AGE, NUTRITION, AND BONE METABOLISM: ANALYSES OF EFFECTS USING A SHORT-TERM *IN VIVO* BONE MODEL

by

Rashmi Sinha

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Name of Candidate: Rashmi Sinha
Doctor of Philosophy, 1987

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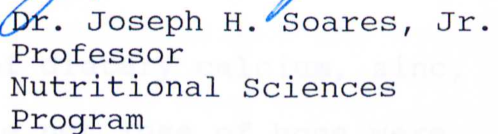

Dr. Joseph H. Soares, Jr.
Professor
Nutritional Sciences
Program

Date Approved: 1/20/87

ABSTRACT

Title of Dissertation: Age, nutrition, and bone metabolism: Analyses of effects using a short-term _in vivo_ bone model

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Dissertation directed by: Dr. J.H. Soares, Jr.
Professor and Chairman of the Graduate Program in Nutritional Sciences, University of Maryland

The preventative effects of dietary calcium, zinc, and vitamin D metabolites on the net loss of bone were assessed using rats of different ages. Biochemical changes were monitored in femurs, humeri, mandibles, scapulas, and tibias. In addition femora were sectioned into epiphyses-metaphyses and diaphyses to evaluate nutritional influences on the trabecular and cortical type bone. Since measurable bone degradation due to aging and nutritional status requires long period of time, a short-term _in vivo_ system was developed to simultaneously examine bone formation and resorption. The system consisted of subcutaneous implantation of demineralized (DB) and mineralized (MB) bone powders. There was evidence of bone formation and resorption in the DB and MB implants respectively, as assessed by marker enzyme (formation-alkaline phosphatase; resorption-acid phospha-

tase) activities, mineral concentrations, radioisotope incorporation, and histological studies.

The results indicated that several different bone samples are required to adequately predict changes occurring in the skeletal system. The epiphyses-metaphyses of long bone is a useful sample site examining changes occurring in trabecular bone while the diaphysis can assess cortical bone status. There was decreased bone formation and resorption as assessed by alkaline and acid phosphatase activity in the MB and DB implants in the 24 month-old rats as compared to 2 month-old rats. Dietary calcium and zinc levels did not affect the overall status of the bones and implants in the aged rats. Conversely, in 2 month-old rats dietary calcium at 1.0% stimulated bone formation in the DB implant, whereas 0.2% calcium increased bone resorption in the MB implants. Furthermore, 75 ng dietary 1,25-dihydroxycholecalciferol (1,25$(OH)_2D$) per day increased resorption in MB implant and inhibited mineralization of DB implants in the young rats. Dietary zinc at 300 ppm reduced bone calcification in 2 month-old. The results of these studies indicated that neither high levels of dietary calcium, nor zinc, act as prophylaxes to counteract bone loss due to aging. The dietary use of 1,25$(OH)_2D$ in old animals needs to be investigated further, since results in young animals are contradictory with reports in older rats.

To my parents
and extended family
without whose love and continued support
none of this would be possible.

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INTRODUCTION

Osteoporosis is a condition of the skeletal system characterized by a decrease in bone mass per unit volume [1]. It is a common ailment in the elderly throughout the world. The condition results in fragile bones that are susceptible to fracture under normal loads. Inadequacies in the diet of such nutrients as calcium, vitamin D, or zinc may be causative agents in the bone loss leading to osteoporosis.

A number of factors influence and interact with the mechanisms by which these essential nutrients affect bone integrity. In general, the intake of calcium by the human population is lower than the Recommended Dietary Allowance [2]. Moreover, a substantial number of patients with osteoporosis do not absorb calcium efficiently [3,4], resulting in a negative calcium balance that is compensated by increased bone decalcification. The regulation of calcium homeostasis is also affected by the hormonal form of vitamin D, $1,25(OH)_2D$ [5]. A decrease in plasma $1,25(OH)_2D$ with age further exacerbates calcium loss from the bone [6]. Finally, zinc has been shown to affect bone formation and repair [7-9], but despite the available data there is little agreement concerning its role in bone calcification.

Nutritional supplementation of calcium, vitamin D metabolites, and zinc may be useful in preventing and treating osteoporosis in the aged population. However,

measurable changes in bone may take years or decades to manifest in humans and many months in laboratory animals. Furthermore, bone undergoes formation as well as resorption and the increase and decrease in one of these processes can influence the overall status of the skeletal system [10]. To overcome these and other problems encountered in bone research, a short-term *in vivo* system which is capable of detecting bone formation and resorption was described and used to examine changes occurring in bone due to age and nutrition.

BACKGROUND

The skeletal system serves as a reservoir for ions, particularly calcium, thereby playing an important role in the control of the body's mineral homeostasis. An intricate and well-coordinated sequence of events within the tissue constantly removes and deposits minerals. An increase in resorption and/or decrease in formation of bone can lead to an overall loss of the skeletal system.

The reasons why bone loss increases with age are not fully understood. A strong relationship between nutritional factors and bone integrity has long been recognized. In particular vitamin D and its metabolites, calcium, and zinc play important roles in bone homeostasis. However, at present the role of these nutrients in the aging of bone remains to be precisely determined.

Physiology, Morphology, and Biochemistry of the Skeleton

Bones can be classified according to their function (weight bearing, articulating, protecting), shape (long, short, flat), or mechanism of formation (endochondral ossification or intramembranous formation) [11]. There is a direct relationship between a bone's shape and its function. For example, the long or weight-bearing bones provide the maximal axial and torque loading properties required for weight bearing and locomotion. The long bones are formed by endochondral ossification and are composed mainly of cortical or compact structures. On the other hand, flat bones of the skull, pelvis, sternum and

scapulae, provide shielding surfaces for the protection of vital organs. The flat bones are formed by intramembranous ossification and contain large quantities of trabecular or cancellous structures.

In a human skeleton approximately 80% of bone mass is cortical and 20% trabecular bone [10]. The amount of cortical and trabecular bone varies between segments of long bones such as the femur, humerus, and tibia. A long bone consists of three main regions: the epiphysis, metaphysis, and diaphysis. The epiphyses are two roughly spherical ends, composed mainly of trabecular bone with a thin shell of cortical bone. In growing animals the epiphysis is separated from the metaphysis by a thick plate of hyaline cartilage known as the growth plate where bone elongates. The metaphysis is primarily composed of trabecular bone with a cortical bone shell. The diaphysis is the central cylindrical shaft composed mainly of cortical bone [10].

All bones, whether cortical or trabecular, have a layered or lamellated microscopic structure [10]. Spaced throughout the lamellar cortical and trabecular bone are small cavities, or lacunae, connected by canals called canaliculi. Osteocytes or entrapped bone cells and their long cytoplasmic processes occupy the lacunae and canaliculi. The main structural unit of cortical bone is an osteon [10] which consists of a central or Haversian canal surrounded by concentric lamellae. Each canal

contains blood vessels, nerves and connective tissue. Trabecular packets are structures in cancellous bone which are functionally similar to osteons of the compact bone. The trabecular packets contain a network of trabeculae with inter-trabecular spaces containing bone marrow [10]. Most trabeculae are less than 0.1mm thick and the osteocytes are nourished by diffusion.

Bone cell biology. Four cell types can usually be recognized in the cortical and trabecular structures of growing and adult skeleton [10]. These are osteoblasts, osteocytes, osteoclasts, and undifferentiated cells.

The **osteoblast** is a mononucleated cell, with a diameter of approximately 15-30 microns [10-13]. This cell contains an extensive network of granular endoplasmic reticulum. The golgi system is well developed and eccentric to the nucleus and produces vesicles which contain amorphous material. Collagen is also synthesized in the osteoblasts and a layer of demineralized Type I collagen is found between the external surface of the cell membrane and the mineralized bone matrix.

In the active osteoblast, various enzymes are localized in the golgi, lysosomes and at the outer surface of the cell membrane [12]. The cell membrane of the osteoblast shows a positive staining reaction for alkaline phosphatase which is a marker enzyme for bone formation [10,12,13].

The osteoblast arises from mesenchyme (mesenchymal cells) in the embryo. In the adult, the osteoblasts

differentiate from marrow stroma cells which may be derived from mesenchymal cells, fibroblasts, reticular cells, preosteoblasts, and/or cells of blood vessel walls [10]. However, the identity and potential of each cell type to transform into osteoblast is not clear.

The **osteocyte** is the principal cell of fully formed bones and its size can range from 20 to 60 microns [10-12]. The osteocyte is an osteoblast that has secreted bone around itself. A young osteocyte is generally located very near the bone surface. Although derived from the osteoblast population, the osteocyte has several different histochemical characteristics [12]. There is a loss of alkaline phosphatase activity along the cell membrane. There is also a gradual loss of reactivity in the golgi as well as the lysosomes. Osteocytes do not divide and eventually they disintegrate and leave empty spaces (lacunae) in the bone matrix.

Osteocytes have many slender cell processes that can extend for considerable distances in canaliculi. These cell processes are in contact with the processes of other osteocytes and bone surface cells. Nutrients can pass into and waste products out of the osteocytes through the canaliculi, and these cells are thought to be responsible for the metabolism of the bone.

Osteoclasts are multinucleated (2 to 50 nuclei) giant cells which are responsible for the resorption of mineralized bone and cartilage [14-18]. These cells range from

20 to over 100 microns in diameter. The nuclei are surrounded by a golgi network. The cytoplasm contains large numbers of mitochondria. The surface of the osteoclast adjacent to the bone has extensive membrane infoldings termed the "ruffled border."

The ruffled border is an unique surface modification of the osteoclast that facilitates bone resorption [10-13]. The plasma membrane of the ruffled border appears to be coated with small bristle-like structures and may contribute to the transport of materials across the membrane. The ruffled border is highly mobile and bone can be seen dissolving beneath it. A clearer zone is present around the ruffled border and seems to be a site of adhesion of the cell to the bone surface. Osteoclasts that lack ruffled borders are not capable of resorbing bone.

The large multinucleated osteoclasts have lysosomal enzyme activity in the golgi, lysosomes, and ruffled border. Lysosomal enzymes are found between the bone matrix and the ruffle borders of these cells. Osteoclasts have a high acid phosphatase activity [10,12,13] and respond rapidly to calcium-regulating hormones such as parathyroid hormone (PTH) by developing or enlarging existing ruffled borders. In the presence of calcitonin (CT) the ruffled borders rapidly disappear.

The osteoclast is believed to be derived from cells of the monocyte-phagocyte line [10,11,16-18]. In fact, macrophages have been shown to behave like osteoclasts *in*

vitro, attaching to bone and causing the hormone-stimulated release of mineral and collagen.

The **undifferentiated mesenchymal cells** are usually found adjacent to cells lining bone surfaces [10,12]. These mononucleated spindle-shaped cells morphologically resemble fibroblasts. They have some of the characteristics of osteoblasts but contain very little organized granular endoplasmic reticulum. The golgi size and number of mitochondria are both less than that of mature osteoblasts. Although some undifferentiated cells give rise to osteoclasts, there are numerically many more cells which give rise to osteoblasts. Enzymes are not very prominent in the golgi system nor in the lysosomes in the undifferentiated cell and the cell membrane reaction for alkaline phosphatase is also negligible.

A foundation of cartilage is necessary for the process of ossification. The primary cells involved in cartilage formation are the **chondroblast** and the **chondrocyte** [10,12,19]. The difference between these two cells is not definitive and is based on the maturity of the cell.

The epiphysial growth plate is a good example for observing discrete stages in the process of cartilage formation and mineralization [20]. Four zones within the growth plate are distinguished on the basis of chondrocytic changes (Figure 1). From the epiphyseal end they are: the zone of reserve or resting cells (R); the zone of cell proliferation (P); the zone of cell hypertrophy (H); and

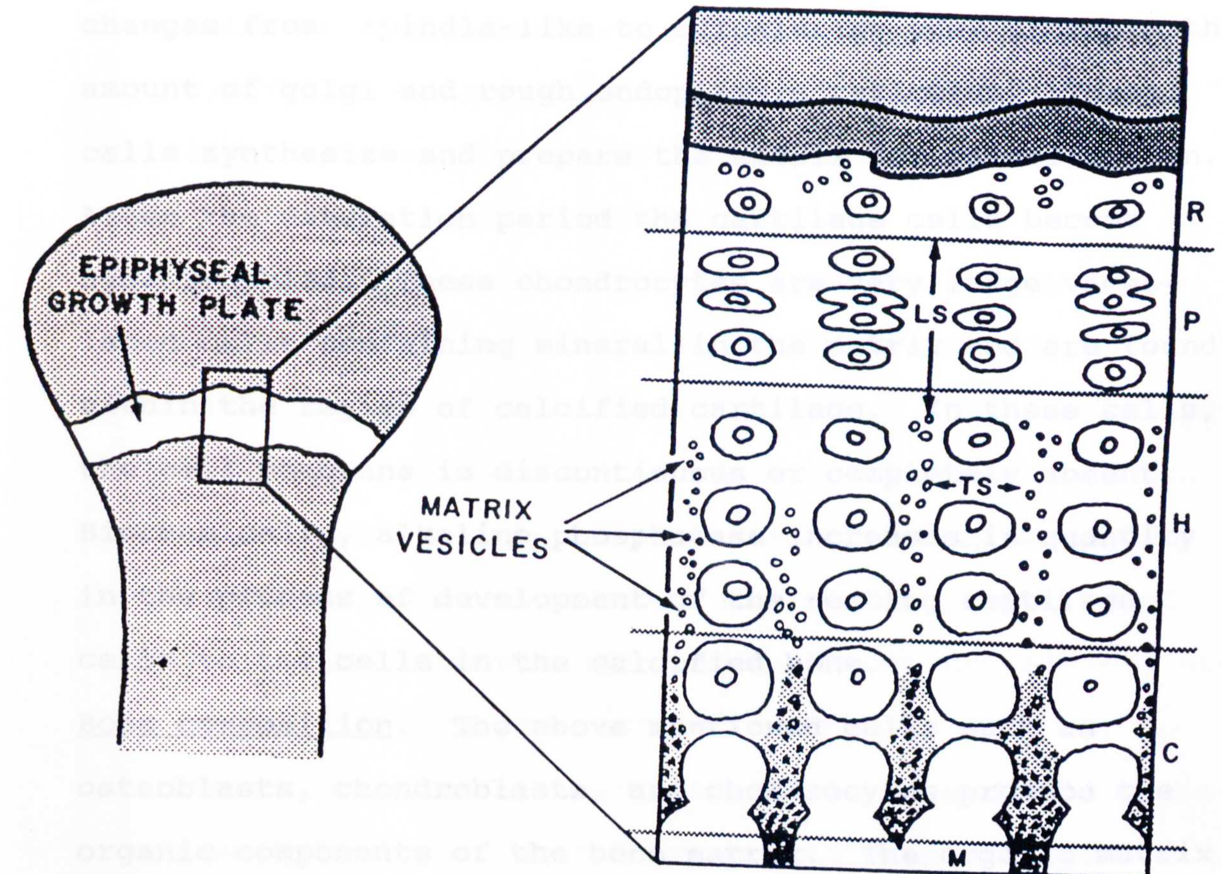


Figure 1: Diagram of the epiphyseal growth plate. The anatomical regions are: The reserve zone (R) which contains resting cells and is composed of only a few chondroblasts. The proliferative zone (P) is a zone where active cell division takes place. The hypertrophic zone (H) contains contain enlarged chondrocytes with the presence of minerals. The calcifying zone (C) contains degenerating chondrocytes [20].

the zone of provisional calcification (C).

The zone of resting cells is the smallest area and is composed of only a few chondroblasts [19]. The next zone is the region of proliferation. There the cells have a high ratio of nuclear to cytoplasmic material. The maturing cartilage cells become larger and their shape changes from spindle-like to circular as they increase the amount of golgi and rough endoplasmic reticulum. These cells synthesize and prepare the matrix for calcification. After the maturation period the cartilage cells become hypertrophied. These chondrocytes are very large vacuolated cells containing mineral in the matrix and are found within the region of calcified cartilage. In these cells, the cell membrane is discontinuous or completely absent. Biochemically, alkaline phosphatase increases in quantity in the process of development of the resting cartilage cells to the cells in the calcified bone.

Bone composition. The above mentioned cells such as osteoblasts, chondroblasts, and chondrocytes produce the organic components of the bone matrix. The organic matrix is broadly subdivided into collagen fibers and amorphous ground substance [21].

Collagen is the predominant organic component of bone. Collagen molecules are composed of a complex helical structure [10,22]. This triple-stranded protein contains the amino acids proline and lysine in the form of hydroxyproline and hydroxylysine. In order to form mature

collagen some of the hydroxylysine molecules are glycosylated.

The cellular and fibrous components of collagen are surrounded by materials collectively labelled as the **amorphous ground substance** [10,21,23]. The constituent molecules of the ground substance include modified (phosphorylated, glycosylated, and sulphated) proteins, and lipids. The predominant type of modified glycoprotein in cartilage and bone are chondroitin sulfate, found in association with type II keratin sulfate.

Mineralization of bone begins in the spaces between the consecutive rods of collagen [10,24]. The major mineral structure of the bone is $Ca_{10}(PO_4)_6(OH)_2$, often referred to as hydroxyapatite. Bone also contains substantial quantities of carbonates, citrate, sodium, magnesium, and fluoride. Trace amounts of iron, zinc, copper, lead, manganese, tin, aluminum, strontium, boron, and silicon are also present in bone [10].

Interconnection of the minerals with the matrix gives bone its stability. The hydroxyapatite crystals are regularly distributed along the length of the collagen fibers. The ground substance surrounds and stabilizes these crystals. The hardness and rigidity of the bone is due to the binding of the hydroxyapatite with collagenous fibers and non-collagenous matrix.

<u>**Ectopic Bone Development**</u>

A number of factors have hindered extensive research

into bone metabolism. As animals age, collagen becomes highly cross-linked and resistant to treatment, making bone a very stable structure. Consequently, <u>in vivo</u> research is often conducted with fetal calvaria or very young bones as detecting net changes in skeletal integrity requires an extended period of study. Another cause for difficulty in bone research is that present techniques make it difficult to study simultaneously bone formation and resorption <u>in vivo</u>. Yet another reason is that there are heterogeneous populations of cells at different stages of differentiation in any one bone. These and other difficulties have led scientists to explore models which would "permit a more synchronous biochemical and morphological examination" [25] of bone metabolism.

Over twenty years ago Urist [26,27] demonstrated that implants of **demineralized bone (DB)** could induce new bone formation. Since that time implantation of decalcified bone has been further researched, and described by Reddi and coworkers [28-33]. In these studies, subcutaneous implantation of acid-insoluble coarse powders of bone matrix rapidly and consistently caused the formation of bone and cartilage [29]. The sequence of events that took place after bone-matrix implantation was as follows. On day one, the irregular fibrin network was enmeshed in polymorphonuclear leukocytes. On day 3, mesenchymal cells were in the vicinity of the matrix. Their multiplication was assessed by ^{3}H-thymidine incorporation and increased

ornithine decarboxylase activity [29,34]. Chondroblasts were seen on days 5 and chondrocytes on days 7-8. Calcification of the hypertrophied cartilage occurred around day 9. On days 12-18, remodeling of bone occurred. This resulted in selective dissolution of implanted bone matrix and formation of new bone [29,35]. Vascularization and hemopoiesis were also observed [31,32].

The changes which took place in various components of the DB implant were examined using the following methods (Figure 2) [29]:

i. The cartilage formation was monitored by determining incorporation of $^{35}SO_4$ into proteoglycans both <u>in vivo</u> [25,35,36] and <u>in vitro</u> [37].

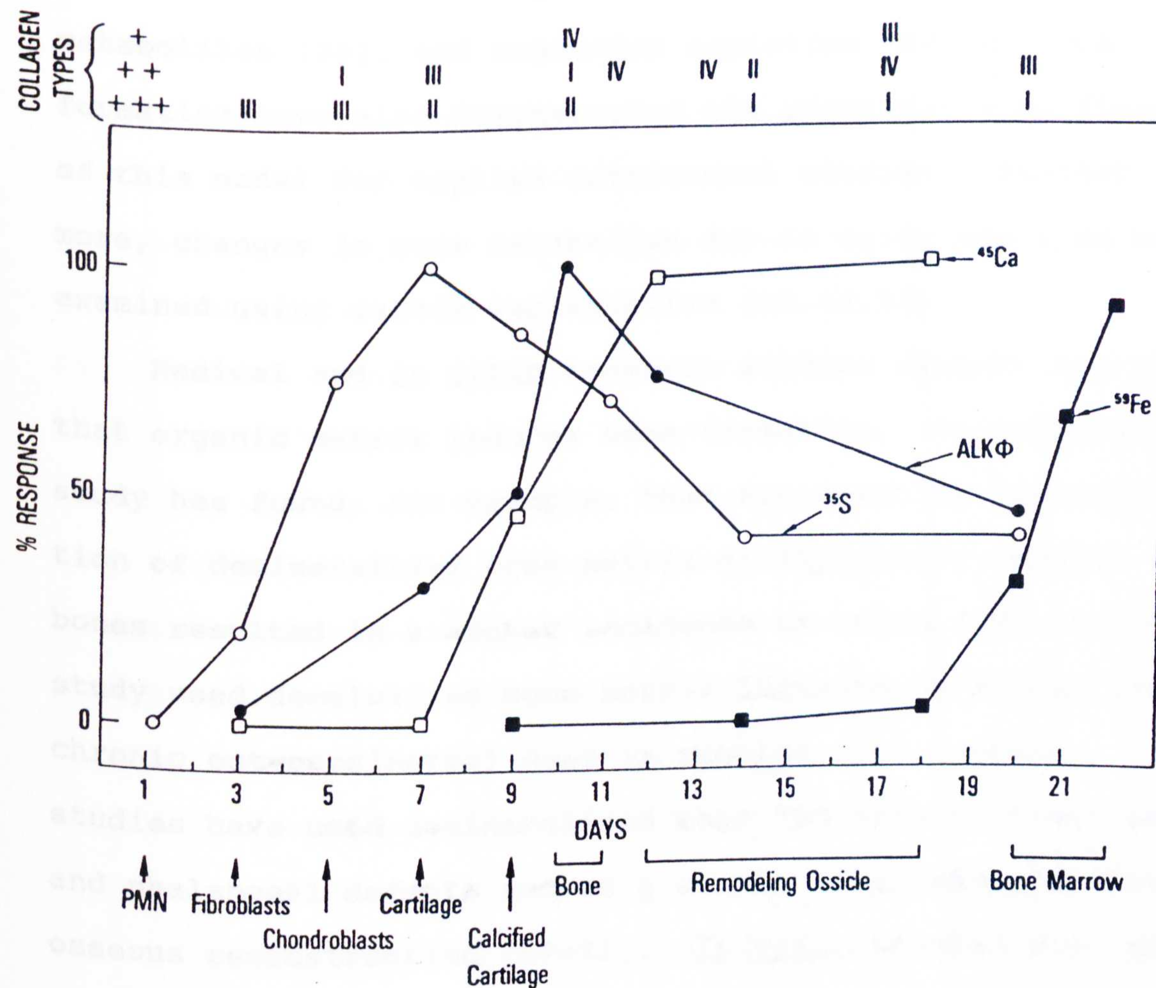
ii. Alkaline phosphatase activity and ^{45}Ca incorporation into bone mineral was used as an indicator for bone mineralization [25,29,35,38,39]. Acid phosphatase activity served as a marker for resorption and remodeling [29,36,40,41].

iii. Collagen formation was monitored by the incorporation of ^{3}H-proline into hydroxyproline [42,43] with prolyl-4-hydroxylase as its marker enzyme [29,41].

iv. Incorporation of ^{59}Fe into hemoglobin was taken as an indicator of erythropoiesis [29,31,44].

