

Bone Elasticity and Ultrasound Velocity Are Affected by Subtle Changes in the Organic Matrix

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ABSTRACT

The mechanical competence of bone can be studied through the measurement of the components of its material elasticity, a property which can vary both in magnitude and in dependence upon orientation (anisotropy). While it is known that the elasticity is largely determined by the mineral constituents of the bone matrix, it is nonetheless clear that it must be also dependent upon the remaining constituents of bone material. In this work, the influence of organic components on the elasticity is explored by altering specific constituents of the bone matrix to varying degrees. This study addresses two questions: first, are the resulting changes in elasticity strongly or weakly dependent upon direction, and second, are they substantially dependent upon the nature and magnitude of the induced matrix alteration? To answer these questions, we performed different chemical manipulations of the bone matrix and measured the changes in elasticity and velocity using the technique of ultrasound critical angle reflectometry. Altering the properties of the organic matrix resulted in substantial and complex changes in the elasticity of bone. The observed changes were strongly dependent upon direction, could not be explained by changes in density alone, and varied strongly with the specific chemical treatment of the matrix. Immersion in urea selectively affected protein components of the organic matrix and resulted in reversible changes in velocity and elasticity, while removal of collagen caused anisotropic decreases and removal of all organic matter caused a collapse of all components of the elasticity. In conclusion, this study confirms that the organic matrix exerts a profound influence on the elasticity and indicates that the measurement of elastic properties at multiple directions is necessary in the assessment of bone mechanical competence. (*J Bone Miner Res* 1998;13:114–121)

INTRODUCTION

THE QUANTITATIVE MEASUREMENT of biomechanical properties of bone in the clinical setting is of considerable interest because it has the potential for allowing a deeper understanding not only of various diseases but also of the effects of different treatment modalities.

The biomechanical competence of bone is assessed indirectly in the clinical setting through the measurement of bone mineral density (BMD). The implied assumption is that the amount of bone mineral is the sole determinant of bone strength. However, the fact that disproportionately large increases in bone strength can correspond to a small increase in bone mineral content (BMC)^(1–3) implies that the biomechanical properties of bone are affected not only

by the total amount of mineral but by other properties, in particular its organization within the matrix.

Theoretical and experimental studies have shown that the mechanical properties of bone arise from an interaction between organic and inorganic components, and not from either constituent alone. Thus, the directional nature of bone mechanical properties⁽⁴⁾ is intrinsically dependent on the ultrastructure and organization of the extracellular matrix components.^(5,6) Furthermore, theoretical models of bone imply that the organic–inorganic interface is a determinant of its mechanical properties.⁽⁷⁾ Experimentally, it has been shown that complete removal of mineral leads to greatly reduced elasticity and strength of the bone matrix, while removal of the organic matrix leaves behind a mineralized material that cannot bear loads in tension or in compression.^(3,8,9)

Because the measurement of BMD is not sufficient for the complete determination of bone mechanical competence,^(10,11) the hypothesis has been put forward that a more comprehensive measurement of the mechanical properties of bone material could be made using ultrasound to measure velocity in bone.^(12–15) There is interest in ultrasound velocity (or speed of sound) because it is a priori a determinant of the well known physical quantity the material elasticity, which measures the resistance of a material to external loads. Values of the elasticity and velocity have been obtained using various ultrasound techniques:⁽¹⁶⁾ These are not, however, entirely equivalent and may in fact measure different properties of bone.^(15,17)

