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Pyridoxine Deficiency Affects Biomechanical Properties of Chick Tibial Bone

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The mechanical integrity of bone is dependent on the bone matrix, which is believed to account for the plastic deformation of the tissue, and the mineral, which is believed to account for the elastic deformation. The validity of this model is shown in this study based on analysis of the bones of vitamin B_6-deficient and vitamin B_6-replete chick bones. In this model, when B_6-deficient and control animals are compared, vitamin B_6 deficiency has no effect on the mineral content or composition of cortical bone as measured by ash weight (63 ± 6 vs. 58 ± 3); mineral to matrix ratio of the FTIR spectra (4.2 ± 0.6 vs. 4.5 ± 0.2), line-broadening analyses of the X-ray diffraction 002 peak (β_{002} = 0.50 ± 0.1 vs. 0.49 ± 0.01), or other features of the infrared spectra. In contrast, collagen was significantly more extractable from vitamin B_6-deficient chick bones (20 ± 2% of total hydroxyproline extracted vs. 10 ± 3% $p \leq 0.001$). The B_6-deficient bones also contained an increased amount of the reducible cross-links DHLNL, dehydro-dihydroxylysinonorleucine, (1.03 ± 0.07 vs. 0.84 ± 0.13 $p \leq 0.001$); and a nonsignificant increase in HLNL, dehydro-hydroxylysinonorleucine, (0.51 ± 0.03 vs. 0.43 ± 0.03, $p \leq$ 0.10). There were no significant changes in bone length, bone diameter, or area moment of inertia. In four-point bending, no significant changes in elastic modulus, stiffness, offset yield deflection, or fracture deflection were detected. However, fracture load in the B_6-deficient animals was decreased from 203 ± 35 MPa to 151 ± 23 MPa, $p \leq 0.01$, and offset yield load was decreased from 165 ± 9 MPa to 125 ± 14 MPa, $p \leq$ 0.05. Since earlier histomorphometric studies had demonstrated that the B_6-deficient bones were osteopenic, these data suggest that although proper cortical bone mineralization occurred, the alterations of the collagen resulted in changes to bone mechanical performance. (*Bone 18:567–574; 1996*)

Key Words: Vitamin B_6; Bone biomechanics; Collagen cross-links; Chicken bone; Mineral analysis.

Introduction

The mechanical properties of bone tissue depend both on the mineral and the matrix (primarily type I collagen fibrils) constituents.[31,43,48] Bone has been shown to exhibit a region of elastic (or recoverable) deformation, followed by a region of plastic (or permanent) deformation.[43] Based on tensile testing of progressively decalcified bovine bone tissue, Burstein et al.[7] hypothesized that the elastic deformation of bone is governed principally by the mineral, while the plastic deformation of bone is governed by a combination of elastic-perfectly plastic behavior of the mineral, and purely elastic behavior of the collagen matrix.

Studies of the effect of alterations of the ultrastructure of bone tissue on mechanical properties have focussed more on the mineral content and/or mineral composition than on the collagen matrix.[10,54] Currey[11] demonstrated that the elastic modulus of bone tissue (the slope of the elastic region of the tensile stress–strain relationship) increases with an increase in mineral content among a variety of species. Variations in mechanical properties of osteopenic tissues have also been associated with alterations in mineral content[25,48] and the bending and torsional stiffness of bones of hypomagnesemic rats have been shown to decrease with reductions in ash content and altered mineral distribution.[6] In these instances, the composition of the organic matrix was not characterized; thus, mineral and matrix effects could not be separated.

The mechanical properties of bone tissue have been shown to be affected by alterations in the collagen matrix, but in these studies there were also abnormalities in mineral content. For example, the importance of collagen intermolecular cross-links to the mechanical performance of bone is apparent in lathyrism, an animal disease caused by eating the seeds of sweet peas (*Lathyrus odoratus*). The seeds contain β-aminopropionitrile (BAPN), which irreversibly inhibits the activity of copper-dependent lysyl oxidase.[55] Thus, BAPN ingestion reduces the formation of collagen cross-links, among other effects. In addition to alternating the collagen matrix, BAPN also decreases bone mineralization in vitro and in vivo.[19,42,46,52] The biomechanical effects of altered bone matrix and mineralization in BAPN treated rats were demonstrated by Spengler et al.[52] and more recently by Lees et al.,[27,28] who tested rat femora in torsion and found both a decrease in torsional stiffness and a decrease in torsional strength. These results support the hypothesis of Burst-

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ein et al.[7] in that a decrease in torsional stiffness is consistent with a decrease in bone mineral and a decrease in torsional strength is consistent with an impaired collagen matrix and/or a decrease in bone mineral.

Collagen cross-linking can also be altered by other lysyl oxidase inhibitors (e.g., isoniazid, thiosemicarbazine)[8,30,55] and copper chelators;[22] but these too may result in defective mineralization as suggested by the lathyritic appearance of these animals. In a nonmineralized model, Opsahl et al. reported a decreased tendon and bone strength in animals given a copper deficient diet to reduce collagen cross-linking.[40] Penicillamine (β,β methylcysteine) and homocysteine form adducts with the reactive aldehydes generated by lysyl oxidase[49] and consequently impair collagen cross-linking.[12,24] Although penicillamine does not prevent the synthesis of aldehyde-derived cross-links like BAPN, it inactivates them by selective binding. Mineral and mechanical studies in animals treated with penicillamine or given a homocysteine-rich diet are not available.

To our knowledge, there have been no studies on bone showing changes in mechanical properties, when the matrix is altered, while the mineral is not. Recent reports of mild skeletal abnormalities in chickens given a moderately vitamin B$_6$-deficient diet 2 weeks after birth suggested these animals might provide a model in which these effects could be separated. The effects of this moderate B$_6$-deficiency on long bones have already been characterized by Massé et al.[32] using histomorphometry and roentgenography. In brief, the morphometric study revealed that the vitamin B$_6$-deficient trabecular bone had a 1.5- to 2-fold decrease in osteoid volume and surface with no change in trabecular bone volume. Preliminary mineral analysis revealed no differences in mineral content or crystal size.

It is believed by many that pyridoxal phosphate (PLP), the coenzyme form of vitamin B$_6$, in addition to copper, is required for lysyl oxidase activity.[18,30,38] According to Fujii et al.,[18] bone collagen cross-link formation is reduced in vitamin B$_6$ deficiency. A previous electron microscopic study revealed that the collagen fibers in articular cartilage of chickens given a vitamin B$_6$-deficient diet, or a homocysteine-rich diet, are abnormally (1.5-fold) enlarged as compared to controls.[33] However, the collagen cross-link analysis on B$_6$-deficient articular cartilage and tendon did not reveal any significant changes.[35] The aim of the present study was to determine whether the loss of structural integrity of connective tissue induced by pyridoxine (vitamin B$_6$) deficiency will affect the biomechanical properties of bone tissue. For this purpose, four-point bend tests were conducted on the tibias of vitamin B$_6$-deficient and normal chicks. Chemical and spectroscopic studies were conducted to confirm the absence of mineral abnormalities and the existence of an altered collagen matrix. Thus, assuming the lack of mineral abnormality could be confirmed, the vitamin B$_6$-deficient chick would provide a model to examine the effect of collagen matrix abnormalities alone on the mechanical performance of bone tissue.

