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The Effect of *In Vitro* Fluoride Ion Treatment on the Ultrasonic Properties of Cortical Bone

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Abstract—The mechanical properties of composites are influenced, in part, by the volume fraction, orientation, constituent mechanical properties, and interfacial bonding. Cortical bone tissue represents a short-fibered biological composite where the hydroxyapatite phase is embedded in an organic matrix composed of type I collagen and other noncollagenous proteins. Destructive mechanical testing has revealed that fluoride ion treatment significantly lowers the Z-axis tensile and compressive properties of cortical bone through a constituent interfacial debonding mechanism. The present ultrasonic data indicates that fluoride ion treatment significantly alters the longitudinal velocity in the Z-axis as well as the circumferential and radial axes of cortical bone. This suggests that the distribution of constituents and interfacial bonding amongst them may contribute to the anisotropic nature of bone tissue.

Keywords—Bone, Ultrasound, Interfacial bonding, Anisotropy, Fluoride.

INTRODUCTION

The mechanical properties of cortical bone have been examined using destructive, mechanical testing as well as nondestructive, ultrasound testing to obtain the elastic constants (1,13–15,30). By measuring the sample density and velocities of sound waves in a number of different directions, ultrasonic testing allows characterization of the anisotropy of a single, small specimen not possible by traditional destructive mechanical testing techniques. A comparison of ultrasonic and mechanical testing results for cortical bone suggests that ultrasonic testing is representative of a high strain rate mechanical test (14,22). Ultrasonic techniques therefore offer a method of examining the anisotropic nature of a single piece of cortical bone at a high strain rate nondestructively.

Cortical bone is a nonhomogenous, anisotropic, viscoelastic, multiphased composite composed of a stiff inorganic bone mineral embedded in a compliant organic matrix (31). Bone mineral, a hydroxyapatite-like material ($Ca_{10}(PO_4)_6(OH)_2$), also contains substantial amounts of CO_3^{2-} (4–6%), citrate (0.9%), Mg^{2+} (0.5%), and Na^+ (0.7%), and trace amounts of Cl^-, F^-, K^+, and Sr^{2+}, and other metal ions (26). Bone mineral has been reported to be a rod-like or an elongated platelet-like shaped with a width or thickness of 25–75 Å by 400–1,000 Å in length (28). The mineral phase is generally oriented parallel to the longitudinal bone axis (28). Type I collagen comprises more than 90% of the organic matrix of bone. The remaining 10% of the organic matrix is composed of a number of noncollagenous proteins and lipids (2). The majority of the noncollagenous proteins are anionic (negatively charged) and bind readily to calcium on the surface of hydroxyapatite (18).

A number of studies have characterized the nonhomogenous and anisotropic nature of cortical bone revealing the complexity with respect to position (proximal, midshaft, and distal) and quadrants (anterior, posterior, medial, and lateral) (13,14). Similar to other composites, the mechanical properties of cortical bone have been demonstrated to depend on a number of factors including: constituent properties, volume fraction, orientation, and more recently, interfacial bonding interactions between the mineral and organic phases (10,31,33–35). Using a detergent and ion-treatment protocol we have demonstrated that interfacial bonding interactions between the mineral and organic phases plays an important role in the tensile and compressive properties of cortical bone. Partial debonding between the constituents of bone have been hypothesized to play a role in the properties of aged and diseased bone (3). Phosphate or fluoride ions are able to modify electrostatic and other types of bonding forces (25) between the mineral and organic constituents of bone leading to an alteration in the mechanical properties (10,31,33–35).

The orientation and properties of the bone mineral (28) as well as collagen fiber orientation (20) have also been shown to be important parameters in the mechanical analysis of bone. The *c*-axis of the mineral phase (21) of bone is generally oriented parallel to the longitudinal axis lead-

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ing to the greatest mineral reinforcement in this direction. In addition to crystallographic orientation, mineral-organic interfacial bonding (between the faces of the HA and the organic constituents) may also play an important role in determining the anisotropic properties of cortical bone tissue. The present study explores the role of interfacial bonding between the mineral and organic constituents of bone and its role in the anisotropic character of bone using ultrasonic testing and a fluoride ion treatment.

MATERIALS AND METHODS

Two adult bovine femurs were obtained from a local slaughterhouse. Specimens were prepared from the medial quadrant of the central diaphysis to minimize biological variability due to position and quadrant with respect to density and mineral content (14). The specimens were wrapped in 0.145 M NaCl soaked paper towels and stored at −20°C until use. This storage procedure does not alter the mechanical properties of bone tissue. Samples remained completely wet throughout the preparation. Forty cortical bone cubes (5 mm × 5 mm × 5 mm) were prepared by cutting on a low speed saw with a diamond disc blade (Buelher Ltd., Lake Bluff, IL, U.S.A.) under dripping distilled water. The long bone (*Z*), circumferential (*T*), and radial (*R*) orientations were marked with a pencil. Samples were randomly separated into two groups (20 samples per group).

The longitudinal ultrasonic velocities were obtained using 5 MHz piezoelectric transducers (Ultran Laboratories, Boalsburg, PA, U.S.A.) in pulse-echo mode. Rectangular pulse width of 110 ns and amplitude of 200 V were employed for optimal reflection detection (14). The longitudinal velocities in the *Z*, *R*, and *T* directions were measured on all specimens at the start of the experiment. Transducers were placed directly on the bone with a coupling agent and the reflections were recorded with a personal computer using an Ultran NDC 7000 System. The dimensions of the cubes were measured in triplicate using a digital caliper. Ultrasonic velocities were calculated by taking twice the thickness of the bone and dividing by the time of flight determined from the ultrasound reflections. Density was calculated as the hydrated tissue mass divided by the bone tissue volume. Bone tissue volume was calculated as the hydrated weight less the submerged weight using an analytical balance. The ultrasonic modulus was calculated by multiplying the square of the velocity by the specimen density (13)

$$c_{11} = \rho v^{2_1},\ c_{22} = \rho v^{2_2},\ c_{33} = \rho v^{2_3}, \qquad (1)$$

where ρ and v_1, v_2, and v_3 represent density and longitudinal ultrasonic velocity in the radial, circumferential, and longitudinal directions, respectively.

The detergent–ion treatment protocol developed by Walsh and Guzelsu (32) was used in this study. This protocol has been shown to allow ions access to the mineralized bone matrix (32) and alter mineral-organic interfacial bonding (10,31,33–35). Cubes were treated in 0.1% Nonidet P40 (Sigma Chemical Co., St. Louis, MO, U.S.A. No. N-3516), a nonionic alcohol-type detergent, for 24 hr under constant shaking at room temperature. Samples were rinsed in copious amounts of distilled water and their ultrasonic velocities in all three directions (*Z*, *R*, and *T*) were measured (day 0). Samples were placed into 500 ml of 0.145 M NaCl or 2.0 M NaF adjusted to pH 7.5. Samples were treated in these equilibration solutions for 20 days under constant stirring at room temperature. The pH of the treatment solutions were measured every 24 hr using a pH electrode. The ultrasound velocities were measured at 3, 6, 13, and 20 days in treatment solutions.