Previous studies have established the ultrasound critical angle reflectometry (UCR) technique in the laboratory and in the clinic.^(18,19) UCR differs in several aspects from widespread transmission and pulse-echo modalities, as shown in previous work.^(15,17) The advantages of the UCR technique include the ability to map bone heterogeneity at variable resolutions (typically between 0.5 mm and 5 mm) and to determine the directional dependence of velocity allowing the unambiguous determination of the principal axes of bone material. These capabilities have been utilized in previous studies in this laboratory⁽¹⁸⁾ and elsewhere,⁽²⁰⁾ demonstrating the validity of this technique in measuring bone material anisotropy. Velocities measured by UCR and transmission ultrasound in a variety of isotropic homogeneous media coincide to better than 3% over a wide range of velocities (2200–6300 m/s).⁽¹⁸⁾ In cortical bone samples, the UCR method gave velocities typically 11% higher than the transmission method, due to the different effects of intrinsic bone heterogeneity and geometry on the two techniques.⁽¹⁸⁾

The velocity and elasticity in a material are intrinsically dependent on its organization and composition and are, in principle, described by a matrix with a maximum of 81 independent elements. Due to the heterogeneity of bone, the material anisotropy cannot be measured accurately by mechanical testing in a single specimen because samples must be cut so as to cover the various orientations. The nondestructive technique of the ultrasound measurement has been used in the past to measure material anisotropy by analyzing either the reflection from the surface of a sample of enamel or bone^(18,20) or the time-of-flight through the specimen using transmission or pulse-echo methods in conjunction with special specimen preparation techniques.^(6,21–27)

The present work was primarily directed at investigating whether the changes in material elasticity vary with the nature of the change induced in the organic matrix or whether they are nonspecific, e.g., dominated by the resulting changes in density. To this end, changes in stiffness (elasticity) were measured after different specific modifications of the principal constituents of the bone matrix. As a consequence, this study makes it possible to obtain experimental evidence for the undoubtedly multifactorial relationship between the intrinsic physicochemical properties of bone material and the value of the ultrasound velocity. A secondary aim of this work was to measure the relationship between the different components of the elasticity. This question is of importance in experimental studies because depending on the presence or absence of unique relation-

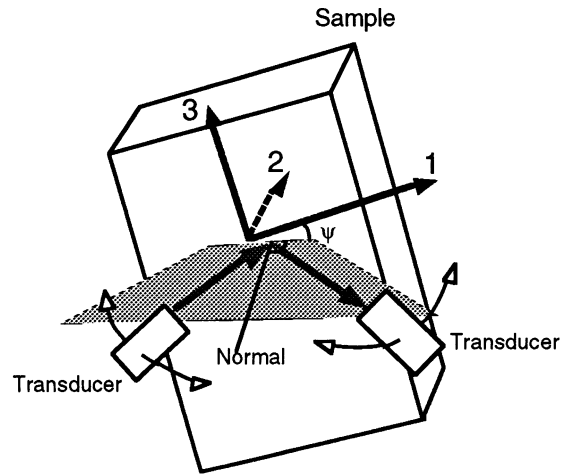


FIG. 1. Measurement of anisotropy using UCR. Pressure and shear velocities are measured in the plane defined by the normal of the material surface and the direction of propagation of the incident wave. The plane of measurement is shown here at a rotation angle (ψ) to the Cartesian sample axes. When the plane of measurement is parallel to the 3-axis (the 3–2 plane; velocity along the 3 or major axis) $\psi = 0^\circ$ and $\psi = 90^\circ$ when it is parallel to the 1-axis (the 1–2 plane, velocity along the 1 or minor axis).

ships, it may be either necessary to measure velocities at multiple angles or sufficient to measure them at one or two orientations.

MATERIALS AND METHODS

The bone samples used in this study were cortical bone specimens from the diaphysis of a bovine femur. Samples were cut with the z axis (direction 3) parallel to the long bone, the x axis (1) in the transverse direction of the bone and the normal to the surface (y , 2) pointing inward along the radial direction (toward the endosteum) and with dimensions 3 cm \times 1 cm \times 0.3 cm along the respective axes, as shown in Fig. 1. Between studies, the samples were stored in a solution with pH and ionic strength adjusted to prevent demineralization. UCR measurements were made at a surface as described below and as depicted in Fig. 1.

