COMPARISON OF THE MACROSCOPIC VISCOELASTIC PROPERTIES OF CORTICAL BONE TO ITS NANO-INDENTATION RESPONSE

A Thesis

Submitted to the Graduate School
of the University of Notre Dame
in Partial Fulfillment of the Requirements
for the Degree of

Master of Science
in Mechanical Engineering

by

Tara N. Shepherd, B.S.

Glen L. Niebur, Director

Graduate Program in Aerospace and Mechanical Engineering
Notre Dame, Indiana
April 2007
Cortical bone provides a rigid structure allowing for the attachment of skeletal muscles. It makes up approximately 80% of skeletal mass and is a damaging viscoelastic material. Damage is an important aspect to evaluate bone quality since damage accumulation affects cortical bone strength. Fatigue and monotonic strength studies have indicated that damage accumulation mechanisms depend on structural and compositional properties. However, the role of the viscoelastic behavior of cortical bone has been overlooked. Bone is a hierarchical composite material. As such, measurements of multiple length scales are useful in order to evaluate bone viscoelastic properties in different microstructural units. To understand the effect of viscoelasticity of bone at small length scales, cortical bone specimens were evaluated under torsional and tensile loads, by ultrasound and by nanoindentation.

Twelve cortical bone specimens were tested using tension, torsion, ultrasonic wave propagation, and indentation test. These tests resulted in the specimens showing stress relaxation and creep effects, which are a key component of viscoelastic behavior. Nanoindentation and torsion mechanical properties were highly correlated, suggesting microscopic properties are reflected at the macroscopic level.
CONTENTS

FIGURES ........................................................................................................................................ iv

TABLES .......................................................................................................................................... vii

ACKNOWLEDGMENTS ........................................................................................................ viii

CHAPTER 1 INTRODUCTION ........................................................................................................ 1
  1.1 Cortical Bone ......................................................................................................................... 1
  1.2 Viscoelasticity of Bone .......................................................................................................... 2
  1.3 Nanoindentation .................................................................................................................... 5
  1.4 Motivation ............................................................................................................................ 7
  1.5 Objectives ............................................................................................................................ 8
  1.6 Overview ............................................................................................................................. 8

CHAPTER 2 MATERIALS AND METHODS .............................................................................. 10
  2.1 Introduction .......................................................................................................................... 10
  2.2 Macroscopic Sample Protocol ............................................................................................ 10
  2.3 Preparation of Nanoindentation Samples .......................................................................... 13
  2.4 Density Measurement .......................................................................................................... 16
  2.5 Conclusions ........................................................................................................................ 17

CHAPTER 3 MECHANICAL TESTING ..................................................................................... 18
  3.1 Macroscopic Testing Methods ............................................................................................ 18
    3.1.1 Sample Preparation ....................................................................................................... 18
    3.1.2 Mechanical Testing ...................................................................................................... 19
    3.1.3 Data Analysis ............................................................................................................... 20
  3.2 Nanoindentation Testing ...................................................................................................... 21
    3.2.1 Sample Preparation ....................................................................................................... 21
    3.2.2 Data Collection ............................................................................................................. 21
    3.2.3 Data Analysis ............................................................................................................... 23
  3.3 Ultrasonic Characterization ................................................................................................. 24
    3.3.1 Mechanical Testing ....................................................................................................... 24
    3.3.2 Data Analysis ............................................................................................................... 25
  3.4 Summary ............................................................................................................................. 26

CHAPTER 4 NANOINDENTATION DRYING EFFECTS .......................................................... 27
  4.1 Introduction .......................................................................................................................... 27
  4.2 Data Analysis ....................................................................................................................... 28
4.3 Discussion.................................................................................................................................29

CHAPTER 5 RESULTS........................................................................................................................................................................30
5.1 Introduction ..................................................................................................................................................................................30
5.2 Torque – Time Measurements ..................................................................................................................................................30
5.3 Force – Time Measurements ...................................................................................................................................................32
5.4 Nanoindentation .........................................................................................................................................................................33
5.5 Ultrasonic Characterization ....................................................................................................................................................34
5.6 Macroscopic Experiments vs. Microscopic Experiments .....................................................................................................37
5.7 Discussion ................................................................................................................................................................................39
5.8 Summary .................................................................................................................................................................................40

CHAPTER 6 CONCLUSIONS ......................................................................................................................................................................41
6.1 Goal of Study ...............................................................................................................................................................................41
6.2 Summary .................................................................................................................................................................................41
6.3 Future Work ............................................................................................................................................................................43
6.4 Conclusion ............................................................................................................................................................................43

APPENDIX A1. PROCEDURE FOR MILLING CORTICAL BONE SPECIMENS ..................................................................................45
APPENDIX A2. PROCEDURE FOR TURNING CORTICAL BONE SPECIMENS ..................................................................................48
APPENDIX A3. PROCEDURE FOR POLISHING CORTICAL BONE SPECIMENS ........................................................................52
APPENDIX A4. DATA COLLECTED TO ANALYZE NANOINDENTATION DRYING EFFECTS .................................................................................................................................55
APPENDIX A5. NANOINDENTATION MODULUS DATA ................................................................................................................58
BIBLIOGRAPHY ..................................................................................................................................................................................61
FIGURES

Figure 1.1 Hierarchical structure organization of bone: (a) cortical bone; (b) osteons; (c) lamellae; (d) collagen fibers; (e) bone mineral crystals (Rho et al., 1998) ........................2

Figure 1.2 Stress effects on viscous, viscoelastic, and elastic materials (a) Stress vs. time; (b) typical creep recovery curves for elastic, viscous, and viscoelastic materials (Lakes, 1998)......................................................................................................................4

Figure 1.3 A typical hysteresis curve; the energy absorbed during one loading-unloading cycle is given by the area within the hoop ........................................................................................................................................5

Figure 1.4 Rheological models used to predict viscoelastic behavior. (a) Maxwell Model; (b) Voigt Model; (c) Standard linear solid model with $\eta = \text{coefficient of viscosity}, F = \text{force}, u = \text{displacement}, \mu = \text{spring constant}$ (adapted from Fung 1998)..........................6

Figure 1.5 A typical nanoindentation load vs. displacement curve..............................................7

Figure 2.1 Final geometry of a cortical bone waisted cylinder for macroscopic tests .....11

Figure 2.2 Average final cross-sectional dimensions of milled specimens including standard deviation (N=12)........................................................................................................................................12

Figure 2.3 Sherline 5400 Series Mill..............................................................................................13

Figure 2.4 Fixture to hold specimen while submerged in buffered saline bath during machining ............................................................................................................................................14

Figure 2.5 Sherline lathe showing placement of cutting tool, irrigation tool, and specimen during machining.................................................................................................................................14
Figure 2.6 Outer diameter and gauge length dimensions of turned down specimen including standard deviation (N=12) ........................................................................................................15

Figure 2.7 Cortical bone specimen (a) Unpolished specimen; (b) Polished specimen; (c) haversian canal; (d) osteon .............................................................................................................15

Figure 2.8 Polishing tool used to hold sample during the polishing process .................16

Figure 3.1 EnduraTEC ELF 3300 testing machine used to perform macroscopic test with saline bath attached for testing .........................................................................................19

Figure 3.2 Commands used to program EnduraTEC for macroscopic tests (a) Torsional command; (b) Axial command ....................................................................................................20

Figure 3.3 Hysitron automated nanomechanical TriboIndenter used for nanoindentation test (adapted from www.hysitron.com) .................................................................22

Figure 3.4 Indent loading protocol for nanoindentation test ........................................22

Figure 4.1. Elastic modulus (GPa) vs. time (minutes) of cortical bone samples measured using nanoindentation (N=4) .........................................................................................28

Figure 4.2. Creep rate (nm/s) vs. time (minutes) of cortical bone samples measured using nanoindentation (N=4) ...............................................................................................29

Figure 5.1 Torque vs. time data of bovine cortical bone samples (N=12) measured over four twist intervals ............................................................................................................31

Figure 5.2 Force vs. time data, measured over four intervals, collected from tension test for all samples (N=12) ........................................................................................................32

Figure 5.3 Examining different sections of a nanoindentation unloading curve to determine the correct range for determining S(h_{max}), the derivative of the unloading curve ................................................................33

Figure 5.4 Elastic modulus (std.dev) of cortical bone samples measured using nanoindentation (N = 20 indents) ...............................................................34
Figure 5.5 Elastic modulus (GPa) calculated for sample #8 using different values for Poisson’s ratio ........................................................................................................................................35

Figure 5.6 Ultrasonic vs. nanoindentation elastic modulus (GPa) for cortical bone samples (N=12) ........................................................................................................................................36

Figure 5.7 Ultrasonic vs. torsion shear modulus (GPa) for cortical bone sample (N=12) ........................................................................................................................................36

Figure 5.8 Creep time constant, $\tau_\sigma$ (s), nanoindentation vs. tension experiments ..........37

Figure 5.9 (a) Creep constant nanoindentation (s) vs. torsion (s); (b) Relaxation constant nanoindentation (s) vs. torsion (s) ........................................................................................................38

Figure A1.1: Metal spacer used to level cortical bone specimen in the vise for a flat surface cut........................................................................................................................................46

Figure A1.2. Vise screwed into vice holder and mounted on the x-axis of the mill to make square samples ........................................................................................................................................47

Figure A2.1: Buffered saline solution container containing valve for controlled saline drip........................................................................................................................................49

Figure A2.2: Placement of buffered saline drip, cutting tool, and specimen during the turning process........................................................................................................................................50

Figure A3.1: Polishing tool used to hold the cortical bone specimen and produce a flat surface during polishing ........................................................................................................................................53

Figure A3.2: Polishing tool path in a counter clockwise direction, opposite of the polishing plate direction ........................................................................................................................................54

Figure A3.3: A polished cortical bone sample viewed at 50x showing visible features; osteons, and haversian canal........................................................................................................................................54
TABLES

TABLE 2.1 DENSITY MEASUREMENTS OF CORTICAL BONE SAMPLES USING ARCHIMEDES’ PRINCIPLE INCLUDING MEAN AND STANDARD DEVIATION (N=12) ..............................................................................................................................17

TABLE 5.1 MEAN (STD.DEV) SHEAR MODULUS, STRESS RELAXATION TIME CONSTANTS AND CREEP TIME CONSTANTS FOR CORTICAL BONE SPECIMENS (N=12) .......................................................................................................31

TABLE A4.1 CALCULATED ELASTIC MODULUS AND P-VALUES FOR 15 INDENTS PREFORMED OVER 56 MINUTES IN WITH OSTEONAL AND INTERSTITIAL TISSUE (N=4)......................................................................................56

TABLE A4.2 CALCULATED CREEP RATES AND P-VALUES FOR 15 INDENTS PREFORMED OVER 56 MINUTES IN WITH OSTEONAL AND INTERSTITIAL TISSUE (N=4)..................................................................................................................57

TABLE A5.1 MEAN ELASTIC MODULUS (STD.DEV) MEASURED USING NANOINDENTATION (N=12) ........................................................................................................................................59
ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Glen Niebur, for all of his support and guidance with this project. I would also like to thank Leon Hluchota, Jingzhou Zhang, Gabriel Converse, and Matthew Landrigan for all of their contributions to this project. Finally, I would like to thank all of my family and friends that supported me throughout this research process.
CHAPTER 1
INTRODUCTION

Bone is a complex and highly organized biological material of the skeletal system. It provides attachment for various muscle groups and provides a protective function to vital organs. Bone is a composite material, containing hydroxyapatite, collagen, proteins and water, whose material properties are determined by the arrangement of these constituents.