Figure 2 also shows the types of collagen synthesized during matrix-induced bone formation. The location of the different types of collagen was examined by indirect

Figure 2: Developmental sequence of the extracellular matrix-induced bone formation. Changes in $^{35}SO_4$ incorporation into proteoglycans indicates cartilage formation. ^{45}Calcium uptake into the mineral phase and alkaline phosphatase activity indicates bone formation. The ^{59}Fe incorporation into hemoglobin is an index for erythropoiesis. The transition in collagen types I to IV were determined by immunofluorescent localization [29].



immunofluorescence [44] and quantified by gel electrophoresis [29,42].

The feasibility of matrix-induced, endochondral bone formation as a model for sequencing events in bone homeostasis has been extensively documented [35,45-52]. Some nutritional studies using vitamin A [25], vitamin D metabolites [38], and magnesium depletion [40] on bone formation have also demonstrated the practical significance of this model for applied nutritional studies. Furthermore, changes in bone metabolism due to aging has also been examined using matrix implantation [45,53,54].

Medical and _in vitro_ research studies support the view that organic matrix induces bone formation. An orthopedic study has found, for example, that treatment by implantation of demineralized bone matrix of improperly mended bones resulted in a higher incidence of union [55]. Another study used decalcified bone matrix implants to bridge large chronic osteoperiosteal gaps in rabbits [56]. Other studies have used demineralized bone implants to treat jaw and phalangeal defects and as a source of biomaterial for osseous reconstruction [57-71]. _In vitro_ studies also show that embryonic skeletal muscle when cultured on bone matrix can differentiate into chondrocytes [62]. Thus, bone matrix contains a factor which is capable of initiating bone formation.

The purification of the active components from bone matrix, which causes auto-induction of endochondral bone

formation is currently being pursued [63-72]. The ability of demineralized bone matrix to induce bone formation has been attributed by Urist [63-65] to a low-molecular weight (17,500 daltons) protein called Bone Morphogenic Protein (BMP). BMP is a hydrophobic glycoprotein with 20.8% acidic amino acids [73]. It is found in dentine and osteosarcoma tumors as well as demineralized bone [74]. BMP was found to stimulate calvarial DNA synthesis and cell replication, but it did not induce collagen synthesis or alkaline phosphatase activity. This indicates that BMP does not affect osteoblastic function directly [65,74]. The mechanism of action of BMP at a molecular level in initiating the differentiation of mesenchymal type cells in cartilage and bone remains to be elucidated.

The relationship between BMP and the osteoinductive protein, isolated by Reddi from cell-free demineralized bone matrix, is not clear [66-72]. The induction property is attributed to a protein with an apparent molecular weight of 50,000 daltons [71]. Further studies which have involved the extraction of bone matrix have revealed two active principles: a chemotactic factor [70] and a mitogen [73].

Although demineralized bone implants have been studied in some detail, implantation of **mineralized bone (MB)** powder to examine resorption has received little attention. Reddi and Huggins [75] demonstrated that implanted bone ash (bone mineral) induced resorption as reflected by increased

concentration of citric and lactic acids. A vast number of multinucleated osteoclastic giant cells were found around the graft, suggesting bone breakdown. In contrast to the results for DB implants no increase in alkaline phosphatase activity was evident.

Two reports using fish [76] or rats [77] have expanded the feasibility of implanting MB powder to study bone resorption. While these studies were limited to histological evaluations, they did confirm the presence of osteoclasts and demonstrated a decrease in the size of bone particles, both indicative of bone resorption. The multinucleated cells on the surface of the bone particles had characteristic features of osteoclasts found in the bone [15,78,79]. Clear zones of attachment and ruffled borders were present. Other studies have used MB powders as controls for their investigations in order to elucidate the effects induced by DB powder [59,80].

The use of subcutaneous implantation of demineralized and mineralized powders may solve some of the problems encountered in bone research. Such a system provides a controlled environment which allows the study of factors influencing bone metabolism.

<u>**Factors influencing bone metabolism**</u>

The influence of factors such as age, vitamin D, calcium, and zinc on bone development has been studied extensively in clinical and laboratory situations. A strong association has been established between these

factors and osteoporosis. Minerals and organic matrix are lost from bones in this condition [81-84]. As a result the bone becomes fragile and fractures under normal stresses. The main areas susceptible to fractures are hips, wrists, and vertebrae.

Depending on sex, age, and fracture pattern, osteoporosis in the older population can be described as postmenopausal (Type 1) [85,86] or senile (Type 2) [86,87]. Patients with vertebral fractures (Type 1) have a disproportionate loss of trabecular bone compared with cortical bone [86,88]. The loss of ovarian function and the consequent decrease in estrogen levels plays an important role in this form of osteoporosis [85]. Patients with hip fracture (Type 2) have slow but continuous loss of both trabecular and cortical bone [86]. This type of bone loss could result from decreased absorption of calcium, secondary hyperparathyroidism or impaired vitamin D metabolism [90,91].

There are two current hypotheses concerning the development of osteoporosis [81-91]. First, the bone accumulates in less than optimal quantities during growth and development, and/or second, a faster rate of bone loss takes place. A number of questions concerning these interpretations remain unanswered. In particular, the reasons why osteoporotics have a low volume of bone formed during development and growth are unclear. The reasons why bone loss increases with age is also unknown. Similar

questions were raised by the members of the National Institutes of Health Consensus Development Panel for Osteoporosis [1]. Their recommendation for future studies was "to elucidate further the mechanisms of bone growth and remodeling, their local and systematic regulation and their alteration in osteoporosis [1]."

Calcium. The body of an average-size adult female contains approximately 1,000 to 1,200 grams of calcium [92]. Over 99% of body calcium resides in the skeleton. When appropriately stimulated by a variety of hormones and metabolic agents, the skeletal system serves as a guardian of the circulating calcium pool. In a normal adult the dynamic process of bone turnover releases calcium into blood and then reaccumulates between 250 milligrams to 1 gram of calcium a day [92].

The normal range of plasma calcium concentration fluctuates between 9 and 11 mg per deciliter [93]. Calcium ions are continuously exchanged between bone tissue and extracellular fluids. This exchange is independent of the formation or resorption taking place in the bone [10]. During hypocalcemia there is an increase in the outward flux of calcium from bone mineral into surrounding extracellular fluid. Alternately, during hypercalcemia there is an inward flux of calcium from the extracellular fluid into bone mineral.

The regulation of plasma calcium involves at least three hormones -- 1,25$(OH)_2$D, PTH, and CT [6]. Higher

circulating levels of PTH increase the conversion of vitamin D to its active metabolite, $1,25(OH)_2D$. $1,25(OH)_2D$ enhances the uptake of calcium from the intestine and also increases the net rate of bone resorption. PTH also increases plasma calcium by decreasing urinary loss of this mineral. The overall effect of CT is to decrease the plasma concentration of calcium by inhibiting bone resorption and increasing urinary excretion.

The daily intake of elemental calcium in the United States is 450 mg to 550 mg, well below the National Research Council's recommended dietary allowance of 800 mg [1]. Calcium metabolic balance studies indicate that 1,000 mg of calcium per day is necessary for premenopausal and estrogen-treated women to maintain calcium balance [1]. Moreover, there is evidence that postmenopausal women require 1,500 mg of calcium per day [1,81,94].

Increasing the dietary calcium intake in the aging population may be one of the crucial factors in preventing bone loss. However the use of calcium supplementation in treating osteoporosis is highly controversial. The National Institutes of Health Consensus Panel on Osteoporosis [1] recommended that increased intake of calcium may prevent age-related bone loss. According to this view, calcium supplementation may be successful in halting bone loss but not in itself lead to increased bone mass [82]. Contrary to this recommendation is the belief that calcium intake in adulthood may have nothing to do with osteoporo-

sis [95].

Vitamin D. This nutrient is involved in the regulation of calcium metabolism and skeletal remodeling [6]. Vitamin D or cholecalciferol (D_3) is ingested in the diet, but the main route by which it becomes available to the body is by synthesis in the epidermis [5, 96-99]. The compound 7-dehydrocholesterol is converted into D_3 in the presence of ultraviolet rays (290-320 mu) in the epidermis. D_3 itself is not active and must be metabolically altered before it is functional [5,96-102]. The first step in the metabolic activation of vitamin D is its hydroxylation to 25-hydroxy-cholecalciferol (25OHD) in the liver. This is the major circulating form of this vitamin. 25OHD is transported to the kidney where it is converted into $1,25(OH)_2D$, in what is considered to be the rate limiting step in vitamin D metabolism [5].

Vitamin D and its active metabolites are initially required to increase the absorption of calcium from the intestine, thereby maintaining adequate levels of this mineral in the extracellular fluid [5]. The $1,25(OH)_2D$ increases the active transcellular transport of calcium in the mucosal cells of the small intestine [5,98-100]. $1,25(OH)_2D$ is thought to initially bind to the cytoplasmic receptor in the target cells in the intestine. The receptor-hormone complex is transferred to the nucleus, where the $1,25(OH)_2D$ binds to specific receptors on the chromatin and stimulates gene expression. This leads to an

increased synthesis of vitamin D dependant proteins such as calcium-binding protein. This protein may facilitate the transfer of calcium across the brush border membrane. The calcium is stored and transported, by either mitochondrial sequestration or by vesicle formation, to the basal-lateral membrane where a sodium gradient is involved in the expulsion of calcium [98-100].

The evidence concerning the role of $1,25(OH)_2D$ in bone metabolism is mixed. The effect of $1,25(OH)_2D$ on bone resorption is well documented [17,102-107]. Various studies have shown enlarged ruffle borders of osteoclasts [105]; and higher acid phosphatase activity [108] when $1,25(OH)_2D$ was supplemented in experimental animals or in the culture medium. A number of studies have also shown that $1,25(OH)_2D$ has a positive effect on bone formation [106,109-111]. Bone matrix implant showed that $1,25(OH)_2D$ had an inhibitory effect on mesenchymal cell growth but was stimulatory for osteoblasts [111]. _In vitro_ evidence indicates that the anabolic effect [112-115] of $1,25(OH)_2D$ may be due to the stimulation of osteoblast differentiation. The contradictory effects of $1,25(OH)_2D$ on bone formation and resorption may be related to the time of exposure or to dosage levels.

A low level of $1,25(OH)_2D$ has been found in osteoporotic women [116]. The role of $1,25(OH)_2D$ in maintaining the normal calcium homeostasis through increasing intestinal absorption may be impaired in these individuals. Despite

the lack of evidence that 1,25$(OH)_2$D is directly responsible for development of postmenopausal osteoporosis, it has been used in therapeutic trials, alone and in combination with calcium, estrogen, and fluoride [116-119]. In general, the results of these studies suggest that 1,25 $(OH)_2$D alone does not result in consistent, long-term improvement in calcium balance, skeletal density or trabecular bone volume [116]. When used in combination with calcium or estrogen/progesterone, on the other hand there was significant improvement in all parameters assessed.

Zinc. There is evidence to show a link between zinc and bone formation and repair [7-9]. Researchers found higher quantities of zinc in the bones and teeth of rats which were fed sufficient amounts of zinc in diets as opposed to deficient diets. The connection between bone and zinc is further strengthened by the finding that zinc uptake is increased in healing bones of rats [120,121]. Similarly, the effect of zinc deficiency on ectopic bone caused retardation in bone formation which was reversed by replenishing the dietary zinc [122].

Contradictory evidence regarding the effect of zinc on bone metabolism was reported by Yamaguchi *et al*.[123-126]. Rats when supplemented with 0.1 to 10 mg zinc per 100 g body weight for 3 days, showed decreased calcium levels in serum and femoral diaphyses and epiphyses [124,125]. In a separate study the above mentioned researchers found that

doses of 1.0 mg/100 g body weight of zinc produced an increase in the dry weight of bone tissue, DNA and calcium content [123,127] in weanling rats. It appears that low doses of zinc stimulated growth and calcification in bones of weanling rats whereas the same doses increased bone resorption in older rat. These results suggest that age and dose of zinc are important factors in determining the positive and negative effects of this essential trace element on bone.

There is some evidence to show a correlation between zinc and bone loss in osteoporosis. High levels of urinary zinc and hydroxyproline were excreted by osteoporotic patients [128]. The authors suggest that zinc may be involved in the process of osteoporosis in patients with spinal cord injury, however, no mechanism was outlined. Another study on human bone obtained at autopsy showed that bone density was inversely related to age, but directly related to the trabecular bone calcium:zinc ratio [129]. They concluded that because there was a decline in the calcium:zinc ratio with age, osteoporosis develops in humans in association with zinc accumulation rather than zinc deficiency. In contrast, serum and bone zinc in patients with senile osteoporosis were found to be lower than in the control subjects [130]. This result suggests that zinc depletion may play a role in the pathogenesis of type II osteoporosis.

Furthermore, a relationship between zinc and vitamin D

has been reported [109,131-134]. A study conducted in our laboratory concluded that the hormonal form of vitamin D enhanced femoral calcium accretion [109]. However, the enhanced bone formation was negated by high dietary zinc level. Vitamin D may have its effect at the level of absorption in the intestine, because an increase in ^{65}Zn was found in the bones of rats supplemented with vitamin D after an oral administration of ^{65}Zn rather than via an injection [131]. Alternatively, the increased absorption of dietary zinc attributed to vitamin D was not due to a direct effect of the vitamin on zinc absorption, but rather to a homeostatic response to the increased need for zinc, which accompanies enhanced skeletal growth and calcification [134].

RATIONALE AND OBJECTIVES

The studies on bone metabolism reviewed in the previous section indicate that bone response patterns may vary with characteristics of the bone examined, particularly its function and composition. Measuring these responses in bone integrity requires extensive periods of study. Furthermore, with present techniques, it is difficult to study bone formation and resorption simultaneously. Both these bone functions need to be examined in order to predict net bone loss that may result from aging and nutritional factors.

The present studies examined the effects of diets and age on bone metabolism using different representative bones, as well as bone subsections. A further important feature of these studies was the use of a short-term *in vivo* model utilizing subcutaneous implantation of DB and MB powders. This enabled the simultaneous investigation of bone formation and resorption as independent processes.

The studies had two major objectives: The first was to assess and develop *in vivo* models for examining bone metabolism. The second objective was to determine the effects of age (2, 8, and 24 month-old rats) and nutrients (calcium, zinc, and vitamin D metabolites) on bone integrity.

MATERIALS AND METHODS

Animals and Diets

Sprague-Dawley[1] and Long-Evans[2] rats were housed individually in hanging wire-bottomed stainless steel cages in a room controlled for temperature, humidity, and light (12-hours light-dark cycle). Deionized water was dispensed from glass bottles with stainless steel sippers.

The commercial rat diet[3] (CRD) used contained 1.5% calcium and 0.8% phosphorus. The composition of the basal zinc and vitamin D deficient semi-purified egg-white diet [109] contained 0.2% calcium and 0.8% phosphorus (Table 1). Additional calcium carbonate was added to the basal diets in experiments 5 and 6 to increase the calcium content to 1.0%. The basal diet was supplemented with either 20 micrograms of D_3[4] or 5 micrograms of $1,25(OH)_2D$[5] per kilogram of diet [135].

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1. Charles River Laboratories, Inc., Wilmington, MA, 01887.

2. Charles River Laboratories, Inc., Portage, MI, 49081.

3. Agway Pro Lab RMH 3200 Meal, Agway, Inc., Country Foods Division, P.O. Box 4933, Syracuse, NY, 13221.

4. United States Biochemical Co., Cleveland, OH 44128.

5. $1,25(OH)_2D$ was kindly provided by Hoffmann-LaRoche, Nutley, NJ, 07110.

Table 1. Composition of semi-purified egg-white diet [109]

Ingredients	% Diet
Dried egg white	18.0
Corn Starch	20.0
Glucose	45.9
Cellulose	4.0
Corn Oil	5.0
Vitamin Mix[a]	1.0
Mineral Mix[b]	6.0
Trace Mineral Mix[c]	0.1

[a]Vitamin Mix provides the following in mg/kg of diet: Biotin 5.0; Calcium pantothenate 12.0; Folic acid 1.5; Menadione 5.0; Nicotinamide 30.0; Pyridoxine hydrochloride 7.0; Riboflavin 6.0; Thiamin hydrochloride 6.0; Cobalamin 0.05; Retinyl acetate 6.9; Tocopheryl acetate 30.0; Alpha Tocopherol 30.0; Choline dihydrogencitrate 2000.0.

[b]Mineral Mix provides the following in g/kg of diet: KH_2PO_4 15.0; Na_2HPO_4 15.0; $CaHPO_4$ 6.79; KCl 7.0; $MgSO_4$ 3.0; $MnSO_4.H2O$ 0.220; $FeC_6H_5O_7$ 0.098; KIO_3 0.010.

[c]Trace Mineral Mix provides the following in mg/kg of diet: $Co(C_2H_3O_2)_2 \cdot 4H_2O$ 21.2; $Na_2MoO_4 \cdot 2H_2O$ 5.0; Na_2SeO_3 0.3; NaF 44.4; $CrCl_3 \cdot 6H_2O$ 10.2; $NiCl_2.6H2O$ 4.0; $Na_2B_4O_7$ 23.3; $NaHASO_4 \cdot 7H_2O$ 1.3; $VoSO_4 \cdot 2H_2O$ 3.9; $Sn(C_4H_4O_6)$ 2.2; $CuSO_4$ 7.0.

Experiment 1. The specific objective of this experiment was to determine if 10 and 300 ppm dietary zinc had a beneficial effect on bone metabolism of 2 and 8 month-old rats. Femurs, humeri, scapulas and tibias were examined to estimate the reliability of using one bone for predicting changes in the skeletal system. The femoral epiphyses-metaphyses and diaphyses were examined separately to determine changes occurring in the cortical and trabecular bone.

Thirty female 2 month-old and thirty female retired-breeders (8 month-old) Sprague-Dawley rats were allowed free access to CRD and deionized water for 5 days. The skeletal tissue of these animals was deep labelled with a total of 7.50uCi/100g body weight with ^{3}H-tetracycline[6] (^{3}H-TC) over a two week period in six subcutaneous injections. Tetracycline has a high affinity for calcium and is thought to parallel the action of this mineral [136-139]. One major advantage of using tetracycline is that once it is removed from the bone, it does not enter the general calcium pool and become redeposited in the bone as would be the case for ^{45}Ca. Therefore, by using labelled tetracycline the net loss of calcium from the bone can be estimated.

Two days after the completion of labelling with ^{3}H-TC both age groups of animals were randomly assigned to two

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6. New England Nuclear, Boston, MA, 02118. ([7-^{3}H(N)] - Tetracycline, specific activity 635mCi/mmol).

dietary regimens (10 and 300 ppm zinc) with $1,25(OH)_2D$ the only vitamin D source in the basal semi-purified egg-white diet.

Eighteen hours before sacrifice the rats were injected intraperitoneally with 3.6uCi/100g of ^{14}C-Proline[7]. Incorporation of ^{14}C-proline was a crude estimate for collagen synthesis. The rats were anesthetized using CO_2 and exsanguinated by heart puncture. The four limbs were removed and frozen at $-20^{0}C$.

The femurs, tibias, humeri, and scapulas were hand cleaned to remove soft tissue. Both the epiphyses-metaphyses were removed from the right femurs and the marrow flushed away with deionized water. Cleaned bones were dried at $100^{o}C$ for 12 hours, weighed and digested overnight in 3 mls of 60% nitric acid. Hydrogen peroxide was added to the digest and heated at $80^{o}C$ until a clear solution was obtained. This solution was filtered through a no.2 Whatman filter paper [140]. ^{3}H-TC and ^{14}C-proline content was quantitated in a scintillation counter[8] using a 0.5 ml aliquot of the bone digest mixed with scintillation

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7. ICN Chemicals and Radioisotope Division, Irvine, CA, 92715. ([U-^{14}C] L-Proline, specific activity 255mCi/mmol).

8. Packard Prias Model PLD Tri-Carb Liquid Scintillation Counter, Packard Instrument Co., Inc., 220 Warrenville Rd., Downers Grove, IL, 60515.

cocktail.[9]

The serum was obtained by allowing the blood to clot at room temperature for 2-4 hours. The samples were centrifuged for 20 mins at 1100xg and the serum drawn off and stored at -20^0C.

Another 0.5 ml of the bone digest and 0.5 ml of serum were diluted separately in 0.5% lanthanum oxide for the determination of calcium. One ml of the bone digest was diluted in 10% nitric acid for zinc analysis. The calcium and zinc content was determined using an atomic absorption spectrophotometer[10].

Preliminary Experiment 2A. The specific objective of this experiment was to develop the implantation technique using DB powder and also to determine whether $1,25(OH)_2D$ with 4.5 and 10 ppm dietary zinc had a beneficial effect on bone development.

This experiment was performed in two blocks. Each block contained 36 female weanling Long-Evans rats which were randomly assigned to one of the four dietary treatments. The basal semi-purified egg-white diet varied in their level of zinc (0.5 or 10 ppm), type of vitamin D (D_3 or $1,25(OH)_2D$) and three days (11, 14, and 21 days after implantation) when the implants were harvested. To ensure

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9. Scinti-Verse I, Fisher Scientific Co., Fairlawn, NJ, 07410.

10. Perkin-Elmer Model 503 Atomic Absorption Spectrophotometer, Perkin-Elmer, Norwalk, CT, 06856.

the survival of the animals fed 0.5 ppm zinc, 4 ppm zinc was added to the basal diet 7 days prior to implantation of DB powder.