Ultrasound UCR methods

The UCR technique is described in detail elsewhere⁽¹⁸⁾; the principles used here are briefly summarized below. Changes in the amplitude and phase of an ultrasound beam reflected from a bone–soft tissue interface are analyzed over a range of incidence angles, in the plane defined by the direction of the incident beam and the normal to the interface (referred to as the plane of scattering) (Fig. 1), to obtain the pressure and shear wave bulk velocities in the solid with a precision of 0.6% and 1.2%, respectively (10 trials of one sample with repositioning between trials).

Two pressure-wave transducers (5 MHz, planar with 0.373" and 0.5" diameter; Panametrics, Waltham, MA,

U.S.A.), a transmitter and a receiver, are mounted on arms that rotate about a center where the sample is situated. A mechanical positioning system is used to align the sample. Computer-controlled stepper motors (model M061-LS02, Superior Electric, Bristol, CT, U.S.A.) move the transducer arms along a circle in 0.3° steps. An ultrasound wave train of four cycles, generated by a signal generator (model AFG-5101, Tektronix, Beaverton, OR, U.S.A.) at 5 MHz, is reflected from the face of the sample and allowed to reach the receiving transducer, positioned symmetrically to the transmitter with respect to the normal. As the transducers are moved synchronously apart, the entire waveform is sampled and stored at each angle using a digital storage oscilloscope (model 2430A, Tektronix). The phase spectrum is obtained by measuring the phase shift of the waveform for each angle increment.

The critical angles of incidence for total internal reflection of the pressure and shear waves are obtained by analyzing the changes in the reflected amplitude and phase spectra over the range of incidence angles in the scattering plane.^(18,28) The velocities are thus obtained from only the measurement of an angle and the velocity of sound in water, by using Snell's law.

By rotating the scattering plane around a point on the surface (about the 2-axis) by a variable angle ψ , the anisotropy of the pressure and shear wave velocities can be assessed (Fig. 1). Thus, when the rotation angle $\psi = 0^\circ$, the velocities are measured along the major (3, long) axis, in the 3–2 plane of scattering and at $\psi = 90^\circ$, along the minor (1, transverse) axis, in the 1–2 plane of scattering. As the sample is rotated, the pressure and shear velocities are measured at each rotation step. When multiple (10) measurements of pressure and shear velocity were made at several orientations at a point on one sample after repositioning it each time, no difference was seen between the 10 sets of velocity measurements (analysis of variance [ANOVA], $p < 0.001$). The accuracy of the measurement of material anisotropy by UCR was verified by comparing this measurement with the results of transmission ultrasound measurements (an accepted method) in the same bone sample. The sample was cut in an irregular hexagonal shape, making possible transmission measurements along two orthogonal directions and at two intermediate directions; UCR measurements were taken at several orientations along one face. The two methods showed similar values at each matched orientation (Fig. 2), demonstrating the accurate measurement of anisotropy in bone material by UCR.

These UCR velocities (V_{IJ}), measured at a particular rotation angle (ψ), are related⁽¹⁸⁾ to the elasticity or material stiffness constants (C_{IJ}) in that plane, and the density (ρ), as

$$V_{IJ} = (C_{IJ}/\rho)^{1/2} \quad (1)$$

The constants C_{IJ} are identified by so-called "abbreviated" indices $I, J = 1 \dots 6$ each of which represent a pair of indices of the Cartesian axes ($x = 1, y = 2, \text{ and } z = 3$): $I = 1$ corresponds to $i, j = 1, 1$; 2 to the pair 2, 2; 3 to 3, 3; 4 to 2, 3; 5 to 1, 3; 6 to 1, 2. Cortical Haversian bone has hexagonal symmetry (transversely isotropic),^(24,29,30) which constrains the 81-element stiffness matrix required to characterize an anisotropic material's elastic behavior to five independent