Following ultrasound testing the density and ash fraction (mineral content) were determined. Samples were dehydrated in acetone for 3 days and ashed in a muffle furnace at 650°C for 24 hr. The ash fraction was calculated by dividing the ashed weight by the dry weight. A fluoride ion electrode (Orion Laboratories, Boston, MA, U.S.A.) was used to obtain the concentration of fluoride ion uptake in cortical bone specimens (19). Thirty-six bone wafers (18 wafers 0.5 mm × 5 mm × 5 mm and 18 2 mm × 5 mm × 5 mm) were cut and equilibrated (3 wafers per group) in 2 M NaF for 0, 1, 3, 6, 9, and 12 days. Following treatments, specimens were washed twice in deionized water, dried for 1 hr at 60°C, weighed, ashed at 650°C for 24 hr, and weighed again. Each specimen was crushed with a mortar and pestle and dissolved in 1 ml of 6 N HCl. Fifty milliliters of this solution was added to 0.95 ml of distilled water. One milliliter of total ionic strength adjustment buffer (Orion) was added (final pH = 5.2), and the fluoride content of the sample was measured in parts per million (ppm) using the fluoride ion electrode. Furthermore, mass and volume from density measurements were used to normalize the fluoride concentrations of different specimens to be expressed as ppm per bone weight per ml HCl used (ppm/g/ml) and ppm per bone volume per ml HCl used (ppm/cm^3/ml).

Fourier Transform Infrared Spectroscopy (FT-IR) was carried out in order to examine the effects that the treatment solutions had on the mineral phase of bone. Bone wafers (0.5 mm thick) were cut from the medial quadrant of a bovine femur and treated with the NP40 detergent and 0.145 M NaCl or 2.0 M NaF for 20 days. IR spectra were obtained at days 0, 3, 6, 9, 12, and 20 of equilibration. Samples for FT-IR spectra analysis were ground with a mortar and pestle and prepared using approximately 0.6 mg of sample per 300 mg of KBr in pellet form. FT-IR spectra were recorded with a Perkin Elmer (U.S.A.) FT-IR Spectrometer Model 1725X. Spectra were recorded from 4,000 cm^{-1} to 400 cm^{-1} with a resolution of 2 cm^{-1}.

X-ray diffraction (XRD) was carried out on cortical bone wafers in the *Z*, *R*, and *T* orientations for NP40 + 2.0 M NaCl and NP40 + 2.0 M NaF treated samples for 3 days. XRD was also performed on intact bone powder treated in NP40 for 24 hr followed by 3 days of equilibration in either 2.0 M NaCl or 2.0 M NaF (4 mg bone powder/400 ml of solution), bone mineral powder and hydroxyapatite (SIGMA Chemical Co, St. Louis, MO, U.S.A.). Intact bone powder was obtained by cutting a piece of bone on a Buehler low speed saw with a diamond disc and collecting the bone particles as they were cut followed by lyophilization. Bone mineral powder was obtained through deproteinization with sodium hypochlorite (NaOCl) (32) or hydrazine treatment (36). XRD was carried out using a Siemens Diffractometer D5000 using a 0.015° step size for 3 sec between 23° and 56° 2O.

STATISTICAL ANALYSIS

Statistical analysis was carried out using Statistical Analysis Software (SAS, Cary, NC, U.S.A.). Analysis of variance (ANOVA) and multiple comparison *post hoc* analyses (Duncan's Multiple Range Test, Least Significant Difference (LSD) and Bonferroni t-tests, Tukey's, and Scheffe's to control for type I and type II errors) were carried out to determine the differences and levels of significance between the elastic constants and time points. Coanalyses of variance (COANOVA) were performed using density and ash weights as covariates to account for any biological variation between groups and its influence on the differences observed.

RESULTS

The pH of the NaCl solution remained at pH 7.5 (±0.2) throughout the 20 day experimental period. The pH of the sodium fluoride solution rose steadily from pH 7.5 to near 10.0 after 3 days and remained constant. This reflects a hydroxyl ion exchange with fluoride ions consistent with our previous results (10,31,33–35) and the exchange between fluoride and hydroxyl ions (5). The densities and ash fraction of the samples before and after the 20 day treatment did not significantly differ. Fluoride ion probe measurements revealed fluoride ion concentration per dry weight of mineral (ppm/gm ashed bone) reached a steady state by 3 days for 0.5 mm and 2.0 mm wafers (Fig. 1). Histological evaluation of a number of demineralized samples stained with hematoxylin and esoin and evaluated under light microscopy revealed the microstructure to be mainly haversian and similar between samples.

Infrared (IR) spectra did not reveal any significant differences between the sodium chloride or sodium fluoride treated groups *versus* time (Figs. 2 and 3). The spectra between 1,200–900 cm^{-1} (ν_1 and ν_3 stretching mode PO_4 regions) and 700–500 cm^{-1} (ν_4 bending modes PO_4 region) did not vary with respect to resolution, which suggests no effect of treatment on the crystal size. No signif-

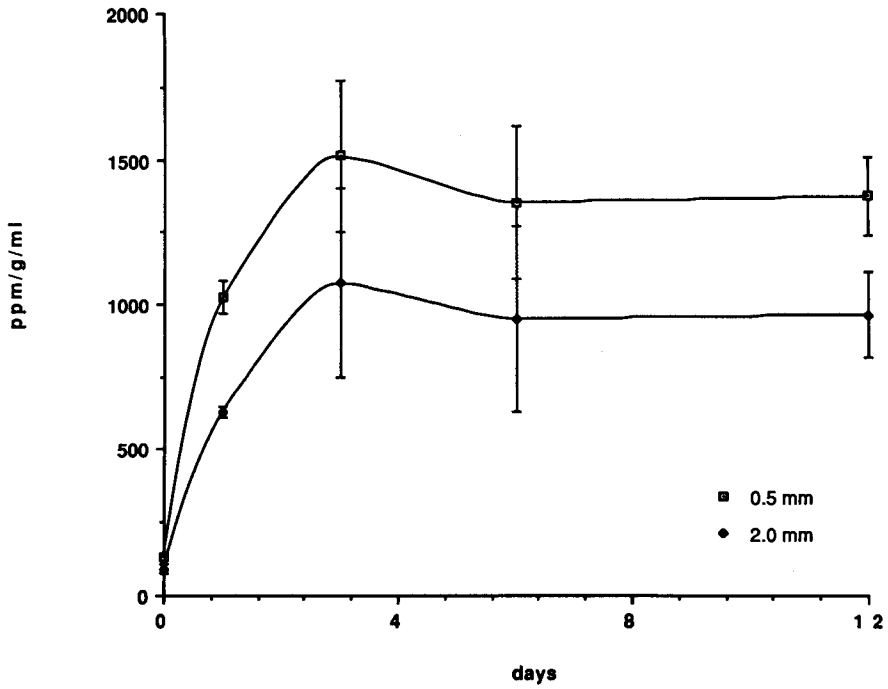


FIGURE 1. Fluoride ion probe measurements revealed fluoride ion concentration per dry weight of mineral reached steady state by three days. Fluoride ion uptake was minimal, suggesting that only surface hydroxyl or carbonate domains of the mineral phase were available for fluoride ion exchange.