Bone can be classified as either cortical bone or trabecular bone. Almost 80% of the skeletal mass in the adult skeleton is cortical bone, while the rest is trabecular bone (Jee 2001). In this thesis, the macroscopic viscoelastic properties of cortical bone were studied by applying torsional loads to bone specimens. A portion from these specimens was subjected to a nanoindentation test to determine the viscoelastic properties. Finally, the results of these two experiments were compared.

1.1 Cortical Bone

Cortical bone, also known as compact bone, is a dense bone that provides a structure allowing for the attachment of skeletal muscles. It forms the outer shell at the end of joints and the vertebrae, and is found primarily on the shafts of long bones. As with many biological materials, cortical bone has a hierarchical structure. The various levels of organizations include the macroscopic level: cortical bone as a solid composite material, the microscopic level: osteons, the submicroscopic level: lamellae, and the nanostructure: collagen fibrils and hydroxyapatite crystals (Fig 1.1; Rho et al., 1998). In
order to understand the mechanical properties of cortical bone as a whole it is important to understand the properties at these different levels.

Cortical bone can be regarded as a damaging viscoelastic material, where damage is defined as a permanent change in structure that results in material property degradation (Jepsen and Davy 1997). Cortical bone structure and microstructure comprise, to a certain extent, bone’s mechanical capabilities and its weaknesses (Augat and Schorlemmer, 2006) thus it is an important orthopaedic concern because of the increased risk of fracture among adults.

1.2 Viscoelasticity of Bone

Materials, both biological and engineering materials, can be classified as either elastic, viscous, or viscoelastic materials. The creep and recovery response of each of these materials is very different. Elastic materials exhibit recovery to zero strain
immediately following load release while viscoelastic materials exhibit recovery after sufficient time (Fig 1.2).

A viscoelastic material is one in which the relationship between stress and strain depends on time. In these types of materials, if the strain is held constant the stress decreases with time (stress relaxation), if the stress is held constant the strain increases with time (creep), and if cyclic loading is applied a phase lag occurs (hysteresis) (Lakes 1998). Hysteresis occurs in a material subjected to cyclic loading whose loading process is somewhat different from the unloading process and it leads to a dissipation of mechanical energy (Fig. 1.3.) These features of creep, hysteresis, and relaxation collectively create a viscoelastic material.

Rheological models, the Maxwell model, Voigt model, and standard linear solid model, are often used to describe the viscoelastic behavior of a material. These models are a combination of linear springs and dashpots. The linear spring represents an instantaneous deformation proportional to the load, while the dashpot represents an instantaneous velocity proportional to the load (Fung 1993).

The Maxwell model (Fig 1.4) is represented by a linear spring and dashpot connected in series. With this combination of the spring and dashpot in series, the constant strain produces stresses that gradually relax. When this combination is put under constant stress the strain will have two components, elastic and viscous. The elastic component will relax immediately upon stress being released and the viscous component will continue to grow with time when the stress is applied (Fung 1993). Thus the Maxwell model predicts that stress decays exponentially with time

$$\sigma = E \varepsilon_0 e^{-t/\tau}$$

where $E$ is the Young’s modulus, $\varepsilon_0$ is the initial strain and $\tau$ is the viscoelastic constant, but is unable to predict creep in materials (Fung 1993).
The Voigt model (Fig 1.4) is represented by a linear spring and dashpot connected in parallel. With the spring and dashpot in parallel, constant stress causes the material to deform at a decreasing rate until the material approaches the steady-state strain. When the stress is relaxed the material gradually relaxes to its undeformed shape

\[ \varepsilon = \frac{\sigma_0}{E} [1 - \exp\left(-\frac{t}{\tau}\right)] \] (1.2)

where \( E \) is the Young’s modulus, \( \varepsilon \) is strain, \( \sigma_0 \) is the initial stress, and \( \tau \) is the viscoelastic constant (Lakes 1998). Thus this combination of the spring and dashpot in parallel is accurate when used to predict creep but does not produce stress relaxation.

The standard linear solid model is a combination of the Maxwell model with a spring in parallel (Fig 1.4). As with the Maxwell model, the standard linear solid model predicts that constant strain produces stresses that will gradually relax. The addition of the spring predicts that under an instantaneous release of stress, the strain will decrease.
Thus, the standard linear solid model is the simplest model that predicts both creep and stress relaxation

\[
\sigma = E_R \left[ 1 - \left( 1 - \frac{\tau \sigma}{\tau_e} \right) e^{-t/\tau_e} \right] \varepsilon_o \tag{1.3}
\]

where \( E_R \) is the reduced Young’s modulus, \( \varepsilon_o \) is intial strain, \( \sigma \) is stress, and \( \tau \) is the viscoelastic constant (Fung 1993).

The study of the viscoelastic behavior of materials is of interest because it can affect the performance of the material either intentionally or unintentionally. Specifically, in bone, viscoelastic behavior has an influence on material properties and studying this viscoelastic behavior will help understand material performance. Viscoelasticity is also of interest because there is a possible link between viscoelasticity and microstructure (Lakes 2001). Finally, bone is known to remodel in response to mechanical load (Mow and Huiskes 2005) and the mechanical energy transfer to the cells causing remodeling may be probed by viscoelastic studies (Lakes 2001).

1.3 Nanoindentation

Nanoindentation is a method to quantify the mechanical properties of materials at very small length scales (Oliver and Pharr 1992). It measures the force during
indentation of the surface by pressing a hard tip into the sample, with a known load. As the load is increased, the indenter sinks into the specimen. When the load is held constant, the indenter continues to sink into the material due to time dependent deformation, and finally when the indenter is unloaded from the specimen the material recovers (Fig 1.5). The slope of the curve during extraction of the indenter tip is subsequently used to calculate the elastic modulus of the material.

Nanoindentation has been used to characterize cortical bone properties at the microstructural level (Rho and Pharr, 1999) without taking into account the viscoelastic effects. However since cortical bone is a viscoelastic material, characterization of its viscoelastic properties is an important mechanical parameter. Although nanoindentation is not normally used to discover viscoelasticity in materials, recently, it was found that nanoindentation can actually detect the viscoelasticity of cortical bone (Fan and Rho, 2003).
A strong motivation behind the use of nanoindentation on cortical bone specimens is the potential to understand the mechanical abilities of a whole bone at smaller length scales such as through the properties of its structural units.

1.4 Motivation

Skeletal function depends critically on bone structural integrity. Diseases, such as osteoporosis, diminish the load bearing capabilities of bone, leading to fracture and other related clinical complications. Understanding how properties degrade provides an important, indirect tool to monitor damage accumulation and its affect on bone properties (Reifsnider, 1991).

Defects in cortical bone arise from damage accumulation as a result of repetitive loading (Les et al 2004). Generally, strength and modulus are used to determine changes in cortical bone due to this damage accumulation. But since bone, in its structural role in the body, exhibits viscoelastic behavior it has an influence on material performance that can not be ignored. Through torsion experiments (Jepsen and Davy 1997) it was found that viscous properties were sensitive measures of the damage process.

Understanding the viscoelastic behavior of bone will help to better understand the damage accumulation process in bone through changes in viscous and elastic
properties. Efforts have been made to understand the viscoelasticity of cortical bone on a macroscopic level, but microstructural measurements of viscoelastic behavior may provide additional insight. Comparison of the macroscopic viscoelastic properties and the nano scale viscoelastic properties will allow a connection to be made between cortical bone as a whole and the structural units that comprise cortical bone. This connection will also allow other structures, such as trabecular bone, to be understood based on its structural units of individual trabeculae.

1.5 Objectives

The objective of this thesis was to compare viscoelastic properties measured at the macroscopic level to those measured by nanoindentation.

The specific aims of this work were to:

- Develop a technique to accurately produce cortical bone specimens of specific size with the specimen long axis parallel to the longitudinal axis of the tibia
- Develop a testing protocol for torsional testing of the specimens
- Measure the viscoelastic properties of cortical bone
- Measure the viscoelastic properties of the cortical bone specimens using nanoindentation techniques
- Compare the viscoelastic properties from macroscopic test with results from nanoindentation test

1.6 Overview

In Chapter two of this thesis, a protocol to produce waisted cylindrical shaped specimens for macroscopic testing is described. A protocol to produce specimens for nanoindentation test is also developed. These protocols cover specimen preparation and the different machining process used to produce samples. Chapter three describes the
testing protocols developed for performing macroscopic tests and nanoindentation tests as well as the theory used to analyze the results. These protocols covered the loading rates and gripping mechanism used in the test. The effect of drying on cortical bone samples during nanoindentation are discussed in chapter four. In chapter five the results of the mechanical test are presented. Finally, conclusions and future directions for this research are described in chapter six.
2.1 Introduction

Cutting and machining of bone samples can be a very time consuming step in preparation of bone specimens for mechanical testing. Certain factors, such as overheating and dehydration, need to be considered during this process. To account for these factors a saline drip was constantly used to keep the cortical bone specimens hydrated and slow cutting speeds were used to reduce the likelihood of overheating. Sample preparation for nanoindentation also has several factors that need to be addressed. Factors such as sample surface roughness and inhomogeneities have a significant effect on the measured mechanical properties during indentation. In order to reduce the presence of these factors samples were finely polished.