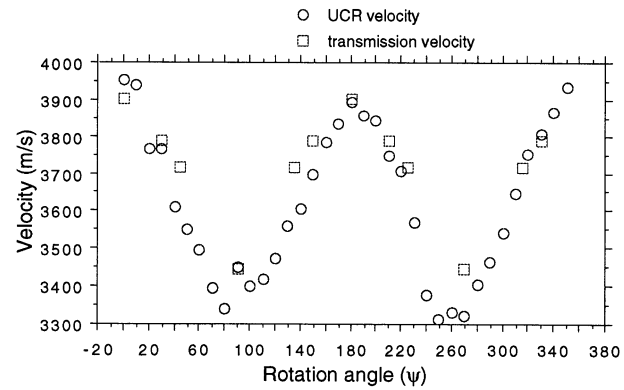


FIG. 2. UCR anisotropy measurements validated by transmission methods in the same bone sample. The bovine cortical bone sample was cut in an irregular hexagonal shape, making possible transmission measurements along two orthogonal directions and at two intermediate directions; UCR measurements were taken at several orientations along one face. Transmission velocity measurements were taken with two pressure wave transducers placed on parallel-cut faces on opposite sides of the sample, and the time-of-flight of a pulse was divided by the distance between the two transducers. The two readings were in agreement at matched orientations.

elements. Fitting the experimentally measured dependence of pressure and shear velocities upon orientation using transformations appropriate for the hexagonal symmetry group,^(18,28,31,32) all five independent stiffness constants can be obtained.

The stiffness constants are, in turn, formally related to the technical constants (elastic moduli, Poisson's ratios), which are the mechanical properties measured during bulk mechanical testing.^(21,25,28,31) The technical constants for bone, determined through an inversion of the stiffness matrix,^(21,25,28,31) (and the precision with which they are determined) are the principal (Young's) moduli in the major and minor directions, E_{33} (3%) and E_{11} (2%), respectively, the Poisson's ratios ν_{13} (10%), ν_{12} (8%), and ν_{31} (13%), and the shear moduli, G_{12} (6%) and G_{23} (3%).

Velocity measurements and elasticity (technical) constants at specific sites on each sample after treatment were compared with the pretreatment (control) values at the same location. The samples were measured from a single surface at a selected, marked location. The density was measured using Archimedes' principle, by weighing the sample when dry and while suspended in water so that:

material density = density of water

$$\times (\text{dry weight/difference between dry and wet weights}) \quad (2)$$

Mechanical testing methods

Since the mechanical tensile testing technique allows only a unidirectional assessment of a bone sample, only changes in the principal elasticity constant measured along the long

axis (3-axis, 3-2 plane, $\psi = 0$) were followed by UCR and nondestructive mechanical testing.⁽²⁸⁾ The samples were tested in tension along their long axis (3-axis) to 20% of the ultimate load, with strain rates of $\sim 60 \mu\text{s}^{-1}$. Strain gauge extensometers were placed covering the area sampled by the UCR measurements, and stress-strain curves were analyzed to obtain the elastic modulus (Young's modulus, E_{33}). To eliminate artifacts due to bending induced in the tensile tests, strains were measured on opposite sides of the sample, and load-strain curves were averaged.⁽³³⁾ Cortical bone samples loaded for 20 cycles under these conditions showed no change in elastic modulus.

Bone material modification methods

Since bone is a composite material, each of its components may be expected to contribute uniquely to the elastic properties of the tissue and their anisotropy. The major organic components were perturbed using the biochemical methods described below to induce different physicochemical changes, and the effects on UCR velocity were measured.

First, to examine the effects of a limited denaturation of the organic matrix, the bone samples ($n = 12$) were placed in a 3 M (pH 7.5) solution of urea at room temperature for up to 72 h. After completion of the UCR measurements, the urea was washed off using the original storage solution, and the measurements were repeated.

