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13. ABSTRACT *(Maximum 200 words)*

This research is part of the recent AFOSR initiative in Biomimetics, which has the broad overall goal of seeking to elucidate and understand the salient characteristics of naturally occuring biological composite materials, with an aim toward mimicking these features in devising improved man-made composite materials. The biological composite of interest in this effort is bone tissue, or more specifically Haversian cortical bone tissue. A major underlying task in this project is to develop a more deterministic understanding of the relationships between microstructural details and macroscopic mechanical behavior. The specific objectives of the project are: To conduct extensive dynamic mechanical tests on cortical bone specimens from a variety of sources to determine the dynamic elastic moduli and material damping; To quantitatively characterize the microstructural features of all bone specimens tested; To correlate mechanical behavior and microstructure through statistical analysis as well as theoretical modeling studies. Recent efforts have focused upon developing methods for extending dynamic mechanical testing to include 3-point bending in addition to torsional loading. Effort has also been directed at developing more detailed characterization of material microstructure in two particular areas. One of these areas is bone tissue composition, or more specifically, determining calcium content, phosphorous content, and collagen content. Another area of interest has been in developing a more complete understanding of the geometric microarchitecture of Haversian bone tissue. To this end, 3-D computer-based models of the void space and osteons have been constructed and analyzed.

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RESEARCH SUMMARY

OBJECTIVES

This research is part of the recent AFOSR initiative in *Biomimetics*, which has the broad overall goal of seeking to elucidate and understand the salient characteristics of naturally occurring biological composite materials, with an aim toward mimicking these features in devising improved man-made composite materials. The biological composite of interest in this effort is bone tissue, or more specifically Haversian cortical bone tissue. A major underlying task in this project is to develop a more deterministic understanding of the relationships between microstructural details and macroscopic mechanical behavior. The unique fiber/matrix interphase material, which is distinctive of this type of bone tissue, is of particular interest because of the recognized significance of interfaces in developing new higher performance composites. The approach combines composite micromechanics modeling techniques to analytically model structure/property relationships with companion experimental testing of tissue samples to evaluate and validate the modeling.

The specific objectives of the project are:

- To conduct extensive dynamic mechanical tests on cortical bone specimens from a variety of sources to determine the dynamic elastic moduli and material damping
- To quantitatively characterize the microstructural features of all bone specimens tested; and
- To correlate mechanical behavior and microstructure through statistical analysis as well as theoretical modeling studies.

The primary focus of recent efforts has been upon developing methods for extending dynamic mechanical testing to include 3-point bending in addition to torsional loading. Considerable effort has also been directed at developing more detailed characterization of material microstructure in two particular areas. One of these areas is bone tissue composition. More specifically, techniques have been developed and implemented for determining calcium content, phosphorous content, and collagen content, as well as estimating the quality of collagen cross-linking. Another area of interest has been in developing a more complete understanding of the geometric microarchitecture of Haversian bone tissue. To this end, three-dimensional computer-based models of the void space (Haversian canals) and secondary osteons have been constructed and analyzed.

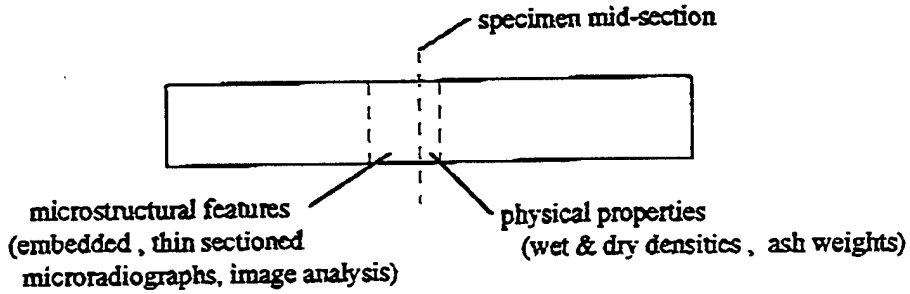
DYNAMIC MECHANICAL PROPERTIES

Twenty-two equine cortical bone specimens have been tested. Flat slab specimens (aprox. 63x10x2 mm) were tested in torsion in a dynamic mechanical testing machine (Rheometrics RDS-II). The oscillating frequency was swept through 3 decades (0.1 to 100 rad/s) at a constant peak strain amplitude of 0.015%. The storage modulus, loss modulus, and loss factor (or tan δ) were measured for each specimen. Following testing, the tissue densities (wet, dry, and ash) were measured and microstructural features quantified through image analysis of microradiographs of specimen cross-sections. Techniques were developed to measure the percent porosity, the percent secondary Haversian (or osteon) area, and the cement line perimeters from eight image fields from each cross-section. Characterization of bone tissue microstructure for the purposes of relating to mechanical properties has not been reported in the literature to this level of detail and quantification.

The figures on the following pages highlight the testing procedures and results. The findings so far can be summarized as:

- methods have been developed and established for dynamical mechanical testing of cortical bone tissue specimens;
- detailed and quantitative image analysis of tissue microstructure gives new insight into material constituents and arrangement;
- storage moduli generally increase with % secondary Haversian area, but decrease for predominantly Haversian bone (i.e. "older" bone);
- loss moduli are relatively insensitive to microstructure;
- loss factor decreases with density and remodeling (% secondary Haversian area), but increases with porosity.

- **specimen sectioning**



- **image analysis**

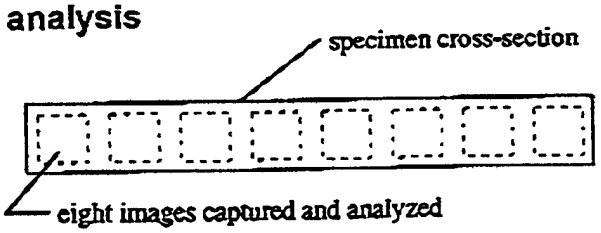


Figure 1. Highlights of procedures for post-test specimen analysis.

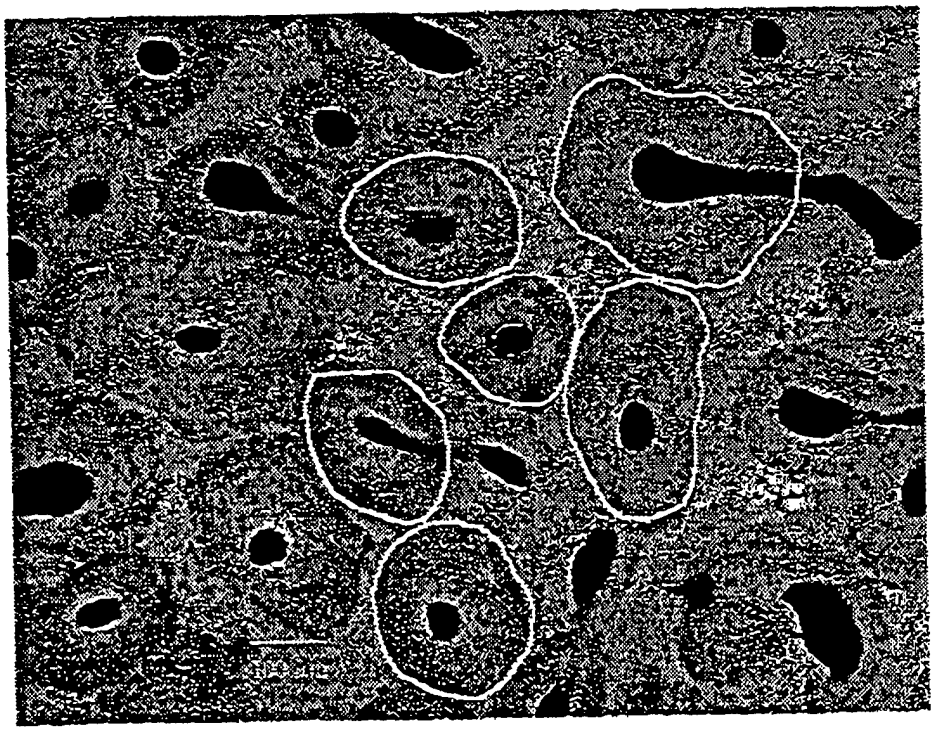


Figure 2. Microradiograph of transverse section with some osteon perimeters marked.