Materials and Methods

Animals

One day-old male Lohmann chicks (rapidly growing commercial broilers) were randomly assigned to a control (adequate vitamin B$_6$ diet) or a vitamin B$_6$-deficient diet. There were ten animals in the experimental group and four in the control group. Chicks were weighed at the beginning of the experiment; each group had a similar mean initial body weight (43 ± 5 g).

The experimental and control basal diets, based on corn-

soyamin, were vitamin and mineral supplemented to fulfill all nutritional requirements during growth.[34] The vitamin D level was kept constant and the Ca:P molar ratio of 1.5:1 was optimal for chick bone growth. Microbiological analysis revealed that the experimental diet contained 0.4 mg vitamin B$_6$/kg, while the control contained 3.0 mg vitamin B$_6$/kg. In order to assure a normal growth and an anabolic state, the vitamin B$_6$-deficient diet was initiated only after the chicks were 2 weeks old. Feed and water were provided ad libitum throughout the 6 week experiment. Chicks were weighed twice weekly and examined to make sure they did not suffer a loss of appetite, growth retardation or neurological symptoms characteristic of a severe vitamin B$_6$ deficiency.[47]

At the end of the experiment, 4 mL of fasting blood was collected by jugular venipuncture using a heparinized syringe. The blood was centrifuged for 10 min at 1000g and plasma was collected for biochemical analyses. The plasma calcium and phosphorus contents were determined by means of an automated COBAS Bio-autoanalyzer (Hoffmann-LaRoche, Switzerland). Plasma amino acids were determined by using a standard method on a Beckman amino acid analyzer. Erythrocytes were resuspended in an equal volume of isotonic saline and centrifuged for 10 min at 1000g. The supernatant was discarded and the erythrocytes were analyzed for the PLP content according to Coburn and Mahuren's method.[9] Plasma was analyzed for PLP using the same method. Results are reported for erythrocyte PLP, since these are more indicative of tissue levels. In plasma, PLP is rapidly dephosphorylated by alkaline phosphatase.[17]

Immediately after the sacrifice of the animals by cervical dislocation, the tibias were dissected, cleaned of soft tissue, wrapped in saline soaked gauze, and stored frozen at −70°C until testing. Radiographic and biomechanical studies were followed by chemical analyses of mineral content and composition, amino acid composition, and collagen solubility of tested bones.

Radiographic Evaluation

The tibias were radiographed (60 kV, 4.5 min) in the anterior–posterior plane prior to mechanical testing. To consider the influence of geometrical variation on whole bone mechanical properties, the endosteal (d) and periosteal (D) diameters were measured at the approximate location of each of the four loading points in the four-point bend test. Tibial cross sections at each of the four locations were then calculated assuming that the cross sections were circular. Area moment of intertia (I) was calculated assuming a circular cross section, $(\pi/64) * (D^4 - d^4)$, and averaged for the four loading point locations. The tibial length was also measured. A comparison of geometrical cross sections and of tibial lengths of the control and B$_6$-deficient tibias was conducted.

Mechanical Testing

Left and right tibias were tested in four-point bending. Prior to testing, the bones were thawed to room temperature. Bones from four control group animals and from nine experimental animals were tested. The tests were performed in ambient air on an MTS closed-loop servohydraulic test apparatus at a displacement rate of 6.25 mm/sec. Testing to failure was performed in the medial-lateral (ML) plane, using an outer support span of 33 mm and an inner span of 11 mm. Load versus displacement of the loading ram was recorded on a personal computer. Specimens were selected randomly for testing and were kept wet with distilled water throughout testing. The whole bone properties of fracture

load (N) and fracture deflection (mm) were determined directly from the load vs. displacement curve for each specimen. Offset yield load and deflection were determined at the intersection of the line parallel to the slope of the initial linear region of the load versus displacement curve offset by 0.05 mm.

Prior to failure testing, each specimen was loaded elastically in load control to 5.62 N at a loading rate of 2.81 N/sec. Stiffness (N/m) and elastic modulus (GPa) were calculated from this pre-failure test according to ASTM D790.[1] In calculating the elastic modulus, the average of the endosteal and periosteal cross sections from the four loading points was used.

Mineral and Matrix Composition

Following mechanical testing, bone from the fracture site was lyophilized, ground in a freezer mill (Spex, Metuchen, NJ, USA) at liquid nitrogen temperatures, and three sets of aliquots set aside for mineral analyses. For analysis of mineral content, triplicate aliquots (2–4 mg) were transferred to weighed dry crucibles. Dry weight was determined following heating to constant weight at 110°C. Ash weight was determined following heating to constant weight at 600°C. Mineral content was calculated as the ratio of ash weight to dry weight. Ten milligram aliquots of ground bone were subjected to wide-angle X-ray diffraction using Cu $K\alpha$ radiation. The line width at half-maximum of the c-axis 002 reflection was measured as an index of crystallite size.[26] Two milligram aliquots of ground bone were mixed with 100 mg KBr, and pellets prepared for infrared spectroscopy. The pellets were analyzed on a Mattson Cygnus Fourier Transform Infrared (FTIR) Spectrometer. Typically, the relative scans at 4 cm⁻¹ resolution were collected. The spectra were analyzed to provide information on the relative mineral to matrix ratio [ratio of integrated areas of the phosphate ν_1, ν_3 mode (900–1200 cm⁻¹) to the amide I band (1580–1650 cm⁻¹)] and the carbonate (840–890 cm⁻¹) to phosphate ratio.

The remaining bone was dissected into metaphyseal and diaphyseal regions, ground as described above, and aliquots used for analyses of amino acid composition, collagen solubility and cross-links. Amino acid analyses were performed on HCl hydrolysates (6N, 18 h, 110°C) of EDTA-demineralized ground bone using a Beckman auto analyzer.

To determine the relative solubility of the collagen in the ground undecalcified cortical bones, ~100 mg aliquots were extracted sequentially in 0.5M EDTA (pH 7.4), 1 mol/L NaCl (pH 7.4), and 3% acetic acid.[20,36] Each extraction was carried out at 4° for 3 days, after which the solution was centrifuged (17 000 rpm, 30 min, 4°C), the pellet washed with the extracting solution (which was pooled with the original extract), water washed, lyophilized, and weighed before extraction with the next solvent. The 3 day demineralization was selected based on preliminary experiments, which demonstrated 95% of the mineral was removed in this time. The extracts, dialyzed against water and lyophilized, were weighed and used for analysis of hydroxyproline (Hyp) according to the method of Hutterer and Singer,[21] as was the final pellet and starting material.