Preparation of bone matrices

Long-Evans rats were anesthetized with CO_2 and sacrificed [141]. The femurs, humeri, and tibias were excised, the extremities were cut with a dremel saw[11], and the bone marrow was flushed with tap water. The diaphyses were scrubbed with a stiff brush and the adherent soft tissues removed meticulously. The bones were washed with copious amounts of tap water for 2 hours; absolute ethanol for 1 hour; and ethyl ether for 0.5 hour. The washed bones were dried overnight at room temperature and stored in a bottle. The cleaned bones were processed as outlined:

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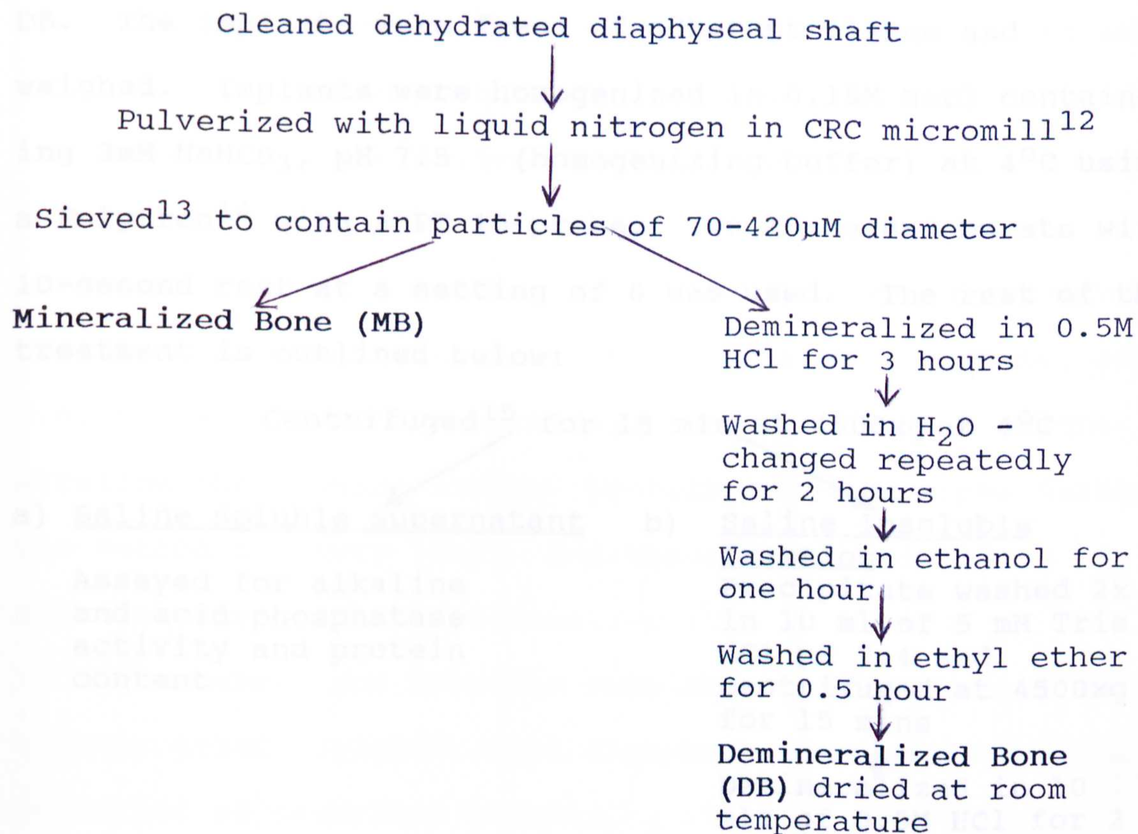
11. Dremel Saw, Division of Emerson Electric Co., Racine, WI, 53405.



The rats were anesthetized with ether, their thoracic area shaved. A 1-cm incision was made in the epidermis at the center of the thorax under sterile conditions and pockets prepared by blunt dissection. A spatula tip full (approximately 20-30mg) of DB powder was placed as a compact deposit on either side of the thorax. The incision was closed with metallic stainless steel clips and the day of implantation designated as day 0.

The rats were anesthetized with CO_2 and exsanguinated by heart puncture 11, 14 and 21 days after implantation of

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12. The Chemical Rubber Co., Cleveland, OH.

13. Fisher Scientific Co., Silver Spring, MD, 20910.

DB. The implants were freed of adherent tissue and weighed. Implants were homogenized in 0.15M NaCl containing 3mM $NaHCO_3$, pH 7.5 (homogenizing buffer) at 4^oC using a Polytron[14] with a PT 10 probe. Two 15-second bursts with 10-second rest at a setting of 6 was used. The rest of the treatment is outlined below:

Centrifuged[15] for 15 min at 4500xg at 4^oC

a) Saline Soluble Supernatant

Assayed for alkaline and acid phosphatase activity and protein content

b) Saline Insoluble fraction

Precipitate washed 2x in 10 ml of 5 mM Tris HCl pH 7.4 and centrifuged at 4500xg for 15 mins

↓

Demineralized in 10 mls of 0.5M HCl for 2 hrs at 25^oC

↓

Centrifuged at 4500xg for 15 mins

↓

Supernatant

↓

Calcium concentration determined by atomic absorption spectroscopy

One ml of the demineralized supernatant was assayed for calcium and zinc as described earlier.

Saline soluble fraction: The supernatant was used in

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14. Brinkman Instruments Co., Divison of Sybron Corp., Westbury, NY, 11590.

15. Sorvall RC-5B Centrifuge, Ivan Sorvall, Inc. Norwalk, CT, 06470.

the assay for determining alkaline (E.C. 3.1.3.1.) and acid (E.C. 3.1.3.2.) phosphatase activities. One unit of enzyme activity was defined as the amount of enzyme which liberated 1 umole of p-nitrophenol from p-nitrophenyl phosphate[16] per 0.5 hour at 37°C. The buffer solution used in the acid phosphatase assay was 0.1M sodium acetate, pH 5.0, while 0.1M sodium barbital, pH 9.3, was used in the alkaline phosphatase assay. Protein was determined using the method of Lowry [142] and the enzyme activities expressed as units/mg protein.

The humeri and scapulas were manually cleaned of soft tissue, dried, weighed, acid digested and mineral analyses performed as described earlier.

Experiment 2. The specific objective of this experiment was to determine the possible beneficial effects of $1,25(OH)_2D$ with 4.5 and 10 ppm dietary zinc on bone development using implants and bones. The femurs, humeri, mandibles, scapulas, and tibias were examined to estimate the reliability of using one bone for predicting changes in the skeletal system.

Seventy-two female weanling Long-Evans rats were obtained from the same commercial breeder as above and housed as described earlier. The experimental animals were randomly assigned to one of the 12 treatment groups. The experimental design was a 2x2x3 factorial with the basal

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16. Sigma Chemical Co., St. Louis, MO, 63178.

semi-purified egg-white diet containing two levels of zinc (4.5 or 10 ppm), two types of vitamin D (D_3 or $1,25(OH)_2D$) and three days (11, 14 and 21 days after implantation) when the implants were harvested.

Thirty-six rats were fed the basal diet with no added zinc for 3 weeks to deplete their stores of this essential trace mineral. The diet was switched to one containing 4.5 ppm zinc (marginally deficient) 2 days before implantation of DB powder. The vitamin D source (D_3 or $1,25(OH)_2D$) was kept constant throughout the experimental period.

Fourteen hours prior to sacrifice, the rats were injected intraperitoneally with 0.5μCi/g body weight with $^{45}CaCl_2$[17] (^{45}Ca) in physiological saline. The animals were anesthetized with CO_2 and exsanguinated by heart puncture. The implants were harvested and bones and serum collected as described earlier.

The implants were homogenized and centrifuged as described previously. The precipitate was equilibrated for ^{45}Ca in 5 mls of 0.1M $CaCl_2$ in 0.02M Tris HCl, pH 7.4, at 20°C for 20 minutes. The equilibration mixture was centrifuged at 4500xg for 15 minutes. The precipitate was washed and demineralized as described earlier. The enzyme assays as well as calcium and zinc analyses were performed as previously described. Demineralized supernatant of the saline insoluble fraction (0.5 ml) was mixed with scintil-

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17. New England Nuclear, Boston, MA, 02118 (specific activity 4-50 Ci/g Calcium).

lation fluid and counted to quantitate ^{45}Ca incorporation. ^{45}Ca incorporation into the implanted bone matrix during the pulse of labelled calcium is expressed as CPM/g tissue. The calcium and zinc level was measured using atomic absorption spectroscopy.

A small portion of two implants from the same experimental group were fixed in Bouin's compound [143]. The implant sections were embedded in JB-4 plastic medium and one micron thick sections were cut with an ultramicrotome and stained with toluidine blue[18] [144].

The right femurs, humeri, scapulas, and tibias, were hand cleaned, and acid digested. The mandibles were cleaned by allowing Dermestes beetles to feed on soft tissue. Calcium, zinc, and the uptake of ^{45}Ca by these bones were measured as described earlier.

Experiment 3. The purpose of this experiment was to determine if implantation of MB powder would induce resorption of the implants.

Twenty-five male Long-Evans rats weighing approximately 150g were fed CRD ad libitum. Rats were anesthetized with ether, the thoracic area was shaved, and two 1-cm incisions were made in the epidermis at the base of the thorax under sterile conditions, and pockets were prepared by blunt dissection. Two #5 gelatin capsules containing approximately 100 mg MB were inserted over the pectoral

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18. American Histochemical, Gaithersburg, MD 20879.

muscle of the surgically prepared pockets as shown in Figure 3. The incisions were closed with metallic skin clips and the day of implantation designated as day 0. The gelatin capsule was used to make a more compact implant.

The implants were harvested at 4, 6, 8, 10, and 12 days after implantation. Alkaline and acid phosphatase activities as well as calcium content of the implants were determined.

Experiment 4. The specific objective of this experiment was to determine if bone formation and resorption could be examined as separate processes in the same animal using DB and MB powder implants.

Forty male Long-Evans rats weighing approximately 150 g were fed CRD ad libitum. Each rat was implanted with one #5 gelatin capsule containing approximately 30mg of DB powder and the other capsule containing 100mg of MB powder (which is an equivalent amount of organic matrix). Five animals in each group had the DB powder implanted on the left thorax while the other five had DB implants on the right side. Using this procedure the influence on implant development of right verses left side was eliminated. The implants were harvested 7, 9, 11 and 13 days after implant-ation.

The alkaline and acid phosphatase activities as well as the calcium content were measured. Histological sections were also prepared for the four time periods.

Figure 3A: Schematic drawing of a rat implanted with two #5 gelatin capsules containing bone powders.

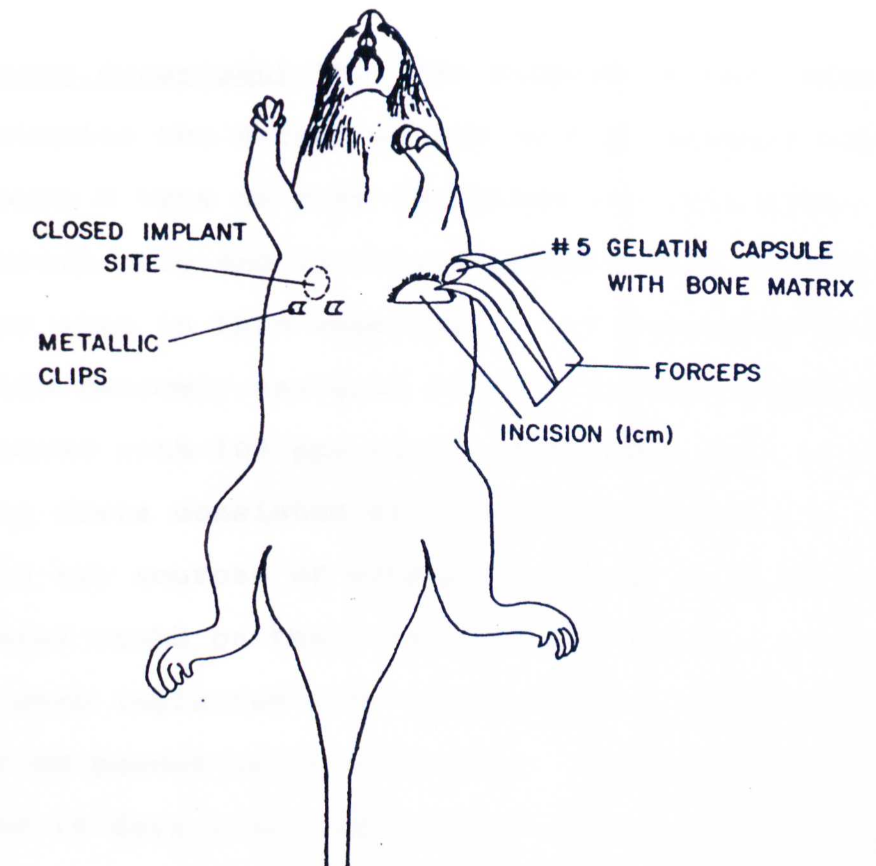
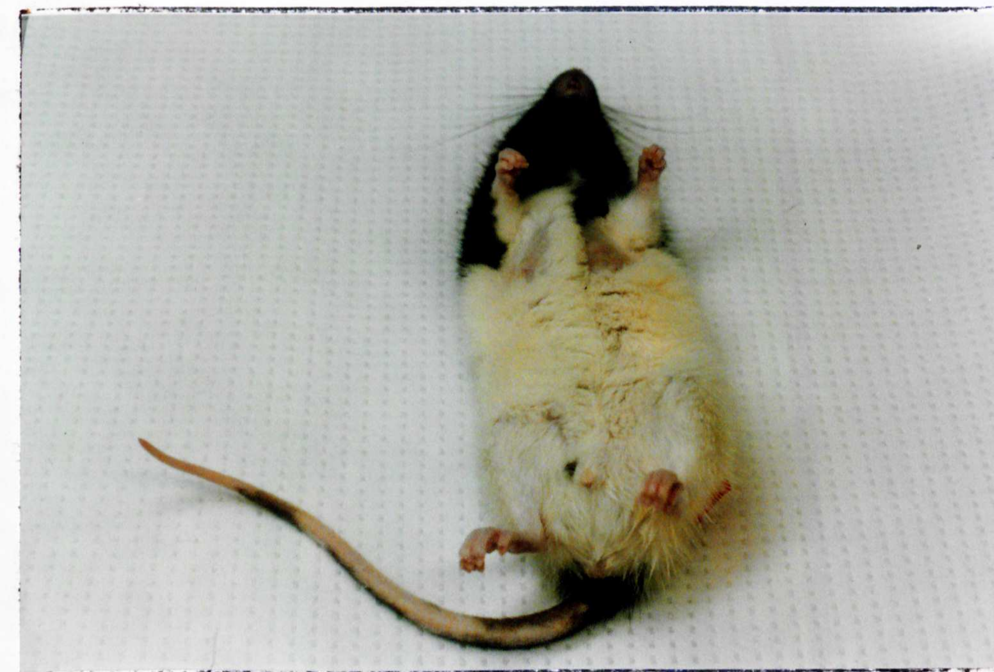


Figure 3B: Photograph of a rat 14 days after implantation. The metallic clips have fallen out and the incisions have healed.



Preliminary Experiment 5A. The purpose of this experiment was to examine the effect of low or high dietary calcium and vitamin D type on bone formation and resorption.

Sixteen male and 22 female offspring of 5 Long-Evans dams were used in this experiment. Litter-mates as well as sexes were randomly assigned to four dietary treatments supplemented with 100 ppm zinc. The basal semi-purified egg-white diets consisted of two levels of calcium (0.2% or 1.0%) and two sources of vitamin D (D_3 or $1,25(OH)_2D$). After three weeks on their respective diet the experimental animals were implanted with approximately 30mg of DB and 100mg of MB powder using a spatula. The implants were harvested 16 days after implantation, and enzyme assays and mineral analyses were performed.

Experiment 5. The specific objective of this experiment was to examine the effect of low or high dietary calcium and vitamin D type on bone formation and resorption. The epiphyses of the femurs and tibias were analyzed in order to compare the biochemical changes in the implants to those in the bones.

A total of 47 Long-Evans 42 day-old male rats were placed on one of four diets for three weeks. The basal semi purified egg-white diets contained two levels of calcium (0.2% or 1.0%) and two sources of vitamin D (D_3 or $1,25(OH)_2D$). All the four diets contained 100 ppm zinc.

Each rat was implanted with two #5 gelatin capsules, one containing approximately 30 mg of DB and the other

containing 100 mg of MB powder. The implants were harvested after 12 days and enzyme activities and calcium content determined as described earlier. The right femurs and tibias were hand cleaned and the femoral distal epiphyses and the tibial proximal epiphyses were separated. The epiphyses were placed in cold homogenizing buffer and treated as described for the implants. Enzyme assays and calcium content were determined as described.

The right humeri were hand cleaned, dried, weighed, acid digested and the calcium content measured.

Experiment 6. The specific objective of this experiment was to determine the effect of low and high dietary calcium on bone formation and resorption in young and old rats. The femoral epiphyses were analyzed in order to compare the changes in the implants with those in the bones. The reliability of using one bone for predicting changes in the skeletal system were examined using the femurs, humeri, scapulas, and tibias.

Twenty-four 18-24 month-old and 24 two month-old female Long-Evans rats were randomly assigned to the basal semi-purified egg-white diet supplemented with either 0.2% or 1.0% calcium. The rats were fed their respective diet for eleven weeks and then implanted with a #5 gelatin capsule containing approximately 30mg of DB and another containing 100mg of MB powder.

Fourteen hours before sacrifice the animals were injected intraperitoneally with 0.1μCi/g body weight with

$^{45}CaCl_2$. The implants were harvested 14 days after implantation and enzyme activities, ^{45}Ca uptake and calcium content were determined. Histological sections of the DB and MB were prepared from each treatment group. The femoral distal epiphyses were also assayed for enzyme activity, ^{45}Ca uptake and calcium concentration.

The right femurs, tibias, humeri, and scapulas were hand cleaned and acid digested. The uptake of ^{45}Ca as well as calcium content of these bones were examined.

Statistical Analyses

The two basic designs used in these experiments were Randomized Complete Block (RCB) and Completely Random Design (CRD) [145]. The details are presented in Table 2 with sources of variation, degrees of freedom, and their respective error terms. The designs for different experiments are summarized below:

Experiment 1: RCB (blocked by position of cage)
2x2 factorial (2 ages and 2 dietary zinc levels);

Experiment 2A: RCB (blocked by time)
2x2x3 factorial (2 types of vitamin D, 2 dietary zinc levels and 3 days of sacrifice)
2 samples (2 implants);

Experiment 2: CRD
2x2x3 factorial (2 types of vitamin D, 2 dietary zinc levels and 3 days of sacrifice)
2 samples (2 implants);

Experiment 3:	CRD Time (harvested implants at 4 times) 2 samples (2 implants);
Experiment 4:	CRD with Split-Plot 2x4 factorial (2 types of implants per rat harvested at 4 times);
Experiment 5A:	RCB (blocked by litter-mates) with Split-Plot 2x2x2 factorial (2 levels of calcium, two types of vitamin D and 2 types of implants per rat);
Experiment 5:	CRD with Split-Plot 2x2x2 factorial (2 levels of calcium, two types of vitamin D and 2 type of implants per rat);
Experiment 6:	CRD with Split-Plot 2x2x2 factorial (2 levels of calcium, two ages and 2 types of implants per rat).

Multivariate analyses were performed in experiments 1, 2, and 6 for the different bones examined [146]. The multivariate procedure analyzed the data for all the bones together, therefore an overall effect of the treatments could be determined. The bone parameters were analyzed as a split-plot design, where the different bones were a factor within the sub-plot. This procedure determine if bones responded similarly to treatments.

Least square means (LSM) were reported for each treatment group. The standard error (SE) was the SE of the least square means. LSM is an estimate of the arithmetic

mean which would be expected had there been no missing observations in a treatment group.

TABLE 2. Sources of variability and respective error terms used in the statistical analyses[1].

Sources of Variation	Exp.1	Exp.2A	Exp.2	Exp.3	Exp.4	Exp.5A	Exp.5	Exp.6
Main plot	Blk(5)[2] Age(1) Zn(1) Age*Zn(1)	Blk(1) D(1) Zn(1) D*Zn(2) Day(2) Day*D(2) Day*Zn(2) Day*D*Zn(2)	Day(2) Zn(1) D(1) Day*Zn(2) Day*D(2) Zn*D(1) Day*Zn*D(2)	Day(4)	Day(3)	Lit(4) D(1) Ca(1) D*Ca(1)	D(1) Ca(1) D*Ca(1)	Age(1) Ca(1) Age*Ca(1)
Main plot Error		Rat (Blk*D* Zn*Day) (48)	Rat(Day* Zn*D) (62)	Rat(Day) (45)	Rat(Day) (38)	Rat(Lit* D*Ca) (29)	Rat(D*Ca) (49)	Rat(Age*Ca) (49)
Sub-plot					Imp(1) Imp*Day (3)	Imp(1) Imp*D(1) Imp*Ca(1) Imp*D*Ca (1)	Imp(1) Imp*D(1) Imp*Ca(1) Imp*D*Ca (1)	Imp(1) Imp*Ca(1) Imp*Age(1) Imp*Ca*Age (1)
Sub-plot Error					Residual (38)	Residual (34)	Residual (43)	Residual (47)
Residual Error	Residual (30)	Residual (68)	Residual (74)					

1 Abbreviations used are SAS notation [145].
2 Degrees of freedom in parentheses.

RESULTS

Experiment 1

The effects of dietary zinc, age, and their interaction on calcium concentration, zinc concentration, ^{3}H-TC content, and ^{14}C-proline incorporation were analyzed using both univariate and multivariate statistical procedures. Univariate analyses were carried out separately for each of the four bones (femurs, humeri, scapulas, and tibias). A multivariate analyses for all four bones were also carried out.

The results from the multivariate analyses (Table 3) show that the concentration of calcium in the bones was not affected either by the age of the animals or the dietary zinc levels. However, the bones of 2 month-old rats had a greater ^{3}H-TC content and ^{14}C-proline incorporation than the 8 month-old animals. Moreover, lower ^{3}H-TC content was found in the animals fed 300 ppm zinc compared to the ones fed 10 ppm zinc. The ^{14}C-proline uptake was not altered by dietary zinc levels.

Based on the multivariate analyses the bones of 2 month-old rats had a higher zinc concentration than that observed for 8 month-old rats (Figure 4). As expected the animals fed 300 ppm zinc had greater zinc concentration in the bones than those fed 10 ppm zinc. Moreover, a significant ($P<0.05$) interaction between dietary zinc levels and age of rats was observed for zinc concentration in scapulas and humeri (Figure 4). There was a higher concentration of

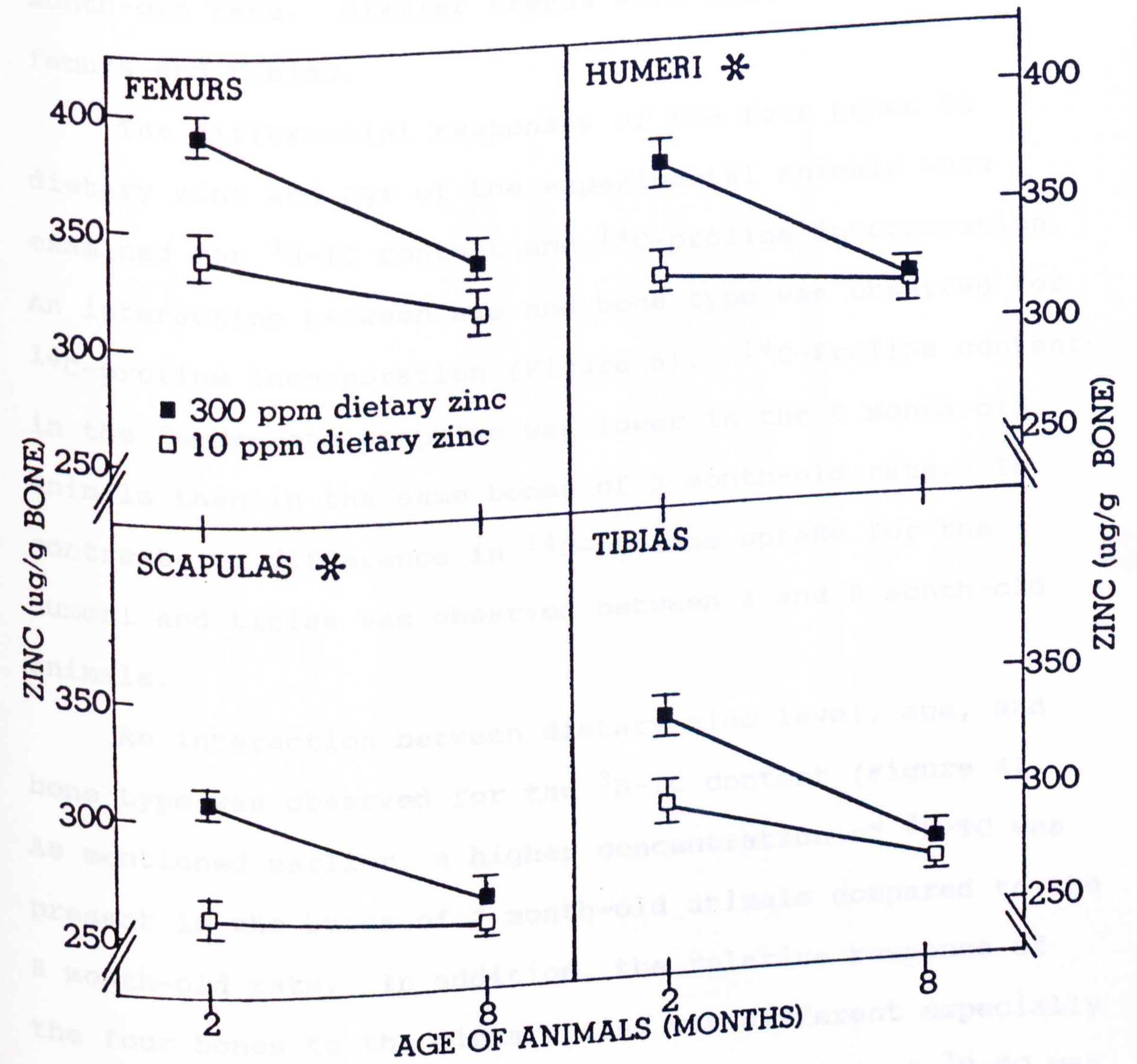
TABLE 3. Calcium concentration, ^{3}H-tetracycline content and ^{14}C-proline uptake in the femurs, humeri, scapulas, and tibias of 2 and 8 month-old female Sprague-Dawley rats fed 10 and 300 ppm zinc (experiment 1).