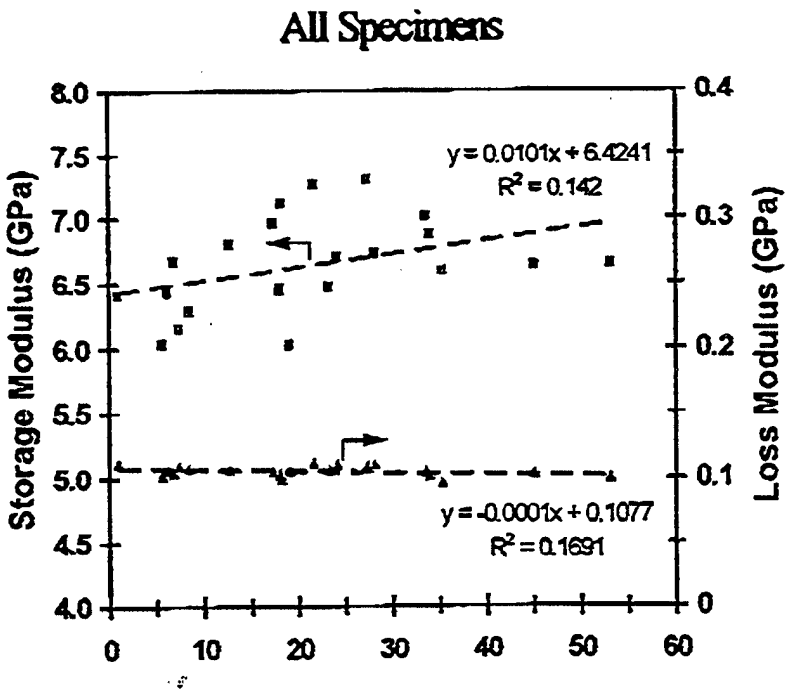


Figure 3. Viscoelastic moduli vs. % secondary Haversian area.

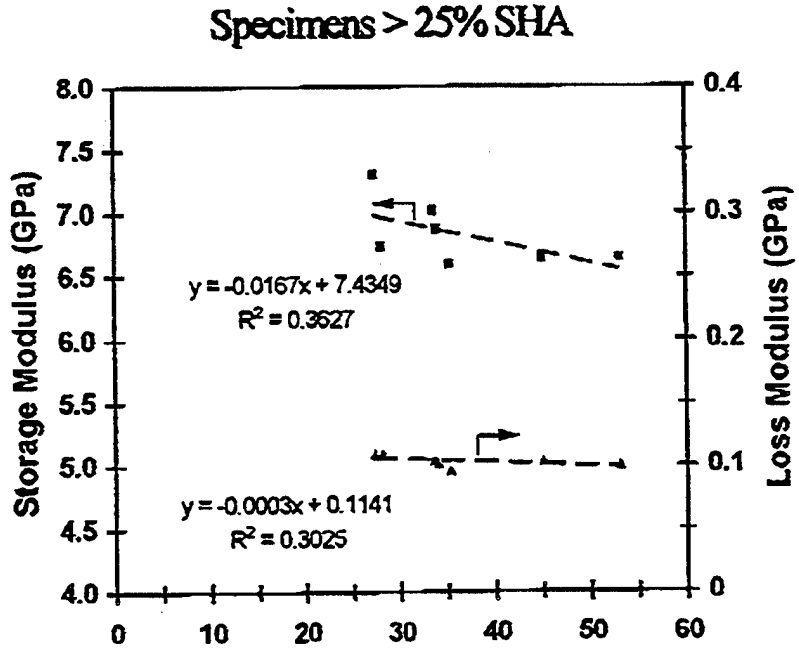


Figure 4. Viscoelastic moduli vs. % secondary Haversian area (only "older" bone).

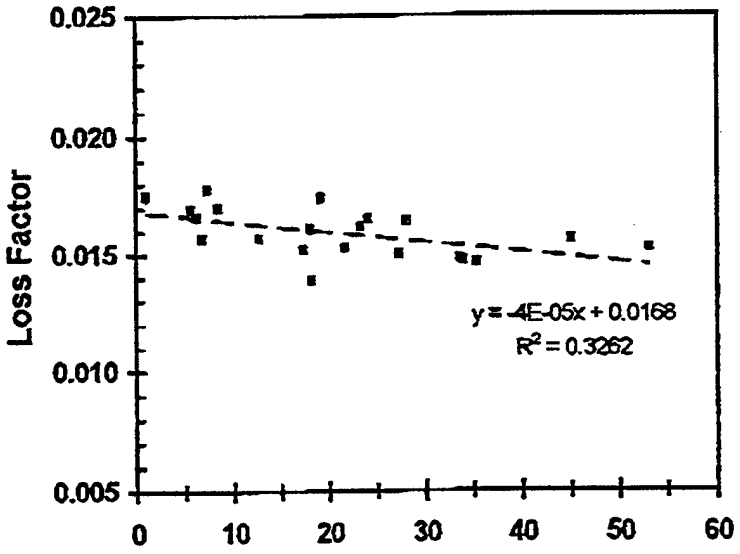


Figure 5. Loss factor vs.% secondary Haversian area.

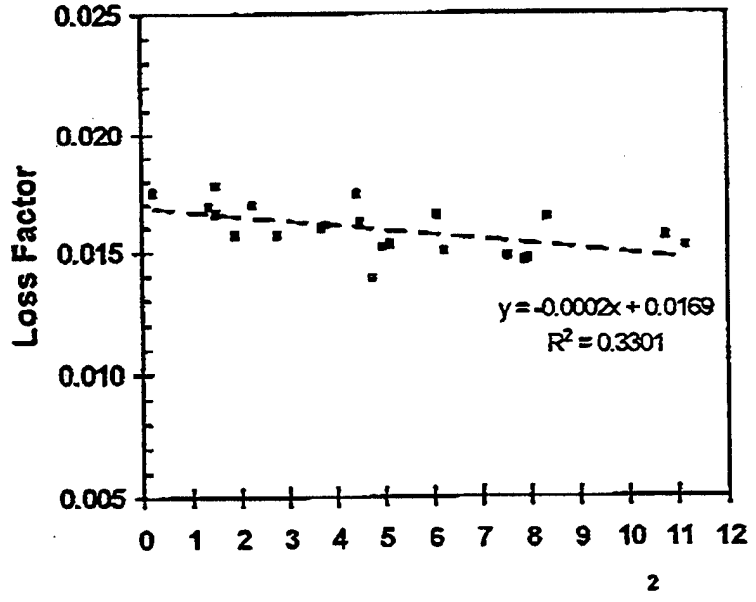


Figure 6. Loss factor vs. cement line perimeter.

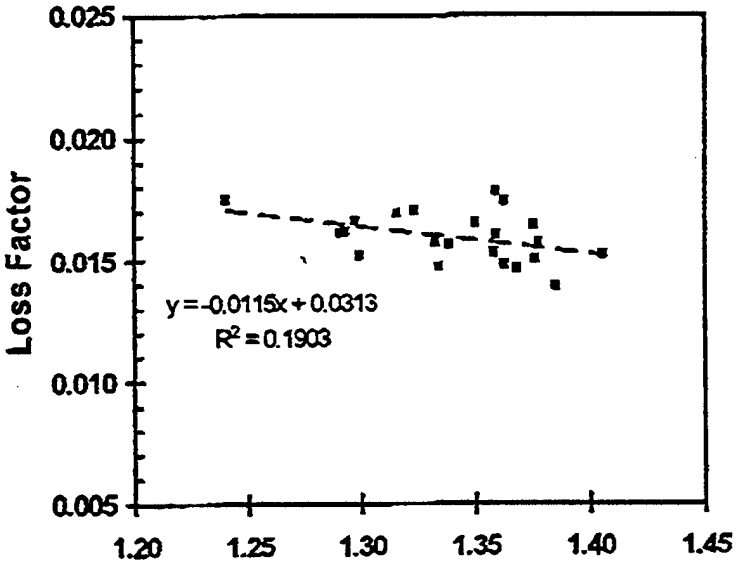


Figure 7. Loss factor vs. ash density.