Second, to examine the effects of total removal of the organic matrix, the organic components were completely dissolved using NaOCl (sodium hypochlorite) solution (Sigma Chemical Co., St. Louis, MO, U.S.A.; >5% available chloride), leaving behind the virtually undisturbed mineral phase.^(8,34,35) The bone samples ($n = 12$) were placed in solution for 24, 48, and 72 h, respectively, and measurements were taken each time. Fourier transform infrared spectroscopy (FTIR) spectra were collected before and after the treatments. The FTIR method is nondestructive and measures the spectrum from a small sample volume of a specimen placed on the face of an attenuated total internal reflection (ATR) crystal. The crystalline state of the mineral can be determined from changes in the shape and position of the phosphate absorption bands.⁽³⁶⁾ Analysis of the spectra showed no significant change in the crystalline environment of bone mineral (specifically, the position and shape of the 560 cm^{-1} and 1032 cm^{-1} peaks were not altered by the treatment).

Third, the major structural component of the organic matrix is Type I collagen. Type I collagenase is an enzyme that specifically acts on the collagen protein. To selectively degrade collagen, the bone samples ($n = 14$) were immersed in a solution of Type I collagenase (Sigma Chemical Co.; 1% w/v, pH 7.5) for up to 30 h at 32°C with mild agitation. Ultrasound, mechanical, and FTIR readings were taken before and after treatment. The IR spectra did not change significantly with the collagenase treatment, indicating that the mineral crystalline state was not affected. No change in the Ca/P ionic concentrations were observed in the collagenase solution before and after the experiment,

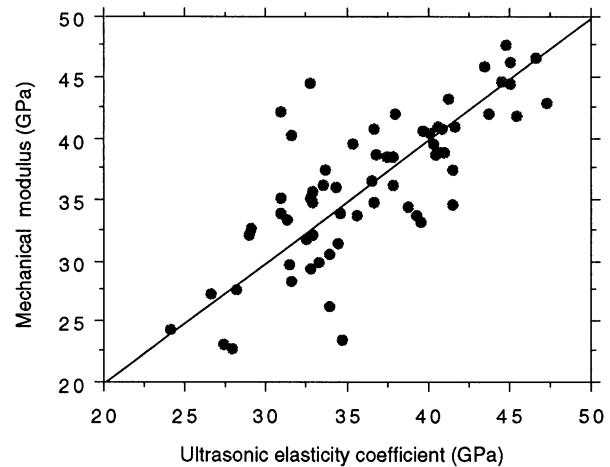


FIG. 3. Linear regression model of two elasticity values, one measured by mechanical testing and the other by ultrasound velocity. The slope of the model fit with these two variables was not significantly different from 1 ($p < 0.01$). A Wilcoxon paired signed ranks test showed no difference (at $p < 0.05$) between the two groups. Data include normal bone and samples treated with the experimental protocols described in this paper.

indicating that no detectable quantities of mineral had been extracted during the treatment.

Statistical analysis

Changes in the density, ultrasound velocity, and elasticity of samples were assessed for statistical significance using the non-parametric Wilcoxon paired signed-ranks test, at significance level of $p < 0.05$.

RESULTS

Unidirectional elasticity values measured along the 3- or major axis by UCR and by mechanical testing before and after treatment were in excellent agreement with means of $36.35 \pm 5.5 \text{ GPa}$ and $36.33 \pm 6.1 \text{ GPa}$, respectively.⁽²⁸⁾ A least-squares linear fit had a slope equal to unity, with an R^2 (correlation coefficient) value of 0.988 as seen in Fig. 3.

Each of the material modification treatments described above had a significant and unique effect on the bone matrix. The changes in density, pressure, and shear velocities and elasticity due to the various treatments are shown in Table 1. These tabulated values are shown as percent changes in the principal elastic moduli, Poisson's ratios, or stiffness constants, Young's modulus from mechanical testing, and in the pressure and shear velocities along two orthogonal directions.