Collagen Cross-links

Bone samples were pulverized under liquid nitrogen using a Spex Freezer Mill (Spex, Inc, Metuchen, NJ, USA), weighed, and demineralized with 0.5M EDTA, pH 7.4, until the solution was Ca-free. The powder was then washed with distilled deionized water and lyophilized. The dried samples (approximately 5 mg) were suspended in 0.15M N-trismethyl-2-aminoethane-

sulfonic acid (TES)-buffer, pH 7.5, and reduced with standardized NaB³H₄. Standardization of NaB³H₄ and reduction was performed by the method described previously.[58,61] The reduced samples were then hydrolyzed with 6N HCl in vacuo after flushing with N₂ at 105°C for 24 h. The hydrolysates were dried under reduced pressure in a vacuum centrifuge (Savant Instruments, Hicksville, NY, USA), and the residue was dissolved in 1 mL of water and filtered. An aliquot of the hydrolysate was subjected to amino acid analysis to determine Hyp content. The hydrolysates with known amounts of Hyp were then analyzed for reducible and nonreducible cross-links on a Waters high-performance liquid chromatograph fitted with a stainless-steel amino acid column (AA911, Interaction, Mountain View, CA, USA). It was linked to an on-line flow liquid scintillation monitor (FIO-ONEβ, Radiomatic Instruments and Chemical, Walnut Creek, CA, USA) and a fluorescence flow monitor (JASCO Instruments Co., Japan).[61] The major cross-links identified were dehydro-dihydroxylysinonorleucine (reduced form, DHLNL), dehydro-hydroxylysinonorleucine (reduced form, HLNL), pyridinoline (Pyr), and deoxypyridinoline (D-Pyr). The former two are reducible, and the latter two nonreducible. Nonreducible cross-links, Pyr and D-Pyr were quantified by fluorescence (E_x 330 nm, E_m 390 nm) standardized by apparently pure Pyr cross-linked peptides isolated from tryptic and/or collagenase digests of bovine bone, Achilles tendon, and dentin collagens.[56,57,59,61,63] Reducible cross-links identified by their chromatographic elution positions on the HPLC were quantified by integrating the respective radioactive cross-link peaks and converting the area counts into a mole/mole of collagen bases using the specific activity of NaB³H₄ and a value of 300 residues of hydroxyproline per collagen molecule.[59]

Data Analyses

Data are reported as mean ± standard deviation (SD). Comparability of the two treatment groups regarding biological, biochemical, and biomechanical variables was assessed by unpaired Student's t-test or nonparametric Mann–Whitney test, whenever data followed a non-Gaussian distribution. Bartlett's test was used to verify equality of SD. The two-tailed criterion at the significance level $P < 0.05$ was employed. For the mechanical data, values beyond two SD of group means were deleted.

Results

Vitamin B₆ deficiency was characterized by a two-third reduction of PLP in erythrocytes (**Table 1**). Deficient animals grew normally and their weight gain at the end of the 6-week experiment was slightly, but not significantly, lower than controls. The

Table 1. Anthropometric and biochemical assessments of vitamin B₆ deficient (−B₆) and control chicks (+B₆)

	Units	−B₆	+B₆
Body weight	(kg)	1.51 ± 0.20	1.67 ± 0.12
Pyridoxal phosphate	(nmol/L) erythrocyte	47.6 ± 9.8[a]	239 ± 71
Ca⁺⁺	(mmol/L) plasma	2.81 ± 0.06	2.75 ± 0.11
PO₄⁻³	(mmol/L) plasma	2.62 ± 0.17	2.61 ± 0.24
Methionine	(nmol/L) plasma	149 ± 51	148 ± 32
Cystathionine	(nmol/L) plasma	45 ± 23[b]	6.5 ± 3.3
Homocysteine	(nmol/L) plasma	213 ± 26[c]	3.8 ± 4.3
Cysteine	(nmol/L) plasma	17 ± 9.7[b]	89 ± 14

[a] $p < 0.01$; [b] $p < 0.05$; [c] $p < 0.001$.

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Table 2. Biological and mineral properties of tibia of vitamin B_6 deficient $(-B_6)$ and control $(+B_6)$ animals

	$-B_6$	$+B_6$
Wet weight (g)	12 ± 1.5^a	14 ± 1.2
Length (cm)	10.0 ± 0.3	9.9 ± 0.1
Diaphyseal diameter (mm)		
Endosteal	4.2 ± 0.7	4.4 ± 0.6
Periosteal	5.3 ± 0.8	5.6 ± 0.5
Area moment of inertia (mm⁴)	25.1 ± 12.5	31.5 ± 7.6
Ash weight (%)	63.1 ± 6.1	58.4 ± 3.1
β_{002} (°2θ)	0.50 ± 0.19	0.49 ± 0.01
Phosphate: Amide I	4.16 ± 0.58	4.52 ± 0.22
(ratio of integrated areas)		
Carbonate: Phosphate	0.0157 ± 0.002	0.0133 ± 0.001
(ratio of integrated areas)		

$^a p \leq 0.05$. All values mean $\pm$ SD for nine experimental and four control animal right and left bones.

B_6-deficient chicks did not show detectable neurological symptoms, although bone and joint abnormalities were usually apparent and have been described in detail elsewhere.[32] As shown in Table 1, the lack of vitamin B_6 did not affect the plasma Ca^{++}/PO_4^{-3} homeostasis, but clearly affected amino acid metabolism, namely the B_6-dependent sulfur amino acid metabolic pathways as reported by several other investigators.[50,51,53] For instance, plasma homocysteine $(p < 0.001)$ and cystathionine $(p < 0.01)$ levels in B_6-deficient animals were significantly increased relative to controls.

Table 2 summarizes the biological and mineral properties of the tibia of B_6-deficient and cortical animals, length, periosteal

and endosteal diaphyseal diameters, area moment of inertia, diaphyseal thickness, ash weight, line width at half-maximum of the c-axis 002 reflection, phosphate: amide I ratio, and carbonate: phosphate ratio. Only the wet weights of the B_6-deficient tibiae were significantly different from the control tibiae. The lengths, diaphyseal inner and outer diameters, and cortical thickness between the two groups were not significantly different. The area moment of inertia was also not significantly different. The ashing of tibial bone diaphyseal segments confirmed that the amount of mineral was not significantly different between groups. X-ray diffraction analysis similarly revealed that the average size of the mineral crystals as measured in the bones by line broadening (β_{002}) was not affected by the vitamin B_6-deficient diet. This was further confirmed by the FTIR analyses which showed no difference in relative mineral to matrix ratio or carbonate: phosphate ratio. The spectra also showed no alterations in the shapes of the amide I or phosphate bands when control and experimental animals were compared (**Figure 1**).