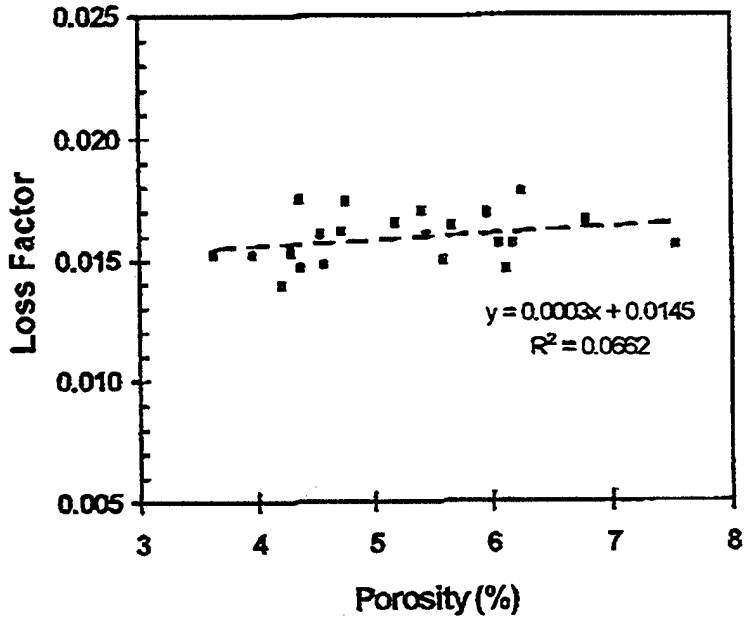


Figure 8. Loss factor vs. porosity.

The most recent efforts in this area have been concerned with developing a method to conduct 3-point bending loading for dynamic mechanical analysis. The Polymer Technology Consortium at Texas A&M University has a Polymer Laboratories Mark II dynamic mechanical tester, but considerable modification and development has been required in order to successfully adapt this instrument for testing cortical bone specimens. New gripping brackets have been constructed and preliminary testing initiated. The results shown in Fig. 9 are repeated tests on a flat slab specimen of equine cortical bone. The storage modulus (E') and dissipation (tan δ) are presented vs. frequency over the range of 0.3 to 50 Hz. The test was repeated for three temperatures (24°C, 25°C, 26°C). Tests are continuing using this method and results will be generated for both equine and human cortical bone.

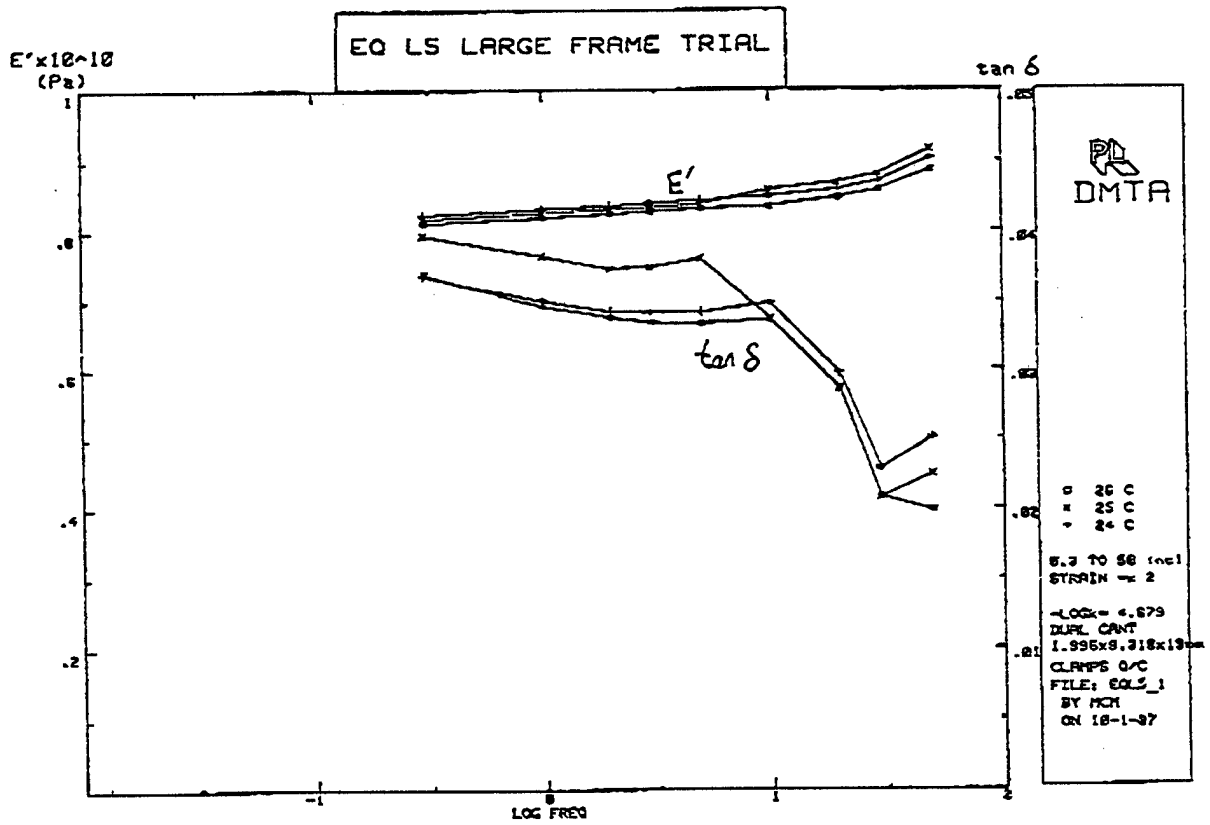


Figure 9. Dynamic mechanical properties in 3-point bending.

MICROSTRUCTURAL CHARACTERIZATION

In considering cortical bone tissue as a fiber-reinforced composite material we have previously modeled [1-3] the secondary osteon, or Haversian system, as the fiber component and the interstitial lamellar bone as the matrix component. Interstitial lamellar bone actually arises from one of two sources: (1) remnants of previous osteons that have been resorbed (incompletely) as part of the continuous turnover of tissue known as "remodeling", or (2) "primary" lamellar bone that has never been remodeled at all and has thus persisted from initial formation. In either case, the overall level of mineralization of interstitial bone is generally higher than secondary osteons because the latter mineralize over time (before being resorbed as well) and are thus less mature. A unique feature of the remodeling process is the deposition of a thin layer of amorphous material at the beginning of new bone formation. Thus, each secondary osteon is surrounded by this material, which is termed the "cement line", although it is not really a line but a layer of material in three-dimensions. A schematic view (not to scale) of Haversian cortical bone is given in Fig. 10.

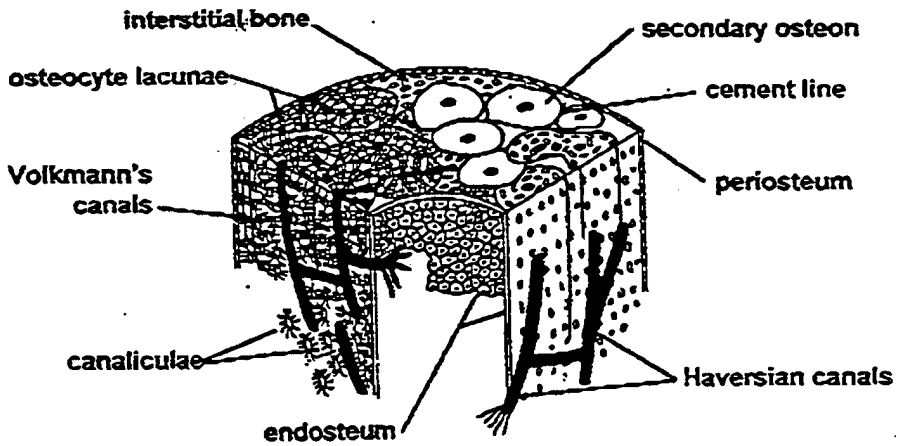


Figure 10. Idealized view of bone mid-shaft showing cortical bone microstructure (adapted from [4]).

