a thin-walled sphere model and an exponential fit to the global pressure-volume curve. The amount of collagen observed with SEM was consistent with differences in the elastic moduli between species but did not reflect changes in these moduli that occurred with age. This indicates that the global pressure-volume curve may not be sufficient to estimate local tissue properties and reveal changes in material properties and ultrastructure. The changes in midwall strain during loading are a better measure of the change in material compliance than slope of the pressure-volume curve, because they are less dependent on ventricular geometry. In this study the changes were not statistically significant for either Krebs or collagenase treatment. However, the slight trend supports a mild stiffening in the fiber direction due to Krebs treatment and an increase in compliance with collagenase treatment. Although midwall strain and the slope of the pressure-volume curve did not change in the current study, LV volume increased with collagen degradation. This indicates that the geometry and structure of the ventricular wall were apparently altered without affecting the material properties. However, other preparations that lead to decreases in collagen density (i.e., β-aminopropionitrile administration or stunning) have shown decreases in myocardial compliance (9), which indicate that a more dramatic or different mechanism of collagen depletion may have enhanced the small trends in strain in the current preparation.

**Structural changes.** The changes observed with SEM and light microscopy suggest that the small and medium collagen fibers were most affected by collagenase treatment. Quantitative morphology using picrosirius red with polarization microscopy revealed a significant decrease in fibrillar collagen, primarily in the medium-sized orange and yellow fibers. SEM showed that this degradation was most pronounced in the endomysial collagen network and struts. A decrease in small collagen fibers could not be demonstrated with the picrosirius-polarization method because the small endomysial fibers are difficult to detect with light microscopy. The area fraction of the small green and teal fibers did decrease 21 and 26%, respectively, but the variation between hearts was relatively large.

Caulfield and Wolkowicz (6) saw similar degradation patterns using the disulfide reagents 5,5'-dithiobis(2-nitrobenzoic acid) and GSSG to induce proteolytic activity of endogenous extracellular collagenases. They saw drastic changes in the ultrastructure using SEM, but the hydroxyproline-to-total protein ratios did not change, suggesting that there were still large nondiffusible collagen fragments present in the tissue. Although the structural organization of the collagen was obviously altered in this study, the collagen molecules, and hence the hydroxyproline residues, may still have been present in the tissue without necessarily providing structural support. Charney et al. (9) found similar ultrastructural changes in stunned myocardium with comparable changes in hydroxyproline concentration, indicating that this level of degradation may be physiologically relevant (26 ± 2 and 28 ± 7 μg/mg tissue dry wt in stunned and normal tissue, respectively).

Both Caulfield and Borg (5) and Robinson et al. (32) have inferred that the intermyocyte struts prevent lateral or transverse slipping of myocytes with respect to each other during passive inflation. However, we did not see changes in the passive 2D strain characteristics after collagen depletion. On the other hand, the zero-pressure reference configuration was clearly different, and we did find increases in segment length from the Krebs state to the collagenase state that corresponded to positive remodeling strains (stretching) in both the circumferential and longitudinal directions. This length change was constant over the entire pressure range, which indicates that the endomysial struts and mesh may be important for determining the unloaded configuration but not passive stiffness. Caulfield and Borg (5) drew their conclusions from observations of the ultrastructural appearance of the myocardium fixed in different phases of the cardiac cycle. When the tissue was fixed in diastole the struts were taut, but when it was fixed in systole they were wavy and appeared unloaded. Rather than preventing lateral or transverse slippage during inflation, the struts may act to keep sarcomeres aligned and at similar lengths near end diastole. The results of our study show that unloaded sarcomere lengths were longer in collagenase-treated hearts than in Krebs-treated hearts, which indicates that the collagen may prevent overstretching of sarcomeres by protecting end-diastolic sarcomere length through decreased unloaded sarco-
mere lengths. This may result in a more uniform contractile strength rather than providing increased stiffness at high loads (32).

Because degradation of the collagen matrix alters the unloaded volume or reference configuration without affecting compliance measurably, there may be an associated change in the residual stress. Residual stress is the stress present in the tissue after all external loads are removed. Rodriguez et al. (33) showed that residual stress affects the transmural gradient in sarcomere length. Intact residually stressed myocardium has a greater gradient than tissue in which residual stress is relieved, although there is no change in the mean sarcomere length. Our study revealed a trend toward a flattening of the transmural gradient of sarcomere length with collagen degradation that is similar to changes seen when residual stress is relieved. This change in residual stress, which results in a change in equilibrium in the unloaded LV, may lead to the observed increases in mean sarcomere length. Hence changes in this unloaded configuration due to collagen degradation indicate that endomysial collagen may serve to maintain the residual stress by decreasing unloaded sarcomere length and coordinate sarcomere alignment. Disruption of this coordination in the beating heart may lead to a decreased strength of contraction. Indeed, in stunned myocardium, which has similar patterns of degradation, contraction is decreased to ~20–30% of the normal strength (9, 40).

Conclusion. In conclusion, fibrillar collagen was found to be an important factor in determining unloaded configuration of the LV but did not affect passive ventricular stiffness. The midwall remodeling that was induced by collagen degradation indicated stretching in both the circumferential (fiber) and the longitudinal (cross-fiber) direction. The increase in ventricular volume was explained, at least in part, by an increase in unloaded sarcomere length with collagen degradation. The results suggest that the medium-sized collagen fibers and the endomysial mesh and struts that attach to the Z line of the sarcomeres prevent overstretching of the sarcomeres at all pressures and serve to maintain the unloaded geometry in the left ventricle.

The authors thank Amy Bloom and Li-Jung Tai for their assistance. We also acknowledge Dr. Richard Podolin for assistance in the initial experiments and development.

This study was supported by National Heart, Lung, and Blood Institute Grants HL-43617 and HL-32583 to J. W. Covell and HL-41663 to A. D. McCulloch. J. H. Omens received American Heart Association Grant CA82–291. A. D. McCulloch was supported by the Whitaker Foundation. D. A. MacKenna was supported by a National Science Foundation Predoctoral Fellowship and an American Heart Association Predoctoral Fellowship.

Address for reprint requests: J. W. Covell, Univ. of California, San Diego, Medicine (Cardiology), 0613-J, 9500 Gilman Dr., La Jolla, CA 92038-0613.

Received 7 July 1993; accepted in final form 18 August 1993.

REFERENCES