Table 3 summarizes the mechanical properties determined from four-point bending of the tibiae from the vitamin B_6-deficient and control animals. The offset yield load and fracture load were significantly reduced in the B_6-deficient group, while the other properties (offset yield deflection, fracture deflection, stiffness, and elastic modulus) were not significantly different.

There were no detectable alterations in bone amino acid composition due to vitamin B_6 deficiency, expressed here as residues/ 1000 residues (**Table 4**). Based on hydroxyproline analysis, there was no difference in the total collagen contents of the bone with 52 ± 14 µg Hyp/mg in the B_6-replete animals and 45 ± 6 µg Hyp/mg in the B_6-deficient bones (**Table 5**). The proportion of Hyp solubilized from the B_6-deficient bones by EDTA, neutral
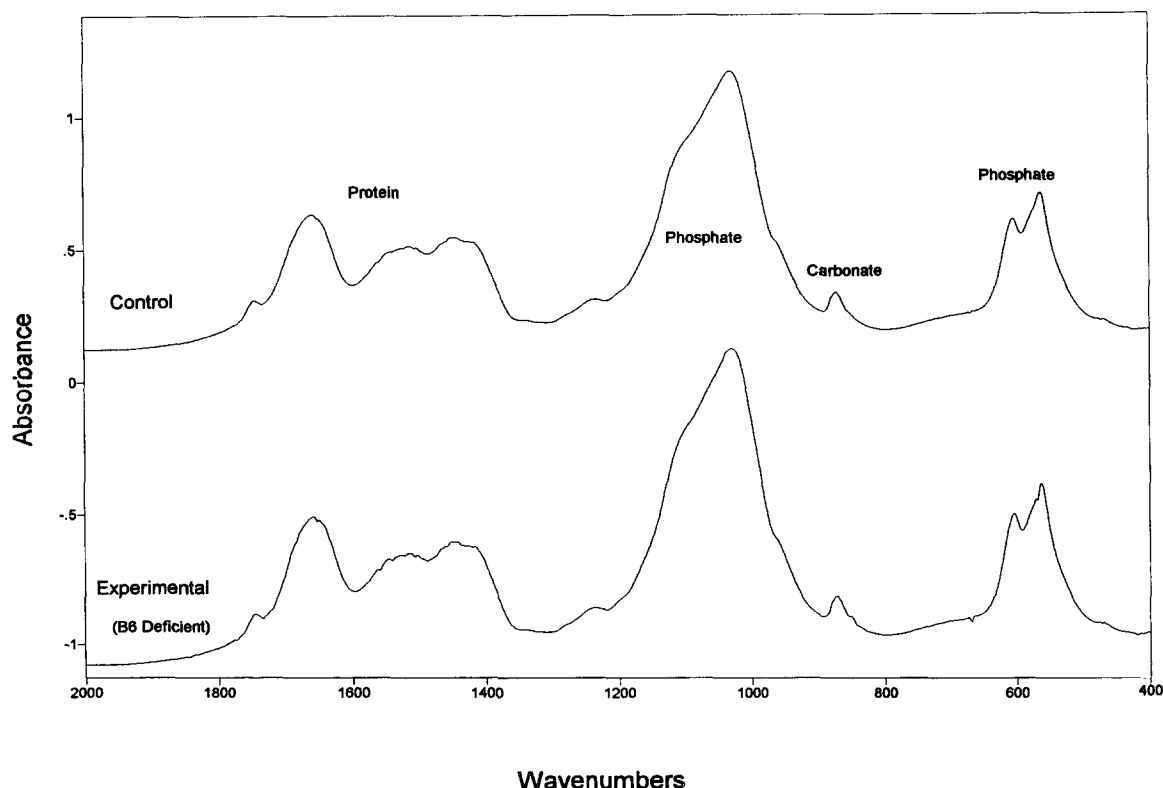

Wavenumbers

Figure 1. FTIR spectra of vitamin B_6-deficient $(-B_6)$ and control $(+B_6)$ bones. The $\nu_1\nu_3$ phosphate modes (phosphate) and the amide I modes (used to calculate the mineral to matrix ratio) are indicated.

Table 3. Biomechanical data

	B_6 deficient	B_6 replete
Offset yield load (N)	124 ± 14[a]	165 ± 9
	n = 8	n = 4
Offset yield deflection	0.97 ± 0.19	0.86 ± 0.19
	n = 8	n = 4
Fracture load (N)	151 ± 23[a]	203 ± 35
	n = 8	n = 4
Fracture deflection (mm)	2.01 ± 0.22	2.30 ± 0.50
	n = 8	n = 4
Stiffness (N/m)	0.13 ± .03	0.15 ± .03
	n = 9	n = 4
Elastic modulus (GPa)	12.05 ± 4.45	10.91 ± 3.8
	n = 9	n = 4

[a]$p \leq 0.05$. All values mean ± SD for n determinations.

Table 5. Comparative solubility of collagen (as hydroxyproline) in the vitamin B_6 deficient (B_6^-) and replete (B_6^+) chick

	B_6^-	B_6^+	p
Collagen content	52 ± 14 μg/mg	45 ± 6	NS
	n = 5	n = 4	
% Soluble	20.1 ± 2	10.1 ± 2.8	0.0014
(Hyp$_{sol}$/Hyp$_{total}$)	n = 5	n = 3	
Fractional solubility (Hyp$_{sol}$/Hyp$_{insol}$)			
EDTA	0.10 ± 0.07	0.022 ± 0.01	0.019[a]
	n = 9	n = 4	
Neutral salt	0.019 ± 0.015	0.0135 ± 0.007	NS
	n = 8	n = 3	
Acetic acid	0.092 ± 0.021	0.083 ± 0.018	NS
	n = 6	n = 4	

[a]Nonparametric test as SDs not equal.

salt, and acetic acid extractions, however, was significantly greater than that extracted from the control bones (25 ± 3% vs. 10.1 ± 3%)($p = 0.001$) (data not shown). These values are based on experiments in which each fraction was analyzed ($n = 4$ and 3, respectively). For a number of experiments, the extract Hyp concentration was too small to allow detection. Thus to portray the extraction profile, data in Table 5 are presented as a ratio of extract Hyp to insoluble Hyp rather than as a function of total Hyp, which could not be determined for all specimens.