Urea: Reversible effects on organic matrix ultrastructure

The density of the samples did not change with urea treatment. The velocities increased reversibly, with a

TABLE 1. EFFECTS OF TREATMENT ON UCR MEASUREMENTS

Parameters	Urea		Collagenase		NaOCl		
	% change	Statistical	% change	Statistical	Parameters	% change	Statistical
Density (g/cc)	-0.2	$p = 0.5$	-0.4	$*p = 0.04$	Density, 24 h	-1.8	$*p = 0.002$
V_{33} (m/s)	1.7	$*p = 0.03$	-3	$*p = 0.02$	Density, 72 h	-2.9	
long axis					V_{33} , 24 h	-15.6	$*p = 0.002$
V_{11} (m/s)	3.2	$*p = 0.005$	-1.2	$*p = 0.04$	V_{33} , 72 h	-23.5	
transverse axis					V_{11} , 24 h	-17.1	$*p = 0.002$
$V_{44} = V_{55}$ (m/s)	11	$*p = 0.004$	-11	$*p = 0.04$	V_{11} , 72 h	-30.1	
V_{66} (m/s)	15	$*p = 0.003$	-2.6	$p = 0.17$	(long axis)		
E_{33} (GPa)	7	$*p = 0.06$	-10	$*p = 0.02$	(transverse axis)		
Mechanical- E_{33}	7.1	$*p = 0.025$	-7.3	$*p = 0.02$	c_{33} (Gpa), 72 h	-43.4	$*p = 0.002$
E_{11} (GPa)	21	$*p = 0.004$	-5	$p = 0.24$	Mech E_{33} , 72 h	-28.1	$*p = 0.002$
G_{12} (GPa)	34	$*p = 0.003$	-3	$p = 0.12$	c_{11} (GPa) 72 h	-52.3	$*p = 0.002$
					($c_{13} + 2c_{44}$)	-49.5	$*p = 0.002$
G_{23} (GPa)	27	$*p = 0.003$	-10	$*p = 0.027$	(GPa) 72 h		
ν_{12}	-41 (urea)	$*p = 0.007$	-4.8	$p = 0.46$			
	-34 (post)	$*p = 0.02$					
ν_{13}	27 (urea)	$p = 0.52$	5	$p = 0.34$			
	56 (post)	$*p = 0.02$					
ν_{31}	9 (urea)	$p = 0.9$	4.2	$p = 0.35$			
	30 (post)	$*p = 0.02$					

Percent changes in measured parameters due to urea, collagenase, and NaOCl treatments of bone samples. Different parameters or times of measurement are shown (as appropriate in the table) for NaOCl treated samples as explained in the text.

*Statistical significance of change assessed using Wilcoxon paired nonparametric test at $p < 0.05$.

greater increase in the transverse direction (ν_{11}) than along the major axis (ν_{33}) (Table 1). The changes in the shear velocities (at all orientations) showed greater sensitivity to the changes in organic ultrastructure caused by urea than the pressure wave velocities.

The elastic moduli (principal and shear) showed reversible increases with greatest changes seen in the shear moduli, but the Poisson's ratios changed irreversibly as shown by the post-treatment results in Table 1. This reversible effect of urea on elastic moduli was confirmed by mechanical testing.

Complete removal of the organic matrix

The reflected amplitude spectra at 24 and 48 h (data not shown here) show the presence of a gradient in mechanical properties occurring as a result of diffusion-limited degradation of organic matter.⁽²⁸⁾ A time-dependent decrease in the pressure-wave velocities was observed (Table 1). As the treatment with NaOCl proceeded, the organic bone matrix was completely dissolved, leaving behind only the mineral so that the samples had to be handled carefully to prevent them from crumbling.

The density after treatment could not be accurately assessed because the ends of the samples were coated with wax to preserve them for attachment to the mechanical testing apparatus. However, a significant overall drop in density was observed (Table 1). The pressure wave velocities decreased significantly, with similar losses along both

principal axes. Shear velocities fell below the threshold for detection by the technique after the first 24 h. Thus, from the anisotropic distribution of the pressure wave velocity, two principal stiffness coefficients and one mixed-mode term coefficient (which includes a shear contribution) were calculated, and the results at 72 h of treatment are listed in Table 1. The significant drop in UCR measurements was confirmed by mechanical testing results along the long axis of the samples.