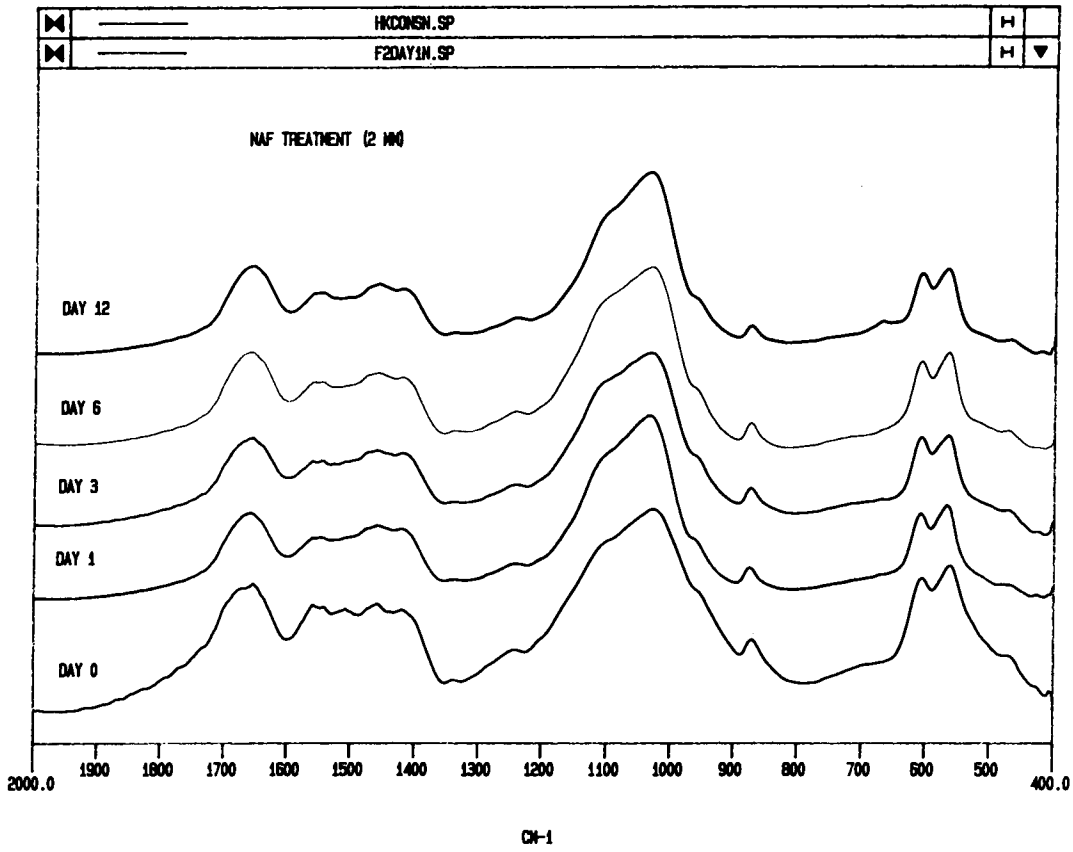


FIGURE 2. Infrared (IR) spectra from 2.0 mm thick bone wafers following NP-40 treatment and various days of equilibration in 2.0 M NaF. The IR patterns did not demonstrate any significant variations.

icant changes were noted in the v_2CO_3 domain (880–860 cm^{-1}) due to fluoride ion interaction with carbonate ions.

The longitudinal ultrasonic velocities and moduli for all time points are summarized in Figs. 4–6 (means and standard deviations). Moduli were greatest in the *Z*-axis followed by *T* and *R* for both groups at all time points ($p < 0.0001$). ANOVA analysis at time 0 did not demonstrate any statistical differences between the NaCl and NaF samples in the *Z* direction. However, at time 0 the *R* and *T* NaF moduli were slightly greater than the sodium chloride controls ($p < 0.05$).

The *Z*, *R*, and *T* NaCl moduli in the sodium chloride sample did not statistically differ during the 20 day equilibration period (Figs. 4–6). ANOVA analysis revealed no statistically significant differences between the NaCl *versus* NaF moduli in the *Z*, *R*, or *T* directions until day 6 ($p < 0.001$), which continued at days 13 and 20. The NaF moduli in the *R* and *T* direction did not significantly change after day 6. The NaF moduli in the *Z* direction continued to demonstrate a reduction at days 13 and 20 of sodium fluoride treatment ($p < 0.001$). A two-way ANOVA revealed time had a significant interactive effect on the NaF group (up to day 6 for *R* and *T* and day 20 for *Z* directions), but not for the NaCl samples. Coanalysis of variance (COANOVA) using ash weight and density as covariates revealed no findings not already predicted by the ANOVA.

X-ray diffraction patterns also revealed the anisotropy of control cortical bone wafers. Bone wafers oriented in the *Z* and *T* axes revealed a strong (002) reflection near 26° 2O which was absent in samples oriented on the *R* axis (Fig. 7). The region between 30° and 35° 2O [(211), (112), (300), and (202) reflections] also revealed a strong reflection for *Z*- and *T*-axis samples but was diminished for *R*-axis samples (Fig. 7). Intact bone powder demonstrated a similar XRD pattern to *Z*-axis bone wafers. Deproteinization of bone wafers with NaOCl or hydrazine followed by crushing into powder with a mortar and pestle revealed similar XRD patterns to the HA powder standard (Fig. 8) and *Z*-axis control wafers.

XRD patterns of bone wafers equilibrated with sodium fluoride (Fig. 9) were similar to the control sodium chloride–treated samples (Fig. 7). The (002) reflection was present for the *Z*-axis oriented specimens, but was reduced in magnitude for the *T*-axis samples (Fig. 9). The reflections between 30° and 35° 2O were present for all sodium fluoride (Fig. 9) samples as in controls (Fig. 7). Treatment of intact bone powder with sodium fluoride ions also re-