Sample preparation procedures were developed to achieve repeatable geometry. The procedures were followed to create 5 x 5 x 50 mm cortical bone beams, which were subsequently turned into specimens with 18 mm gauge length and 3 mm gauge diameter. Another procedure was developed to create polished samples for nanoindentation.

2.2 Macroscopic Sample Protocol

Fourteen cylindrical samples were prepared from seven bovine tibiae. Each tibia was cut with the specimen long axis parallel to the long axis of the bone. For torsional loading, waisted cylindrical specimens were generated from the 5 x 5 x 50 mm cube
specimens. The waisted cylinders had a 5 mm grip diameter, 3 mm gage diameter, and 18 mm gauge length (Fig 2.1; Jepsen and Davy 1997).

A rough cut on the cortical bone was made approximately 5x5x50 mm with a band saw (MSK, Fleet Wood Slicing Machines, Newark, NJ). The rough cut specimens were then measured for accuracy. If the sample was found to not be roughly square or within 1 mm of the final specimen dimensions a diamond saw (Model 650 Low Speed Diamond Wheel Saw, South Bay Technology, San Clemente, CA) was used to make the necessary adjustments.

A perfectly square cross section was needed in order to precisely hold the sample in a lathe. To achieve this within an accuracy of 0.5 mm (Fig 2.2) a CNC mill (5400 Series, Sherline Products, Vista, CA, Fig 2.3) was used. The rough cut specimens were securely mounted in a vise (Mill Vise 3551, Sherline Products, Vista CA). The vise was mounted in a custom container designed to keep the specimens submerged in saline solution during milling (Fig 2.4). The sample container was mounted onto the x-axis of the milling machine and filled with saline solution. A G-code program was generated based on the initial size of the specimen to drive the CNC mill. Each side of the sample was reduced separately using a fly cutter (Fly Cutter 3052, Sherline Products, Vista,
Figure 2.2 Average final cross-sectional dimensions of milled specimens including standard deviation (N=12)

CA). The specimen was rotated and each side was cut until the bone was 5 x 5 mm square.

The squared specimens were mounted onto a CNC lathe (Model 4400, Sherline Products, Vista, CA) using a 4-jaw self-centering chuck (1075, Sherline Products, Vista, CA). The central gage length of the specimen was turned using a spindle speed of 2800 rpm and a federate of 0.001in/min. During the machining process the specimen was under a saline drip, for lubrication (Fig 2.5). The turned down section of the sample had an 18 mm gauge length with a coefficient of variation of 0.02, and a diameter of 3 mm with a coefficient of variation of 0.016 (Fig 2.6).
2.3 Preparation of Nanoindentation Samples

Fourteen cylindrical specimens were cut from the macroscopic samples for use in indentation tests. The samples were cut to approximately 5 mm height from the center of the cylindrical specimens. The sample was mounted to a metal disc that had a slightly larger diameter than the sample. The disc was used to mount the finished sample to the magnetic chuck of the nanoindenter. The specimens were polished to obtain a clear view of features such as osteons and haversian canals and provide a smooth surface for the indenter (Fig 2.7).

For the cortical bone specimens to have a parallel surface the specimen was polished on a polishing wheel (Buehler Ltd., Lake Bluff, IL) using carbide abrasive paper (Carbimet Disc, Buehler Ltd., Lake Bluff, IL) with progressively finer abrasives (600, 800, and 1200 grit). The specimen was held for polishing using a grinding fixture (Fig 2.8). During the grinding process a continuous flow of water covered the surface.
Figure 2.4 Fixture to hold specimen while submerged in buffered saline bath during machining

The rotation speed was set to 200 rpm and polishing was performed by lightly pressing the polishing tool against the carbide disc. The specimen was rotated in a circular pattern in the opposite direction of the carbide disc rotation making sure to keep uniform pressure on the polishing tool. Each specimen was polished for 60 to 90 seconds for each abrasive. The specimens were further polished using nylon cloths (No. 40-7058, Buehler Ltd., Lake Bluff, IL), 2 μm magnesia, and 0.25 μm alumina suspensions. Water
Figure 2.6 Outer diameter and gauge length dimensions of turned down specimen including standard deviation (N=12) was used as a lubricant. The 2 μm magnesia, the first suspension used, was spread uniformly in the inner region of the nylon cloth. The polishing wheel rotation speed was set to 200 rpm and the specimen was polished for two to three minutes. The specimen surface was washed using water and evaluated under a microscope. If specimen features could not been seen the polishing with magnesia was repeated.

Figure 2.7 Cortical bone specimen (a) Unpolished specimen; (b) Polished specimen; (c) haversian canal; (d) osteon
Fine polishing of samples was achieved using a $0.25 \, \mu \text{m}$ alumina suspension. With the polishing wheel rotation speed set to 200 rpm the specimen was polished for one to two minutes. After polishing, the specimen surface was washed using water and evaluated under a microscope. If scratches were still seen on the surface after the alumina was used the last step was repeated. Typically, the entire polishing process took 5 minutes.

2.4 Density Measurement

The density of the cortical bone tissue was measured using Archimedes’ principle. To begin measurements the suspended mass of the sample was measured by placing the specimen in the submerged basket and recording the mass. Next, the saturated mass was measured after excess water was removed from the specimen. The specimen was then placed on the top of the specimen basket and support hanger until a stable weight reading was recorded. The apparent densities of the samples were determined from the volume of fluid displaced when the sample was immersed in DI water.

The cortical bone samples were stored in a freezer and -20°C, thus before density measurements could begin the samples were allowed to thaw by sitting in DI water for approximately 20 minutes. The samples had an average density of 2.02 g/cm$^3$ and a standard deviation of 0.07 g/cm$^3$ (Table 2.1).
TABLE 2.1

DENSITY MEASUREMENTS OF CORTICAL BONE SAMPLES USING ARCHIMEDES’ PRINCIPLE INCLUDING MEAN AND STANDARD DEVIATION

(N=12)

<table>
<thead>
<tr>
<th>Specimen ID</th>
<th>Density g/cm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>2.02</td>
</tr>
<tr>
<td>9</td>
<td>2.10</td>
</tr>
<tr>
<td>11</td>
<td>2.04</td>
</tr>
<tr>
<td>12</td>
<td>2.07</td>
</tr>
<tr>
<td>14</td>
<td>2.04</td>
</tr>
<tr>
<td>15</td>
<td>1.85</td>
</tr>
<tr>
<td>17</td>
<td>2.03</td>
</tr>
<tr>
<td>18</td>
<td>2.03</td>
</tr>
<tr>
<td>19</td>
<td>2.00</td>
</tr>
<tr>
<td>21</td>
<td>2.04</td>
</tr>
<tr>
<td>23</td>
<td>2.03</td>
</tr>
<tr>
<td>24</td>
<td>1.93</td>
</tr>
<tr>
<td>Average</td>
<td>2.02</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>0.07</td>
</tr>
</tbody>
</table>

2.5 Conclusions

A technique for producing waisted cylindrical specimens aligned with the longitudinal axis of the cortical bone structure was developed. This technique produced accurate specimens for torsional loading and nanoindentation. Specifically, the major points of this chapter are that:

- A protocol for producing cylindrical waisted cylinder shaped samples using a CNC mill and lathe was developed
- A protocol for polishing cylindrical cortical bone samples for nanoindentation was developed
3.1 Macroscopic Testing Methods

Mechanical testing was performed on an EnduraTEC ELF 3300 (EnduraTEC, Minnetonka, MA) testing instrument under displacement control (Fig 3.1). A WinTest™ custom load configuration was used to control the functions of the load frame. In this setting the user can specify the loading rate, final displacement, twist angle, and sampling rate. This program recorded the force data from the load cell and saved the data in a file format compatible with Microsoft Excel™. All tests were preformed in a saline solution bath at 37° C to keep the bone hydrated during testing and to control for the known temperature dependence of viscoelastic properties (Lakes 1998).

3.1.1 Sample Preparation

Twelve waisted cylindrical shaped specimens were prepared from seven bovine tibiae as described in the previous chapter. Briefly, the specimens were machined to 5 x 5 x 50 mm while submerged in a saline solution bath using a tabletop mill. The specimens were then machined into waisted cylinders while under a constant saline drip. The specimens were machined to approximately 3.00 mm in diameter with an 18 mm gauge length. Except during
3.1.2 Mechanical Testing

Specimens were held in grips, which were attached to the load frame, during testing. The grips were placed so that the gauge length of the specimen was exposed. The specimens were loaded in axial torsion and tension. They were first loaded in torsion using a ramp-hold-unload protocol. They were ramped to 3.5°, 50% of the shear yield, at a rate of 40°/s, then held at this position for 120 seconds, and finally unloaded at the same rate of 40°/s (Jepsen, Davy 1994). This cycle was repeated four times on each sample with a 4 minute recovery period between cycles (Fig 3.2). The 4 minute recovery period was introduced to relieve residual internal stresses.

Samples were then tested in tension using a similar protocol of ramp-hold-unload with a recovery period (Fig 3.2). The sample was ramped to 0.012 mm, 5% of the yield,
3.1.3 Data Analysis

After all tests were completed the data was used to calculate viscoelastic properties of the specimens. The interpretation of the macroscopic data included calculating the shear modulus, elastic modulus, and relaxation time constant and the creep time constant.

To calculate the relaxation constants the cortical bone samples were assumed to follow a standard linear solid viscoelastic model. Using this model the constants can be determined using the following equation:

$$\sigma = E_R \left[ 1 - \left( 1 - \frac{\tau_\sigma}{\tau_\varepsilon} \right) e^{-t/\tau_\varepsilon} \right] \varepsilon_0, \quad (3.1)$$

where $\sigma$ is stress, $E_r$ is reduced modulus, $\tau_\sigma$ is relaxation constant under constant stress and $\tau_\varepsilon$ is relaxation constant under constant (Fung 1993).
3.2 Nanoindentation Testing
Nanoindentation testing was performed on a Hysitron TriboIndenter (Hysitron, Minneapolis, MN) testing machine under load control (Fig 3.4). A custom load pattern was used to control the indentation placements. This pattern consisted of 20 indents in a osteons in all parts of the cortical bone samples. The triboindenter uses a real-time data collection program that recorded the force data from the load cell as well as the displacement, and saved the data in a file format compatible with Microsoft Excel™.