Selective removal of collagen proteins by collagenase

The mean density of the samples decreased by 0.4%. The mean pressure and shear velocity decreased in all samples, with a greater loss along the major axis (ν_{33}) than along the minor axis (ν_{11}). A representative pressure velocity distribution at a location on one sample before and after the collagenase action is shown in Fig. 4.

The shear velocity along the major axis changed to a greater extent than did the pressure velocity, underlining the sensitivity to changes in material structure and composition of this ultrasound propagation mode. The shear velocity along the minor axis, however, did not change (Table 1).

The elastic modulus (E_{33}) along the major axis decreased to a greater extent than along the minor axis (E_{11}), reducing the anisotropy in the material. Mechanical testing also demonstrated a similar decrease in E_{33} . The 10% reduction in the shear modulus G_{23} was significant, while the smaller

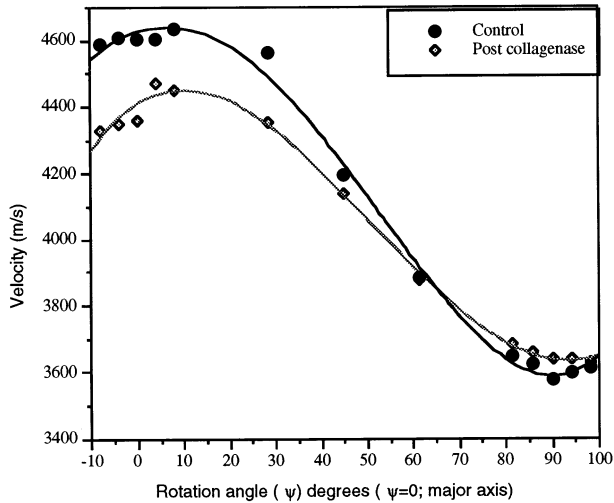


FIG. 4. Collagenase–anisotropic changes in pressure wave velocity. The graph demonstrates the effects of collagenase on the pressure wave velocity angular distribution for a representative sample of bone, measured at a single location. Each point is the pressure wave velocity obtained at the specified rotation angle (ψ), as described in Fig. 1 and in the text. Polynomial fitted lines are shown only to separate the two different data sets. The velocity is maximum in the direction of the long axis of the bone and minimum in the transverse direction. The anisotropic decrease along the major axis is clearly seen, indicating the directional influence of collagen fibers. The shear wave velocities showed similar trends (data not shown). This graph also demonstrates the anisotropic nature of bone material and the ability of UCR to measure this anisotropy at a single location.

decrease in G_{12} was not statistically significant. Changes in the Poisson's ratios were not statistically significant (Table 1).

DISCUSSION

Ultrasound velocity and elasticity are strongly correlated with material elasticity measured by mechanical testing both before and after the different modifications of bone matrix as shown in Fig. 3, validating the use of the UCR technique for making this measurement.⁽²⁸⁾ The equivalence of other ultrasound methods and mechanical testing for determination of elasticity have been demonstrated by other researchers,^(13,14,18,21,37,38) who have also shown a strong positive relationship between bone strength and ultrasound velocity (or elasticity).^(13,38–44) The high precision and accuracy (Figs. 2 and 3) of the nondestructive UCR measurement make it possible to study the directional dependence of changes in elasticity due to progressive changes in bone material in the same sample and at the same point.