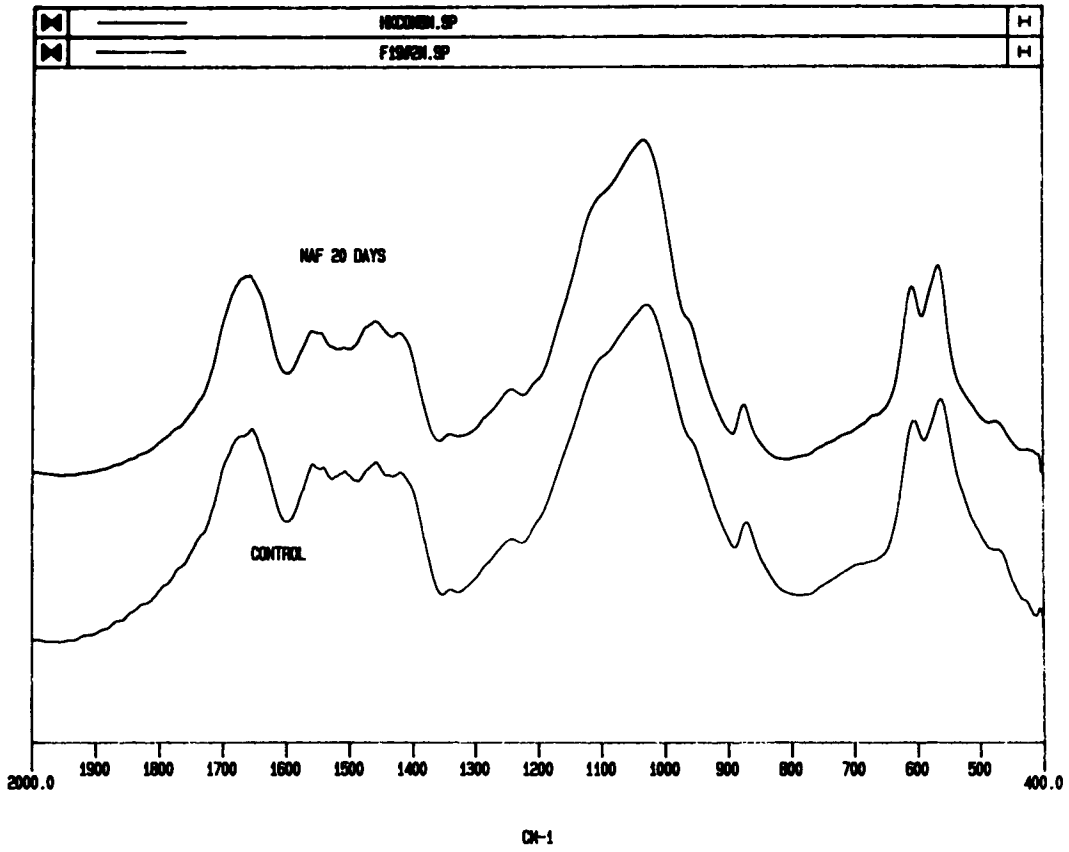


FIGURE 3. IR spectra for controls and NaF treated at day 20 did not demonstrate any significant variations.

vealed reflections near 29° 2O which was not present in the control sodium chloride–treated bone powder (Fig. 10). These peaks correspond to the (102) and (210) reflections of hydroxyapatite (29).

DISCUSSION

The heterogeneous and anisotropic nature of bone is well suited for the loads imposed during functional activity. The wide range of mechanical properties for bones from different species, age, and type of bone reflect the variety of loading conditions that bone as a structural material must endure. The heterogeneous and anisotropic mechanical properties have been well documented (13,14, 16). Structural evidence explaining the anisotropy of bone considered bone mineral crystallographic orientation within the type I collagenous matrix (28) as well as collagen fiber orientation (18). The work from this study suggests that interfacial bonding interactions between the mineral and organic constituents may also play a role in the anisotropic nature of bone tissue.

Ultrasound testing, a nondestructive technique, offers an advantage over destructive mechanical testing by enabling the properties of a single specimen to be obtained in many directions. In addition, the nondestructive nature of ultrasound enables the same specimens to be used throughout the time course of the experiment. Considering our experiment was an *in vitro* equilibration, the biological effects of sodium fluoride on bone cells were not considered. Slow strain rate destructive mechanical testing has shown that *in vitro* sodium fluoride equilibration results in a reduction in the tensile (10,34) and compressive properties (6,34) of adult bovine cortical bone oriented on the long bone (*Z*) axis. Carter and Beaupres (4) have also hypothesized that fluoritic bone would have a reduction in tensile properties. The results obtained in the present study indicate that *in vitro* equilibration of bovine cortical bone with sodium fluoride also lowers the ultrasonic properties in the *Z*-, *R*-, as well as the *T*-direction.

The reduction in the longitudinal velocities and ultrasonic moduli following fluoride ion treatment did not arise as a result of a reduction in density, partial demineralization or deproteinization, or modification in the organic phase of bone (10,31,33–35). Previous tensile experiments on demineralized adult bovine cortical bone samples, where only the type I collagenous organic phase is present, equilibrated with sodium fluoride ions demonstrated no alteration in the mechanical properties. The re-

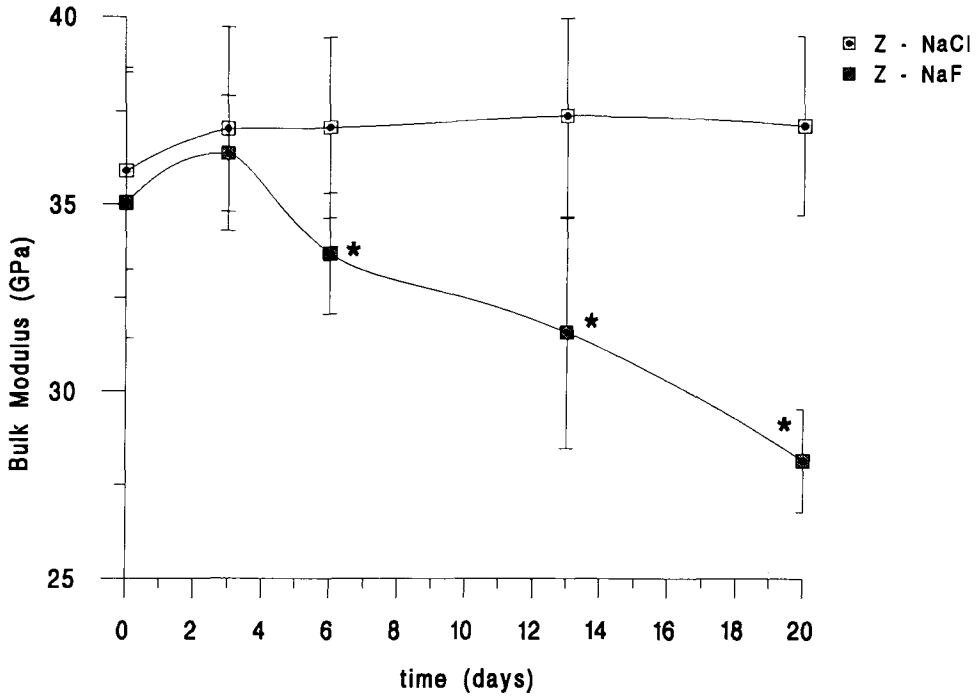


FIGURE 4. Ultrasonic moduli in the long bone (*Z*) direction is presented for 0.145 M NaCl (controls) and 2.0 M NaF treated *versus* time. The NaCl moduli did not vary *versus* time. However, NaF treatment caused a reduction that becomes significant after day 6 ($p < 0.001$). The modulus continued to demonstrate an increase in the reduction even at day 20 ($p < 0.001$).