3.2.1 Sample Preparation
After macroscopic testing the samples underwent nanoindentation. The mid section of the gage length of each specimen was cut for use during indentation. This section had a 3 mm diameter and was approximately 5 mm in height. Samples were polished using a polishing wheel (Buehler Ltd., Lake Bluff, IL) as described in Chapter 2. The polished samples allowed places for indentation to be chosen. Pictures of the polished sample were taken using a microscope at 50x magnification which allowed the indentation sites to be examined after nanoindentation was performed. After the samples were polished and a picture was taken using the microscope, the samples were wrapped in gauze, hydrated with buffered saline solution, and stored in the freezer in new containers. The rest of the sample was also rewrapped in gauze, hydrated with buffered saline solution, and restored in the freezer at -20°C in a different container.

3.2.2 Data Collection
Before testing, specimens were glued to a metal disc with Loctite™ (Loctite, Rocky Hill, CT) adhesive, for stability during indentation, and allowed to thaw while wrapped in gauze damped with buffered saline solution. After thawing, the specimen
was inserted into the TriboIndenter for nanoindentation. Twenty indents were placed within the osteons of each of the samples.

![Hysitron automated nanomechanical TriboIndenter used for nanoindentation test](adapted from www.hysitron.com)

Using a Berkovich indenter tip, each indentation had a loading to 10 mN at a rate of 2 mN/s, followed by a 10 second hold time, then the load was released at 2 mN/s (Fig 3.4). Once the loading rate was loaded the indentation pattern was loaded and indentation began. The indentation experiment took approximately 45 minutes. Afterward the samples were wrapped in gauze, rehydrated, and restored in the freezer at -20°C.

![Indent loading protocol for nanoindentation test](Fig 3.4)

22
3.2.3 Data Analysis

Young’s modulus, relaxation time constant, and creep time constant were calculated from the indentation data. The Pharr Oliver equation (Eqn 3.2) was used to calculate the young’s modulus

\[ S(h_{\text{max}}) = \frac{2}{\pi} E_r \sqrt{A_c(h_{\text{max}})} , \]  

(3.2)

where \( S(h_{\text{max}}) \) is the derivative of the unloading curve at the point of initial unloading, \( h_{\text{max}} \), \( E_r \) is the reduced modulus, and \( A_c(h_{\text{max}}) \) is the contact area over which the material and the indenter are in instantaneous contact (Oliver and Pharr 1992). This relationship has been shown to be a good approximation for a Berkovich indenter tip (Pharr et al 1992). The reduced modulus, \( E_r \), depends on the deformation of the material and the diamond indenter tip through the following relationship:

\[ \frac{1}{E_r} = \frac{1 - \nu_{\text{specimen}}^2}{E_{\text{specimen}}} + \frac{1 - \nu_{\text{tip}}^2}{E_{\text{tip}}} , \]  

(3.3)

where \( \nu_{\text{specimen}} \) is the Poisson ratio of the specimen, \( E_{\text{tip}} \) the elastic modulus of the indenter tip, \( \nu_{\text{tip}} \) the Poisson ratio of the indenter tip (Hengsberger et al 2002). The indentation modulus:

\[ E_{\text{indentation}} = \left( \frac{1}{E_r} - \frac{1 - \nu_{\text{tip}}^2}{E_{\text{tip}}} \right)^{-1} \]  

(3.4)

can be calculated with \( \nu_{\text{tip}} = 0.07 \) and \( E_{\text{tip}} = 1140 \text{ GPa} \) (Hengsberger et al 2002). This variable, \( E_{\text{indentation}} \), can then be used to determine the elastic modulus of the specimen:

\[ E_{\text{indentation}} = \frac{E_{\text{specimen}}}{1 - \nu_{\text{specimen}}^2} \]  

(3.5)

where \( \nu_{\text{specimen}} \), the Poisson’s ratio of the specimen is assumed (Hengsberger et al 2002).
3.3 Ultrasonic Characterization

Ultrasonic methods can be used to characterize the elastic properties of bone and to analyze its mechanical behavior. Previous experimental methods for measuring the elastic modulus may not reflect the true stiffness of the bone due to bias introduced by machine error, edge effects, and heterogeneity bias, amongst other biases (Burstein et al 1975; Abendschein and Hyatt, 1970). To fix these biases, ultrasonic experiments investigated the elastic modulus of bone (Abendschein and Hyatt, 1970). Ultrasonic methods were found to be accurate measures for the elastic properties of bone (Hunt et al 1998).

The same samples used in the nanoindentation test were characterized by ultrasound. The samples were tested to determine the elastic and shear modulus for comparison with the values found using nanoindentation, torsion and tension experiments.

3.3.1 Mechanical Testing

Before testing, specimens were allowed to thaw in a buffered saline solution bath for 45 – 60 minutes. The longitudinal and transverse wave velocities traveling in the longitudinal direction for each bone specimen were measured.

In order obtain consistent measurements; a calibration run was first performed with a gauge block. The gauge block was placed between the transducers with DI water as the couplant fluid in the specimen-transducer interfaces. Calibration was performed until the same time delay result was consistently recorded for the steel gauge. After the calibration, the cortical bone samples were tested using a longitudinal wave and DI water as the couplant fluid. Following this all samples were testing using shear a shear wave and honey as the couplant fluid. The lower energy carried by the shear waves, prompts the use of a higher viscosity couplant.

Based on the plane wave equation
\[ C_{11} \frac{d^2u}{dx^2} = \rho \frac{d^2u}{dt^2} \]  

(3.6)

and assuming a wave of the form

\[ u(x, t) = \sin(x - \omega t) \]  

(3.7)

the stiffness coefficient was determined by the relationship

\[ C_{11} = \rho \nu^2. \]  

(3.8)

### 3.3.2 Data Analysis

To determine the values for the stiffness tensor the cortical bone samples were assumed to be orthotropic, creating the following stiffness tensor (Bartel et al. 2006):

\[
 C_{ij} = \begin{bmatrix}
 c_{11} & c_{12} & c_{13} & 0 & 0 & 0 \\
 c_{12} & c_{22} & c_{23} & 0 & 0 & 0 \\
 c_{13} & c_{23} & c_{33} & 0 & 0 & 0 \\
 0 & 0 & 0 & c_{44} & 0 & 0 \\
 0 & 0 & 0 & 0 & c_{55} & 0 \\
 0 & 0 & 0 & 0 & 0 & c_{66} \\
\end{bmatrix}, 
\]

(3.6)

where the constants of the stiffness matrix are calculated as follows:

\[ c_{ij} = \rho \cdot \nu_{ij}^2, \]

(3.7)

where \( \rho \) is apparent tissue density and \( \nu \) the wave velocity (Pithioux et al 2002). The elastic modulus and shear modulus were calculated using Hooke’s law:

\[ C_{11} = \frac{1 - \nu_{23} \nu_{32}}{E_2 E_3 \Delta}, \]

(3.8)

where

\[ \Delta = \frac{1 - \nu_{12} \nu_{21} - \nu_{23} \nu_{32} - \nu_{31} \nu_{13} - 2 \nu_{21} \nu_{13} \nu_{32}}{E_1 E_2 E_3}, \]

(3.9)

and
\[ C_{12} = 2G_{12}, \quad (3.10) \]

where \( G_{12} \) is the shear modulus (Bartel 2006).

### 3.4 Summary

In this chapter, the testing protocols used in both the macroscopic and microscopic experiments were developed. Specifically, the procedures that were developed were a:

- torsional testing protocol, which loaded the sample to 3.5° at 40°/s with a 120 second hold time followed by the sample being unloaded at the same rate followed by a 4 minute recovery period, this pattern was repeated for 4 cycles

- tensile testing protocol, which loaded the sample to 0.12mm at 40mm/s with a 120 second hold time followed by the sample being unloaded at the same rate followed by a 4 minute recovery period, this pattern was repeated for 4 cycles

- nanoindentation testing protocol, which indented the specimen 20 times; each indent was loaded into the sample to 10 mN at 2 mN/s with a 10 second hold time followed by the sample being unloaded at the same rate
CHAPTER 4
NANOINDENTATION DRYING EFFECTS

4.1 Introduction

Viscoelastic properties of cortical bone depend on water content as well as temperature (Sasaki, Enyo 1995). The relaxation phenomenon of bone is changed by the water content of the bone specimen (Lakes et al 1979). Drying also increases the modulus of elasticity, increases the tensile and bending strength and makes bone much more brittle and less tough (Currey 1988).

To determine if cortical bone samples became dehydrated during a series of 15 indents, data from 5 ovine cortical bone samples were analyzed. The samples were polished and stored using the procedures described in Chapter 2. Before the indentation test, the samples were allowed to completely thaw while submerged in buffered saline. Four of the samples had all indents placed in osteons, and one sample had all indents placed in the interstitial tissue. Based on the physical features of the cortical bone seen through the nanoindenter microscope, locations for indents were randomly chosen. Indents were initiated every four minutes for a total of 56 minutes. The elastic modulus and creep rate of the samples was determined by analyzing the nanoindentation data recorded.
4.2 Data Analysis

The data was analyzed to determine the elastic modulus, as discussed in Chapter 3, and to determine the creep rate of each indent. The creep rate was calculated by taking the change in depth vs. change in time at the middle of the hold time (5 seconds).

The modulus of all of the samples showed no trend ($R^2 = 0.031$, $p > 0.15$) indicating that the cortical bone properties were not affected by drying over the 56 minutes (Fig 4.1, Table A4.1).

![Elastic modulus (GPa) vs. time (minutes) of cortical bone samples measured using nanoindentation (N=4)](image)

Figure 4.1. Elastic modulus (GPa) vs. time (minutes) of cortical bone samples measured using nanoindentation (N=4)
The creep rate of the samples also showed no trend ($R^2 = 0.0003$, $p > 0.12$) indicating that the time period had no effect on creep measurements (Fig 4.2, Table A4.2).