DIETARY ZINC (ppm)	Calcium (mg/g)		^{3}H-TC($x10^{3}$DPM/g)		^{14}C-Proline ($x10^{3}$DPM/g)	
	AGE OF ANIMALS (months)					
	2	8	2	8	2	8
Femur						
10	272±6.3(10)[1]	271±6.2(9)	51±7.4(10)	37±7.2(9)	64±2.8(10)	54±2.8(9)
300	277±6.7(10)	264±6.1(10)	59±7.8(10)	25±7.5(10)	64±3.3(10)	** 58±2.7(10)
Humeri						
10	234±5.3(10)	239±5.2(9)	64±8.8(10)	25±8.6(9)	101±4.8(10)	101±4.7(9)
300	235±5.6(10)	238±5.1(10)	102±9.3(10)	** 19±9.0(10)	108±5.6(10)	107±4.7(10)
Scapulas						
10	233±2.9(10)	238±2.9(9)	79±5.5(10)	49±5.4(9)	85±4.7(10)	71±4.6(9)
300	237±3.1(10)	240±2.8(10)	62±5.8(10)	** 33±5.6(10) a	100±5.4(10)	** 71±4.6(10)
Tibias						
10	236±6.0(10)	232±5.9(9)	52±5.7(10)	25±5.6(9)	93±3.5(10)	89±3.4(9)
300	234±6.4(10)	223±5.8(10)	49±6.1(10)	** 9±5.8(10)	99±4.0(10)	92±3.4(10)

Sources of variation	Multivariate analyses of variance for the four bones, P-value		
Zinc	NS	0.01	NS
Age	NS	0.01	0.01
Zinc*Age	NS	NS	NS

1 The values presented are least square means (LSM)±SEM (number of rats per group).
** Significant ($P<0.01$) difference in the 2 and 8 month-old rats when the univariate analysis was performed.
a Significant ($P<0.01$) difference in the rats fed 10 and 300 ppm zinc when the univariate analysis was performed.

Figure 4: The interaction between 10 or 300 ppm dietary zinc and 2 or 8 month-old female rats on the zinc concentration of the femurs, humeri, scapulas, and tibias (experiment 1).
* P<0.05



zinc in the humeri and scapulas of the 2 month-old rats fed 300 ppm zinc than those fed 10 ppm zinc. However, dietary zinc level had no effect on bone zinc concentration in 8 month-old rats. Similar trends were also observed for the femurs and tibias.

The differential responses of the four bones to dietary zinc and age of the experimental animals were examined for ^{3}H-TC content and ^{14}C-proline incorporation. An interaction between age and bone type was observed for ^{14}C-proline incorporation (Figure 5). ^{14}C-Proline content in the femurs and scapulas was lower in the 8 month-old animals than in the same bones of 2 month-old rats. In contrast, no difference in ^{14}C-proline uptake for the humeri and tibias was observed between 2 and 8 month-old animals.

An interaction between dietary zinc level, age, and bone type was observed for the ^{3}H-TC content (Figure 6). As mentioned earlier, a higher concentration of ^{3}H-TC was present in the bones of 2 month-old animals compared to the 8 month-old rats. In addition, the relative response of the four bones to the dietary zinc was different especially in the 2 month-old animals. A lower retention of ^{3}H-TC was observed in the scapulas and tibias of 2-month old rats fed 300 ppm zinc as compared to rats of the same age which were fed 10 ppm zinc. In contrast, the 2 month-old rats fed 300 ppm zinc had higher ^{3}H-TC content in the humeri than the 2 month-old rats fed 10 ppm zinc. The significant ($P<0.05$)

Figure 5: An interaction ($P<0.05$) between bone type and age of female rats for ^{14}C-proline content (experiment 1).

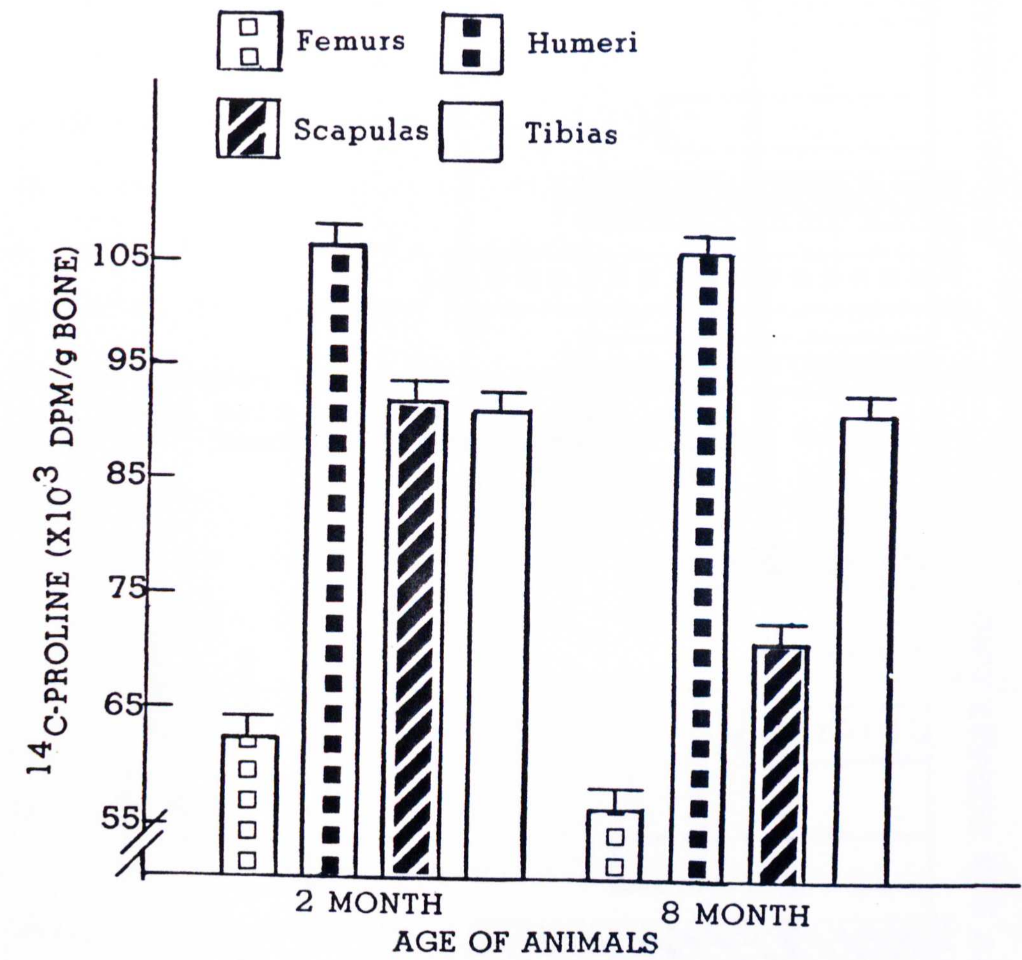
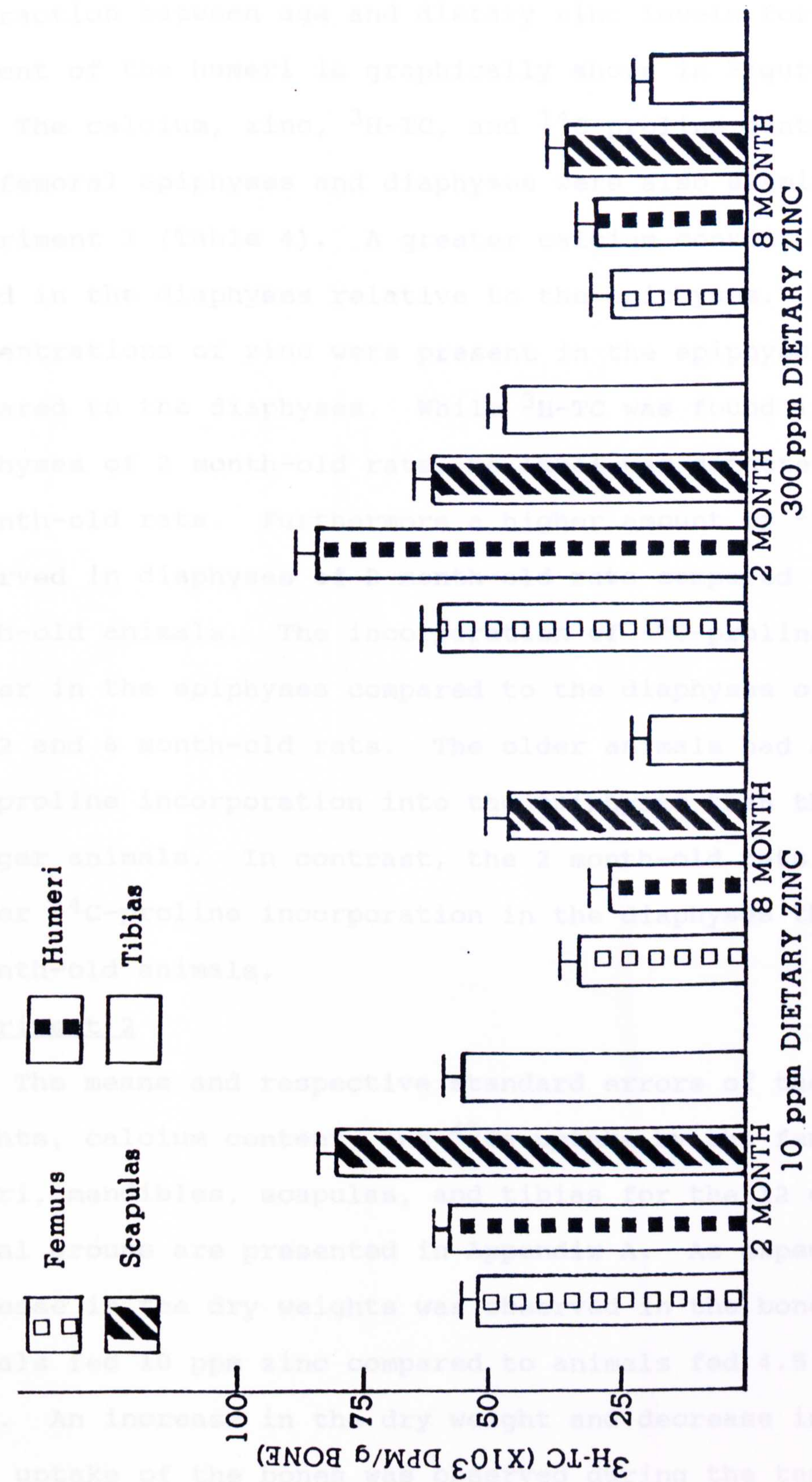


Figure 6: An interaction ($P<0.05$) between bone type, age, and dietary zinc level on the ^{3}H-tetracycline content (experiment 1).



interaction between age and dietary zinc levels for ^{3}H-TC content of the humeri is graphically shown in Figure 7.

The calcium, zinc, ^{3}H-TC, and ^{14}C-proline content of the femoral epiphyses and diaphyses were also examined in experiment 1 (Table 4). A greater calcium content was found in the diaphyses relative to the epiphyses. Higher concentrations of zinc were present in the epiphyses compared to the diaphyses. While ^{3}H-TC was found in epiphyses of 2 month-old rats, no label was detected in the 8 month-old rats. Furthermore a higher amount of ^{3}H-TC was observed in diaphyses of 2 month-old rats compared to 8 month-old animals. The incorporation of ^{14}C-proline was higher in the epiphyses compared to the diaphyses of both the 2 and 8 month-old rats. The older animals had a higher ^{14}C-proline incorporation into the epiphyses than the younger animals. In contrast, the 2 month-old rats had higher ^{14}C-proline incorporation in the diaphyses than the 8 month-old animals.

Experiment 2

The means and respective standard errors of the dry weights, calcium content, and ^{45}Ca uptake by the femurs, humeri, mandibles, scapulas, and tibias for the 12 experimental groups are presented in Appendix A. As expected, an increase in the dry weights was observed in the bones of animals fed 10 ppm zinc compared to animals fed 4.5 ppm zinc. An increase in the dry weight and decrease in the ^{45}Ca uptake of the bones was observed during the ten day

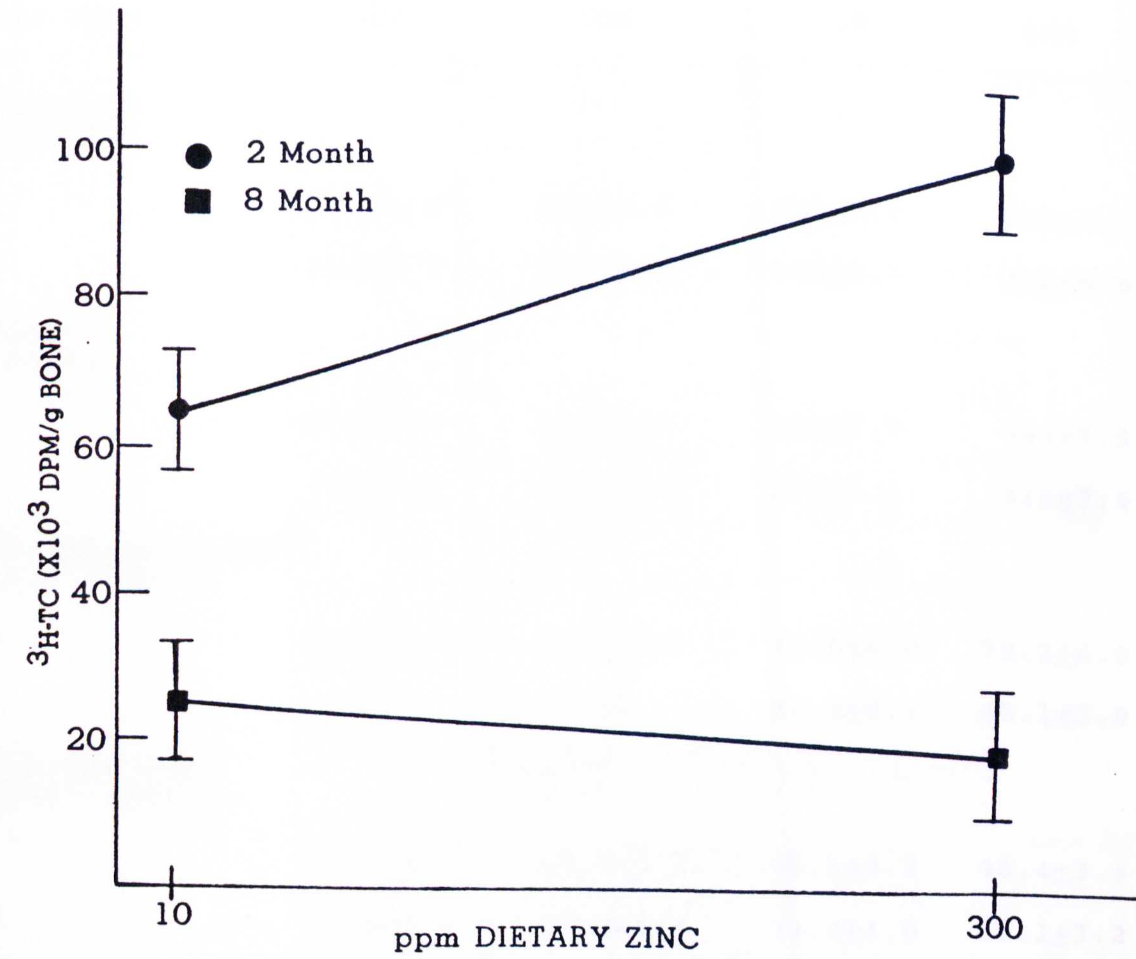


Figure 7: The interaction ($P<0.05$) between 10 or 300 ppm dietary zinc and 2 or 8 month-old female rats on the ^{3}H-tetracycline content of the humeri (experiment 1).

TABLE 4. Calcium concentration, zinc concentration, ^{3}H-tetracycline content and ^{14}C-proline incorporation in the femoral epiphyses-metaphyses and diaphyses of 2 and 8 month-old female Sprague-Dawley rats fed 10 and 300 ppm zinc (experiment 1).

	Epiphyses-Metaphyses		Diaphyses	
	DIETARY ZINC LEVEL (ppm)			
Age (month)	10	300	10	300
Calcium[1] (mg/g)				
2	257±5.4[4]	257±5.5	282±5.4	290±5.5
8	255±5.2	250±5.5	285±5.2	272±5.5
Zinc[1] (µg/g)				
2	358±7.7	420±7.9	324±7.7	393±7.9
8	326±7.4	341±7.9	296±7.4	318±7.9
^{3}H-Tetracycline[2] ($x10^{3}$ DPM/g)				
2	30.0±8.5	6.6±9.4	75.0±6.0	72.2±6.0
8	ND	ND	56.2±5.7	45.1±5.8
^{14}C-Proline[3] ($x10^{3}$ DPM/g)				
2	79.4±3.2	80.6±3.4	48.8±3.2	48.4±3.4
8	88.4±3.0	80.5±3.2	33.3±3.0	38.1±3.2

1. The calcium and zinc concentration was significantly ($P<0.01$) different in the epiphyses and diaphyses.

2. Not detectable (ND), factorial ANOVA not appropriate.

3. Interaction between age and bone section significant at $P<0.01$.

4. Mean ±SEM. The number of rats in each group are as follows: 2 month, 10 ppm zinc - 14; 2 month, 300 ppm zinc - 14; 8 month, 10 ppm zinc - 15; 8 month, 300 ppm zinc - 14.

period.

A multivariate analyses of calcium concentration of the bones indicated that a trend ($P<0.07$), due to dietary vitamin D source was present for the five bones examined (Figure 8). A lower calcium content was found in the femurs when the diets were supplemented with $1,25(OH)_2D$. A similar trend was observed for the scapulas and mandibles, but conflicting results were found for the tibias and humeri.

An interaction between dietary zinc levels and vitamin D type was observed for ^{45}Ca uptake in the five bones examined. The difference in ^{45}Ca uptake between the vitamin D types was reduced at 10 ppm zinc for the humeri, scapulas, and mandibles, but not for the femurs and tibias. For the latter two bones, there was higher ^{45}Ca uptake for the $1,25(OH)_2D$ compared to D_3 at 10 ppm zinc.

In experiment 2, the results from the enzyme assays performed on the implants showed an increase in the alkaline phosphatase activity from day 11 to 14 followed by a decrease from day 14-21 (Figure 9A). A similar pattern was present for the uptake of ^{45}Ca. An accumulation of calcium and zinc from day 11 to 21 was also observed (Figure 9B).