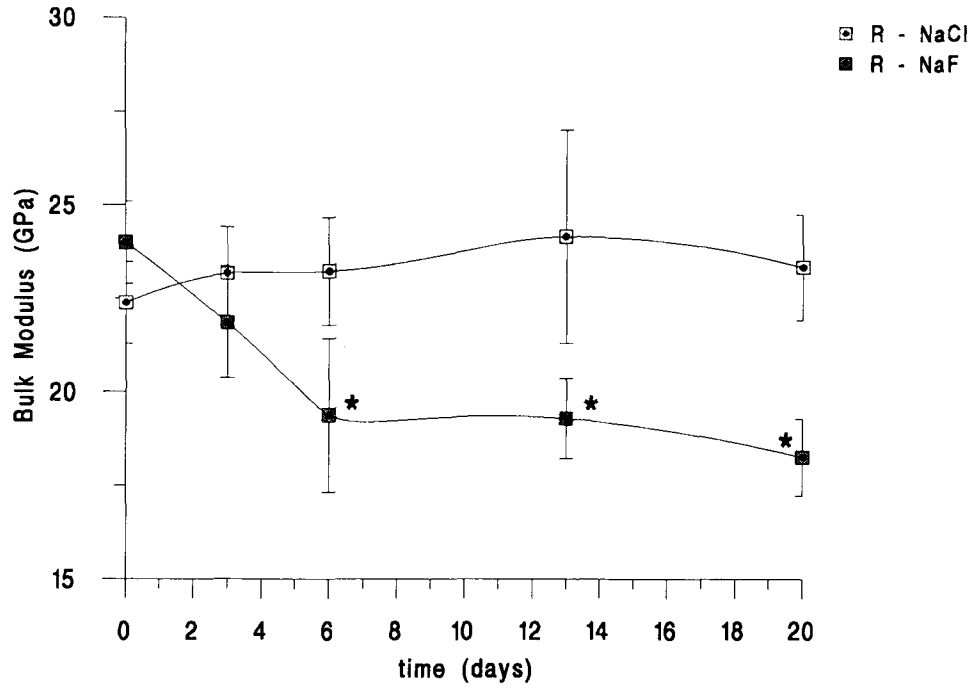


FIGURE 5. Ultrasonic moduli in the radial (*R*) direction is presented for 0.145 M NaCl (controls) and 2.0 M NaF treated versus time. The NaCl moduli did not vary versus time. However, NaF treatment caused a reduction which becomes significant after day 6 ($p < 0.001$) and stabilized.

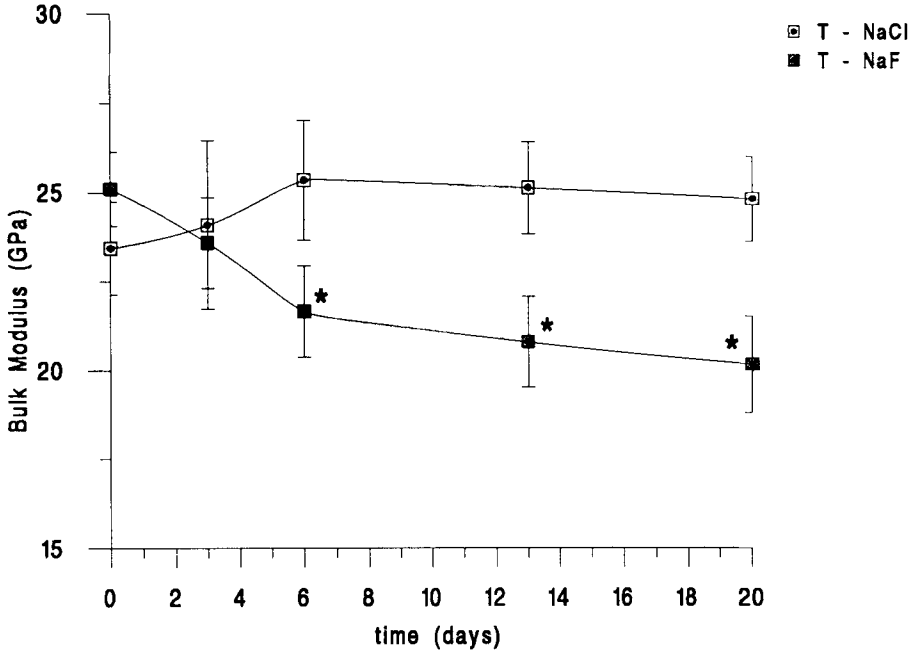


FIGURE 6. Ultrasonic moduli in the circumferential (*T*) direction is presented for 0.145 M NaCl (controls) and 2.0 M NaF treated *versus* time. The NaCl moduli did not vary *versus* time. However, NaF treatment caused a reduction that becomes significant after day 6 ($p < 0.001$) and stabilized.