The contents of nonreducible collagen cross-links pyridinoline (Pyr) and its lysyl analogue (D-Pyr) of control and altered B_6-deficient bones, were similar, whereas the reducible cross-link contents differed **(Table 6)**. In the B_6-deficient animal's bones, DHLNL was found to be significantly higher than in controls (by 18%). While the 20% elevation in B_6-deficient animal's bones HLNL content was not statistically significant, data showed a clear trend of elevation. Another nonreducible cross-link histidinohydroxylsysinonorleucine[62] was virtually absent from these samples. Although significant amounts of free aldehydes of lysine and hydrosylysine were also found in both B_6 deficient and control bones, they were not quantified due to the complexity of chromatographs around their elution position. The

Table 4. Amino acid content of vitamin B_6 deficient ($-B_6$) and vitamin B_6 replete ($+B_6$) chick tibia (residues per 1000)

Amino acid	($+B_6$) n = 4	($-B_6$) n = 4
Asp + Hyp[a]	743 ± 80	807 ± 102
Thr	255 ± 38	245 ± 13
Ser	338 ± 36	329 ± 42
Glu	796 ± 69	804 ± 24
Pro	1144 ± 59	1210 ± 114
Ala	1152 ± 57	1276 ± 96
Gly	2980 ± 141	3139 ± 92
Val	229 ± 133	202 ± 126
Met	60 ± 15	51 ± 5
Ile	170 ± 14	179 ± 16
Leu	399 ± 29	415 ± 30
Tyr	89 ± 6	44 ± 45
Phe	214 ± 14	233 ± 21
His	95 ± 12	103 ± 13
Lys	345 ± 19	372 ± 17
Arg	494 ± 80	469 ± 52
Cysta[b]	71 ± 8	332 ± 314

[a]Hydroxyproline.
[b]Cystathionine.

ratio of reducible cross-links, DHLNL/HLNL, and the ratio of mature (nonreducible) cross-links, Pyr/D-Pyr were increased but not significantly, indicating hydroxylysine content was not significantly increased.

Discussion

This study demonstrates that the vitamin B_6-deficient fast-growing male chick may provide an animal model in which the effect of a matrix abnormality on bone mechanical properties can be separated from those of mineral defects. The vitamin B_6-deficient chicks were found to show no changes in plasma ion markers of mineral homeostasis (calcium and phosphate concentrations). The mineral content and other characteristics of B_6-deficient tibia as measured by X-ray diffraction and FTIR were unaltered. Noncollagenous proteins were not studied because they are not considered to be major components contributing to mechanical properties. The only changes detected were the increased collagen solubility and the altered collagen cross-links. These, along with the previously reported trabecular histomorphometric alterations and cortical thinning[32] confirm the validity of the model.

The lack of a significant difference in elastic modulus between the B_6-deficient and control tibias is consistent with the lack of a significant difference in mineral content and composition. The decreased offset yield and fracture loads in the B_6-deficient tibia may be due to the alterations in the collagen cross-links, as discussed below. Additionally, though not significant, the trend toward smaller endosteal and periosteal diameters, and decreased area moment of inertia in the B_6-deficient tibias may have also contributed to these decreased mechanical properties.

The mineral component in the B_6-deficient cortical bone was not significantly different from that in the control chick cortical bone in terms of amount, composition, and crystal properties. Two independent methods were used to measure the proportion of mineral in the ground bone, ash weight determination and measurement of the ratios of the integrated areas of mineral and matrix components in the FTIR spectra. Similarly, based on X-ray diffraction measurement of the line broadening of the c-axis reflection which is inversely proportional to the crystallite size and perfection in the needle (c axis) direction, the crystals were of comparable size/perfection. The shape of the phosphate bands in the infrared spectra were identical, confirming the X-ray results, and establishing that there were no differences in the relative amounts of acid phosphate, nonstoichiometric apatites,

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Table 6. Collagen cross-links (mol/mol collagen)

	DHLNL[a]	HLNL[b]	DHLNL/HLNL	Pyr[c]	D-Pyr[d]	Pyr/D-Pyr
−B$_6$	1.03 ± 0.07[e]	0.509 ± 0.033	2.04 ± 0.22	0.055 ± 0.017	0.028 ± 0.006	1.93 ± 0.45
+B$_6$	0.84 ± 0.13	0.428 ± 0.029	1.86 ± 0.25	0.049 ± 0.011	0.028 ± 0.006	1.68 ± 0.39

[a]Dehydro-dihydroxylysinonorleucine.
[b]Dehydro-hydroxylysinonorleucine.
[c]Pyridinoline.
[d]Deoxypyridinoline.
[e]$p \leq 0.001$.

etc. that contribute underlying components to the phosphate absorption band. Additionally, the carbonate to phosphate ratio calculated from the infrared spectra was unaltered, again confirming the similarity of the mineral in the powdered bone specimens.

Collagen intermolecular cross-linking is essential for the mechanical properties of the collagen fibril matrix such as stability and tensile strength. Vitamin B$_6$ deficiency has been associated with altered cross-link formation in both collagen and elastin.[18,39] Fujii et al.[18] using severely B$_6$-deficient rats reported, as we do, an increase in the amount of soluble collagen in the bones of B$_6$-deficient animals as contrasted with control. They found twice as much collagen in the neutral salt extract, and 1.25 times as much in the acetic acid extract. They did not measure the collagen extracted during EDTA decalcification. However, they found that the proportions of DHLNL and HLNL to Hyp were reduced in the insoluble fraction of the B$_6$-deficient rat bone. In contrast, the moderately B$_6$-deficient chick had increased proportions of these reducible cross-links. It should be noted here that chicken bone is known to contain high concentrations of reducible cross-links and very low concentrations of Pyr D-Pyr.[15,16] It is also well documented that an increase in reducible cross-links occurs in metabolically active tissues, such as granulation tissues,[2] osteogenic osteoblastoma,[3] and acute disuse osteoporosis.[64] A similar increase in metabolic activity may well be taking place in the B$_6$-deficient chick bones, as suggested by the histomorphometric data,[32] and the observation that while total cross-link content was unchanged, the B$_6$-deficient bones had increased reducible (i.e., newly formed) cross-links.

Vitamin B$_6$ is essential for most amino acid synthesis and turnover, thus its deficiency may have general systemic effects as implied by the plasma amino acid composition in the vitamin B$_6$-deficient animals. No significant alterations in bone amino acid composition were detected in this study. However, significant changes in plasma amino acids related to metabolic sulfur amino acid interconversions were observed. The elevation of those amino acids may have contributed to bone matrix abnormalities. Several previous studies also showed that plasma homocysteine concentrations are elevated in vitamin B$_6$-deficient animals.[37,45,50,51] Excess quantities of homocysteine and cystathionine accumulate because of the requirements of cystathionine synthase (E.C.4.2.1.22) and cystathionase (E.C.4.4.1.1) for vitamin B$_6$.[50,53] The collagen defect in homocystinuria is attributed to blocking of the aldehyde groups by homocysteine thereby blocking cross-link formation.[24,45] In contrast to the aldehyde-deficient lathyritic collagen, the penicillamine containing collagen (and probably, by analogy, homocystinuric collagen) has an aldehyde content greater than normal.[12,13]