This cement line material is generally regarded as a highly compliant interface between the osteon and interstitial bone, but its precise function and role in determining overall macroscopic mechanical properties is largely unknown. We have conducted initial studies [5,6] correlating dynamic mechanical properties with cement lines but the results were inconclusive. In evaluating these studies, two potentially confounding factors emerged that need further investigation: (1) the specific contents of mineral (calcium, phosphorous) and collagen and their relative amounts; and (2) the three-dimensional architecture of the void space (Haversian canals) and the cement line surfaces. The influence that these factors may have on dynamic mechanical properties can be more fully understood by including their characterization in subsequent studies. Thus,

developments related to these two issues are highlighted in the remainder of this section. More specifically, techniques have been developed and implemented for determining calcium content, phosphorous content, and collagen content, as well as estimating the quality of collagen cross-linking. Another area of interest has been in developing a more complete understanding of the geometric microarchitecture of Haversian bone tissue. To this end, three-dimensional computer-based models of the void space (Haversian canals) and secondary osteons have been constructed and analyzed.

Compositional Analysis

Methods of compositional analysis for mineral and collagen contents were developed as part of a study of loading effects on the tibia of the mouse. Mouse bones lack the osteonal microstructure of larger mammals but are nevertheless composed of collagen fibers with embedded mineral crystals. An advantage in studying these bones is that the relative effects of separate mineral (calcium and phosphorous) and collagen components can be determined more directly and apart from the influences of the more complex osteonal, or Haversian, microstructure. Furthermore, bone is also a 'smart' composite material that can adapt to loading in several possible ways. One way would be to increase or decrease the degree of mineralization and/or collagen formation at the tissue level. Another way would be to increase the mass or amount of bone tissue present by forming larger bones (or at least increasing the bone cross-section). Pilot research being conducted in collaboration with colleagues in the Department of Health and Kinesiology at Texas A&M University [7,8] provided an excellent opportunity to begin addressing some of these issues. The focus of the present treatment is upon the compositional analysis methods, but basic findings will also be included as appropriate.

Calcium and phosphorous contents. Each tibia bone was dried at 100 °C for 24 h and then weighed to obtain dry weights. The bones were then hydrolyzed in 1.8 ml of 6 N HCl for 18 h at 110 °C. The hydrolysates were neutralized with 4.32 ml of 2.5 N NaOH and the final volumes brought to 15 ml. The neutralized hydrolysates were stored at 4 °C until assays were performed. Calcium content was assayed using spectrophotometry and the calcium-sensitive dye, Arsenazo III. First, an aliquot of the neutralized hydrolysate was adjusted to pH 5 with HCl to exclude calcium binding to inorganic phosphate. The sample was then diluted 7-fold with H_2O and centrifuged for 5 min at 11,600 g. A 15 µl aliquot of the supernatant was mixed with 1 ml of 0.1 mM Arsenazo III (pH 6.5). After 5 min, the solution absorbance was measured at 600 nm and compared against the absorbance of calcium standards (0-1.5 µg). Eight neutralized hydrolysates were also assayed for calcium content by inductively-coupled plasma (ICP) analysis. The two methods compared very well: the mean deviation of the dye-based method from the ICP technique was -0.14% while the precision was 1.75%. For phosphorous contents, aliquots (125 µl) of the neutralized hydrolysates were mixed with 2 ml of 20% trichloracetic acid and brought to 5 ml volume with H_2O. After standing for 10 min the samples were centrifuged at 1500 g for 10 min. Two ml of the supernatant, 3 ml of H_2O, and 1 ml of acid molybdate were mixed and 0.25 ml of Fiske & Subbarow reducer was added. After standing for 10 min, the solution absorbance was measured at 660 nm and compared to phosphorous standards (0-25 µg).

Collagen and amino acid analysis. The collagen content of each tibia bone was estimated by determining the hydroxyproline content in the neutralized hydrolysates. This is a fairly standard technique as described by Woessner [9]. In order to assess whether differential protein expression may occur, the relative amino acid distribution in the neutralized hydrolysate was determined. Aliquots (0.5 ml) of the hydrolysates were first dried under vacuum and then reconstituted in 0.025% EDTA. They were further diluted 10-fold with the EDTA solution immediately prior to assay. Samples were derivatized with phenylisothiocyanite and subsequently analyzed via UV detection using an ABI 420 hydrolyzer/derivatizer with on-line 130A HPLC system connected to a 920A data collection system.

Briefly, female mice 6 weeks of age initially were studied for 4 weeks in four different groups. One group (n=11) served as a control and all 3 other groups had their ovaries surgically removed at the beginning of the study. One of these groups (n=10) was administered estrogen through implanted capsules containing estradiol (designated the OVX+E2 group). The other two groups remained estrogen deficient. One was designated OVX (n=10) while the other was designated OVX+TRAIN (n=10) because the latter also were subjected to a regular muscle exercise protocol designed to mimic heavy resistance training. At the conclusion of the 4 week study period, the left tibia of each euthanized animal was removed and tested for mechanical properties in 3-point bending. The estrogen deficiency reduced the calcium and phosphorous contents of both the OVX and OVX+TRAIN groups significantly (Fig. 11), but this was a direct result of smaller bones in these groups (Fig. 12). When normalized for the dry weight or wet weight of each bone, the calcium and phosphorous contents were the same for all four groups. The collagen contents followed a similar pattern, as apparent in the total and normalized contents presented in Fig. 13. Mechanical properties for the whole bones were not significantly different (Fig. 14), but the modulus of elasticity (Fig. 15) of the bone material was higher for the OVX+TRAIN group.

An important finding of these results is that the training did not prevent smaller bones due to estrogen deficiency but it did improve the quality of the bone tissue enough to prevent a decrease in whole bone mechanical properties. This improvement is clearly evidenced by the improvement in the elastic modulus and 'normalized' properties as shown in Fig. 15. The mechanisms underlying these differences in material properties are not known at this time, but it is clear from the compositional analysis that differences in the amount, or quantity, of mineral and/or collagen are not the cause. This also emphasizes the importance of measuring these compositional parameters. Bone can also be considered as a two-phase composite on the collagen-mineral level and these measures provide insight necessary for quantifying effects on this size scale as well. Possible explanations for the observed results may be differences in the quality of the minerals at the crystal level, differences in the quality of the collagen (cross-link maturity), or perhaps differences in the binding between the collagen and mineral phases. The relative amino acid contents are shown in Fig. 16 and suggest similarities between the OVX+TRAIN and the OVX+E2 groups, but the significance of these is not clear. Further investigation is planned. The ability to make these more detailed compositional measurements will be exploited in the next phase of study to include microstructural characterization on the collagen-mineral level as well as on the osteon level (as in our previous studies).

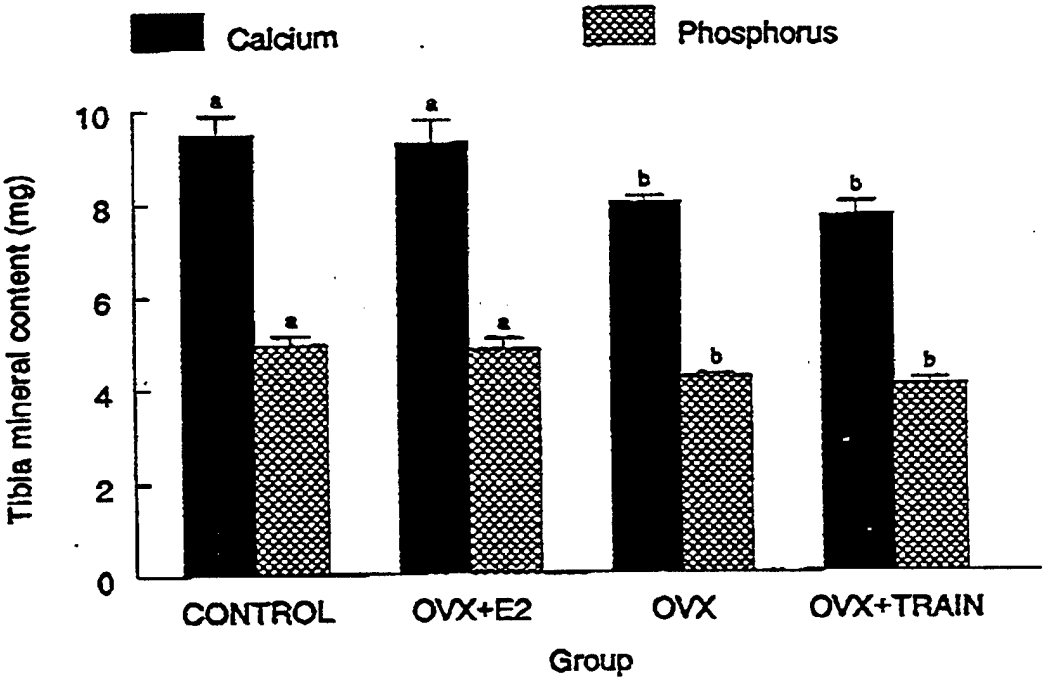


Figure 11. Calcium and phosphorous contents for the four study groups.