Histological sections were prepared from the implants harvested 11, 14, and 21 days after implantation. Chondroblasts and chondrocytes were present in the implant obtained 11 days after implantation, indicating formation

Figure 8: Calcium concentration of the femurs, humeri, mandibles, scapulas, and tibias of female rats fed D_3 or $1,25(OH)_2D$ (experiment 2).
* $P<0.05$

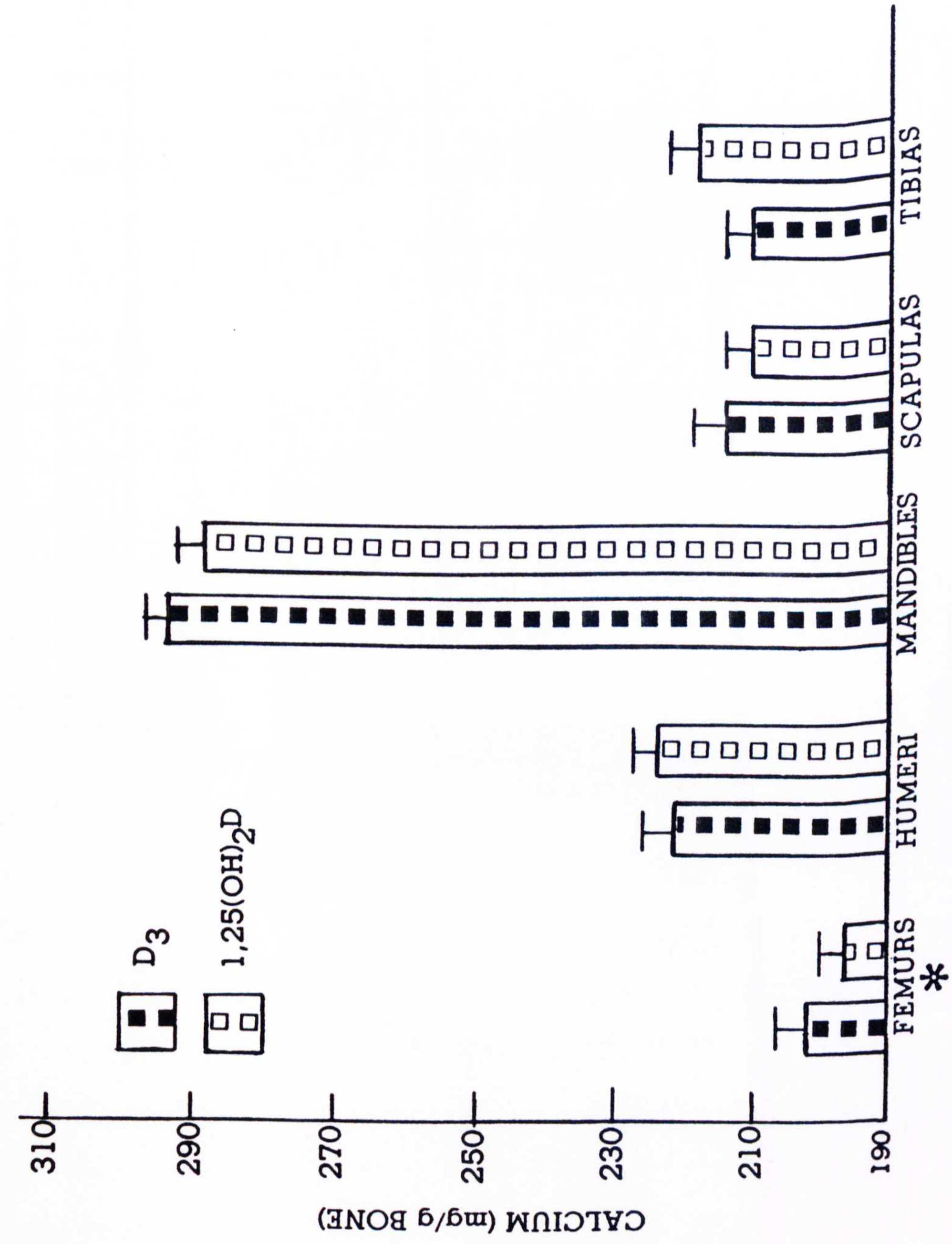
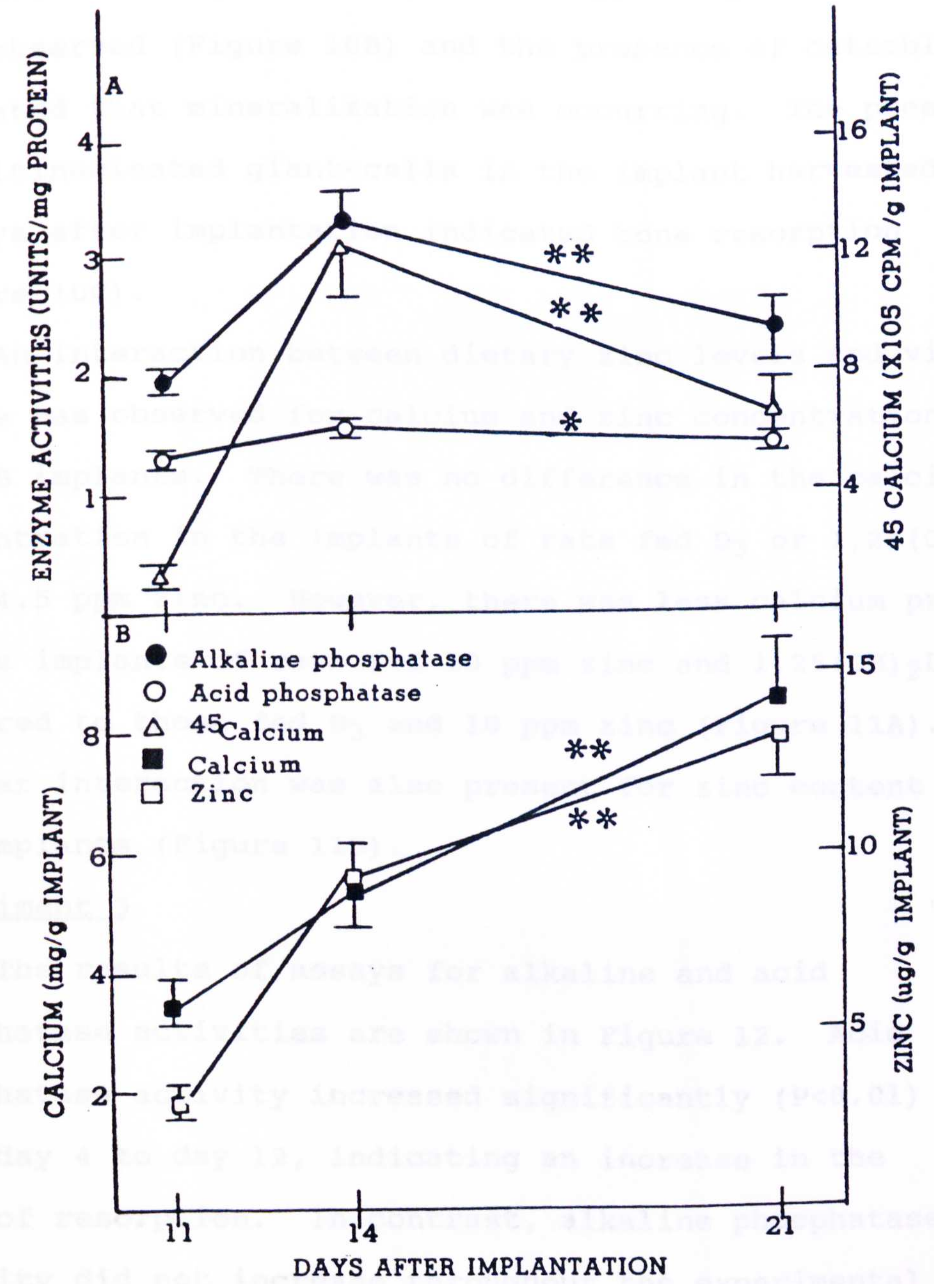


Figure 9A: Alkaline phosphatase, acid phosphatase, and ^{45}calcium uptake in demineralized implants 11, 14, and 21 days after implantation (experiment 2).
** P<0.01
* P<0.05

Figure 9B: Calcium and zinc content in demineralized implants 11, 14, and 21 days after implantation (experiment 2).
** P<0.05



of cartilage (Figure 10A). Fourteen days after implantation degenerating chondrocytes and hypertrophied cartilage were observed (Figure 10B) and the presence of osteoblasts indicated that mineralization was occurring. The presence of multinucleated giant-cells in the implant harvested at 21 days after implantation indicated bone resorption (Figure 10C).

An interaction between dietary zinc levels and vitamin D type was observed for calcium and zinc concentrations in the DB implants. There was no difference in the calcium concentration in the implants of rats fed D_3 or $1,25(OH)_2D$ with 4.5 ppm zinc. However, there was less calcium present in the implants of rats fed 10 ppm zinc and $1,25(OH)_2D$ as compared to those fed D_3 and 10 ppm zinc (Figure 11A). A similar interaction was also present for zinc content of the implants (Figure 11B).

Experiment 3

The results of assays for alkaline and acid phosphatase activities are shown in Figure 12. Acid phosphatase activity increased significantly ($P<0.01$) from day 4 to day 12, indicating an increase in the rate of resorption. In contrast, alkaline phosphatase activity did not increase throughout the experimental period, reflecting a low rate of bone formation in the MB implants.

Experiment 4

In experiment 4, the two types of implants used in

Figure 10A: Photomicrograph of demineralized bone implant harvested 11 days after implantation (x160). Chondroblasts and chondrocytes in close apposition to the implanted matrix (experiment 2).

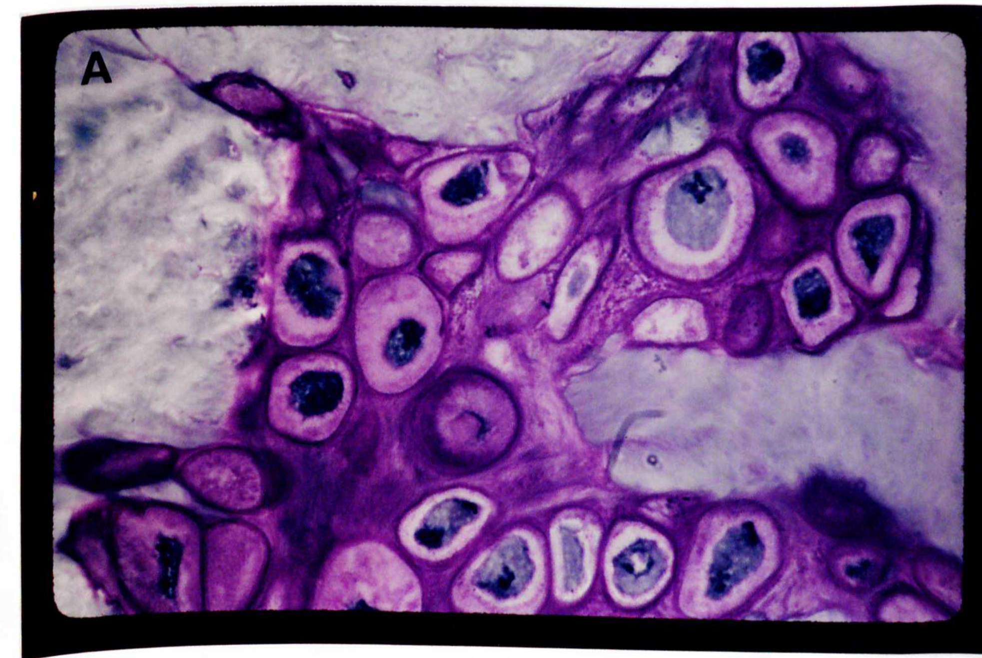


Figure 10B: Photomicrograph of demineralized bone implant harvested 14 days after implantation (x160). Degenerating chondrocytes and hypertrophied cartilage are seen (experiment 2).

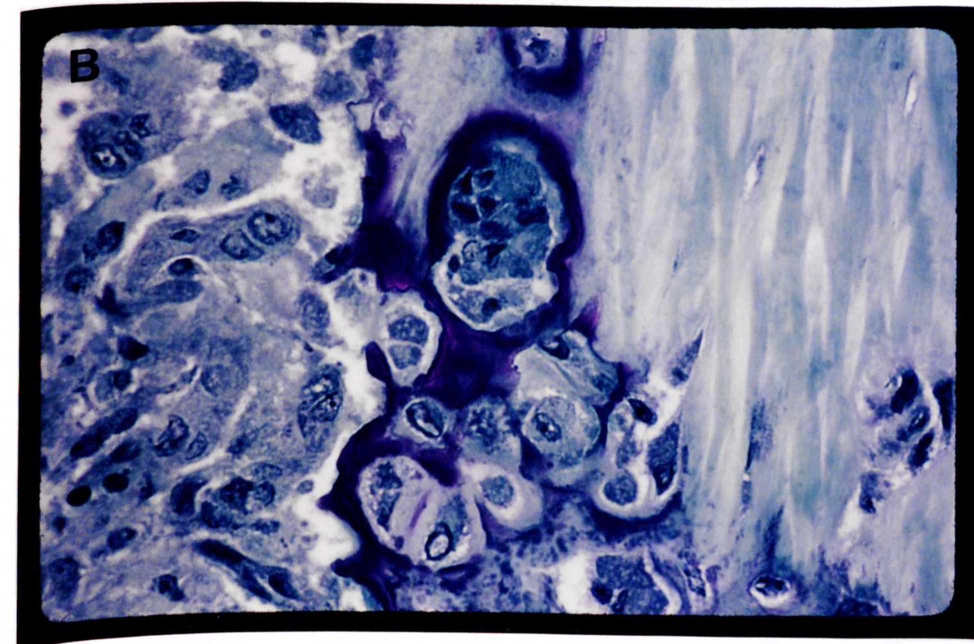


Figure 10C: Photomicrograph of demineralized bone implant harvested 21 days after implantation (x160). Multinucleated giant cells (arrows) were present in these implants (experiment 2).

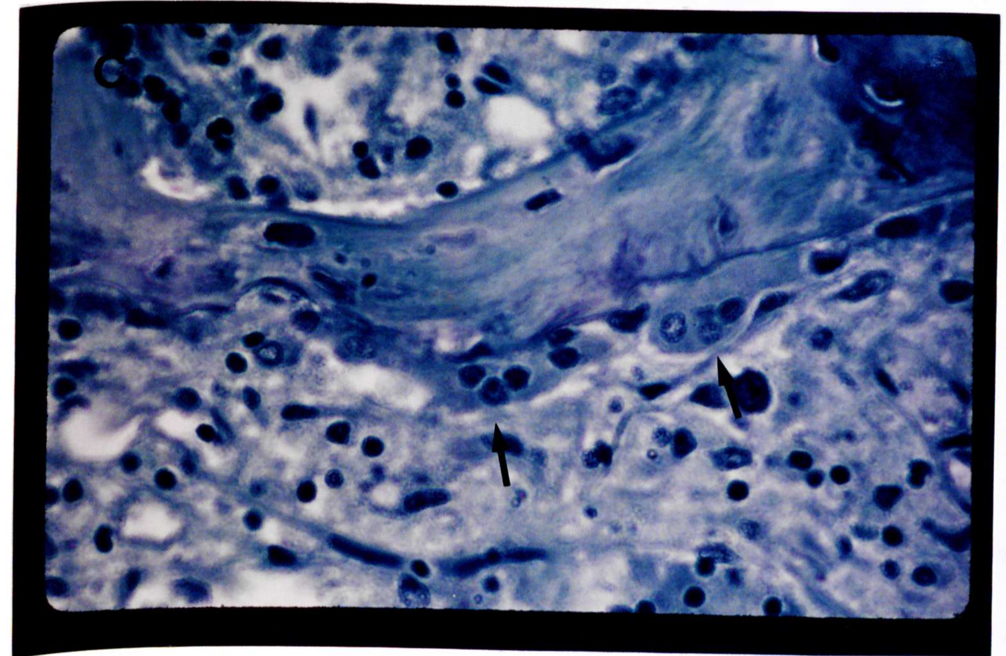
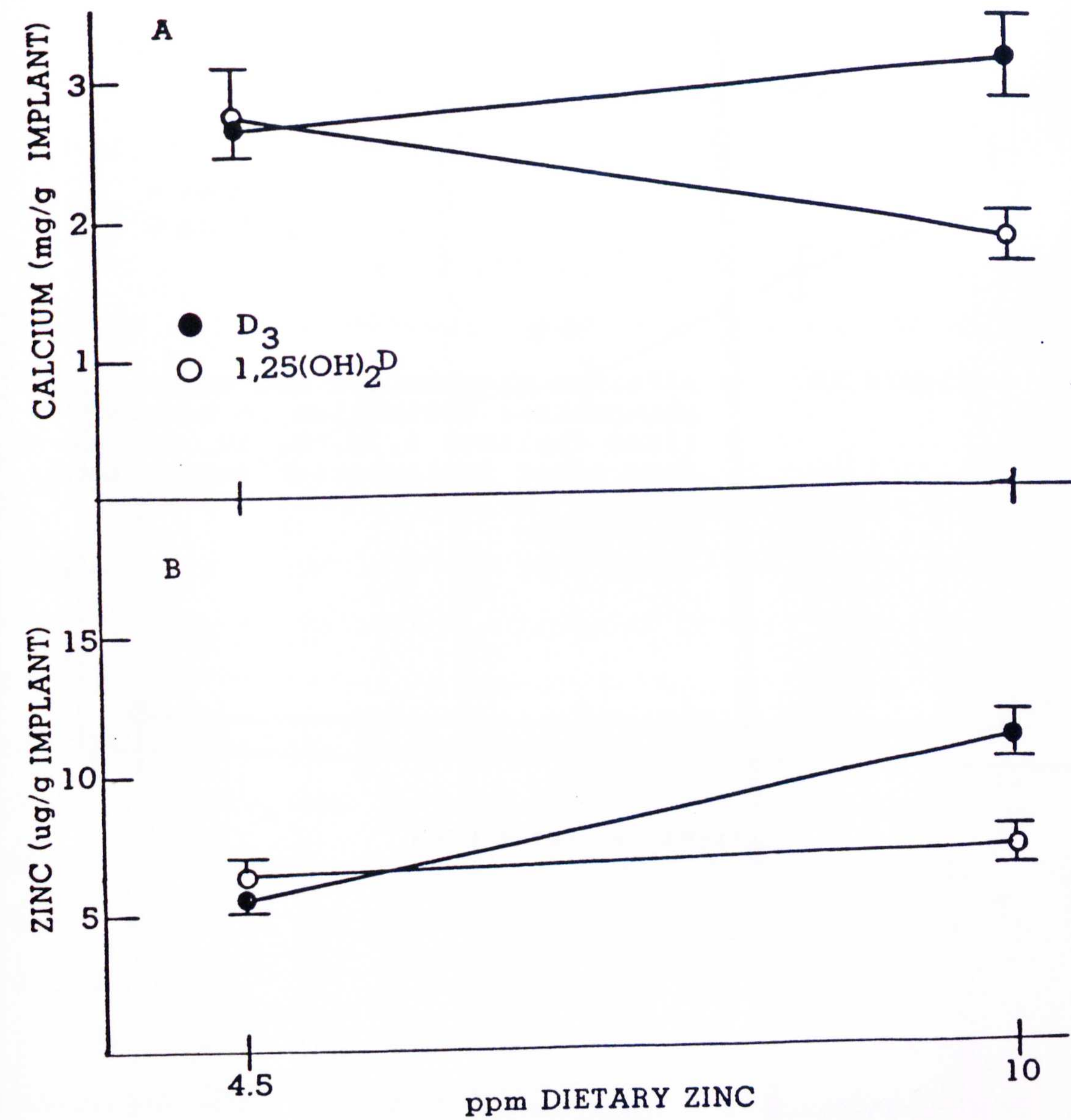


Figure 11A: An interaction ($P<0.05$) between dietary zinc level and vitamin D type for calcium concentration of the demineralized implants (experiment 2).

Figure 11B: An interaction ($P<0.05$) between dietary zinc level and vitamin D type on the zinc concentration of the demineralized implants (experiment 2).



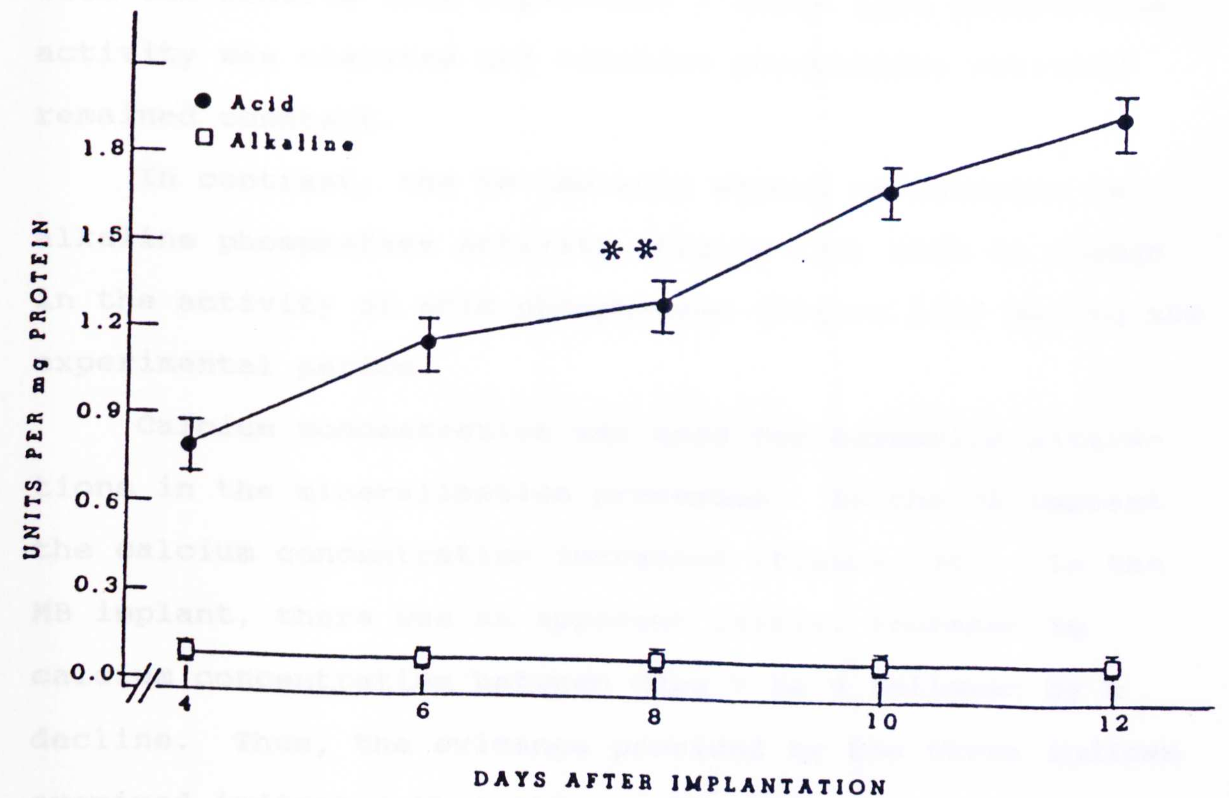


Figure 12: Alkaline phosphatase and acid phosphatase activities in mineralized implants 4, 6, 8, 10, and 12 days after implantation (experiment 3).
** P<0.01

experiments 2 and 3 were combined in each experimental animal to study bone dynamics. Thus, both bone formation and resorption were examined simultaneously.

In the MB implants acid phosphatase activity increased dramatically (Figure 13A), while alkaline phosphatase activity remained low (Figure 13B). This is consistent with the results from Experiment 3 where acid phosphatase activity was elevated and alkaline phosphatase activity remained constant.

In contrast, the DB implants showed an increase in alkaline phosphatase activity (Figure 13B) with no change in the activity of acid phosphatase (Figure 13A) during the experimental period.

Calcium concentration was used for assessing alterations in the mineralization processes. In the DB implant the calcium concentration increased (Figure 13C). In the MB implant, there was an apparent initial increase in calcium concentration between days 7 to 9 followed by a decline. Thus, the evidence provided by the three indices examined indicates that this system did reflect bone dynamics and allows assessment of bone formation and resorption.

Histological observations from experiment 2 were consistent with the biochemical data. Chondrocytes were visible on day 9 in the DB implant indicating early stage of endochondral bone formation, (Figure 14A). Giant multinucleated cells of a MB implant were evident by day 9

Figure 13A: Acid phosphatase activity in mineralized and demineralized implants 7, 9, 11, and 13 days after implantation. The interaction between implant type and time was significant at P<0.01 (experiment 4).

Figure 13B: Alkaline phosphatase activity in mineralized and demineralized implants 7, 9, 11, and 13 days after implantation. The interaction between implant type and time was significant at P<0.01 (experiment 4).

Figure 13C: Calcium content of mineralized and demineralized implants 7, 9, 11, and 13 days after implantation. The interaction between implant type and time was significant at P<0.05 (experiment 4).