Our previous chick studies repeatedly showed that vitamin B$_6$-deficient collagen differed from normal. The total aldehyde content (measured spectrophotometrically based on benzothiazolone hydrazone[41]) in anterior cruciate ligament from B$_6$-deficient

chicks was found to be significantly higher as compared to controls (unpublished data). Collagen fibers in articular cartilage (predominantly Type I in the chick according to Eyre et al.[16]) appeared to be swollen when examined by electron microscopy.[33] This fibrocartilage contained a more soluble collagen as revealed by the higher proportion of neutral salt-soluble collagen.[32] Higher proportions of neutral salt soluble collagen are similarly found in ovo when chicks are injected with a vitamin B$_6$ antimetabolite or the lathrogen BAPN.[5] Increased tissue hydration and collagen solubility are also hallmarks of BAPN-induced lathyrism. BAPN- and penicillamine-treatment result in the production of more soluble collagen as reflected in the increase in neutral salt soluble collagen fractions from chick embryo and rat skin.[29,30] The present study on bone, in contrast to a previous study on cartilage,[32] did not show a significantly higher proportion of salt soluble collagen. However, the total collagen in the B$_6$-deficient bones was more soluble and the EDTA-solubilized collagen fraction greater than in normal bones. Similarly, Fujii et al.[18] reported that more collagen in vitamin B$_6$-deficient rat bone and skin could be solubilized in neutral salt and acid solvents as compared to controls. In Deshmukh et al.'s study,[12] significantly larger amounts of all soluble collagen fractions were present in the bone of this penicillamine-treated rats. A high hydroxylysine content of the acid soluble collagen was also a feature of this penicillamine treatment. Moreover, the incorporation of 4,5 ^{3}H-L-lysine into aldehyde derivatives present in acid soluble collagen following reduction with NaBH$_4$ and base hydrolysis revealed a higher amount (cpm/mg collagen) of dihydroxynorleucine (reduced form of hydroxylysyl aldehyde) in treated rats than in normal animals. Assuming that lysyl oxidase activity is not increased, this aldehyde precursor of DHLNL can only be produced in higher amounts if more substrate (namely hydroxylysyl residues) is present; an event that is unlikely to occur in vitamin B$_6$ deficiency. The hydroxylysine content of bone was not measured in the present study. However, the ratio of reducible cross-links, DHLNL/HLNL, and the ratio of mature cross-links, Pyr/D-Dpyr, indicated a trend (in the same direction in both cases) but no significant differences, indicating that hydroxylation of lysine residues involved in cross-linking was not significantly changed.

There are several alternative mechanisms which might account for the matrix defects and the lowered bone strength, although they are less likely. Pyridoxal phosphate is a substrate for alkaline phosphatase,[17,23] an enzyme key in mediating bone formation.[17] In the presence of decreased pyridoxal phosphate, local phosphate concentrations might have been reduced, and as a consequence, the amount of mineral might have been diminished. However, the absence of mineral abnormalities in the present study suggests that the decreased availability of one of many such alkaline phosphatase substrates had little effect. The earlier biochemical study[32] did however show a significantly reduced activity of the bone alkaline phosphate isoenzyme in plasma.

Since a single substrate deficiency is not apt to affect the enzyme activity, the reported reduction in alkaline phosphatase activity may be indicative of a reduction in osteoblastic activity or in protein synthesis. In general, protein synthesis does not seem to be affected by vitamin B$_6$ deficiency, particularly in the case of a moderately deficient diet with normal growth rate as in this study. Since the total protein content was not measured in the present or earlier studies, we cannot be sure which one of the two, synthetic activity or number of osteoblasts, was reduced.

One possible cause for altered matrix synthesis and turnover might be the destabilization of DNA associated with a decreased concentration of polyamines. Ornithine decarboxylase, the rate limiting enzyme in the synthesis of polyamines, has an absolute requirement for PLP.[14] The polyamines stabilize DNA, and thus could affect cell and calcium, and phosphate metabolism, as well as matrix synthesis. Alterations in polyamine production does not seem to be important in the vitamin B$_6$-deficient model studied here, since neither plasma ornithine values nor serum calcium and phosphate levels were significantly affected.

The other possible mechanism, which we cannot totally exclude, is altered matrix turnover. The previous study on histomorphometry of B$_6$-deficient trabecular bone revealed a significantly lower osteoid tissue surface and volume and a greater eroded surface compared to controls.[32] The first morphometric change suggests that there is less total trabecular bone matrix in general. Similar data are not available for cortical bone. However, the unmodified mineral and collagen (Hyp) content of the cortical bone in the current study indicates that if there is less bone, what there is, mineralized normally. The second major morphometric change in the trabecular bone suggests that bone collagen turnover is increased with degradation exceeding synthesis. A high turnover rate in trabecular bone would preclude formation of nonreducible (mature) cross-links in a collagenous tissue, since a short residence period of reducible cross-links does not favor their formation. The end result would be the net increase in DHLNL and HLNL, as is seen in the immobilized monkey experimental model of osteoporosis.[64] In agreement with this model, no significant changes were observed in the contents of mature cross-links in the cortical bone analyzed. Only with a rapid synthesis of new collagen can there be an increase in the reducible cross-links. The higher solubility of collagen found previously in cartilage[32] and currently in cortical bone is consistent with a higher amount of newly synthesized collagen. Similarly, periodontal ligament, known as an exceptionally high turnover collagenous tissue, has an abundant amount of reducible cross-links and few if any mature cross-links.[61]

Thus B$_6$ deficiency in the chick, either because of direct effects on collagen cross-link formation, or indirect systemic effects on osteoblast metabolism, results in bone in which the architecture is slightly albeit significantly modified, and collagen is more soluble. The vitamin B$_6$-deficient chick bones have reduced fracture strength, in the absence of impaired mineralization. An osteoporotic-like bone disorder induced by severe vitamin B$_6$ deficiency was described roentgenographically by Benke et al.[4] in rats. Abnormalities in fracture healing in these animals were also reported.[14] It is of interest to note that hip fracture patients have been reported to be vitamin B$_6$ deficient[44] suggesting that B$_6$ deficiency may be a risk factor for osteoporotic fractures.

Conclusion

In summary, vitamin B$_6$ deficiency affects the structural integrity of collagen and consequently modifies the fracture strength of bone in the absence of alterations to the mineral. The present study underscores the contribution of the collagen matrix to the mechanical properties of bone.

References

1. ASTM D790M-86. Standard Test Methods for Flexural Properties of Unreinforced and Reinforced Plastics and Insulating Materials (Metric). In: Annual Book of ASTM Standards. Vol. 8.01. Philadelphia, PA: ASTM; 1990; 284–292.

2. Bailey, A. J. Changes in the nature of the collagen during development and resorption of granulation tissue. Biochem Biophys Acta 328:383–390; 1973.