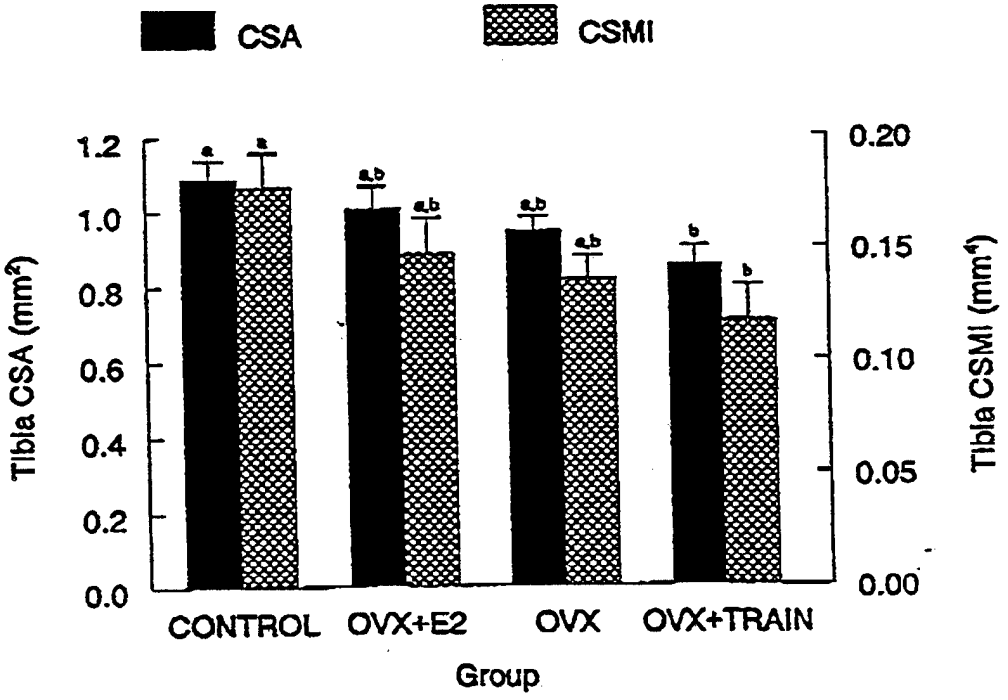


Figure 12. Cross-sectional area (CSA) and moment of inertia (CSMI) for the four groups.

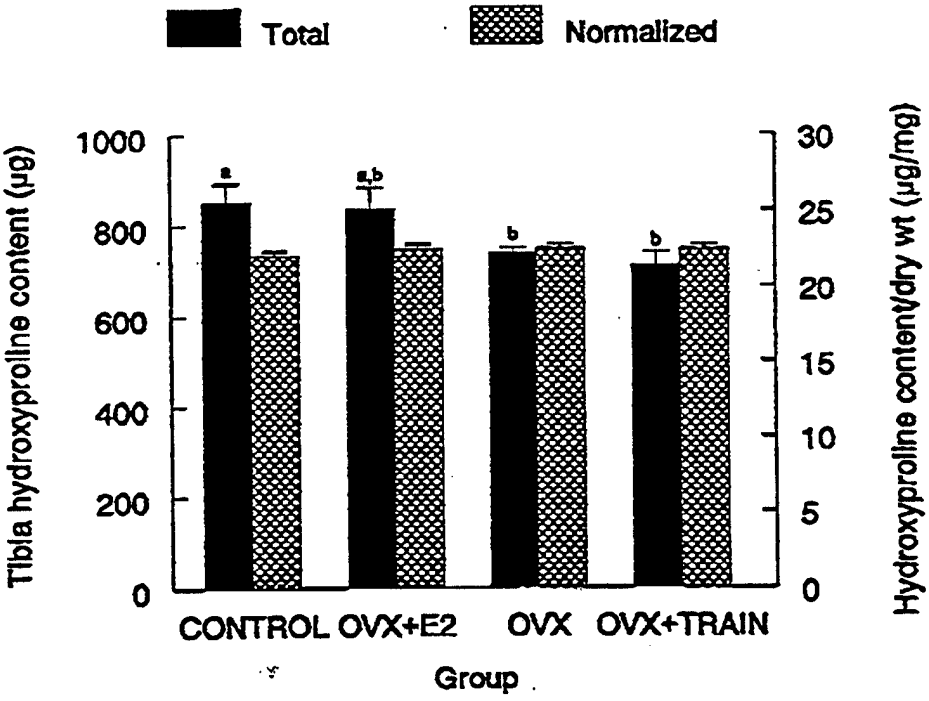


Figure 13. Collagen contents (total & normalized) for the four groups.

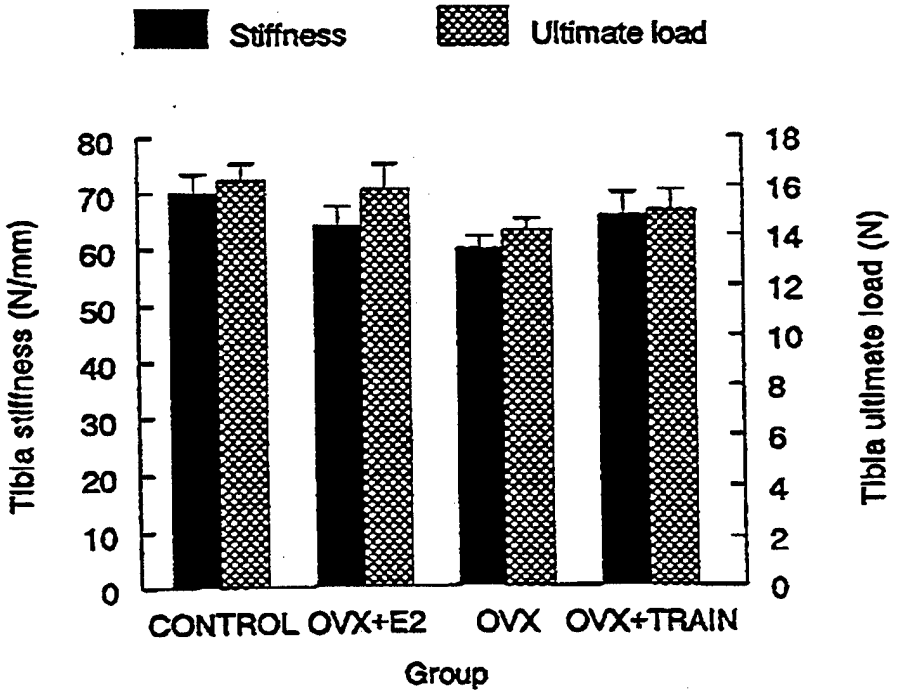


Figure 14. Whole bone mechanical properties for the four groups.