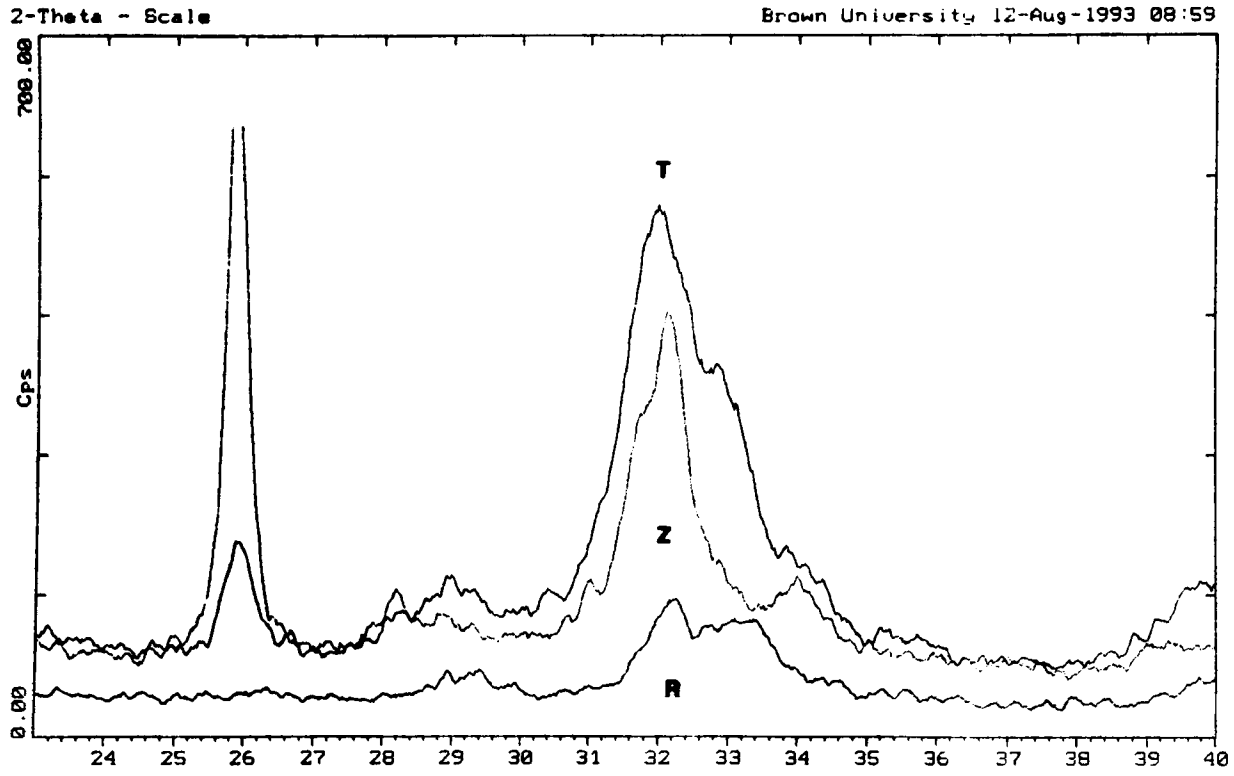
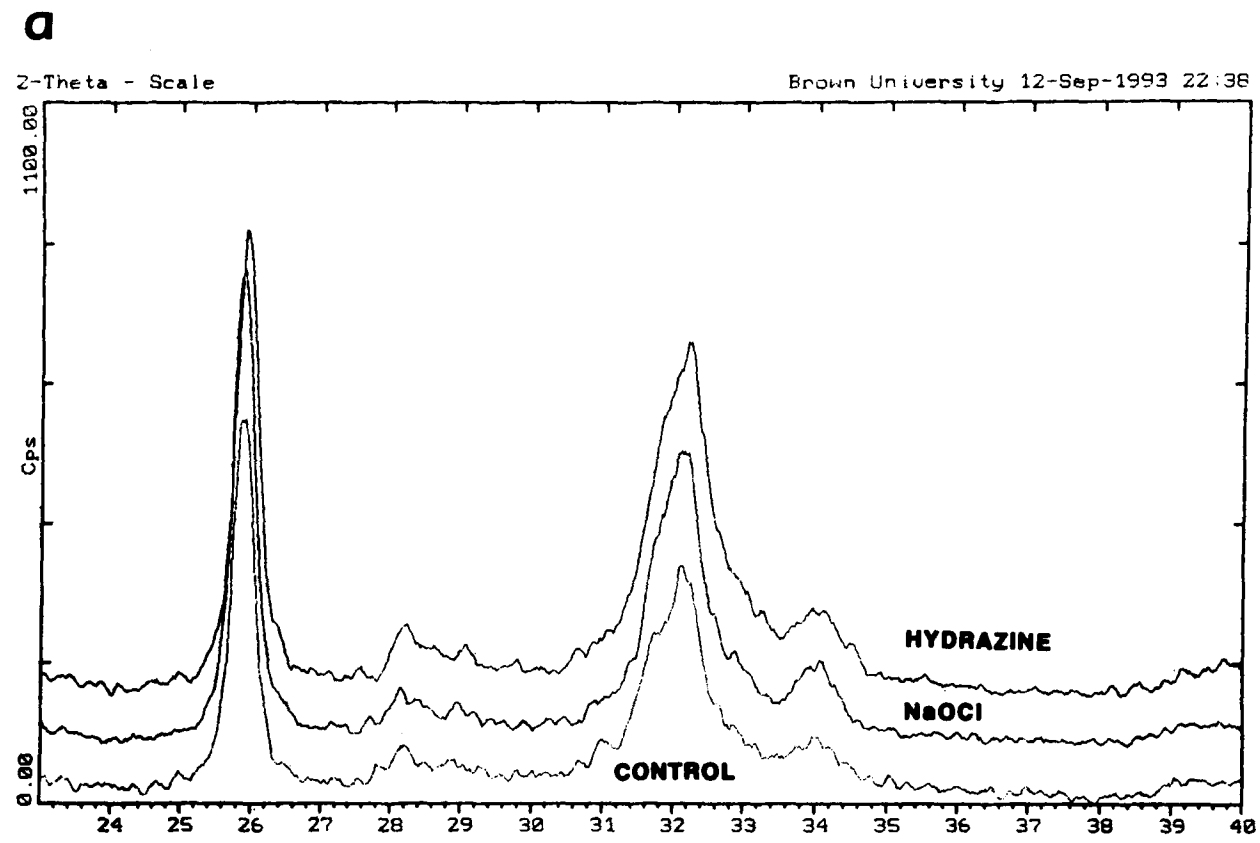