![Figure 4.2. Creep rate (nm/s) vs. time (minutes) of cortical bone samples measured using nanoindentation (N=4)](image)

4.3 Discussion

Nanoindentation tests were preformed on 4 ovine, cortical bone samples. The samples underwent a series of 15 indents over a 56 minute time period in either osteonal or interstitial tissue. No trend was seen in the elastic modulus calculated nor the creep rates calculated for any of the specimens. As such, nanoindentation protocols that take as long as one hour should not be affected by dehydration of the bone.
CHAPTER 5
RESULTS

5.1 Introduction

This chapter contains the results from macroscopic and microscopic experiments. Torque-time, force-time, and depth-time data was collected using torsion, tension, and nanoindentation protocols and procedures previously described in Chapter 3. The data was analyzed to calculate young’s modulus, shear modulus and, using the standard linear solid model, to calculate the relaxation constants under constant stress and strain.

5.2 Torque – Time Measurements

Each sample reached a peak torque, showed stress relaxation, and fully recovered during the 4 minute recovery period (Fig 5.1). The samples reached a peak torque of 0.159 ± 0.033 Nm.

Using the torque-time data the shear modulus and relaxation constants were calculated (Table 5.1). The average shear modulus was 5.86 ± 1.14 GPa which falls into the range of cortical bone (Bonfield and Li 1967). The relaxation time constant was 24.84 ± 3.75 s and the average creep time constant under constant stress was 22.08 ± 3.55 s.
Figure 5.1  Torque vs. time data of bovine cortical bone samples (N=12) measured over four twist intervals

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>G (GPa)</th>
<th>$\tau_\sigma$ (s)</th>
<th>$\tau_\epsilon$ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>7.35</td>
<td>18.82</td>
<td>20.72</td>
</tr>
<tr>
<td>9</td>
<td>6.00</td>
<td>27.96</td>
<td>31.16</td>
</tr>
<tr>
<td>11</td>
<td>6.97</td>
<td>24.50</td>
<td>27.32</td>
</tr>
<tr>
<td>12</td>
<td>6.26</td>
<td>25.14</td>
<td>28.63</td>
</tr>
<tr>
<td>14</td>
<td>6.49</td>
<td>17.76</td>
<td>19.55</td>
</tr>
<tr>
<td>15</td>
<td>6.73</td>
<td>25.32</td>
<td>28.31</td>
</tr>
<tr>
<td>17</td>
<td>5.16</td>
<td>20.35</td>
<td>22.75</td>
</tr>
<tr>
<td>18</td>
<td>5.63</td>
<td>22.08</td>
<td>24.59</td>
</tr>
<tr>
<td>19</td>
<td>4.82</td>
<td>23.22</td>
<td>26.18</td>
</tr>
<tr>
<td>21</td>
<td>6.85</td>
<td>23.21</td>
<td>25.54</td>
</tr>
<tr>
<td>23</td>
<td>4.88</td>
<td>21.04</td>
<td>23.91</td>
</tr>
<tr>
<td>24</td>
<td>3.35</td>
<td>15.55</td>
<td>19.41</td>
</tr>
</tbody>
</table>

Average 5.87  22.08  24.84
Std.Dev 1.15      3.55     3.75
5.3 Force – Time Measurements

Samples showed typical stress relaxation behavior in tension (Fig 5.2). The peak force was 31.26 ± 3.37 N on average. Using the force – time data the elastic modulus, stress relaxation time constant and creep time constant were calculated.

Figure 5.2 Force vs. time data, measured over four intervals, collected from tension test for all samples (N=12)

Using the standard linear solid model as previously described in Chapter 3, the relaxation time constant under constant strain was 37.78 s ± 3.56 s and the creep time constant under constants stress was 36.03 s ± 2.95 s.
5.4 Nanoindentation

Each bone sample was indented twenty times over the course of 45 minutes. Using the depth – time data and load – displacement data, the elastic modulus and relaxation constant under constant stress and strain for each specimen was calculated.

Typically, 40% - 95% of the unloading curve from the nanoindentation load – displacement data is used to calculate the elastic modulus of a sample (Hengsberger et al 2002). To determine the appropriate data range for determining the derivative of the unloading curve, $S(h_{\text{max}})$, three different parts of the unloading curve for sample #23 were examined: 10000 μN – 8000 μN; 9000 μN – 7000 μN; 40% of the unloading curve starting from 10000 μN, and the entire unloading curve (Fig 5.3). The elastic modulus for each section of the unloading curve was calculated for the four subranges of data investigated, the modulus varied from 9.7 to 16.8 GPa. It was determined that 40% of the upper portion of the unloading curve was a good portion to use to determine the elastic modulus for the rest of the samples.

![Figure 5.3 Examining different sections of a nanoindentation unloading curve to determine the correct range for determining $S(h_{\text{max}})$, the derivative of the unloading curve](image-url)
The elastic modulus for each of the samples was in the range of 13.64 GPa – 17.99 GPa with an average of 16.13 GPa which falls into the previously reported range for cortical bone (Fig 5.4; Table A5.1; Reilly et al 1974). Using the standard linear solid model the relaxation constants under constant stress and strain were determined. The average relaxation constant under constant stress was $2.69 \text{ s} \pm 0.12 \text{ s}$ and the relaxation time under constant strain was $2.41 \text{ s} \pm 0.08 \text{ s}$.

### 5.5 Ultrasonic Characterization

The elastic and shear modulus of the cortical bone specimens were analyzed using ultrasonic methods to determine the correlation between values found using nanoindentation and mechanical testing experiments. To calculate the elastic modulus a value of Poisson’s ratio was assumed. To determine an appropriate value, the elastic
modulus with Poisson’s ratio ranging from 0.2 to 0.35 was calculated (Fig 5.5). It was observed that the calculated Young’s Modulus was sensitive to Poisson’s ratio. It was then assumed that Poisson’s ratio would be 0.3 for all samples since this is the value typically used. Using this value the elastic modulus calculated from ultrasonic methods was found to be similar to nanoindentation calculations ($R^2 = 0.88868, p = 7.61\times10^{-5}$; Fig 5.6).

![Figure 5.5 Elastic modulus (GPa) calculated for sample #8 using different values for Poisson’s ratio](image-url)
The shear modulus values calculated from ultrasonic methods correlates to the values found from torsion experiments ($R^2 = 0.9613$, $p = 2.37 \times 10^{-8}$; Fig 5.7).

Figure 5.6 Ultrasonic vs. nanoindentation elastic modulus (GPa) for cortical bone samples (N=12)

Figure 5.7 Ultrasonic vs. torsion shear modulus (GPa) for cortical bone sample (N=12)
5.6 Macroscopic Experiments vs. Microscopic Experiments

To determine if the viscoelastic behavior from macroscopic experiments correlate to microscopic experiments, the relaxation constants were compared. When comparing the relaxation constant under constant stress, \( \tau_\sigma \), there was no correlation seen between nanoindentation and tension experiments (\( R^2 = 0.0101 \); Fig 5.8). When comparing \( \tau_\sigma \) from nanoindentation to torsion test a correlation was observed (\( R^2 = 0.7228 \), \( p = 0.00046 \); Fig 5.8). When comparing the relaxation constant under constant strain, \( \tau_\varepsilon \), again no correlation was found between nanoindentation experiments and tension experiments. When comparing \( \tau_\varepsilon \) for nanoindentation experiments and torsion experiments a correlation existed (\( R^2 = 0.6465 \), \( p = 0.001621 \); Fig 5.9).

Figure 5.8 Creep time constant, \( \tau_\sigma \) (s), nanoindentation vs. tension experiments
Figure 5.9 (a) Creep constant nanoindentation (s) vs. torsion (s); (b) Relaxation constant nanoindentation (s) vs. torsion (s)
5.7 Discussion

Torsion, tension, and nanoindentation experiments were performed to determine the viscoelastic response of twelve cortical bone samples and to determine if the viscoelastic behavior on a macroscopic level was similar to that calculated on a microscopic level. The elastic modulus and shear modulus of the samples were calculated using these techniques and were compared to values obtained using ultrasonic characterization techniques. Using the standard linear solid model the relaxation constants under constant stress and strain were able to be determined.

When comparing the elastic modulus measured using nanoindentation, ultrasonic characterization, and tension test a correlation was existed between the nanoindentation data and the ultrasonic characterization data but no correlation was seen with the tension data. One reason that none of tension data showed any correlation with the other data, from torsion, nanoindentation or ultrasonic experiments could be due to slippage present during testing. When looking at the tension data the force values collected during testing are very scattered. This scatter could be due to the specimens not being securely tightened in the grip ends which caused slippage during the testing. Another reason why the tension data is so inconsistent with the other data could be because of the low values of force that were being reached throughout the testing. The low force values that were reached on a machine that can reach forces well above the ones experienced in the testing could cause the LVDT measurements to not be as accurate as they would be if we were experiencing higher forces. To correct for this error an extensometer should have been used, yielding the most accurate displacement results. The correlation between the calculated nanoindentation modulus and the modulus calculated using ultrasonic characterization techniques indicates that either of the two experiments will produce an accurate result.

When comparing the shear modulus measured using torsion experiments to the shear modulus measured using ultrasonic characterization techniques a correlation was
seen. This indicates that either experiment can be used to determine the shear modulus. Ultrasonic techniques produced an accurate result for both the elastic modulus and shear modulus. This allows ultrasonic characterization to be used as a tool to determine the elastic and shear properties of cortical bone specimens.

When comparing the relaxation constant under constant stress and strain the nanoindentation data correlated with the torsion data; but again no correlation was seen with the tension data. A correlation existed between these two experiments despite the fact that they produced constants on different time scales. The different time scales seen between these two experiments could be due to the level at which they are being tested. Torsion test are testing on a macroscopic level, millimeters, while nanoindentation test are on a microscopic level, micrometers. We would expect the cortical bone samples to relax faster on a microscopic scale because during nanoindentation individual lamella are being testing. Another reason that the time scales are different could be due to the type of behavior that the bone is experiencing during the testing. During the torsion test the cortical bone samples stayed within the elastic region and were thus only experiencing viscoelastic effects, while during the nanoindentation experiments the cortical bone samples were in the plastic deformation region and thus experienced viscoplastic effects.