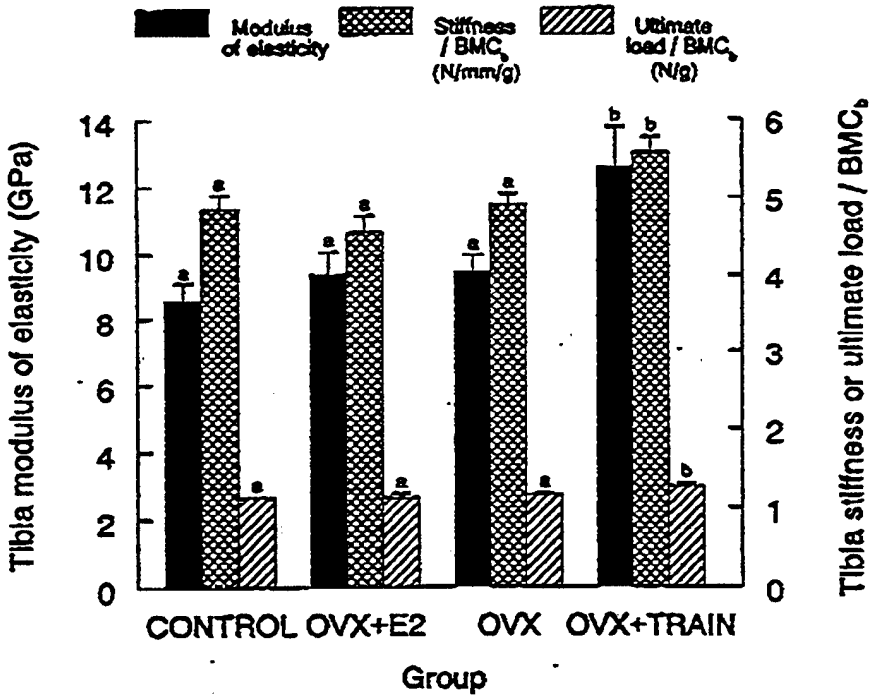


Figure 15. Bone tissue level material properties for the four groups.

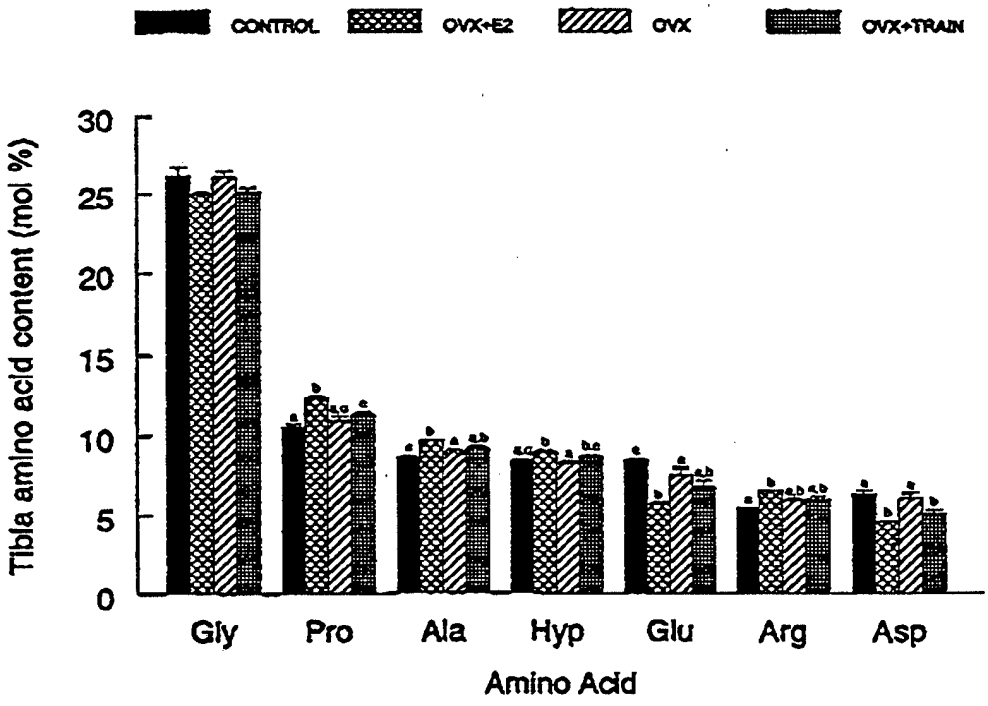


Figure 16. Relative amino acid contents for the four groups.

3-D Microarchitecure Modeling

The three-dimensional geometry of the Haversian systems of equine cortical bone tissue has been modeled using computer-aided design (CAD) software. The problem was studied by a senior undergraduate mechanical engineering student as part of an independent study course [10]. The raw data had been acquired previously as part of a recent M.S. thesis [11] dealing with this same issue. These consist mainly of serial sections of decalcified bone cross-sections, which were digitized and enhanced to isolate the Haversian canals and cement lines surrounding each secondary osteon. A sample section is shown in Fig. 17. Eight of these sections were analyzed initially, but this was increased to 30 sections in the more recent work. The goals of more recent efforts have been to compare two different software packages, to evaluate the effects of slide distortion from section-to-section, and to quantify the longitudinal variations in size and orientation of specific osteons. The purpose of comparing and evaluating two software packages was to seek to reduce the substantial labor and computer resources needed for constructing these models. The more streamlined and time-efficient the procedures can be made, then the more useful these models will be as tools for additional structure/property studies.

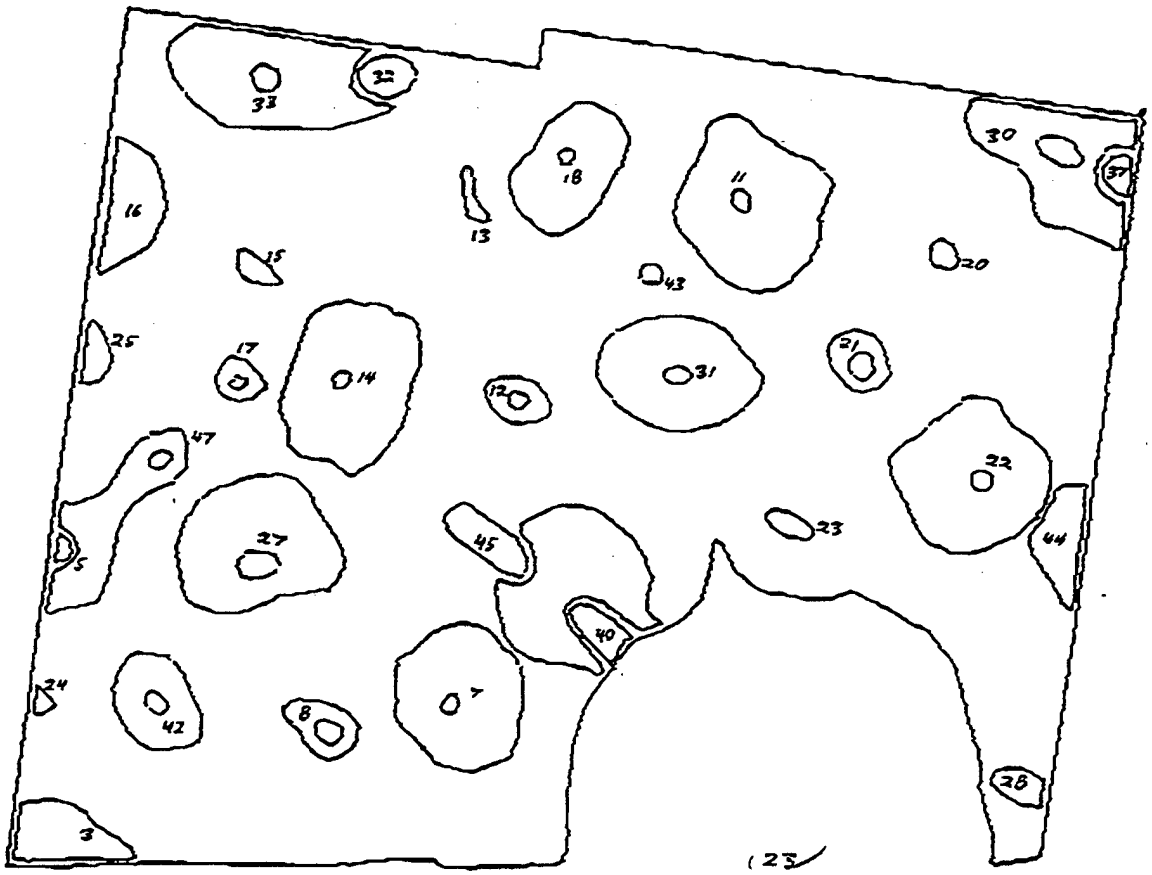


Figure 17. Sample image of lines representing Haversian canals and osteon perimeters.