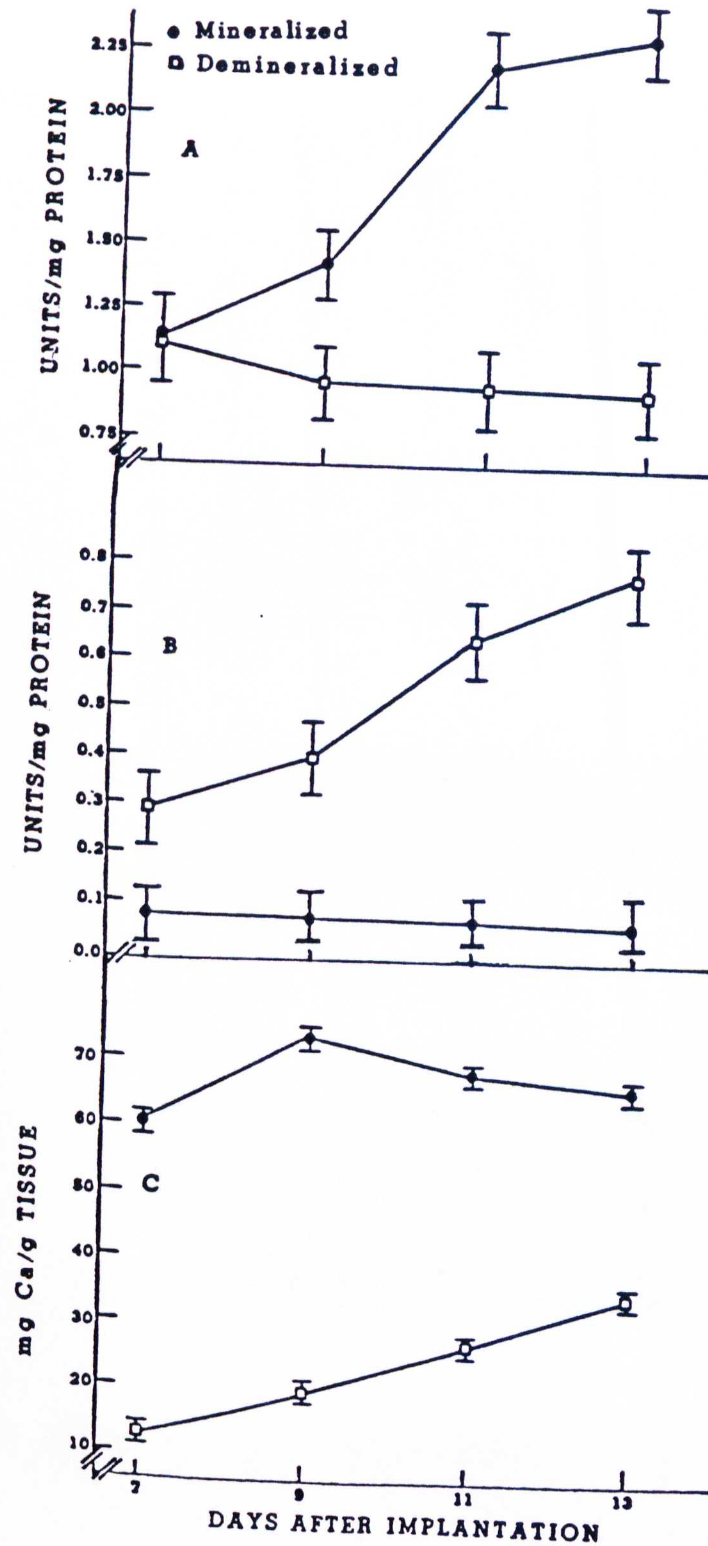
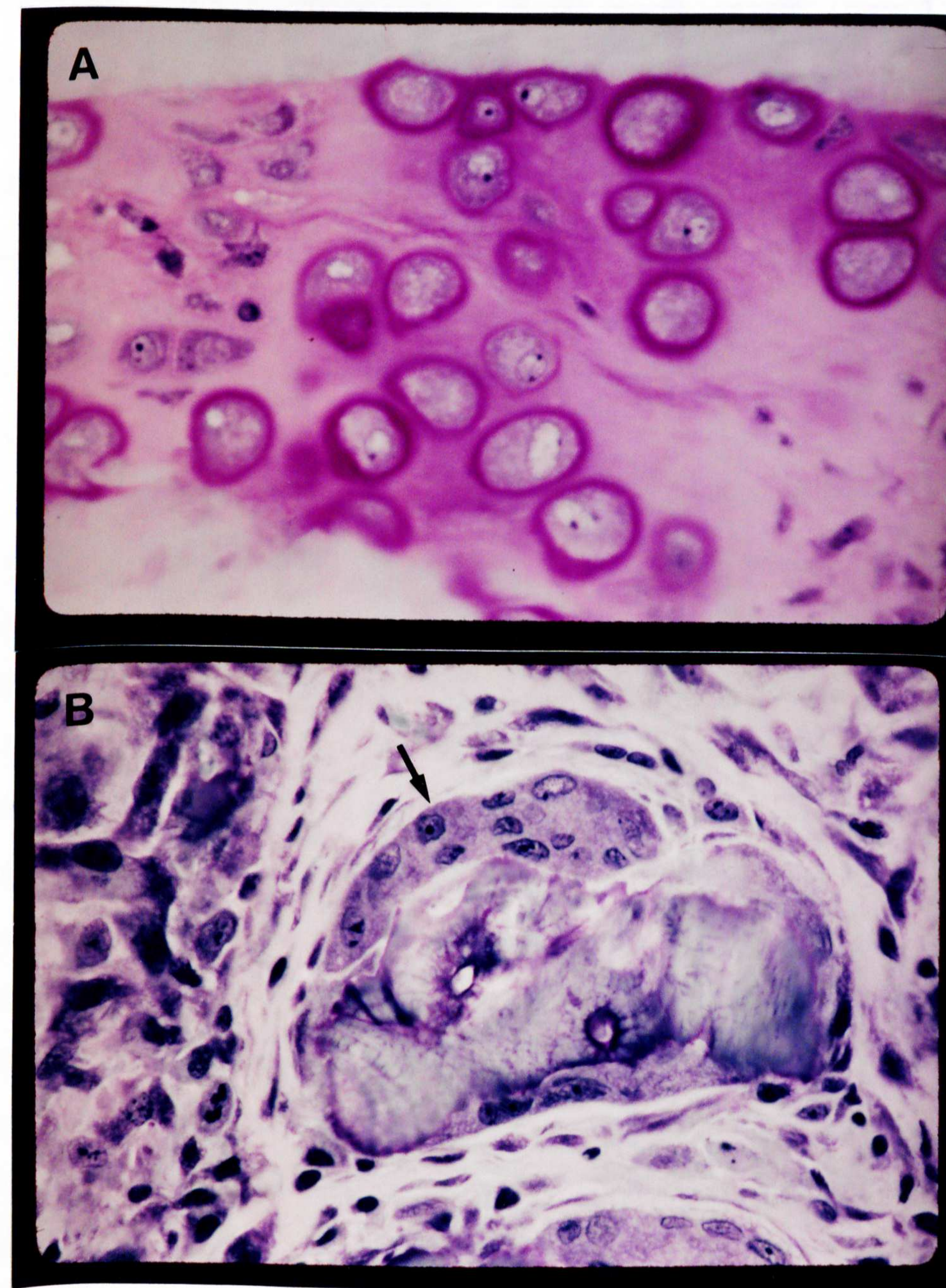


Figure 14A: Photomicrograph of demineralized bone implant 9 days after implantation (x160). Cartilage cells in close apposition to the implanted matrix (experiment 4).

Figure 14B: Photomicrograph of mineralized bone implant 9 days after implantation (x160). Multinucleated osteoclastic giant cells (arrow) in the vicinity of bone mineral (experiment 4).



(Figure 14B). These cells are generally considered to be osteoclastic as they play a role in bone resorption and produce acid phosphatase. Four days later (day 13) the DB implant demonstrated osteoblasts with the occurrence of bone formation and was consistent with increased alkaline phosphatase activity (Figure 14C). In contrast, on the same day the MB implant had visible osteoclastic giant cells (Figure 14D) involved in erosion of bone mineral and production of acid phosphatase.

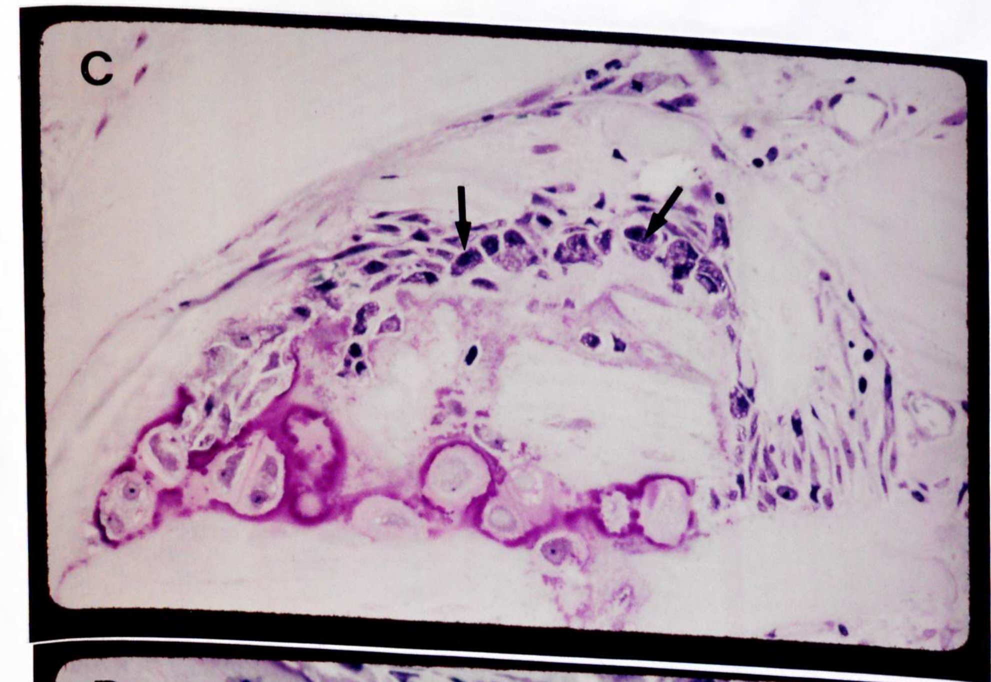
Experiment 5

A significant interaction ($P<0.05$) between the type of implant (DB and MB) and the dietary calcium level was observed for the acid phosphatase activity (Figure 15A). When rats were fed a 0.2% calcium diet, there was a greater difference between DB and MB implants than when these rats were fed 1.0% calcium diet. This is due to increased resorption of MB implants in the animals fed 0.2% compared to 1.0% calcium. No significant interactions for alkaline phosphatase activity or calcium concentrations were observed. The arithmetic means and standard errors for the enzyme activities and calcium concentration of the MB and DB implants in the four experimental groups are shown in Appendix B.

A significant interaction ($P<0.05$) between the type of implant and the dietary vitamin D was observed (Figure 15B) for calcium concentration. Rats fed D_3, showed a greater difference between the DB and MB implants than when fed

Figure 14C: Photomicrograph of demineralized matrix 13 days after implantation (x100). Osteoblasts (arrows) were also seen (experiment 4).

Figure 14D: Photomicrograph of mineralized bone implant 13 days after implantation (x100). Osteoclastic giant cells (arrows) were observed (experiment 4).



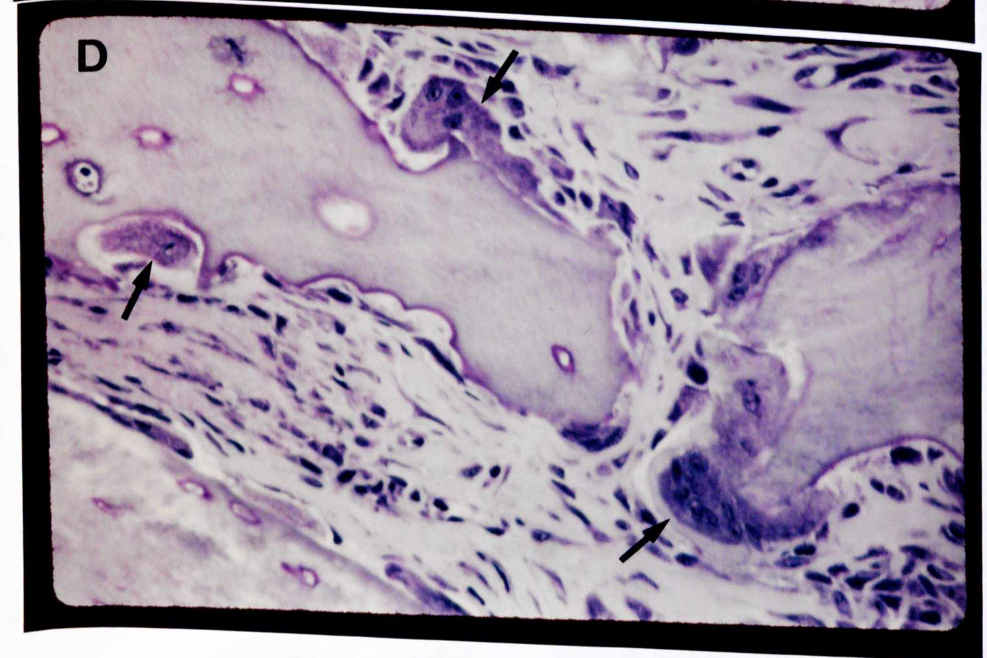
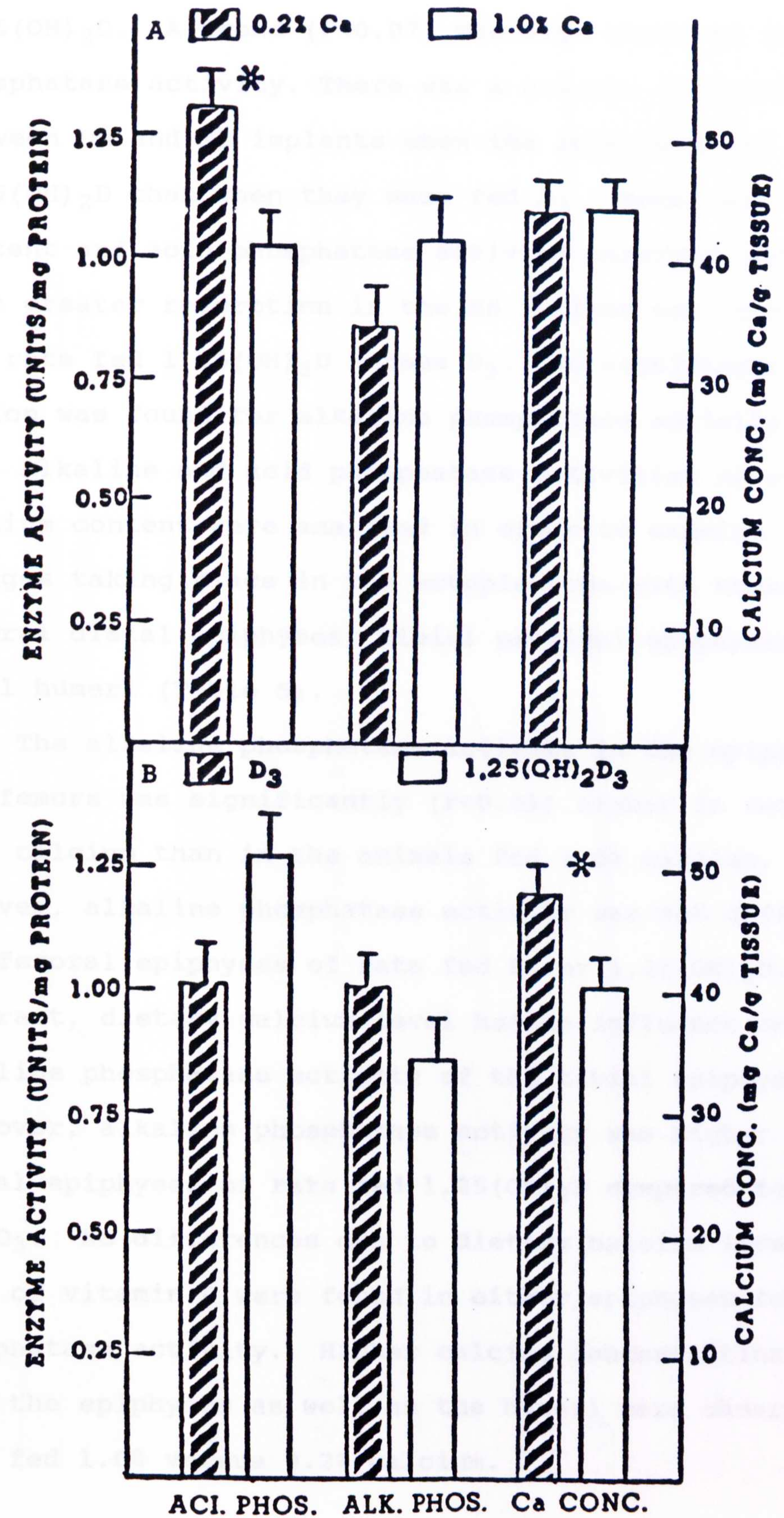


Figure 15A: The net difference between demineralized and mineralized implants for acid phosphatase ($P<0.05$), alkaline phosphatase activity and calcium concentration when rats were fed 0.2% versus 1.0% calcium (experiment 5).

Figure 15B: The net difference between demineralized and mineralized implants for acid phosphatase ($P<0.07$), alkaline phosphatase activity, and calcium concentration ($P<0.05$) when rats were fed D_3 versus $1,25(OH)_2D$ (experiment 5).



$1,25(OH)_2D$. A trend ($P<0.07$) was also observed for acid phosphatase activity. There was a greater difference between DB and MB implants when the rats were fed $1,25(OH)_2D$ than when they were fed D_3. Both calcium content and acid phosphatase activity observed indicates that greater resorption in the MB implant was occurring in the rats fed $1,25(OH)_2D$ versus D_3. No significant interaction was found for alkaline phosphatase activity.

Alkaline and acid phosphatase activities as well as calcium content were analyzed in order to examine the changes taking place in the ectopic bone with those in the femoral distal epiphyses, tibial proximal epiphyses, and total humeri (Table 5).

The alkaline phosphatase activity in the epiphyses of the femurs was significantly ($P<0.01$) higher in rats fed 0.2% calcium than in the animals fed 1.0% calcium. However, alkaline phosphatase activity was not different in the femoral epiphyses of rats fed D_3 or $1,25(OH)_2D$. In contrast, dietary calcium level had no influence on the alkaline phosphatase activity of the tibial epiphyses. Moreover, alkaline phosphatase activity was higher in the tibial epiphyses of rats fed $1,25(OH)_2D$ compared to rats fed D_3. No differences due to dietary calcium level or the form of vitamin D were found in either epiphyses for acid phosphatase activity. Higher calcium concentrations in both the epiphyses as well as the humeri were observed for rats fed 1.0% versus 0.2% calcium.

TABLE 5. Alkaline phosphatase, acid phosphatase and calcium concentration in the femoral and tibial epiphyses and calcium content of the humeri in animals fed D_3 or $1,25(OH)_2D$ and 0.2% or 1.0% calcium (experiment 5).

	Alkaline phosphatase (units/mg protein)		Acid phosphatase (units/mg protein)		Calcium (mg/g)	
	PERCENT DIETARY CALCIUM					
	0.2	1.0	0.2	1.0	0.2	1.0
Femoral Epiphyses						
D_3	2.09±.114	1.54±.114	1.23±.043	1.14±.043	79±4.5	90±4.5
$1,25(OH)_2D$	1.74±.114	1.49±.119	1.10±.043	1.12±.045	78±4.7	93±4.7
	**					
Tibial Epiphyses						
D_3	1.85±.157	1.72±.157	1.09±.040	1.13±.040	90±3.2	97±3.2
$1,25(OH)_2D$	2.25±.157	2.06±.164	1.15±.040	1.18±.042	92±3.2	102±3.3
	a					
Humeri						
D_3	-	-	-	-	234±3.4	242±3.4
$1,25(OH)_2D$	-	-	-	-	238±3.4	245±3.6
					**	

1. The values presented are LSM±SEM. There are 12 rats in each group except the group fed 1.0% calcium and $1,25(OH)_2D$ which had 11 rats.

** Significant ($P<0.01$) difference in the implants and bones of rats fed 0.2% and 1.0% calcium.

a Significant ($P<0.05$) difference in the implants and bones of rats fed D_3 and $1,25(OH)_2D$.

Experiment 6

Based on the multivariate analyses, no influence of dietary calcium level was observed on the dry weight, calcium concentration, or ^{45}Ca uptake (Table 6). As expected the bones of the old rats had significantly ($P<0.05$) higher dry weights than the bones of young animals. Lower calcium concentration and ^{45}Ca uptake were also observed in the bones of the old rats compared to the bones of the young animals.

The differential response due to the age of the animal and dietary calcium level was examined for the femurs, humeri, scapulas, and tibias. An the interaction between the age of rats and bone type was significant ($P<0.05$) for calcium concentration (Figure 16A). A trend ($P<0.07$) was observed for zinc content (Figure 16B). The femurs, humeri, and tibias of the young animals had higher concentrations of calcium and zinc as compared to the old animals. Equivalent calcium content and higher amount of zinc was present in the scapulas of old rats compared to the scapulas of young animal.

An interaction between dietary calcium, age, and bone type was observed for ^{45}Ca uptake (Figure 17). The major effect was due to the age of rats as all four bones of the old animals had a lower uptake of ^{45}Ca compared to the young rats. The four bones of the young and old rats responded differently for ^{45}Ca uptake when these animals were fed the two levels of calcium. A lower uptake of ^{45}Ca

TABLE 6. *Dry weight, calcium concentration and ^{45}calcium uptake in the femurs, humeri, scapulas and tibias of young and old female Long-Evans rats fed 0.2% and 1.0% calcium* (experiment 6).

	Dry Weight (mg)		Calcium (mg/g)		^{45}Calcium (x10^{4} CPM/g)	
	PERCENT DIETARY CALCIUM					
	0.2	1.0	0.2	1.0	0.2	1.0
Femur						
Young	473±15.3(14)[1]	508±15(14)	256±3.3(14)	256±3.3(14)	360±9.5(14)	326±9.5(14)
Old	588±16.6(12)	**[2] a[3] 617±16(13)	253±3.5(12)	** 250±3.4(13)	262±10.3(12)	** b 246±9.8(13)
Humeri						
Young	231±6.7(14)	242±6.7(14)	269±2.2(14)	266±2.2(14)	390±11.6(14)	345±10.6(14)
Old	287±7.2(12)	** 288±7.0(13)	259±2.3(12)	268±2.2(13)	251±11.5(12)	**a 253±11.0(13)
Scapulas						
Young	125±5.8(14)	124±5,8(14)	237±5.3(14)	250±5.3(14)	355±12.2(14)	239±12.2(14)
Old	150±6.3(12)	** 155±6.0(13)	250±5.7(12)	256±5.5(13)	256±13.2(12)	** 228±12.6(13)
Tibias						
Young	361±1.7(14)	370±1.7(14)	250±3.8(14)	251±3.8(14)	328±10.4(14)	292±10.4(14)
Old	421±1.8(12)	** 343±1.7(13)	241±4.2(12)	* 243±4.0(13)	213±11.2(12)	** a 200±10.8(13)

Sources of variation	Multivariate analysis of variances for the four bones, P-value		
Calcium	NS	NS	NS
Age	0.01	0.01	0.01
Calcium*Age	NS	0.05	0.05

1. The values presented are LSM±SEM (number of rats per group).
2. Significant (*-P<0.05, **-P<0.01) difference in the bones of young and old rats when univariate analyses were performed.
3. Significant (a-P<0.05, b-P<0.01) difference in the bones of rats fed 0.2% and 1.0% calcium when univariate analyses were performed.