3. Banes, A. J., Bernstein, P. H., Smith, R. E., and Mechanic, G. L. Collagen biochemistry of osteoporotic bone: Quantitative changes in bone collagen cross-links in virus-induced avian osteopetrosis. Biochem Biophys Res Commun 81:1390–1397; 1978.

4. Benke, P. J., Fleshood, H. L., and Pitot, H. C. Osteoporotic bone disease in the pyridoxine-deficient rat. Biochem Med 6:526–535; 1972.

5. Bird, T. A. and Levene, C. I. The effect of a vitamin B$_6$-antagonists, 4-deoxypyridoxine, on the cross-linking of collagen in the developing chick embryo. Biochem J 21:633–638; 1983.

6. Boskey, A. L., Rimnac, C., Federman, M., Bansal, M., Lian, J., and Boyan, B. D. Effect of Short-term Hypomagnesemia on the Chemistry, Physical and Mechanical Properties of Rat. J Orthopaed Res 10:774–783; 1992.

7. Burstein, A. H., Zika, J. M., Heiple, K. G., and Klein, L. Contribution of collagen and mineral to the elastic-plastic properties of bone. J Bone Jt Surg 57A:956–961; 1975.

8. Carrington, M. J., Bird, T. A., and Levene, C. I. The inhibition of lysyl oxidase in vivo by isoniazid and its reversal by pyridoxal. Effect on collagen cross-linking in the chick embryo. Biochem J 221:834–837; 1984.

9. Coburn, S. P. and Mahuren, J. D. A versatile cation-exchange procedure for measuring the seven major forms of vitamin B$_6$ in biological samples. Anal Biochem 126:310–317; 1983.

10. Crenshaw, T. D. Reliability of dietary calcium and phosphorus levels and bone mineral content as predictors of bone mechanical properties at various time periods in growing swine. J Nutr 116:2155–2170; 1986.

11. Currey, J. D. Effects of Differences in Mineralization on the Mechanical Properties of Bone. Philos Trans R Soc London B 304:509–518; 1984.

12. Deshmukh, K., Just, M., and Nimni, M. E. A defect in the intramolecular and intermolecular cross-linking of collagen caused by penicillamine. 3. Accumulation of acid soluble with a high hydroxylysine content in bone. Clin Orthop 91:186–196; 1973.

13. Deshmukh, K. and Nimni, M. A defect in the intramolecular and intermolecular cross-linking of collagen caused by penicillamine. II. Functional groups involved in the interaction process. J Biol Chem 244:1787–1795; 1969.

14. Dodds, R. A., Catterall, A., Bitensky, L., and Chayen, J. Abnormalities in fracture healing induced by vitamin B$_6$-deficiency in rats. Bone 7:489–495; 1986.

15. Eyre, D. R., Koob, T. J., and van Ness, K. P. Quantitation of Hydroxypyridinium Crosslinks in collagen by High-Performance Liquid Chromatography. Anal Biochem 137:380–388; 1984.

16. Eyre, D. R., Brickley-Parsons, D. M., and Glimcher, M. J. Predominance of Type I collagen at the surface of avian articular cartilage. FEBS Lett 85:259–263; 1978.

17. Fedde, K. N., Lane, C. C., and Whyte, M. P. Alkaline phosphatase is an ecto-cyznyme that acts on micromolar concentrations of natural substrates at physi-

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ologic pH in human osteosarcoma (SAOS-2) cells. Arch Biochem Biophys 264:400–409; 1988.

18. Fujii, K., Kajiwara, T., and Kurosi, H. Effect of vitamin B₆-deficiency on the cross-link formation of collagen. FEBS Lett 97:193–195; 1979.

19. Gerstenfeld, L. C., Riva, A., Hodgens, K., Eyre, D. R., and Landis, W. J. Post-translational control of collagen fibrillogenesis in mineralizing cultures of chick osteoblasts. J Bone Miner Res 9:1031–1043; 1993.

20. Glimcher, M. J. Studies on the structure, organization and reactivity of bone collagen. In: Gibson, T. Ed. Proceedings of the International Symposium on Wound Healing. Montreux: International Cooperation in Medical Sciences; 1975; 253–70.

21. Hutterer, F. and Singer, E. G. A modified method for hydroxyproline determination. Anal Chem 32:556–558; 1970.

22. Iguchi, H., Kasai, R., Okumura, H., Yamamuro, T., and Kagan, H. M. Effect of dietary cadmium and/or copper on the bone lysyl oxidase in copper-deficient rats relative to the metabolism of copper in the bone. Bone Miner 10:51–59; 1990.

23. Irino, T., Matsushita, M., Sakagishi, Y., and Komoda, T. Phosphorylcholine as a unique substrate for human intestinal alkaline phosphatase. Int J Biochem 26:273–277; 1994.

24. Jackson, S. H. The reaction of homocysteine with aldehyde: an explanation of the collagen defects in homocystinuria. Clin Chim Acta 45:215–217; 1973.

25. Jensen, K. S. and Mosekilde, L. A model of vertebral trabecular bone architecture and its mechanical properties. Bone 11:417–423; 1990.

26. Klug, H. P. and Alexander, L. E. In: X-Ray Diffraction Procedure for Polycrystalline and Amorphous Materials. New York: Wiley; 1974; 687–692.

27. Lees, S., Eyre, D. R., and Barnard, S. M. BAPN dose dependence of mature cross-linking in bone matrix collagen of rabbit compact bone: Corresponding variation of sonic velocity and equatorial diffraction spacing. Connect Tissue Res 24:95–105; 1990.

28. Lees, S., Hanson, D., Page, E., and Mook, H. A. Comparison of dosage-dependent effects of β-aminopropionitrile, sodium fluoride, and hydrocortisone on selected physical properties of cortical bone. J Bone Miner Res 9:1377–1389; 1994.

29. Levene, C. I. and Gross, J. Alterations in state of molecular aggregation of collagen induced in chick embryos by β-aminopropionitrile (Lathyrus factor). J Exp Med 110:771–790; 1959.

30. Levene, C. I., Sharman, D. F., and Callingham, B. A. Inhibition of chick embryo lysyl oxidase by various lathyrogens and the antagonistic effect of pyridoxal. Int J Exp Pathol 73:613–624; 1992.

31. Martin, R. B. and Ishida, J. The relative effects of collagen fiber orientation, porosity, density, and mineralization on bone strength. J. Biomechan 22:419–426; 1989.

32. Massé, P. G., Boskey, A. L., Pritzker, K. P. H., Mendes, M., and Weiser, H. Vitamin B₆ deficiency experimentally-induced bone and joint disorder: microscopic, radiographic and biochemical evidence. Br J Nutr 71:919–932; 1994.

33. Massé, P. G., Colombo, V. E., Gerber, F., Howell, D. S., and Weiser, H. Morphological abnormalities in vitamin B₆ deficient tarsometatarsal chick cartilage. Scanning Microsc 4:667–674; 1990.