The original work in this area [11] used the Intergraph/EMS software package for 3-dimensional reconstruction and surfacing of Haversian canals and osteon perimeters. The same procedures were compared using Pro/Engineer solid modeling software. Several relative advantages and disadvantages of the two packages were identified and evaluated, but the decision was made to continue using EMS for this phase of the project. One major advantage of EMS was the ability to filter the raw data internally to reduce the total number of data points included in each model. The number of points for each closed curve representing Haversian canals and osteon perimeters was filtered by specifying the "chord height tolerance", or CHT. The CHT represents the spacing between points and two values were examined, 1 and 4 microns. The skinned surfaces for osteon #12 are shown in Fig. 18 for all three cases: the unreduced raw data, filtered with a CHT=1, and filtered with a CHT=4. The respective file sizes for just this single osteon were 37.8 MB, 19.9 MB, and 9.3 MB. The fidelity of the model does not appear to be degraded significantly for even the CHT=4 case and the storage savings are substantial. The effects of CHT were further evaluated quantitatively by plotting the areas enclosed by the Haversian canal and osteon perimeters versus length for all 3 cases (Fig. 19). Once again, there are no substantial differences in the values for the CHT=4 case. Another obvious yet undeniable feature of the surfaces in Fig. 18 is the "jaggedness" caused by distortion in the histological slides from one section to the next. This distortion was characterized for the first 16 sections by measuring the change in position within each cross-section for a common landmark feature in each image. The distances between two common points are summarized in Table 1. The variables *deltaX* and *deltaY* are the differences in position between the two points in the repective coordinate directions, and *Distance* is simply the total distance between the two points. Considerable variation is apparent and the standard deviations range from roughly 3 to 7 %. Because the computer resources for fully 3-D modeling (even for surfaces only) are so immense an alternate method was devised for displaying the 3-D variations in microstructure. The 2-D representations in Fig. 20 show the mean Haversian canal diameters (dark lines) and the mean osteon outside diameters (light lines) as a function of longitudinal position for 6 osteons. Definite tapering and branching is evident.

Table 1

Layer	deltaX	deltaY	Distance
1	131.3	351.0	374.8
2	160.0	333.8	370.2
3	141.6	327.2	356.6
4	152.6	341.6	374.1
5	152.2	342.0	374.3
6	121.4	332.5	354.0
7	149.8	313.0	347.0
8	132.4	325.3	351.2
9	129.1	322.8	347.7
10	142.2	334.7	363.7
11	153.9	336.3	369.8
12	149.6	340.0	371.5
13	148.6	353.7	383.6
14	135.8	350.9	376.3
15	150.3	338.7	370.6
16	138.2	355.4	381.3
Average	143.1	337.4	366.7
Std. Dev	10.79	11.86	11.82

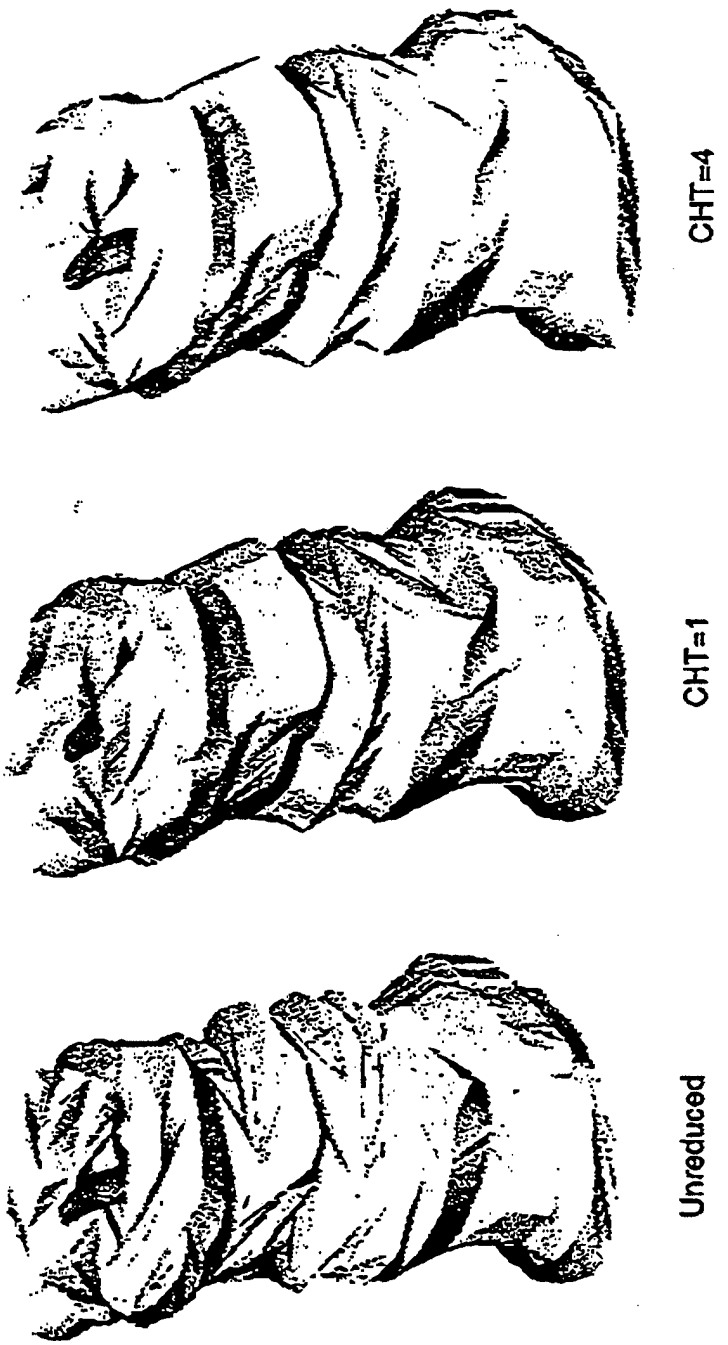


Figure 18. Skinned surfaces for the Haversian canal and osteon cement line for osteon #12.