5.8 Summary

The viscoelastic behavior of cortical bone was determined using nanoindentation, torsion, and tension experiments. The results from these experiments were compared on a macroscopic and microscopic level as well as with ultrasonic characterization techniques. The significant findings from these experiments were:

- Ultrasonic characterization is showed nanoindentation to be an accurate technique for determining the elastic modulus
- Relaxation and creep time constants correlated between macroscopic and microscopic experiments
CHAPTER 6
CONCLUSIONS

6.1 Goal of Study

Elastic and yield properties are used to characterize cortical bone, while viscoelastic effects have received considerably less attention. However, viscoelastic behavior may play an important role in very high or very low loading rates. In this thesis, the role of viscoelastic behavior on cortical bone was examined through tension, torsion, and indentation test. The results from all three experiments were compared to determine if they produced a similar and accurate measure of viscoelastic behavior.

6.2 Summary

A technique for creating waisted cylindrical shaped cortical bone specimens was developed and successfully used to produce cortical bone specimens. The average gauge diameter error of the cortical bone specimens was ± 0.06mm, and the average gauge length error of the specimens was ± 0.29mm. A technique to polish cortical bone samples for nanoindentation test was also developed. This procedure was successfully implemented to produce well polished samples.

Twelve cortical bone specimens were tested in torsion, tension, and nanoindentation, to determine the relaxation constants under constant stress and constant strain using the standard linear solid model. Through these tests and through ultrasonic
characterization the shear modulus and Young’s modulus was also determined for each specimen.

The shear modulus for the bone specimens measured through torsion experiments was 5.87 GPa ± 1.15 GPa, which falls into the range for cortical bone. The shear modulus measured using ultrasonic characterization was 5.75 GPa ± 1.11 GPa. When comparing the results for all samples the shear modulus measure during ultrasonic characterization correlated to the shear modulus measured using torsion experiments.

Using nanoindentation, the elastic modulus was 16.13 GPa ± 1.48 GPa, which also falls into the range for cortical bone. The elastic modulus calculated using ultrasonic characterization was 16.14 GPa ± 1.90 GPa. When comparing the results of the elastic modulus from nanoindentation experiments to ultrasonic characterization there was a correlation.

The creep time constant from nanoindentation experiments was 2.64 s ± 0.33 s. The same creep time constant calculated from torsion experiments was 22.08 s ± 3.55 s. Though the creep time constants are on a different order of magnitude the two experiments did show correlation. The same creep time constant calculated from tension experiments was 34.42 s ± 2.62 s. There was no correlation observed between the torsion and tension experiments, nor between the nanoindentation and tension experiments.

The stress relaxation time constant calculated using nanoindentation experiments was 2.28 s ± 0.23 s. The stress relaxation time constant calculated using torsion techniques was 24.84 s ± 3.75 s. There was a correlation seen between nanoindentation and torsion experiments. The stress relaxation time constant calculated using tension experiments was 36.1 s ± 3.19 s. There was no correlation observed between tension and torsion experiments, nor between tension and nanoindentation experiments.
6.3 Future Work

For better understanding of the viscoelastic behavior and its effect on cortical bone, several factors should be examined. For example, the effect of aging on the viscoelastic properties can be examined to determine if viscoelastic behavior changes with age. Also, the viscoelastic response in diseased bone, for example osteoporotic bone, should be examined. Understanding the role of cortical bone as a viscoelastic material will allow for potential treatment/prevention of the bone diseases and disorders related to age.

The current specimen preparation and experimental protocols developed for cortical bone torsion and nanoindentation testing were validated by results and could be used in future studies. But the results for the tension experiments weren’t validated through this study. Future test could be done to determine if a heated water bath is necessary for the tension experiments. Understanding this factor will allow for the use of an extensometer for more accurate data collection if the heated water bath proves to not have a significant effect.

6.4 Conclusion

Understanding the viscoelastic behavior of cortical bone provides insight into time dependent bone material properties. These experiments have given insight into relaxation constants measured using nanoindentation and torsion experiments. The specific aim of this thesis was to characterize the viscoelastic behavior of cortical bone through torsion, tension, and nanoindentation tests.

- A technique to accurately produce cortical bone specimens of specific size with the specimen long axis parallel to the longitudinal axis of the tibia was developed.
- A testing protocol for torsional testing of the specimens was developed.
• The viscoelastic properties, elastic modulus, and shear modulus of the cortical bone specimens using nanoindentation, tension, torsion and ultrasonic characterization techniques were calculated.

• The viscoelastic and elastic behavior of cortical bone was not affected by drying over a one hour period.

• The measurement of the elastic modulus using ultrasonic techniques verified that nanoindentation results verify macroscopic results.

• A correlation between the macroscopic and microscopic stress relaxation and creep time constants was observed.
APPENDIX A1.

PROCEDURE FOR MILLING CORTICAL BONE SPECIMENS

Tissue Mechanics Laboratory
Department of Aerospace and Mechanical Engineering
University of Notre Dame

Title: Milling Cortical Bone Specimens

A Objective
Mill 5x5x50 mm cortical bone specimens.

B Materials
• Cortical bone specimen
• Buffered saline

C Tools
• Tabletop mill (Sherline Products Inc., Vista, CA)
• Vise (Sherline Products Inc., Vista, CA)
• Vise holder
• Fly cutter (Sherline Products Inc., Vista, CA)
D Procedure

D-1 Rough cutting

*Note: Before cutting, learn how to use the band saw!*

Rough cut the cortical bone specimen to the final dimensions using the band saw. After rough cut, measure the sample to see accuracy. If the sample is not within 1mm of final dimension (5x5x50mm) use the diamond saw to make specimen more accurate. The less extra material the specimen has the faster the milling process will be.

D-2 Milling

Mount the specimen in the vise making sure that it is level by using the metal spacer (Fig A1.1). Mount the vise in the vise holder and fill with buffered saline solution.

![Figure A1.1: Metal spacer used to level cortical bone specimen in the vise for a flat surface cut](image)

Make sure the solution is slightly covering the specimen. After this, mount the vise holder onto the x-axis of the milling machine (Fig A1.2).

To create and run programs make sure to use the Sherline CNC (inch) desktop icon. Once program is open use the g-code `squaresample.ncg` to cut the specimen. This program may need to be adjusted if more than 0.5mm of material need to removed from each side. Do not let the feedrate speed increase 0.5 for the x-axis or the spindle speed...
increase ¼ of the max speed!! This speed will cause the buffered saline solution to spill out of the container.

Align the fly cutter with the side of the sample in the z-axis and the x-axis. Use the fly cutter to square the sample. After all sides are cut sample should be 5mm x 5mm.

![Figure A1.2. Vise screwed into vice holder and mounted on the x-axis of the mill to make square samples](image)

**D-3 G-Code**

squaresample.ncg

% (making square sample cutting .5mm off)
g01 g20 g40 g49 g90 x0 (deletes previous program, puts x to zero coordinate)
z-0.01 f2 (moves spindle down 0.01 inches, sets feedrate)
x-2.0 f0.5 (cuts along x axis)
x0 f5 (returns to home position quickly)
z-0.0197 f2
x-2.0 f0.5
x0 f5
%

47
APPENDIX A2.

PROCEDURE FOR TURNING CORTICAL BONE SPECIMENS

_Tissue Mechanics Laboratory_

_Department of Aerospace and Mechanical Engineering_

_University of Notre Dame_

_Title: Turning Cortical Bone Specimens_

**A Objective**

Turn down 5x5x50 mm cortical bone specimens to a 3mm diameter

**B Materials**

- cortical bone specimen
- Buffered saline

**C Tools**

- Tabletop lathe (Sherline Products Inc., Vista, CA)
- 4 jaw chuck (Sherline Products Inc., Vista, CA)
- Saline Drip
- Cutting tool (Sherline Products Inc., Vista, CA)
D Procedure

D-1 Setup

Begin by filling the saline drip container with buffered saline (Fig A2.1). Attach the 4 jaw chuck onto lathe and insert the square cortical bone specimen. With 2/3 of the cortical bone specimen sticking out of the chuck, tighten chuck until jaws are firmly gripping specimen. Align drip tool with cutting tool so the buffered saline will wet part of the cutting tool and cortical bone specimen during drip (Fig A2.2).

Figure A2.1: Buffered saline solution container containing valve for controlled saline drip

Next, manually move the edge of the cutting tool to the edge of the cortical bone specimen. The specimen and cutting tool are now aligned and ready to be turned.
Figure A2.2: Placement of buffered saline drip, cutting tool, and specimen during the turning process

D-2 Turning

To create and run programs make sure to use the Sherline CNC (inch) desktop icon. Once program is open use the g-code 3mmdiameter.ncg to cut the specimen. This program should not need any adjustments and will turn a 3mm diameter in the cortical bone specimen with gauge length of 18mm.

Once the program has finished take the sample out of the 4 jaw chuck and place the opposite end into the chuck. Repeat the process of aligning the cutting tool and open the 3mmdiameter2.ncg program to finish the turning process. Again this program should not need any adjustments.

The final specimen will have a 3 mm reduced section, 18mm gauge length, and a 9.6mm radius of transition (Fig A2.3).
D-3 G-Code

3mmdiameter.ncg
%(turning down inner 3mm diameter)
g01 g20 g40 g49 g90  x0 y0 (goes to home position, uses inch measurements, cancels previous commands)
x.433 (moves in x direction to position where 9.6mm radius of transition begins)
g02 x0.433 y0 i0.433 j.039 (small steps to final)
g02 x0.433 y0 i0.433 j.0787
g02 x0.433 y0 i0.433 j.118
g02 x0.433 y0 i0.433 j.157
g02 x0.433 y0 i0.433 j.197
g02 x0.433 y0 i0.433 j.236
g02 x0.433 y0 i0.433 j.276
g02 x0.433 y0 i0.433 j.315
g02 x0.433 y0 i0.433 j.354
g02 x0.433 y0 i0.433 j.378 (9.6mm radius of transition)
g01 y0 x.59 (moves to end of transition end to begin cutting inner diameter)
y.02 x1.3 (cuts inner diameter, increment = 0.5mm)
y0 x.59
y.039 x1.3
y0 x.59
y.059 x1.3
y0 x.59
y.0787 x1.3
y0x0
%

3mmdiameter2.ncg
%(turning down transition on opposite end)
g01 g20 g40 g49 g90  x0 y0 (goes to home position, uses inch measurements, cancels previous commands)
x.433 (moves in x direction to position where 9.6mm radius of transition begins)
g02 x0.433 y0 i0.433 j.039 (small steps to final)
g02 x0.433 y0 i0.433 j.0787
g02 x0.433 y0 i0.433 j.118
g02 x0.433 y0 i0.433 j.157
g02 x0.433 y0 i0.433 j.197
g02 x0.433 y0 i0.433 j.236
g02 x0.433 y0 i0.433 j.276
g02 x0.433 y0 i0.433 j.315
g02 x0.433 y0 i0.433 j.354
g02 x0.433 y0 i0.433 j.378 (9.6mm radius of transition)
g01 x0 y0
%
APPENDIX A3.