Figure 16A: Interaction (P<0.05) between bone type and age of rats on the calcium concentration (experiment 6).

Figure 16B: Interaction (P<0.07) between bone type and age of rats on the zinc concentration (experiment 6).

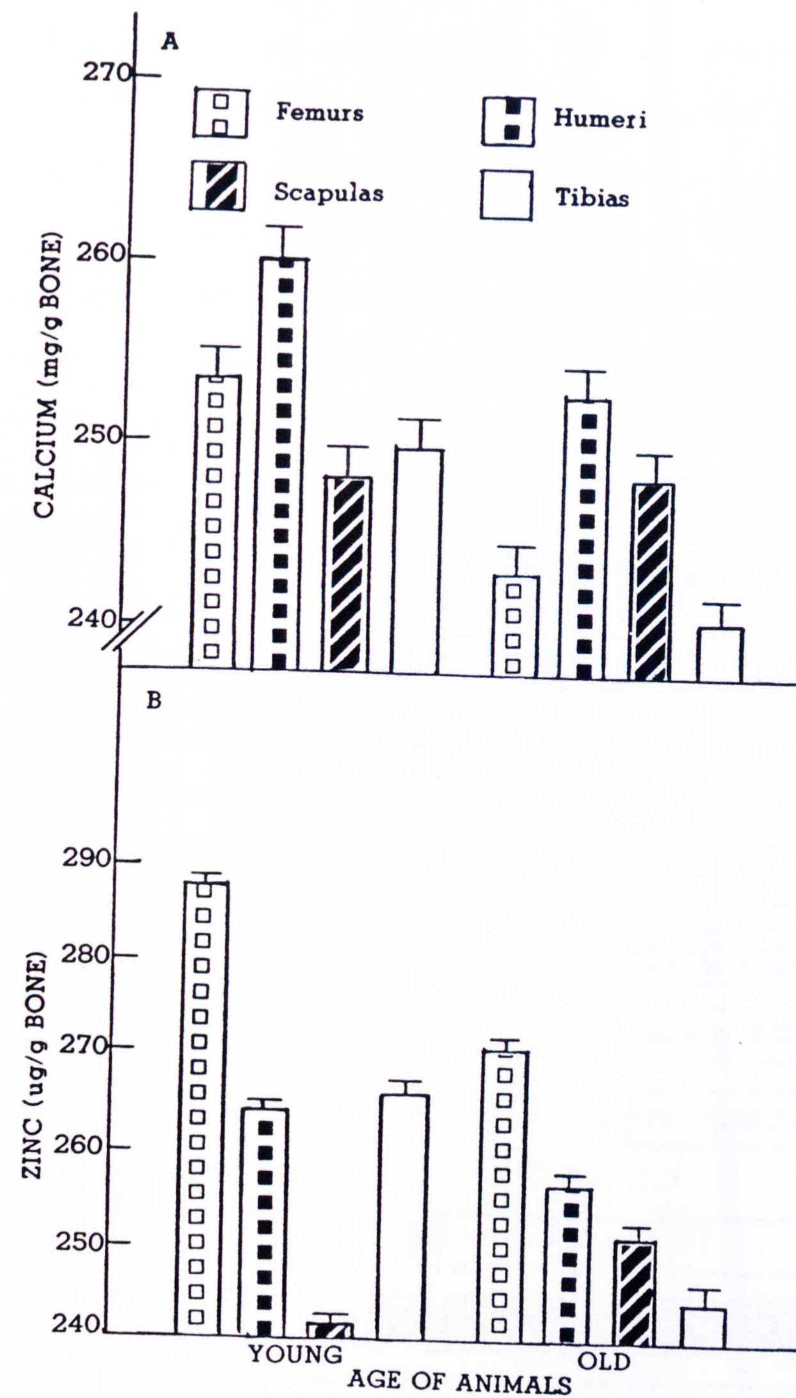
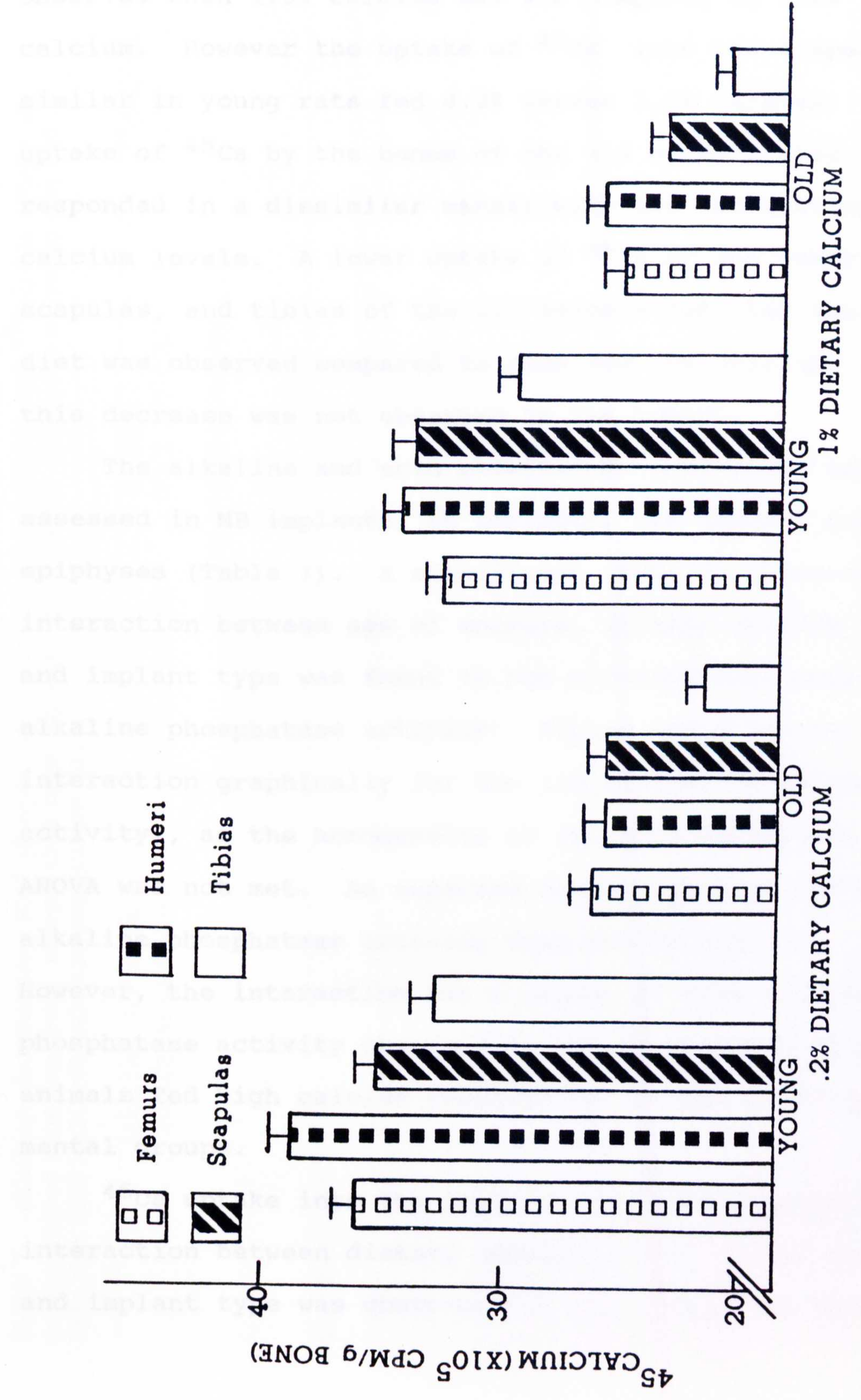


Figure 17: Interaction (P<0.05) between bone type, age of rats, and dietary calcium levels on the ^{45}calcium uptake (experiment 6).



by the femurs, humeri, and tibias of young rats was observed when 1.0% calcium was fed compared to 0.2% calcium. However the uptake of ^{45}Ca into the scapulas was similar in young rats fed 0.2% versus 1.0% calcium. The uptake of ^{45}Ca by the bones of the old animals also responded in a dissimilar manner with the two dietary calcium levels. A lower uptake of ^{45}Ca by the femurs, scapulas, and tibias of the old animals fed high calcium diet was observed compared to ones fed low calcium, whereas this decrease was not observed in the humeri.

The alkaline and acid phosphatase activities were assessed in MB implants, DB implants, and femoral distal epiphyses (Table 7). A significant ($P<0.01$) three-way interaction between age of animals, dietary calcium level, and implant type was found in the untransformed data for alkaline phosphatase activity. Figure 18A shows this interaction graphically for the $\log_{10}$(alkaline phosphatase activity), as the homogeneity of variance assumption for an ANOVA was not met. As expected the DB implants had higher alkaline phosphatase activity than the MB implants. However, the interaction was a result of high alkaline phosphatase activity observed in the DB implants of young animals fed high calcium compared to all the other experimental groups.

^{45}Ca uptake into the implants was also examined. An interaction between dietary calcium level, age of animals, and implant type was observed for the $\log_{10}$(^{45}Ca uptake) as

TABLE 7. Alkaline phosphatase, acid phosphatase, calcium concentration and ^{45}Calcium uptake in demineralized bone implants, mineralized bone implants and femoral epiphyses of young and old rats fed 0.2% and 1.0% calcium (experiment 6).

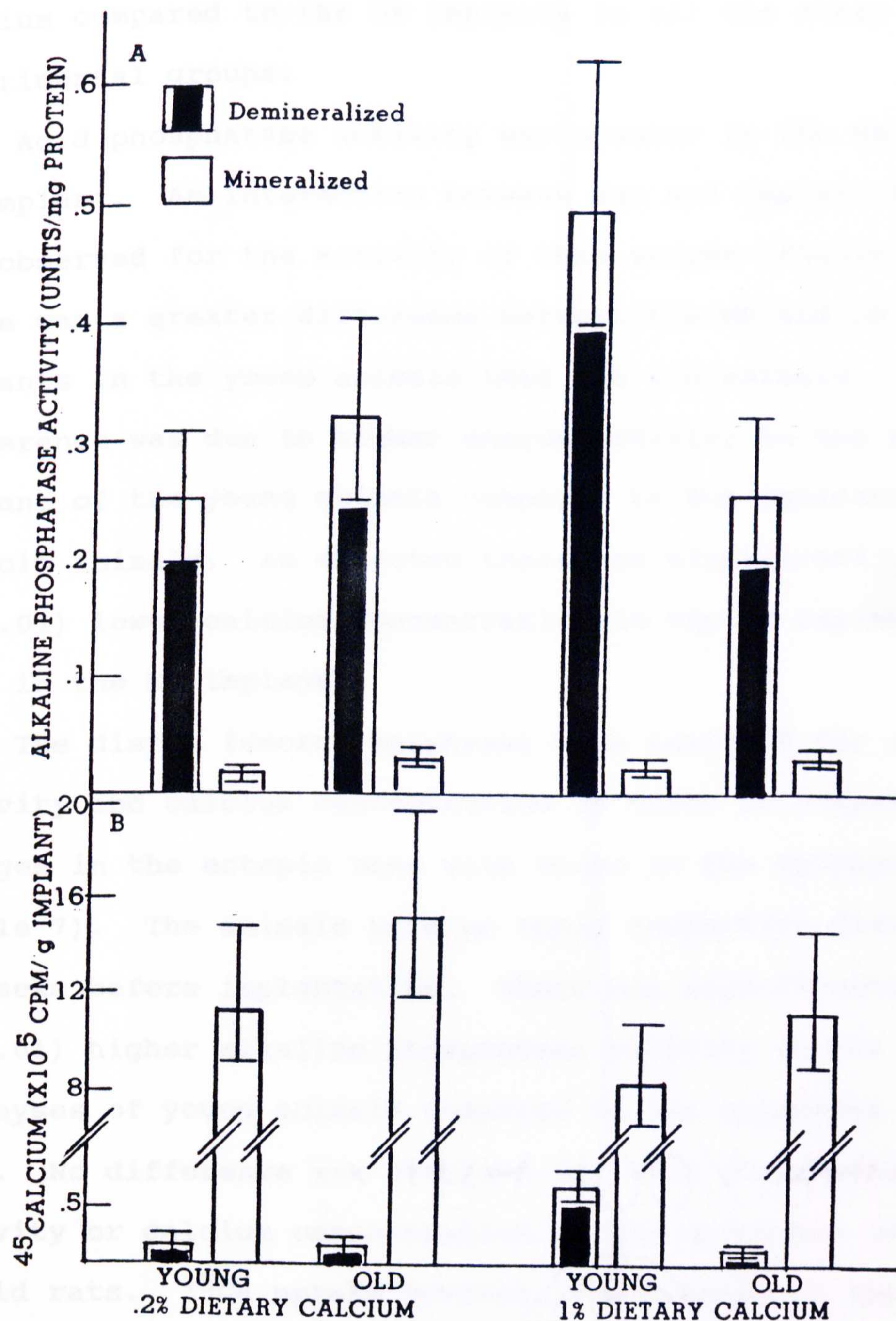
	Alkaline Phosphatase (units/mg protein)		Acid Phosphatase (units/mg protein)		Calcium (mg/g implant)		^{45}Calcium ($\times 10^5$ CPM/g implant)	
	PERCENT DIETARY CALCIUM							
	0.2	1.0	0.2	1.0	0.2	1.0	0.2	1.0
Demineralized[1]								
Young	.28±.038[2]	.65±.117	.87±.026	.92±.079	.12±.019	.20±.048	.35±.15	1.49±.48
Old	.36±.065	.28±.076	.92±.059	.86±.030	.10±.007	.13±.261	.19±.03	.28±.11
Mineralized								
Young	.02±.004	.03±.005	2.40±.156	2.56±.228	70±3.0	70±3.5	12.2±1.24	9.31±1.48
Old	.03±.004	.08±.045	1.92±.165	1.66±.157	67±4.4	60±5.8	15.9 ±1.19	12.9±1.95
Femoral Epiphyses								
Young	1.12±.090	1.02±.090	.93±.029	.94±.029	127±4.1	114±4.1	36.6±1.56	33.6±1.56
Old	.68±.097	.81±.097	.89±.031	.92±.031	122±4.6	127±4.4	24.0±1.76	26.0±1.68
	**						**	

1. The significant interactions for the demineralized and mineralized implants are presented in Figures 18 and 19.
2. The values presented are LSM±SEM. The numbers of rats in each groups are as follows: Young, 0.2% dietary calcium-14; young, 1.0% dietary calcium-14; old, 0.2% dietary calcium-12; old, 1.0% dietary calcium-13.

** Significant (P<0.01) difference in the bones of young and old rats.

Figure 18A: Alkaline phosphatase activity of demineralized and mineralized implants in young or old rats fed 0.2% or 1.0% calcium (experiment 6).

Figure 18B: ^{45}Calcium uptake by demineralized and mineralized implants in young or old rats fed 0.2% or 1.0% calcium. The interaction between implant type, age, and dietary calcium level significant at $P<0.01$ for $\log_{10}(^{45}Ca)$ (experiment 6).

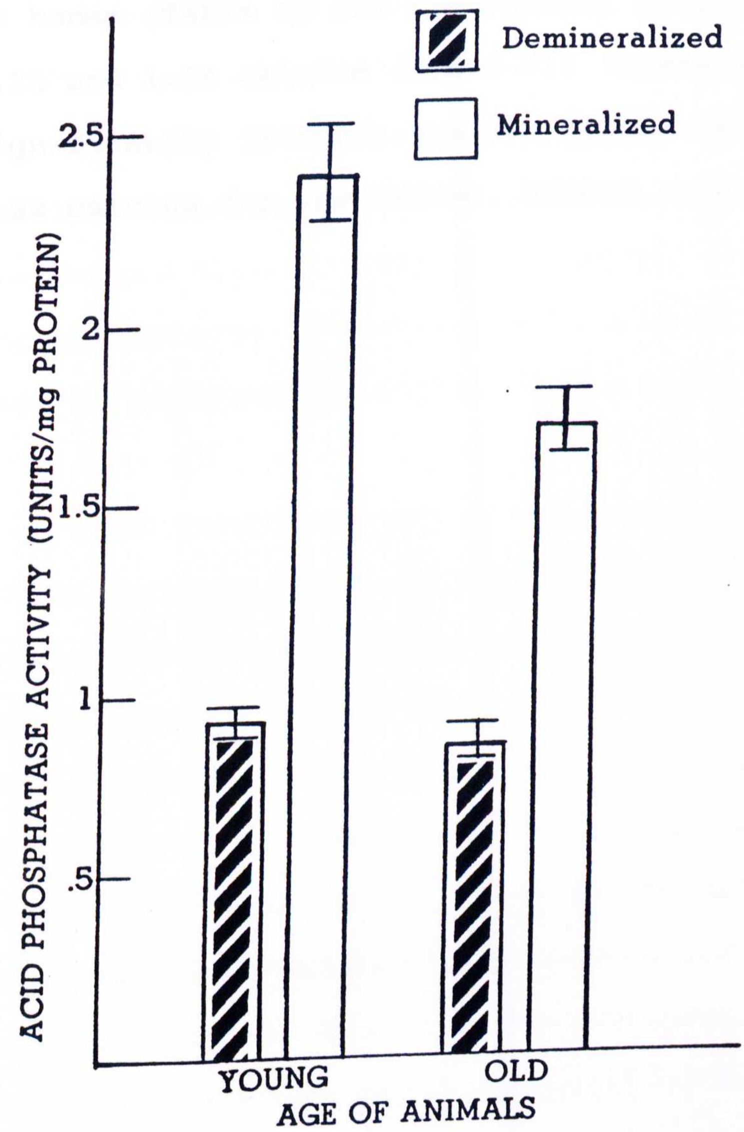


presented in Figure 18B. As for alkaline phosphatase activity the interaction was due to higher uptake of this isotope by the DB implant in the young rats fed 1.0% calcium compared to the DB implants in all the other experimental groups.

Acid phosphatase activity was greater in the MB than DB implant. An interaction between age and implant type was observed for the activity of this enzyme (Figure 19). There was a greater difference between the MB and DB implants in the young animals than the old animals. This difference was due to higher enzyme activity in the MB implant of the young animals compared to the implants of the old animals. As expected there was significantly ($P<0.01$) lower calcium concentration in the DB implants than in the MB implant.

The distal femoral epiphyses were analyzed for enzyme activity and calcium concentration in order to compare the changes in the ectopic bone with those in the epiphyses (Table 7). The animals were on their respective diets for 11 weeks before implantation. There was significantly ($P<0.01$) higher alkaline phosphatase activity in the epiphyses of young animals compared to the epiphyses of old rats. No difference was observed for acid phosphatase activity or calcium concentration in the epiphyses of young or old rats. ^{45}Ca uptake however, was greater in the epiphyses and the four bones of young animals compared to the old rats. The calcium concentration of the four bones

Figure 19: Acid phosphatase activity of demineralized and mineralized implants in young and old female rats. The interaction between implant type and age is significant at P<0.05 (experiment 6).



examined gave mixed results. The calcium content of the femurs ($P<0.01$) and tibias ($P<0.05$) was higher in the young animals as compared to the same bones of the old animals.

There was no difference in the calcium concentration of the four bones (Table 6) and the femoral epiphyses of rats fed 0.2% and 1.0% calcium (Table 7). There was, however, significantly ($P<0.05$) greater uptake of ^{45}Ca in rats fed 0.2% calcium for the femurs, humeri, and tibias.

DISCUSSION

Assessment and Development of an In Vivo Bone Model

Numerous studies have reported effects of aging and nutritional manipulation on bone mineralization. In most of these studies results were based on observations made on a single bone, generally the femur [109,120,133,138,147]. The results from these observations are then assumed to hold for the entire skeletal system. However, an occasional reference is made to the fact that results from the femur may not be representative for all the bones in the body [9].

Based on measurement of calcium and zinc concentrations, and ^{45}Ca incorporation, it was determined that individual bones had differing responses to a variety of experimental treatments. These results may be explained by the fact that two types of bone were examined. The femurs, humeri, and tibias are all weight-bearing long bones which undergo endochondral bone formation, while the scapulas and mandibles are non weight-bearing flat bones and are formed by intramembranous ossification [11,29]. Because of the differences in the functions and development of these two kinds of bone, minerals may accumulate or deplete at different rates [148]. Even though the humerus is a long bone it differs in its response for ^{3}H-TC content and ^{14}C-proline to the other such bones.

Indritz and Heagarty [148] concluded that the femur

may not be a representative sample of the entire skeleton. They also suggested that the age of the experimental animals and the specific dietary element being investigated should be taken into account before selecting bone samples for analysis [148]. However, they do not specify the conditions under which specific bones should be used. The present results showed that individual bones can respond dissimilarly. Hence, more than one bone should be examined when conducting experiments assessing the effects of nutrition and other physiological conditions.

If only one bone is used then subsections of bones can be used to examine changes in the trabecular and cortical structures. Bone fractures such as those observed in osteoporosis occur in areas with high trabecular bone content such as the vertebrae, proximal epiphyses, and distal radial epiphyses [81,83]. Using dual photon absorptiometry it was observed that 57% of the femoral neck and 95% of the femoral shaft were composed of cortical bone [149].

The results showed a higher concentration of calcium and ^{3}H-TC in the diaphyses with a greater zinc and ^{14}C-proline content in the epiphyses-metaphyses. Trabecular bone turnover is thought to be at least one order of magnitude greater than that for cortical bone, therefore, skeletal alterations occur rapidly and are more striking in trabecular bone [149-150]. There is evidence that trabecular bone can be as much as 7 times more active than

cortical bone [152]. Thus, the higher incorporation of zinc and ^{14}C-proline in the epiphyses indicates a greater cellular activity in that part of the femur, with its high content of trabecular bone and presence of growth plate. Lower initial incorporation and higher turnover of trabecular bone may be responsible for the lack of ^{3}H-TC content in the epiphyses of the 8 month-old animals in experiment 1. Thus, use of the epiphyses to examine changes in trabecular bone, and the diaphyses for cortical bone, may provide more relevant information than using the whole bone.

Research involving bone dynamics has been hindered due to the differential response of various bones discussed above as well as other problems:

i. With present techniques it is difficult to study simultaneously bone formation and resorption _in vivo_.

ii. Detectable net changes in skeletal integrity due to aging and nutritional manipulations are manifested over a long period due to the relative stability of bone.

iii. Bone contains a heterogeneous population of cells at varying stages of differentiation. This makes it difficult to study specific processes such as collagen and proteoglycan synthesis and mineralization.

In an effort to minimize these difficulties, a short-term model capable of examining bone formation and resorption

was developed. This model utilized subcutaneous implantation of DB and MB powders [153].

The initial experiment (experiment 2) with DB implants showed similar observations to those obtained by Reddi and coworkers [29-34] for alkaline and acid phosphatase activities, mineral contents, and cellular development. However, in experiment 2 the peak for alkaline phosphatase activity occurred at day 14, whereas Reddi found the peak to be at day 11. Similarly the appearance of osteoblasts in histological sections of implants were delayed. Our results are similar to those reported from other laboratories [63,64,154,155]. For example, Nishimoto et al. [154] also found the peak for alkaline phosphatase activity to occur at day 14 in 1 month-old rats. Moreover, it must be emphasized that Reddi examined the implants at every 2 day intervals from day 0 to day 21, [29] whereas in these studies the implants were examined only at days 11, 14, and 21 and the peak could have occurred on days when samples were not taken.