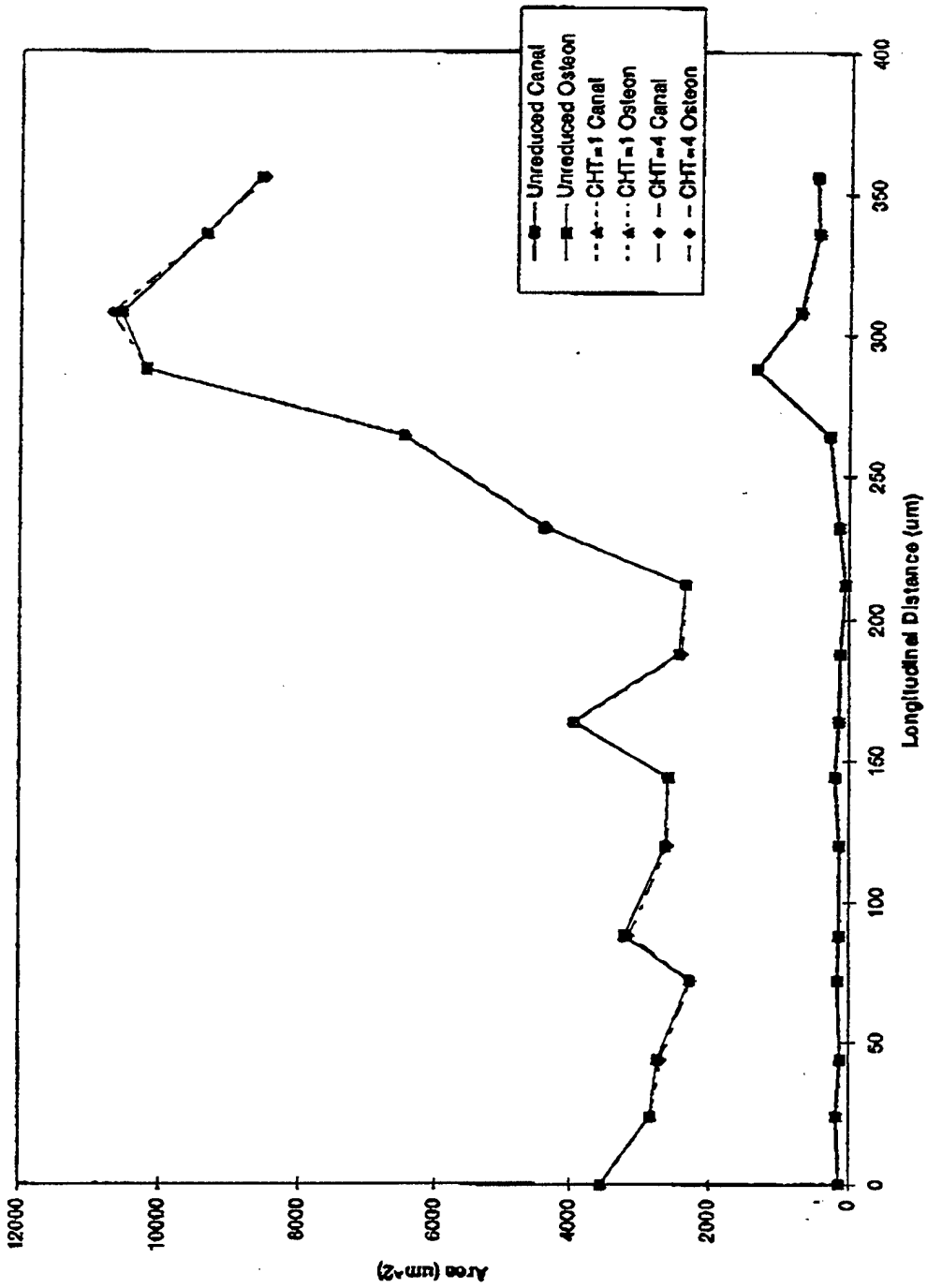


Figure 19. Variation in areas of the Haversian canal and osteon cement line for osteon #12.

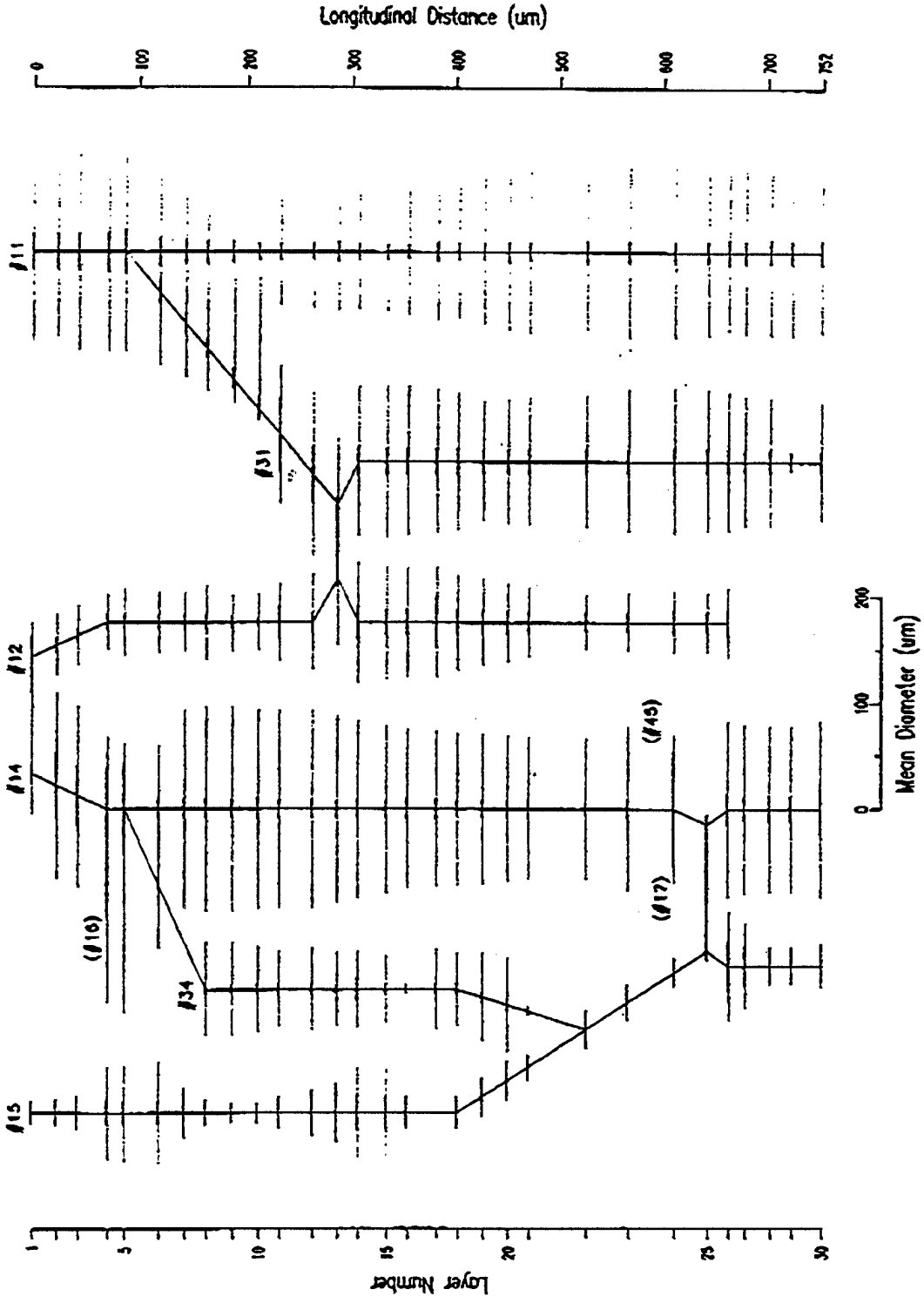


Figure 20. 2-D representation of variation in mean diameters with length for 6 osteons.

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10. Haskett, B., "3-D Solid Modeling and Analysis of Bone Microstructure," MEEN 485 - Special Topics in Mechanical Engineering, senior independent study course, Spring 1996.
11. Deisseroth, K. B., "Three-Dimensional Modeling and Analysis of the Haversian Systems in Cortical Bone Tissue," M.S. thesis , Dept. of Mechanical Engineering, Texas A&M University, May 1995.

ADDITIONAL INFORMATION

PERSONNEL SUPPORTED

Faculty: Harry A. Hogan, Ph.D., Associate Professor

Students: Andrew K. Ayers - M.S. thesis contributed to the overall effort although his research was not directly suported by the grant
Jennifer Groves - undergraduate research assistant
Bradley Haskett - undergraduate independent study course
Megan McCurdy - began pursuing M.S. degree on this project

PUBLICATIONS - none for this period

INTERACTIONS AND TRANSITIONS

Presentations at Meetings. The following presentations were made reporting on research related to the current grant:

Warren, G. L., Hogan, H. A., Groves, J. A., Armstrong, R. B., and Bloomfield, S. A., "The Effect of Training on Tibial Bone Properties in Estrogen-Deficient Mice," 16th Annual Meeting, Society for Physical Regulation in Biology and Medicine, Oct. 9-12, 1996, Chicago, IL.

Deisseroth, K. B., and Hogan, H. A., "Characterization of Three-Dimensional Osteon Geometries in Haversian Cortical Bone Tissue," 1995 Advances in Bioengineering, **BED-Vol. 31**, 1995 ASME International Mechanical Engineering Congress and Exposition, Nov. 12-17, 1995, San Francisco, CA, pp. 341-342.

Hogan, H. A., Gunn, K. S., Ahern, J. C., and Simmons, D. J., "Characterizing Osteonal Microstructure and Its Influence Upon the Anisotropic Moduli of Equine Cortical Bone," 1995 Bioengineering Conference, **BED-Vol. 29**, R. M. Hochmuth, N. A. Langrana, M. S. Hefzy, eds., 1995 ASME/AICHE/ASCE Summer Bioengineering Conference, June 28-July 2, 1995, Beaver Creek, CO, pp. 241-242.

Ayers, A. K., and Hogan, H. A., "Dynamic Mechanical Properties of Equine Cortical Bone and Their Dependence Upon Tissue Density," 1995 Bioengineering Conference, **BED-Vol. 29**, R. M. Hochmuth, N. A. Langrana, M. S. Hefzy, eds., 1995 ASME/AICHE/ASCE Summer Bioengineering Conference, June 28-July 2, 1995, Beaver Creek, CO, pp. 245-246.

Consultative and Advisory Functions. no consultative or advisory functions during this period

Transitions. none during this period

NEW DISCOVERIES, INVENTIONS, OR PATENTS - none during this period