FIGURE 7. X-ray diffraction patterns for intact control samples in the *Z*, *T*, and *R* orientations are presented. The XRD patterns reveal the anisotropy present amongst the *Z*-, *T*-, and *R*-axes of bone. Bone wafers oriented in the *Z*- and *T*-axes revealed a strong (002) reflection near 26° 2-theta. The region between 30 and 35° 2-theta [(211), (112), (300) and (202) reflections] also revealed a strong reflection for *Z*- and *T*-axis samples which was diminished for *R*-axis samples.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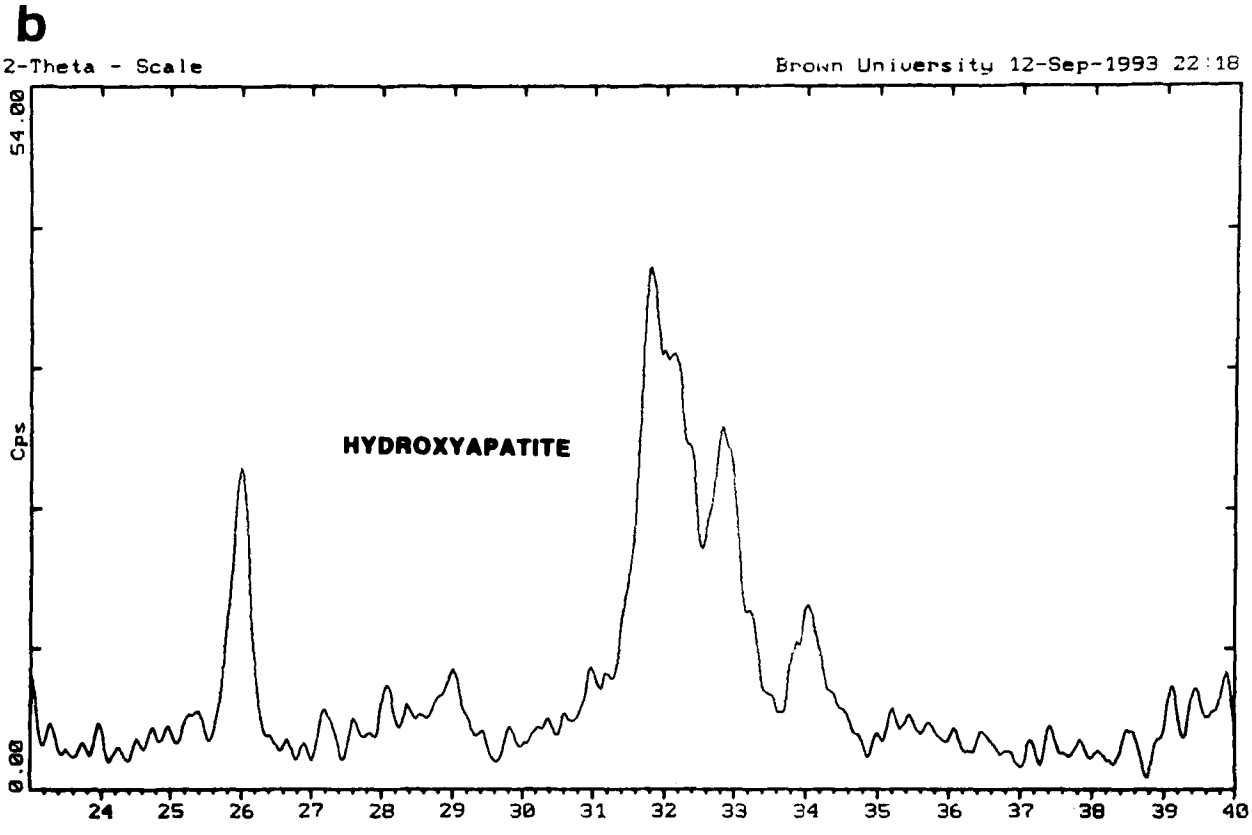
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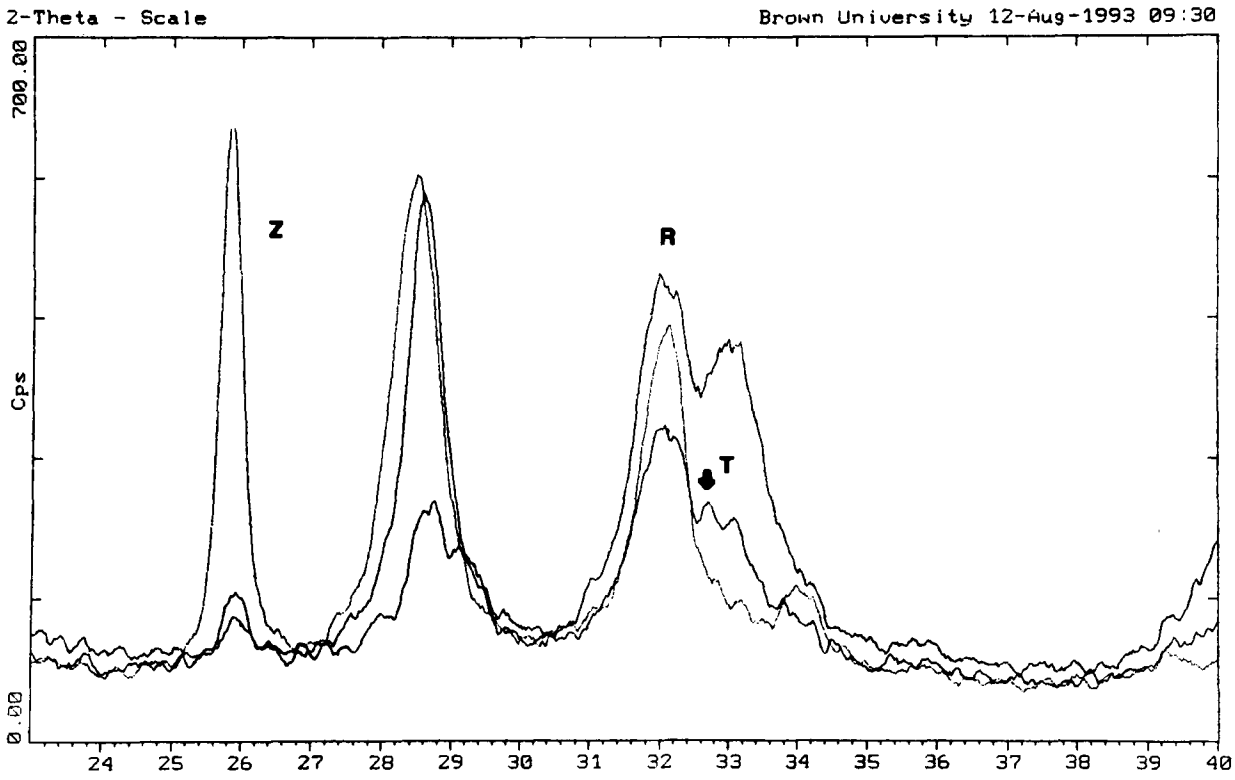

FIGURE 9. X-ray diffraction patterns for sodium fluoride treated wafers in the *Z*-, *T*-, and *R*-axes. The XRD pattern is similar to control, sodium chloride treated samples (Fig. 7).

duction in the properties may be attributed to an alteration in the interfacial bonding between the mineral and organic constituents of bone due to the action of fluoride ions. The detergent and ion-treatment protocol allows ions access to the mineral–organic interface to alter the interfacial bonding between the mineral and organic constituents of bone (10,31,33–35). Partial debonding between the matrix and fibers of a composite will significantly modify the strength and stiffness (5).

The *in vitro* effects of fluoride ions on hydroxyapatites have been extensively discussed in the literature (18): (i) uptake of fluoride ions increases with an increase in fluoride ion concentration in solution (16,26); (ii) the absorption of fluoride is pH dependent (16,26). Fluoride uptake *in vitro* is higher at lower pH (pH 5) than at neutral pH (pH 7) (17). The basic conditions (pH 7.5 which rose to near pH 10) in the present study would not favor maximal fluoride ion uptake by the adult bovine bone. The increase in pH following sodium fluoride treatment suggests a substitution of fluoride ions for hydroxyl ions on the mineral surface. This F-OH substitution may have resulted in conversion of the surface of the mineral from hydroxyapatite to fluorapatite at local sites. Our fluoride ion probe measurements indicate a very small percentage of F-OH exchange in the adult bovine cubes used in the ultrasound portion of the study. Fluoride ion probe and pH measurements suggest fluoride replaced hydroxyl ions of the mineral phase. The FTIR spectrum did not suggest any dramatic alterations in the mineral phase due to fluoride ion treatment in terms of carbonate substitution.

X-ray diffraction patterns on intact adult bovine bone wafers on the *Z*-, *T*-, and *R*-axes following NaCl or NaF equilibration for 3 days further illustrated the anisotropic nature of bone with regard to the orientation of the mineral phase. Strong (002) reflections were present in *Z* and *T* specimens while absent in the *R*-axis samples. These data suggest that the *c*-axis of the mineral phase is oriented parallel to the *Z*-axis (bone axis) as well as a significant amount of the *c*-axis of the mineral phase oriented in the *T* orientation (28). The preferential orientation of the *c*-axis of the mineral phase in the *Z*- and *T*-axis is reflected in the greater mechanical properties in the *Z*- followed by *T*- and *R*-axes (14).

Bone powder was obtained to increase the treatment

FIGURE 8. (a) X-ray diffraction patterns for intact control wafer (*Z*-axis) and samples deproteinated with sodium hypochlorite (NaOCl) or hydrazine are presented. XRD patterns for the deproteinated samples are similar to the *Z*-axis intact control sample. (b) X-ray diffraction pattern for hydroxyapatite standard.