PROCEDURE FOR POLISHING CORTICAL BONE SPECIMENS

Tissue Mechanics Laboratory
Department of Aerospace and Mechanical Engineering
University of Notre Dame

Title: Cortical Bone Polishing

A Objective
Polish cortical bone as clearly as possible for view in microscope.

B Materials
• Round cortical bone specimen

C Tools
• Polisher (Buehler Ltd., Lake Bluff, IL)
• Polishing tool
• 600 grit carbide disc, 8in. diameter (Carbimet® Disc, Buehler Ltd. Lake Bluff, IL)
• Nylon polishing cloth (No. 40-7058, for 800 wheel), Buehler Ltd., Lake Bluff, IL
D Procedure

D-1 Rough polishing

Note: Before polishing, learn how to use the polisher!
Before polishing, cortical bone samples should be approximately 3mm in diameter and 4 – 5 mm in height.

Fit the cortical bone sample onto the tool for polishing (Fig A3.1). The polishing holder is used to keep the polished surface parallel to the bone surface. Adjust the height of the tool so the top portion of the specimen is level with the edge of the tool. This will keep the specimen on the tool during polishing. Mount the #600 grit carbide sandpaper into the polishing pad and use water as lubricant. Mount the disc on the polisher and turn the water spigot on such that continuous flow covers the polishing surface. Set the rotation speed to 200 rpm and begin grinding the surface by lightly pressing the polishing tool against the carbide disc. Move specimen in a circular pattern in the opposite direction of the carbide disc rotation (Fig A3.2). Polish the specimen for 60 s to 90 s. Make sure uniform pressure is being applied while holding the specimen to produce a flat, smooth surface.

![Figure A3.1: Polishing tool used to hold the cortical bone specimen and produce a flat surface during polishing](image)

D-2 Fine polishing

Note: Wear gloves before operation!

Mount the nylon cloth into the polishing pad and use water as lubricant. In the first step, use 3 μm diamond polishing compound. Use your finger to spread the compound on the polishing cloth mainly in the inner 2/3 region of the cloth. Polish the
specimen for 2 minutes at 200 rpm. Low rotation speed needs longer polishing time. After polishing, wash the polished surface with water.

Repeat the process using 0.005μm alumina polishing compound. The polishing time, using the alumina compound should be 60 seconds with a rotation speed of 200 rpm. For each compound, use a new piece of nylon cloth.

![Figure A3.2: Polishing tool path in a counter clockwise direction, opposite of the polishing plate direction](image)

D-3 Finished Specimen

Features of the specimen should be recognizable under the microscope at end of polishing (Fig A3.3). After completing the final polishing step, store the polished sample.

![Figure A3.3: A polished cortical bone sample viewed at 50x showing visible features; osteons, and haversian canal](image)
APPENDIX A4.

DATA COLLECTED TO ANALYZE NANOINDENTATION DRYING EFFECTS

Tissue Mechanics Laboratory

Department of Aerospace and Mechanical Engineering

University of Notre Dame

Title: Nanoindentation Data to Analyze Drying Effects
<table>
<thead>
<tr>
<th>C22 (osteon)</th>
<th>C04 (osteon)</th>
<th>C05 (osteon)</th>
<th>C13 (interstitial)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modulus (GPa)</td>
<td>time (s)</td>
<td>Modulus (GPa)</td>
<td>time (s)</td>
</tr>
<tr>
<td>22.44</td>
<td>0</td>
<td>17.85</td>
<td>0</td>
</tr>
<tr>
<td>34.80</td>
<td>4</td>
<td>41.63</td>
<td>4</td>
</tr>
<tr>
<td>14.10</td>
<td>8</td>
<td>23.57</td>
<td>8</td>
</tr>
<tr>
<td>25.29</td>
<td>12</td>
<td>15.55</td>
<td>12</td>
</tr>
<tr>
<td>17.89</td>
<td>16</td>
<td>27.54</td>
<td>16</td>
</tr>
<tr>
<td>23.70</td>
<td>20</td>
<td>18.97</td>
<td>20</td>
</tr>
<tr>
<td>32.61</td>
<td>28</td>
<td>26.77</td>
<td>28</td>
</tr>
<tr>
<td>29.33</td>
<td>32</td>
<td>9.42</td>
<td>32</td>
</tr>
<tr>
<td>25.48</td>
<td>40</td>
<td>11.21</td>
<td>40</td>
</tr>
<tr>
<td>26.70</td>
<td>44</td>
<td>37.40</td>
<td>44</td>
</tr>
<tr>
<td>23.26</td>
<td>48</td>
<td>34.18</td>
<td>48</td>
</tr>
<tr>
<td>25.65</td>
<td>52</td>
<td>20.77</td>
<td>52</td>
</tr>
<tr>
<td>39.65</td>
<td>56</td>
<td>12.83</td>
<td>56</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>P-value</th>
<th>P-value</th>
<th>P-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>8.03E-06</td>
<td>Intercept</td>
<td>2.67E-04</td>
</tr>
<tr>
<td>X Variable</td>
<td>0.2559</td>
<td>X Variable</td>
<td>0.606</td>
</tr>
</tbody>
</table>
# Table A4.2

## Calculated Creep Rates and P-Values for 15 Indents Performed Over 56 Minutes in With Osteonal and Interstitial Tissue (N=4)

<table>
<thead>
<tr>
<th>C04 (osteon)</th>
<th></th>
<th>C05 (osteon)</th>
<th></th>
<th>C22 (osteon)</th>
<th></th>
<th>C13 (interstitial)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>creep rate (nm/s)</td>
<td>time (min)</td>
<td>creep rate (nm/s)</td>
<td>time (min)</td>
<td>creep rate (nm/s)</td>
<td>time (min)</td>
<td>creep rate (nm/s)</td>
<td>time (min)</td>
</tr>
<tr>
<td>8.7109019</td>
<td>0</td>
<td>8.639902</td>
<td>0</td>
<td>8.710962</td>
<td>0</td>
<td>8.710962</td>
<td>0</td>
</tr>
<tr>
<td>8.7114243</td>
<td>4</td>
<td>8.711022</td>
<td>4</td>
<td>8.710962</td>
<td>4</td>
<td>8.710962</td>
<td>4</td>
</tr>
<tr>
<td>8.7109019</td>
<td>8</td>
<td>8.711022</td>
<td>8</td>
<td>8.644939</td>
<td>8</td>
<td>8.710962</td>
<td>8</td>
</tr>
<tr>
<td>8.6395419</td>
<td>12</td>
<td>8.711022</td>
<td>12</td>
<td>8.644339</td>
<td>12</td>
<td>8.710962</td>
<td>12</td>
</tr>
<tr>
<td>8.7109619</td>
<td>16</td>
<td>8.646798</td>
<td>16</td>
<td>8.711022</td>
<td>16</td>
<td>8.710962</td>
<td>16</td>
</tr>
<tr>
<td>8.7109619</td>
<td>20</td>
<td>8.711022</td>
<td>20</td>
<td>8.710962</td>
<td>20</td>
<td>8.710962</td>
<td>20</td>
</tr>
<tr>
<td>8.7109619</td>
<td>24</td>
<td>8.644999</td>
<td>24</td>
<td>8.711022</td>
<td>24</td>
<td>8.64296</td>
<td>24</td>
</tr>
<tr>
<td>8.7109619</td>
<td>28</td>
<td>8.709703</td>
<td>28</td>
<td>8.711022</td>
<td>28</td>
<td>8.710962</td>
<td>28</td>
</tr>
<tr>
<td>8.7109619</td>
<td>32</td>
<td>8.710962</td>
<td>32</td>
<td>8.710962</td>
<td>32</td>
<td>8.710902</td>
<td>32</td>
</tr>
<tr>
<td>8.7114843</td>
<td>36</td>
<td>8.779863</td>
<td>36</td>
<td>8.711022</td>
<td>36</td>
<td>8.710962</td>
<td>36</td>
</tr>
<tr>
<td>8.6439794</td>
<td>40</td>
<td>8.711022</td>
<td>40</td>
<td>8.776745</td>
<td>40</td>
<td>8.710962</td>
<td>40</td>
</tr>
<tr>
<td>8.7109619</td>
<td>44</td>
<td>8.711022</td>
<td>44</td>
<td>8.711022</td>
<td>44</td>
<td>8.710962</td>
<td>44</td>
</tr>
<tr>
<td>8.7114243</td>
<td>48</td>
<td>8.639722</td>
<td>48</td>
<td>8.710962</td>
<td>48</td>
<td>8.644459</td>
<td>48</td>
</tr>
<tr>
<td>8.6395802</td>
<td>52</td>
<td>8.710962</td>
<td>52</td>
<td>8.711022</td>
<td>52</td>
<td>8.710962</td>
<td>52</td>
</tr>
<tr>
<td>8.6408611</td>
<td>56</td>
<td>8.711022</td>
<td>56</td>
<td>8.711022</td>
<td>56</td>
<td>8.710902</td>
<td>56</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>P-value</th>
<th>P-value</th>
<th>P-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>4E-30</td>
<td>Intercept</td>
<td>1E-28</td>
</tr>
<tr>
<td>X Variable</td>
<td>0.125</td>
<td>X Variable</td>
<td>0.42233</td>
</tr>
</tbody>
</table>
APPENDIX A5.