34. Massé, P. G., Vuilleumier, J. P., and Weiser, H. Is pyridoxine essential for bone metabolism? Int J Vit Nutr Res 58:295–299; 1988.

35. Massé, P. G., Yamauchi, M., Mahuren, J. D., Coburn, S. P., and Howell, D. S. Connective tissue integrity is lost in vitamin B₆-deficient chicks. J Nutr 125:26–34; 1995.

36. Miller, E. J. and Rhodes, R. K. Preparation and Characterization of the Different Types of Collagen. Methods Enzymol 82:33–64; 1982.

37. Miller, J. W., Ribaya-Mercado, J. D., Russell, R. M., Shepard, D. C., Morrow, F. D., Cochary, E. F., Sdowski, J. A., Gershoo, S. N., and Selhub, J. Effect of vitamin B₆ deficiency on fasting plasma homocysteine concentrations. Am J Clin Nutr 55:1154–60; 1992.

38. Murray, J. C., Fraser, P., and Levine, C. I. The effect of pyridoxine deficiency on lysyl oxidase activity in the chick. Exp Mol Pathol 28:301–308; 1978.

39. Myers, B. A., Dubick, M. A., Reynolds, R. D., and Rucker, R. B. Effect of vitamin B₆ (pyridoxine) deficiency on lung elastin cross-linking in perinatal and weanling rat pups. Biochem J 229:153–160; 1985.

40. Opsahl, W., Zeronian, H., Ellison, M., Lewis, D., Rucker, R. B., and Riggins, R. S. Role of copper in collagen cross-linking and its influence on selected mechanical properties of chick bone and tendon. J Nutr 112:708–716; 1982.

41. Paz, M. A., Blumenfeld, O. O., Rojkind, M., Hansen, E., Furfine, C., and Gallop, P. M. Determination of carbonyl compounds with N-methyl benzothiazolone hydrazone. Arch Biochem Biophys 109:548–559; 1965.

42. Reddi, A. H. and Sullivan, N. E. Inhibition of mineralization by experimental lathyrism during matrix-induced endochondral bone differentiation. Proc Soc Exp Biol Med 162:445–48; 1979.

43. Reilly, D. T. and Burstein, A. H. The Mechanical Properties of Cortical Bone. JBJS 56A:1001–1022; 1974.

44. Reynolds, T. M., Marshall, P. D., and Brain, A. M. Hip fracture patients may be vitamin B₆-deficient. Controlled study of serum pyridoxal-5'-phosphate. Acta Orthop Scand 63:635–638; 1992.

45. Rucker, R. B., Myers, B. A., Reynolds, R. D., and Tinker, D. Role of vitamin B₆ of rats in the cross-linking of elastin. In: Reynolds, R. D. and Leblem, J. E. Eds. Vitamin B₆-Its role in Health and Disease. New York: Liss; 1985; 441–446.

46. Sandhu, H. S. and Jande, S. S. Effects of beta-aminoproprionitrile on formation and mineralization of rat bone matrix. Calcif Tissue Int 32:80–85; 1982.

47. Sauberlich, H. E. Vitamin B₆ nutrition. In: Leklem, J. E. and Reynolds, R. D. Eds. Analysis and Status Assessment. New York; Plenum; 1982.

48. Shah, K. M., Goh, C. H., Karunanithy, R., Low, L. S., Das De, S., and Bose, K. Effect of Decalcification on Bone Mineral Content and Bending Strength of Feline Femur. Calcif Tissue Int 56:78–82; 1995.

49. Siegel, R. C. Lysyloxidase. Int Rev Connect Tissue Res 9:73; 1981.

50. Smolin, L. A. and Benevenga, J. Accumulation of homocysteine in vitamin B₆ deficiency: A model for the study of cystathionine β-synthase deficiency. J Nutr 112:1264–1272; 1982.

51. Smolin, L. A. and Benevenga, N. J. Factors affecting the accumulation of homocysteine in rats deficient in vitamin B₆. J Nutr 114:103–111; 1984.

52. Spengler, D. M., Baylink, D. J., and Rosenquist, J. B. Effect of beta-aminopropionitrile on bone mechanical properties. J Bone Joint Surg 59A:670–72; 1977.

53. Sturman, J. A., Cohen, P. A., and Gaull, G. E. Effects of deficiency of vitamin B₆ on transsulfuration. Biochem Med 3:244–251; 1969.

54. Verhaeghe, J., Suiker, A. M. H., Einhorn, T. A., Geusens, P., Visser, W. J., Van Herck, E., Van Bree, R., Magitsky, S., and Bouillon, R. Brittle bones in spontaneously diabetic female rats cannot be predicted by bone mineral measurements: studies in diabetic and ovariectomized rats. J Bone Miner Res 9:1657–1667; 1994.

55. Wilmarth, K. R. and Froines, J. R. In vitro and in vivo inhibition of lysyl oxidase by aminopropionitriles. J Toxicol Environ Health 37:411–423; 1992.

56. Yamauchi, M., Chandler, G. S., and Katz, E. P. In: Collagen Cross-linking and Mineralization. In: Slavkin, H. and Price, P., Ed. Chemistry and Biology of Mineralized Tissues. New York: Elsevier Science; 1992; 39–46.

57. Yamauchi, M., Katz, E. P., Ostsubo, K., Teraoka, K., and Mechanic, G. L. Cross-linking and stereospecific structure of collagen in mineralized and non-mineralized skeletal tissues. Connect Tissue Res 21:159–67; 1989.

58. Yamauchi, M. and Katz, E. P. The post-translational chemistry and molecular packing of mineralizing tendon collagens. Connect Tissue Res 29:81–98; 1993.

59. Yamauchi, M. and Mechanic, G. L. Cross-linking of collagen. In: Nimni, M. E. Ed. Collagen: Biochemistry. Vol. 1. Boca Raton, FL: CRC; 1988.

60. Yamauchi, M., Katz, E. P., and Mechanic, G. L. Intermolecular cross-linking and stereospecific molecular packing in Type 1 collagen fibrils of the periodontal ligament. Biochemistry 25:4907–4913, 4930; 1986.

61. Yamauchi, M., Katz, E. P., and Mechanic, G. L. Intermolecular cross-linking and stereospecific molecular packing. Biochem 26:4907–4913; 1986.

62. Yamauchi, M., London, E. R., Guenat, C., Hashimoto, F., and Mechanic, G. L. Structure and formation of a stable histidine-based trifunctional. J Biol Chem 262:11428–11434; 1987.

63. Yamauchi, M., Young, R. R., Chandler, G. S., and Mechanic, G. L. Cross-linking and new bone collagen synthesis in immobilized and recovering primate osteoporosis. Bone 9:415–418; 1988.