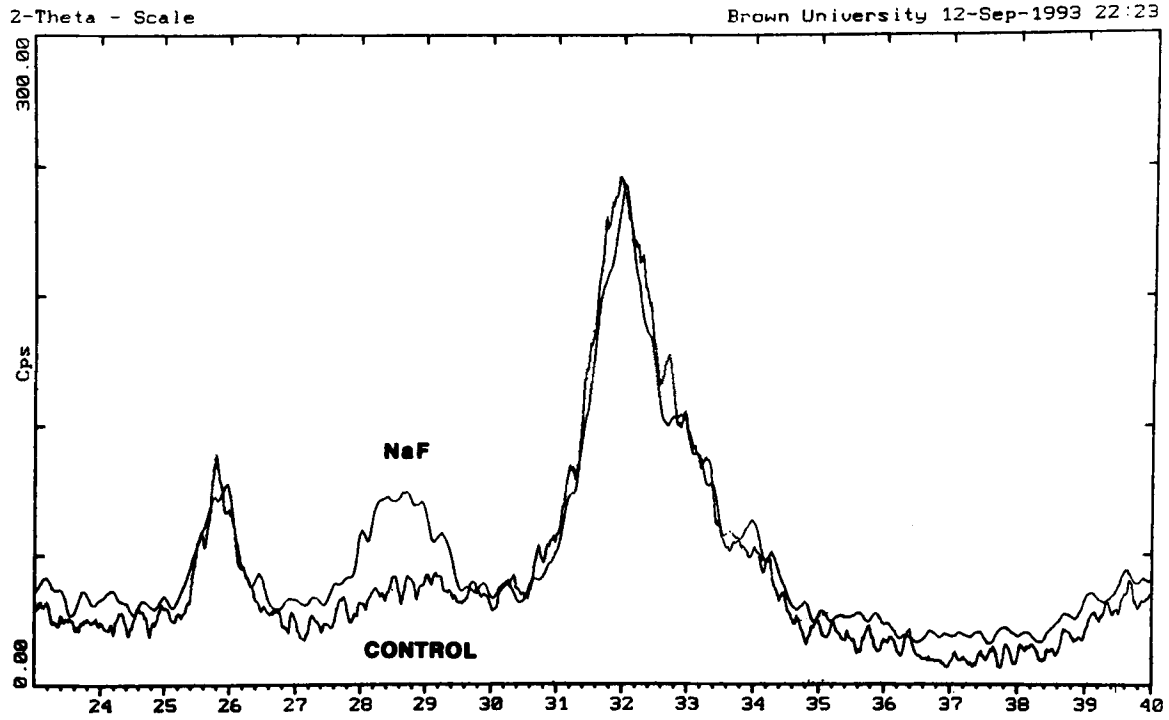

FIGURE 10. X-ray diffraction patterns for control and sodium fluoride treated bone powder are presented. XRD patterns for the bone powder reveal similar findings to *Z*-axis oriented wafers.

surface area to further examine the effect of *in vitro* fluoride ion treatment on adult bovine cortical bone. The XRD pattern for control bone powder was similar to the *Z*-axis control bone wafer sample. Similarly, the XRD pattern for the sodium fluoride treated bone powder resembled the *Z*-axis bone wafer treated with sodium fluoride. The XRD pattern of the fluoride treated powder and wafers showed an increase in resolution compared with control samples. Similar results have been found by LeGeros and coworkers (17) for calf bone powder equilibrated in varying concentration sodium fluoride solutions (at pH 5 and pH 7) at 37°C. The reduction of an intact piece of bone to a powder removes the anisotropic character revealed in intact samples in the *Z*-, *T*-, and *R*-axes.

Lees and Hanson (15) recently demonstrated a reduction in ultrasonic moduli in the radial axis following a fluoride supplemented diet (80 mg/kg body weight/day). Gynpas and Rey (8) fed rats 8 mM NaF/l in their water for 6 months. The percent of fluoride by weight incorporation by 6 months was approximately 0.5%. This amount of fluoride incorporation *in vivo* did not affect the Ca, P, or Ca/P molar ratio or the content of rat cortical bone (7). XRD and IR spectroscopy also did not demonstrate any dramatic differences compared with control cortical samples as was found for IR and XRD results in the present *in vitro* equilibrated samples. Fluoride ion incorporation into the mineral phase of growing rats did demonstrate some carbonate exchange for fluoride. An intense band has been noticed in the v_2CO_3 domain (880–860 cm^{-1}) for fluoridated apatites due to interaction between fluoride and carbonate ions during an *in vivo* fluoride treatment. FTIR on bone powder subjected to *in vitro* equilibration with sodium fluoride did not demonstrate such changes. This suggests that the fluoride interaction with the mineral phase *in vitro* is primarily occurring through hydroxyl ion exchange mainly at the mineral surface. This fluoride interaction may reduce the solubility of the bone crystals (27) which leads to a less active mineral–organic interfacial bonding.

The reduction in the elastic constants following sodium fluoride ion treatment is primarily a result of an alteration in mineral–organic interfacial bonding. The exchange of fluoride for hydroxyl ions does not alter the electrostatic equilibrium since both possess a valence of -1. However, fluoride ions cannot form hydrogen bonds as can hydroxyl ions. Other possibilities accounting for fluoride ion reduction in properties may be due to the increase in pH at the mineral–organic interface which will alter protein secondary and tertiary structures (24). The pH of the NaF solution rose from pH 7.5 to near 10 by the end of the equilibration. From electrokinetic measurements we know that at a pH above its isoelectric point (IEP) a species will carry a net-negative electrostatic charge. The isoelectric point is defined as the pH where the zeta potential is zero (11,12). The IEP of collagen lies near pH 5.0 (9,11) while the IEP of bone mineral is approximately 8.0 (23). There-

fore, near pH 10 both the mineral and organic constituents will possess negative charges (since they are both above their IEPs), which establishes an electrostatically unfavorable condition leading to a reduction in interfacial bonding.

This work presents the first data indicating that interfacial bonding between the mineral and organic constituents of bone play an important role in determining the anisotropic nature of bone. The greatest reduction in the fluoride treated samples compared with controls was observed in the *Z*-axis. The radial and circumferential orientations revealed similar alterations compared with the *Z*-axis, but did not continue to decrease *versus* time. The available mineral surface for organic interactions in all three directions may not be equivalent. This may account for the continued reduction in the *Z*-axis ultrasonic moduli following fluoride ion treatment as more surface area was affected upon long-term equilibration. However, in the *R* and *T* orientations, the effect appeared to reach steady state by 6 days.

It is known that the mineral phase of bone has a preferred orientation along the *z*-axis of bone (28). In addition, the mineral crystals also have an orientation along the long axis of the collagen molecule (30). As a result, the available surface area for bonding between the mineral and organic constituents is greatest along the *Z*-axis. This may also account for the anisotropy of cortical bone and greater mechanical properties along the *Z*-axis. Furthermore, considering the greater surface area of mineral–organic interfacial bonding it is not surprising to find the greatest reduction in ultrasonic moduli following fluoride ion treatment along the *Z*-axis.

The *in vitro* fluoride equilibration model used in the present study does not directly examine the effects of fluoride intake *in vivo*. *In vivo* fluoride ion intake has been shown to influence cell metabolism and to become incorporated into the mineralized matrix during bone remodeling. The present *in vitro* model represents fluoride interaction at the bone mineral surface. However, since the mechanical properties of fluoroapatite and hydroxyapatite have been shown to be similar (7), surface treatment of bone mineral with fluoride will not alter the bulk properties of the mineral phase. Therefore, the *in vitro* model used in the present study is useful in exploring the effects of fluoride ions on the mechanical properties of bone and interfacial bonding between the constituents.

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