NANOINDENTATION MODULUS DATA

Tissue Mechanics Laboratory
Department of Aerospace and Mechanical Engineering
University of Notre Dame

Title: Nanoindentation Modulus Data
TABLE A5.1

1 MEAN ELASTIC MODULUS (STD.DEV) MEASURED USING NANOINDENTATION (N=12)

<table>
<thead>
<tr>
<th>#8 $E_{\text{specimen}}$ (GPa)</th>
<th>#9 $E_{\text{specimen}}$ (GPa)</th>
<th>#11 $E_{\text{specimen}}$ (GPa)</th>
<th>#12 $E_{\text{specimen}}$ (GPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indent 2 15.45</td>
<td>Indent 1 5.01</td>
<td>Indent 1 18.56</td>
<td>Indent 1 20.59</td>
</tr>
<tr>
<td>Indent 3 15.82</td>
<td>Indent 2 14.64</td>
<td>Indent 2 15.51</td>
<td>Indent 2 20.46</td>
</tr>
<tr>
<td>Indent 4 15.45</td>
<td>Indent 3 16.78</td>
<td>Indent 3 12.91</td>
<td>Indent 3 17.89</td>
</tr>
<tr>
<td>Indent 5 14.33</td>
<td>Indent 4 16.61</td>
<td>Indent 4 20.17</td>
<td>Indent 4 19.44</td>
</tr>
<tr>
<td>Indent 6 13.44</td>
<td>Indent 5 16.60</td>
<td>Indent 5 18.34</td>
<td>Indent 5 17.19</td>
</tr>
<tr>
<td>Indent 7 15.12</td>
<td>Indent 6 15.06</td>
<td>Indent 6 18.97</td>
<td>Indent 6 16.84</td>
</tr>
<tr>
<td>Indent 13 11.59</td>
<td>Indent 7 14.08</td>
<td>Indent 7 12.28</td>
<td>Indent 7 19.46</td>
</tr>
<tr>
<td>Indent 14 15.54</td>
<td>Indent 8 15.11</td>
<td>Indent 8 19.76</td>
<td>Indent 8 18.61</td>
</tr>
<tr>
<td>Indent 15 14.47</td>
<td>Indent 9 14.63</td>
<td>Indent 9 15.78</td>
<td>Indent 9 16.92</td>
</tr>
<tr>
<td>Indent 16 16.36</td>
<td>Indent 10 13.72</td>
<td>Indent 10 18.10</td>
<td>Indent 10 17.99</td>
</tr>
<tr>
<td>Indent 17 17.26</td>
<td>Indent 11 25.29</td>
<td>Indent 11 19.27</td>
<td>Indent 11 16.09</td>
</tr>
<tr>
<td>Indent 18 17.15</td>
<td>Indent 12 8.45</td>
<td>Indent 13 19.41</td>
<td>Indent 12 18.70</td>
</tr>
<tr>
<td>Indent 19 15.78</td>
<td>Indent 13 17.12</td>
<td>Indent 14 20.03</td>
<td>Indent 13 15.81</td>
</tr>
<tr>
<td>Indent 20 16.93</td>
<td>Indent 14 13.85</td>
<td>Indent 15 19.49</td>
<td>Indent 14 17.79</td>
</tr>
<tr>
<td>Indent 21 16.33</td>
<td>Indent 15 20.11</td>
<td>Indent 16 20.42</td>
<td>Indent 15 15.83</td>
</tr>
<tr>
<td>Indent 16 16.83</td>
<td>Indent 17 17.03</td>
<td>Indent 16 17.32</td>
<td>Indent 16 17.32</td>
</tr>
<tr>
<td>Indent 18 18.58</td>
<td>Indent 19 20.00</td>
<td>Indent 18 17.27</td>
<td>Indent 19 17.93</td>
</tr>
<tr>
<td>Indent 20 17.27</td>
<td>Indent 20 17.27</td>
<td>Indent 18 17.27</td>
<td>Indent 20 17.27</td>
</tr>
</tbody>
</table>

Average 15.40  Average 15.24  Average 17.99  Average 17.93
Stdev 1.50  Stdev 4.43  Stdev 2.36  Stdev 1.52
<table>
<thead>
<tr>
<th>#14 $E_{\text{specimen}}$ (GPa)</th>
<th>#15 $E_{\text{specimen}}$ (GPa)</th>
<th>#17 $E_{\text{specimen}}$ (GPa)</th>
<th>#18 $E_{\text{specimen}}$ (GPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indent 1</td>
<td>19.67</td>
<td>Indent 2</td>
<td>16.94</td>
</tr>
<tr>
<td>Indent 2</td>
<td>17.69</td>
<td>Indent 3</td>
<td>17.02</td>
</tr>
<tr>
<td>Indent 3</td>
<td>20.71</td>
<td>Indent 4</td>
<td>17.24</td>
</tr>
<tr>
<td>Indent 4</td>
<td>15.98</td>
<td>Indent 5</td>
<td>16.94</td>
</tr>
<tr>
<td>Indent 5</td>
<td>18.96</td>
<td>Indent 6</td>
<td>19.78</td>
</tr>
<tr>
<td>Indent 6</td>
<td>20.05</td>
<td>Indent 7</td>
<td>13.18</td>
</tr>
<tr>
<td>Indent 7</td>
<td>18.07</td>
<td>Indent 8</td>
<td>18.20</td>
</tr>
<tr>
<td>Indent 8</td>
<td>17.78</td>
<td>Indent 9</td>
<td>17.52</td>
</tr>
<tr>
<td>Indent 9</td>
<td>16.52</td>
<td>Indent 10</td>
<td>14.38</td>
</tr>
<tr>
<td>Indent 10</td>
<td>18.52</td>
<td>Indent 11</td>
<td>14.64</td>
</tr>
<tr>
<td>Indent 11</td>
<td>18.34</td>
<td>Indent 12</td>
<td>14.53</td>
</tr>
<tr>
<td>Indent 12</td>
<td>17.17</td>
<td>Indent 13</td>
<td>14.66</td>
</tr>
<tr>
<td>Indent 13</td>
<td>16.91</td>
<td>Indent 14</td>
<td>14.56</td>
</tr>
<tr>
<td>Indent 14</td>
<td>15.31</td>
<td>Indent 15</td>
<td>14.72</td>
</tr>
<tr>
<td>Indent 15</td>
<td>20.44</td>
<td>Indent 16</td>
<td>14.84</td>
</tr>
<tr>
<td>Indent 16</td>
<td>18.93</td>
<td>Indent 17</td>
<td>14.94</td>
</tr>
<tr>
<td>Indent 17</td>
<td>16.48</td>
<td>Indent 18</td>
<td>14.45</td>
</tr>
<tr>
<td>Indent 18</td>
<td>15.97</td>
<td>Indent 19</td>
<td>9.34</td>
</tr>
<tr>
<td>Indent 19</td>
<td></td>
<td>Indent 20</td>
<td>14.18</td>
</tr>
<tr>
<td>Indent 20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>17.96</td>
<td>Average</td>
<td>14.23</td>
</tr>
<tr>
<td>Stdev</td>
<td>1.59</td>
<td>Stdev</td>
<td>3.04</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>#19 $E_{\text{specimen}}$ (GPa)</th>
<th>#21 $E_{\text{specimen}}$ (GPa)</th>
<th>#23 $E_{\text{specimen}}$ (GPa)</th>
<th>#24 $E_{\text{specimen}}$ (GPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indent 1</td>
<td>16.54</td>
<td>Indent 1</td>
<td>15.70</td>
</tr>
<tr>
<td>Indent 2</td>
<td>16.70</td>
<td>Indent 2</td>
<td>16.09</td>
</tr>
<tr>
<td>Indent 3</td>
<td>16.96</td>
<td>Indent 3</td>
<td>16.86</td>
</tr>
<tr>
<td>Indent 4</td>
<td>15.69</td>
<td>Indent 4</td>
<td>13.26</td>
</tr>
<tr>
<td>Indent 5</td>
<td>17.12</td>
<td>Indent 5</td>
<td>12.32</td>
</tr>
<tr>
<td>Indent 6</td>
<td>14.51</td>
<td>Indent 6</td>
<td>14.28</td>
</tr>
<tr>
<td>Indent 7</td>
<td>14.96</td>
<td>Indent 7</td>
<td>17.01</td>
</tr>
<tr>
<td>Indent 8</td>
<td>14.35</td>
<td>Indent 8</td>
<td>14.63</td>
</tr>
<tr>
<td>Indent 9</td>
<td>9.80</td>
<td>Indent 9</td>
<td>16.87</td>
</tr>
<tr>
<td>Indent 10</td>
<td>16.48</td>
<td>Indent 10</td>
<td>17.34</td>
</tr>
<tr>
<td>Indent 11</td>
<td>17.37</td>
<td>Indent 11</td>
<td>15.66</td>
</tr>
<tr>
<td>Indent 12</td>
<td>13.14</td>
<td>Indent 12</td>
<td>16.14</td>
</tr>
<tr>
<td>Indent 13</td>
<td>17.44</td>
<td>Indent 13</td>
<td>15.78</td>
</tr>
<tr>
<td>Indent 14</td>
<td>18.49</td>
<td>Indent 14</td>
<td>15.43</td>
</tr>
<tr>
<td>Indent 15</td>
<td>17.52</td>
<td>Indent 15</td>
<td>15.60</td>
</tr>
<tr>
<td>Indent 16</td>
<td>16.88</td>
<td>Indent 16</td>
<td>14.86</td>
</tr>
<tr>
<td>Indent 17</td>
<td>16.75</td>
<td>Indent 17</td>
<td>13.34</td>
</tr>
<tr>
<td>Indent 18</td>
<td>21.43</td>
<td>Indent 19</td>
<td>15.11</td>
</tr>
<tr>
<td>Indent 19</td>
<td>19.08</td>
<td>Indent 20</td>
<td>19.18</td>
</tr>
<tr>
<td>Average</td>
<td>16.38</td>
<td>Average</td>
<td>15.36</td>
</tr>
<tr>
<td>Stdev</td>
<td>2.43</td>
<td>Stdev</td>
<td>1.43</td>
</tr>
</tbody>
</table>

Average 17.13 Average 13.67