The mineral component is the primary determinant of bone mechanical properties and has been singled out as the most significant factor in bone strength.^(1,2) However, the

organic and inorganic components of bone are intimately conjoined at many levels, not least in determining bone architecture. This in turn influences mechanical properties, and the intrinsic microarchitecture of cortical bone typically results in greater values of elasticity and strength along the major axis of bone than along the transverse direction.^(45–51) Recently, the contribution of the collagen matrix to the elastic anisotropy has been questioned,⁽⁵²⁾ contradictory results^(8,53) coming from studies of completely demineralized bone matrix (mainly collagen). It should be noted, however, that the physical properties of the organic matrix, in particular its structure, can be expected to differ in different bone types (Haversian, plexiform)⁽³⁰⁾ and animal species.⁽⁵⁴⁾

It should also be noted that previous studies of the effects of collagenase^(9,53) on bone matrix properties used destructive mechanical testing, making it necessary to use different samples for the treated and control groups. Thus, treatment-induced effects were masked by intrinsic variation among samples, and the resulting increase in uncertainty made interpretation more difficult. The results obtained here quantitatively demonstrate, for the first time, that collagen contributes to the elasticity of bovine bone material at all orientations and that its contribution is greatest along the major axis of bone (which in these samples coincides with the preferred orientation of collagen fibers).^(45,46,50)

The results of the sodium hypochlorite (NaOCl) treatment demonstrate a progressive breakdown of the organic matrix with increased exposure to the solution and show that the organic matrix exerts a significant influence on the properties of bone. These results are in agreement with data from other laboratories^(8,54) which showed that a significant anisotropy ratio is retained after the organic matter is removed. However, without the organic matrix, the mineral component cannot support tensile or compressive loads, a fact reflected in the collapse of the elastic moduli of the material observed here.

Changes in the organic binding matrix significantly influence the mechanical characteristics of bone.⁽⁷⁾ The results of the urea experiments, discussed below, are further indications of the complexity of the interaction between the organic and the mineral phase in determining the mechanical properties of bone.

In view of the fact that all other manipulations of the organic matrix resulted in decreased elasticity, the increase in material elasticity through the action of urea found in these experiments was surprising, as was its almost complete reversibility. These results were, however, corroborated by those obtained with guanidine HCl, another chaotropic agent (data not shown).

The mode of action of chaotropic agents is not very well understood. Urea has been used to denature collagen *in vivo*^(55,56) and *in vitro*^(57,58) and it would have been expected that by denaturing bone collagen, a trend parallel to the effects of collagenase would be observed. This is not the case, and there are several potential interpretations of these findings. Changes in the conformation of noncollagenous proteins could increase intermolecular bonding between matrix components, leading to higher velocities. Further, as

chaotropic agents affect polar interactions with water, collagen or other molecules with tertiary structures dependent on hydrogen bonding could swell and pack more tightly within the matrix, possibly creating new protein-protein interactions, thus also acting to increase the elastic properties due to better packing and improved stress-strain transfer between components. Indeed, other researchers have also suggested, through experimental^(59,60) and theoretical studies,⁽⁷⁾ that a change in the bonding between the mineral crystals and organic matrix could significantly influence the elastic properties of the material. Our results quantitatively measure this effect. It is interesting to speculate that the increased elasticity observed by us after exposure to urea could be a mechanism for the previously unexplainable increase in pressure wave transmission velocity in the patella seen after excessive loading (marathon race).⁽⁶¹⁾

In summary, the results of these experiments show a complex causal relationship between the organization of the organic and inorganic components of bone material and changes in ultrasound velocity and elasticity coefficients and a strong dependence of the mechanical properties of the tissue on the physicochemistry of the organic components. The results also show that the shear wave velocity is often a more sensitive indicator of changes in material properties than the pressure wave velocity.

Finally, it is clear that the matrix of elasticity does not maintain a fixed relationship either between its components or with bone density, and changes in bone quality have different effects on the various components. This observation, in turn, indicates that measuring the multiple components of bone mechanical status could lead to a more detailed evaluation of bone status.

The UCR technique is particularly suited to evaluate changes in bone status in vivo due to multiple reasons, in particular the limited sample volume it interrogates (typically <1 mm³). The small volume assessed by UCR at various angles of orientation can be scanned over a surface to obtain an image of the material properties of bone.

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