The implantation of MB powders (experiment 3) resulted in increased acid phosphatase with minimal alkaline phosphatase activities indicating the occurrence of bone resorption. A combination of the two implantation techniques were used to examine both bone formation and resorption within the same experimental animal (experiment 4). The results showed that both bone formation and resorption could be examined simultaneously, but as independent

processes. However, lower alkaline phosphatase activity was observed in the DB implants in the rats implanted with both MB and DB powders as compared to ones implanted with only DB. Further studies are needed in order to clarify why the implantation of MB powders decreases the bone formation capacity of DB implants.

To compare the changes taking place in the implants with those in the bone, the femoral distal epiphyses and tibial proximal epiphyses were analyzed for alkaline and acid phosphatase activities as well as calcium content. The cellular activity of the epiphyses due to its trabecular bone content is closest to the high cellular activity observed in the ectopic bone. However, similar changes were not observed in the implants and epiphyseal bones in the study where the animals were fed the experimental diets for only 3 weeks before implantation (experiment 5).

Animals were fed the experimental diets for eleven weeks in experiment 6 before implantation, as a longer time period may be necessary for changes to manifest in the epiphyses compared to the implants (experiment 6). The results showed similar changes in the implants and femoral epiphyses for alkaline phosphatase activity, ^{45}Ca uptake, and calcium concentration. However, a discrepancy in the acid phosphatase activity between the implants and epiphyses was still present. There is no explanation for this observation except that small changes in acid phosphatase activity may not be measurable in intact bones.

The short-term *in vivo* model developed in this project was found to be useful in examining the influence of diets and age on bone dynamics. The implants developed in physiological conditions where the nutritional and hormonal environment was similar to the bones of the skeletal system.

Effects of Age and Nutrition on Bone Metabolism

Osteoporosis is a bone condition observed most frequently in postmenopausal women [1]. The older male population is also prone to this disorder [1]. Nutritional supplementation may be useful in preventing and treating osteoporosis in both population groups. In the present studies the effects of dietary calcium, vitamin D metabolites, and zinc on bone metabolism were determined, thus allowing an assessment of the possible uses of these minerals and vitamin D metabolites in the prevention of bone loss.

Results of alkaline phosphatase activity in the DB implants (experiment 6) indicates that there was higher bone formation in the young animals compared to the old animals. Higher acid phosphatase activity reflecting greater bone resorption was also observed in the MB implants of the young as compared to the old animals. Thus, both bone formation and resorption were greater in the young animals compared to the old animals. Yet there was higher calcium concentration in the bones of the young as compared to old rats.

The coupling of bone formation with resorption explains why the young rats have higher bone calcium content with increased bone metabolism [73,88,156,157]. One group of investigators [73] report the existence of two local mediators that regulate bone volume. One of the mediators couples bone formation and resorption, so that with increased osteoclastic activity a factor is released into the surrounding area which stimulates osteoblastic activity. The other mediator extracted from embryonic and adult bones stimulates bone cell proliferation, collagen synthesis, and bone formation of embryonic chick tibia in culture [73]. Another report [157] suggests that the osteoblasts alter the behavior of osteoclasts making them more or less active depending on physiological conditions.

Some controversy exists about the possible role of defective coupling in the pathogenesis of osteoporosis. Parfitt [156] suggests that "the defect in bone formation that occurs in normal aging (and is exaggerated in osteoporosis) is too small a total work output by each new team of osteoblasts, so that refilling of resorption cavities is incomplete". The present result is in agreement that the decrease in bone formation (as reflected by decreased alkaline phosphatase activity in the DB implants of old rats as compared to young rats) is of primary importance. The decrease in bone formation may result from too few osteoblasts being present due to defective coupling signal, deficiency of osteoblast precursors, or decreased activity

of osteoblasts [88]. However, the exact reasons at present are unknown.

A recent article entitled "How important is dietary calcium in preventing osteoporosis" [95], outlines the controversy regarding the use of calcium supplementation in the treatment of osteoporotic patients. Since the report of the Consensus Committee on Osteoporosis [1], there has been a boom in the commercial market for various types of calcium supplements including calcium-containing antacids. However, many researchers are not convinced that calcium intake in adulthood can help prevent bone loss observed in osteoporosis [95,158,159]. No difference in the alkaline and acid phosphatase activities in the DB and MB implants of old animals fed 0.2% and 1.0% calcium diet was found (experiment 6). This indicates that high dietary intake in old animals affected neither formation nor resorption of bone implants.

The lack of an effect of high calcium on bone dynamics could result from the gradual decrease in intestinal calcium absorption with aging [3,160,161]. Furthermore, the ability of the body to adapt to a wide range of calcium intakes may play a role in this effect [158]. The intestine accomplishes this by increasing the fraction of calcium available for absorption when the intake is low. The adaptation of the intestine is also observed when high dietary calcium is fed by reducing active intestinal calcium transport [160,162]. The absolute amount of

calcium absorbed could still be higher in animals fed high calcium [160]. However, the results from experiment 6 do not support the concept of increased uptake since the plasma [163] or even bone calcium did not change in the old rats fed 1.0% compared to 0.2% calcium.

The use of calcium supplementation in the older population remains controversial. It is still an open question whether loading the skeleton with calcium premenopausally prevents osteoporosis later on in life. The higher alkaline phosphatase activity in the DB implants of young animals fed 1.0% calcium as compared to the ones fed 0.2% calcium implies increased bone formation (experiment 6). Thus, loading the skeleton of young animals with high calcium could be useful in preventing bone loss later on in life. However evidence provided by Gordan and Vaughn [164] would tend to disagree. These researchers provide examples which show that a large premenopausal bone mass does not prevent postmenopausal osteoporosis.

The results from experiment 5 further emphasize the importance of high dietary calcium intake in the young animals. Increased bone resorption as reflected by high acid phosphatase activity was observed in the MB implants of rats fed 0.2% compared to 1.0% calcium. Similar results were obtained in studies where increased active remodeling of the endosteal section of the femoral cortex was observed. A decrease in the total quantity of bone was found in rats consuming a low calcium diet [147,164].

Increased activity of osteoclasts was implicated in bone resorption [166].

The use of calcium as a prophylactic agent cannot be totally eliminated. There is evidence that calcium supplementation in conjunction with estrogen in ovariectomized rats and estrogen-deprived women contributes significantly to changes in calcium homeostasis in a positive manner [158,161,167]. Furthermore, in glucocorticoid-induced osteoporosis, calcium supplementation suppresses bone resorption [168].

Vitamin D in its hormonal form, $1,25(OH)_2D$, plays important roles in calcium homeostasis [5,96,97,100,101]. There have been reports that serum concentrations of $1,25(OH)_2D$ are lower in patients with osteoporosis [116]. Decreased plasma $1,25(OH)_2D$ was also found in hypogonadal men with osteoporosis [169]. The decrease in serum $1,25(OH)_2D$ was implicated in malabsorption of calcium at the intestinal level which ultimately led to net bone loss. The inadequate metabolism of 25OHD to $1,25(OH)_2D$ may be the major cause in decreased absorption of calcium [3].

To compensate for decrease in $1,25(OH)_2D$, studies have been conducted in which oral $1,25(OH)_2D$ was used as a prophylaxis [108,116,119,170]. An advantage of feeding $1,25(OH)_2D$ is that this metabolite bypasses part of the feedback mechanism of the vitamin D-hormonal system involved in the regulation of calcium. Therefore, the effect of the active metabolite of vitamin D alone could be

examined without introducing confounding aspects of conversion of 25OHD to $1,25(OH)_2D$.

Higher acid phosphatase activity and lower calcium concentration in the MB implants of rats in experiment 5 implied that greater resorption was taking place in the animals fed $1,25(OH)_2D$ compared to D_3. This result is in agreement with various studies, where bone resorption was induced by $1,25(OH)_2D$ [17,102,104-107]. One reason could be that at high levels, $1,25(OH)_2D$ induced differentiation of monocytes to osteoclasts [17,18].

The stimulatory action of $1,25(OH)_2D$ on bone formation found in Sprague-Dawley rats by Soares et al. [109] was not observed in these studies which used Long-Evans rats. An important factor may be the age of the animals. The Sprague-Dawley rats used were 4-5 months old by the end of the experimental period while the Long-Evans rats were 2 months old. A specific age- or development-related elevation in circulating $1,25(OH)_2D$ has been reported in both rats and humans [163]. Furthermore, absence of estrogen also has a negative effect on bone metabolism in rats fed $1,25(OH)_2D$ [171,172]. Therefore, the intake of 75ng of $1,25(OH)_2D$/day by these weanling sexually-immature Long-Evans rats resulted in increasing bone resorption rather than enhancing bone formation.

Furthermore, Soares et al. have [109] reported a decrease in bone resorption in rats fed $1,25(OH)_2D$ with either 4.5 or 10 ppm zinc as compared to rats fed D_3 and

4.5 and 10 ppm zinc. The mineral concentrations (experiment 2) show that more zinc and calcium was incorporated into the DB implants of animals fed D_3 and 10 ppm zinc compared to the rats fed $1,25(OH)_2D$. The decreased mineral deposition in rats fed $1,25(OH)_2D$ is in agreement with the results obtained from various laboratories [173-175]. These researchers also found impaired mineralization due to high doses of $1,25(OH)_2D$. Thus, 75ng of $1,25(OH)_2D$/day not only increases bone resorption but also decreases mineralization in young Long-Evans rats as assessed by the bone implant system.

There is a need for standardization of experimental procedures to assess the effects of $1,25(OH)_2D$ on bone metabolism. Varying dosages of $1,25(OH)_2D$ [103] have been administered orally [10,118,129], through intermittent injections [185,186] or via continuous infusion [111,113]. These studies have used different ages as well as species of animals to examine the effect of $1,25(OH)_2D$ on bone metabolism. Procedural uniformity is necessary to elucidate the influence of $1,25(OH)_2D$ on bone formation and resorption.

The major thrust of the present studies was the development of the <u>in vivo</u> model and the examination of the effects of age, calcium, and vitamin D on bone metabolism. A secondary aspect concerned the effects of zinc on bone loss.

The exact role of zinc in bone metabolism and osteopo-

rosis is not clear. The increased concentration of zinc in bones with aging has been implicated in the development of osteoporosis [129]. Results from another report suggest that zinc depletion in both serum and bone may play a role in the pathogenesis of senile osteoporosis [130]. A lower zinc concentration was found in the bones of the old animals in experiment 1 and 6. However, there is no evidence that the lower bone zinc content had a role in bone loss.

The ^{3}H-TC retention in the bones of the 2 month-old rats in experiment 1 is in agreement with a report that high but non-toxic dietary levels of zinc may be a factor in enhancing bone loss in young rats when calcium intake is low [109,177]. In contrast, a greater ^{3}H-TC content was observed in the humeri of 2 month-old rats fed 300 ppm as compared to the ones fed 10 ppm zinc. This agrees with a report that 200 ppm zinc decreased resorption of ^{3}H-TC in the femur [176]. However, higher retention of ^{3}H-TC was not observed in the femurs of rats in experiment 1. Thus, 300 ppm dietary zinc did not have a beneficial effect on bone integrity in old animals, whereas the same level of zinc was found to be detrimental for the bones integrity in young animals.

CONCLUSIONS

When assessing the effects of nutrients, age and other factors on the skeletal system, more than one bone should be sampled. The results of these studies show that analysis of the epiphyses-metaphyses is useful for examining changes occurring in trabecular bone while similar analysis of the diaphyses is useful for assessing the metabolic states of cortical bone.

A short-term *in vivo* model system was described in which both DB and MB implants were effectively used to examine bone formation and resorption. This model system maintains the physiological conditions needed to evaluate effects of nutritional, hormonal, and physiological factors on the skeletal system.

Alkaline phosphatase activity of the DB implants was lower in the 24 month-old rats as compared to 2 month-old rats. This indicates that bone formation is reduced in aging. Dietary calcium (0.2% and 1.0%) and zinc (10 and 300 ppm) levels did not appear to affect the overall status of the bones and implants of 8 and 24 month-old animals.

Higher calcium concentrations were observed in the bones of the 2 month-old rats. Both formation (as indicated by higher alkaline phosphatase activity in the DB implants) and resorption (suggested by the high acid phosphatase activity in the MB implants) were greater in these animals. In the young animals, 1.0% calcium stimulated bone formation (suggested by high alkaline phospha-

tase activity in DB implants) and 0.2% calcium, bone resorption (indicated by the higher acid phosphatase activity in MB implants). Dietary supplementation of vitamin D in the form of $1,25(OH)_2D$ at (75ng per day) decreased calcium concentration in MB implants reflecting higher bone resorption. Mineralization was inhibited as lower quantities of calcium and zinc were found in DB implants of young rats fed $1,25(OH)_2D$ as compared to the ones fed D_3. High (300 ppm) levels of zinc reduced bone calcification of 2 month-old rats as indicated by lower ^3H-TC retention in the bones of rats fed 300 ppm as compared to 10 ppm zinc.

The results of these studies indicate that neither high levels of dietary calcium nor of zinc can be recommended as a prophylaxis to counteract bone loss due to aging. The dietary use of $1,25(OH)_2D$ in old animals needs to be investigated further, since results in young animals conflict with other reports in older rats. The age and sexual development of the animals may be crucial in the type of effect $1,25(OH)_2D$ has on bone metabolism.

Appendix A

Dry weight, calcium concentration and ^{45}calcium uptake by the femurs, humeri, mandibles, scapulas and tibias of weanling female Long-Evans rats fed 4.5 or 10 ppm zinc and D_3 or $1,25(OH)_2D$ in bones collected 11, 14 and 21 days after implantation of demineralized bone powder (experiment 2).

	Dry Weight (mg)		Calcium (mg/g)		$^{45}Ca (X10^3 CPM/g)$	
Dietary Zinc (ppm)	D3	$1,25(OH)_2D$	D3	$1,25(OH)_2D$	D3	$1,25(OH)_2D$
Day 11						
Femurs						
4.5	195±9.2(6)	220±9.2(6)	204±9.7(5)	201±8.8(6)	434±17.9(5)	403±16.3(6)
10	236±8.5(7)	226±8.5(7)	205±9.7(5)	197±8.8(6)	402±17.9(7)	403±16.3(7)
Humeri						
4.5	99±4.4(6)	150±4.4(6)	217±12.5(5)	223±11.4(6)	419±17.1(5)	395±15.6(6)
10	116±4.1(7)	107±4.1(7)	215±12.5(5)	238±11.0(6)	384±17.1(7)	418±14.5(7)
Mandibles						
4.5	-	-	290±10.0(5)	286±9.1(6)	317±2.4(5)	276±2.2(6)
10	-	-	285±10.0(5)	283±9.1(6)	295±2.4(7)	277±2.0(7)
Scapulas						
4.5	44±2.9(6)	46±2.9(6)	224±10.5(5)	222±9.6(6)	468±2.9(5)	456±2.6(6)
10	57±2.7(7)	50±2.7(7)	215±10.5(5)	198±9.6(6)	434±2.9(7)	435±2.4(7)
Tibias						
4.5	170±8.0(6)	173±8.0(6)	209±9.8(5)	211±9.0(6)	367±16.8(5)	332±15.6(6)
10	202±7.4(7)	177±7.4(7)	204±9.8(5)	204±9.8(6)	342±16.8(7)	366±14.2(7)

Appendix A (continued)

Dietary Zinc	Dry Weight (mg)		Calcium (mg/g)		^{45}Ca (x10^3 CPM/g)	
	D3	$1,25(OH)_2D$	D3	$1,25(OH)_2D$	D3	$1,25(OH)_2D$
Day 14						
Femurs						
4.5	207±9.2(6)	216±9.2(6)	231±9.7(5)	208±9.7(5)	430±16.3(6)	385±16.3(6)
10	228±9.2(6)	237±10.0(5)	203±9.7(5)	192±6.7(5)	374±17.9(5)	369±17.9(5)
Humeri						
4.5	106±4.4(6)	204±4.4(6)	206±12.5(5)	238±12.5(5)	401±15.6(6)	354±15.6(6)
10	118±4.4(6)	120±4.9(5)	217±12.5(5)	202±12.5(5)	348±17.1(5)	368±17.1(5)
Mandibles						
4.5	-	-	289±10.0(5)	283±10.0(5)	280±2.2(6)	268±2.2(6)
10	-	-	292±10.0(5)	282±10.1(5)	276±2.4(5)	262±2.4(5)
Scapulas						
4.5	49±2.9(6)	52±2.9(6)	201±10.5(5)	221±10.5(5)	482±2.6(6)	409±2.6(6)
10	52±2.9(6)	56±3.2(5)	209±10.5(5)	209±10.5(5)	377±2.9(5)	374±2.9(5)
Tibias						
4.5	176±8.0(6)	181±8.0(6)	202±9.8(5)	218±9.8(5)	358±15.3(6)	324±15.3(6)
10	194±8.0(6)	185±8.7(5)	212±9.8(5)	220±9.8(5)	301±16.8(5)	321±16.8(5)

Appendix A (continued)

Dietary Zinc	Dry Weight (mg)		Calcium (mg/g)		^{45}Ca(x10^{3} CPM/g)	
	D3	1,25(OH)$_2$D	D3	1,25(OH)$_2$D	D^3	1,25(OH)$_2$D
Day 21						
Femurs						
4.5	218±9.9(5)	228±9.2(6)	215±9.8(4)	194±8.8(6)	369±20.0(4)	354±16.3(6)
10	239±9.2(6)	256±9.2(6)	210±8.8(6)	191±8.8(6)	362±16.3(6)	390±16.3(6)
Humeri						
4.5	109±4.9(5)	115±4.4(6)	243±14.0(4)	232±11.4(6)	365±19.1(4)	331±15.6(6)
10	118±4.4(6)	122±4.4(6)	226±11.4(6)	201±11.4(6)	354±17.1(6)	374±15.6(6)
Mandibles						
4.5	-	-	293±11.1(4)	300±9.1(6)	241±2.7(4)	241±2.2(6)
10	-	-	301±9.1(6)	293±9.1(6)	284±2.2(4)	292±2.2(6)
Scapulas						
4.5	5.1±3.2(5)	57±2.9(6)	222±11.8(4)	198±9.6(6)	433±3.2(4)	350±2.6(6)
10	5.4±2.9(6)	58±2.9(6)	214±9.6(6)	211±9.6(6)	408±2.6(6)	396±2.6(6)
Tibias						
4.5	188±8.7(5)	194±8.0(6)	219±8.0(4)	233±9.0(6)	335±18.7(4)	296±15.3(6)
10	201±8.0(6)	211±8.0(6)	216±9.0(6)	201±9.0(6)	312±15.3(6)	324±15.3(6)

Appendix A (concluded)

Multivariate analyses of variance five bones, P-value

Source of Variation	Dry Weight	Calcium	^{45}Calcium
Zinc	0.01	NS	NS
D	NS	NS(0.07)	NS
Time	0.05	NS	0.05
Zinc	NS	NS	0.05
Zinc*Time	NS	NS	NS
D*Time	NS	NS	NS
Zinc*D*Time	NS	NS	NS

1. LSM±SE of LSM. The number of rats in each group in parentheses.

APPENDIX B

Alkaline phosphatase, acid phosphatase and calcium concentration of demineralized and mineralized bone implants from male Long-Evans rats fed 0.2% or 1.0% calcium and D_3 or $1,25(OH)_2D$ (experiment 5).

	Alkaline Phosphatase (units/mg protein)		Acid Phosphatase (units/mg protein)		Calcium (mg/g implant)	
	PERCENT DIETARY CALCIUM					
	0.2	1.0	0.2	1.0	0.2	1.0
Demineralized						
D_3	.93±.130[1]	1.20±.171	1.40±.038	1.51±.103	25.5±1.0	25.0±1.30
$1,25(OH)_2D$	.85±.590	.97±.074	1.46±.052	1.48±.059	26.2±1.33	29.1±1.40
Mineralized						
D_3	.04±.003	.05±.008	2.60±.113	2.41±.129	71.8±3.43	75.9±2.96
$1.25(OH)_2D$	.05±.004	.04±.004	2.87±.137	2.62±.126	68.2±3.19	68.0±3.32

1. LSM±SE. There were 12 rats in each group except the group fed 1.0% calcium and $1,25(OH)_2D$ which had 11 rats.
Significant interactions are plotted in Figures 15A and 15B.

Appendix C

Initial weight, final weight and feed intake of Sprague-Dawley rats (experiment 1).

Experimental Groups	Initial Weight (g)	Final Weight (g)	Feed Intake (g/day)
2 month-old			
10 ppm zinc	235±3.5[1]	300±6.8	16.7±0.61
300 ppm zinc	240±2.0	311±4.0	18.4±0.96
8 month-old			
10 ppm zinc	393±6.9	408±7.4	16.6±0.71
300 ppm zinc	386±7.5	389±10.0	18.0±0.74

1. Mean±SEM.

Appendix D

Initial weight, final weight and feed intake of Long-Evans rats (experiment 2).

Experimental Groups	Initial Weight (g)	Final Weight (g)	Feed Intake (g/day)
Day 11			
D_3			
4.5 ppm zinc	81±1.9[1]	118±2.0	11.4±0.52
10 ppm zinc	84±2.3	137±1.8	12.2±0.20
$1,25(OH)_2D$			
4.5 ppm zinc	81±3.5	118±1.3	10.2±0.57
10 ppm zinc	79±2.5	132±2.2	12.4±0.34
Day 14			
D_3			
4.5 ppm zinc	77±2.3	120±5.8	10.5±0.51
10 ppm zinc	79±2.6	130±3.9	11.5±0.25
$1,25(OH)_2D$			
4.5 ppm zinc	81±3.2	128±3.8	10.0±0.54
10ppm zinc	76±2.3	127±2.2	11.6±0.32
Day 21			
D_3			
4.5 ppm zinc	80±1.8	123±1.8	10.0±0.56
10 ppm zinc	79±3.4	134±3.8	11.0±0.18
$1,25(OH)_2D$			
4.5 ppm zinc	83±1.8	120±2.4	9.7±0.53
10ppm zinc	78±3.2	138±2.6	11.1±0.30

1 Mean±SEM.

Appendix E

Initial weight, final weight and feed intake of Long-Evans rats (experiment 5).

Experimental Groups	Initial Weight (g)	Final Weight (g)	Feed Intake (g/day)
D_3			
0.2% calcium	190±2.4[1]	262±3.7	16.5±0.81
1.0% calcium	187±2.8	248±5.0	15.9±0.93
$1,25(OH)_2D$			
0.2% calcium	189±1.6	260±3.1	16.9±0.88
1.0% calcium	182±2.0	258±3.0	16.5±0.83

1 Mean±SEM.

Appendix F

Initial weight, final weight and feed intake of Long-Evans rats (experiment 6).

Experimental Groups	Initial Weight (g)	Final Weight (g)	Feed Intake (g/day)
2 month-old			
0.2% calcium	195±2.8[1]	266±4.0	14.8±0.85
1.0% calcium	192±3.1	265±4.0	14.2±0.78
24 month-old			
0.2% calcium	346±16.9	362±16.4	18.6±1.10
1.0% calcium	390±8.8	371±14.1	17.9±0.93

[1] Mean±SEM

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