The Role of Vegetables in the Maintenance of Acid-Base Balance and Bone Structure

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Abstract

Metabolic acidosis may over time lead to osteoporosis by causing a release of calcium and other mineral phases from bone. The regular consumption of fruits and vegetables is believed to be associated with higher bone mineral density. In the last ten years various population-based studies have found positive effects of fruit and vegetable intakes on bone health. The aim of the present study was to investigate the effects of broccoli, onion, and potato on bone density and strength in male rats.

Forty male Sprague-Dawley rats were randomized into four equal groups of ten each. The animals were fed either a base, broccoli, onion, or potato diet for a period of eight weeks. The apparent percentage calcium and phosphorus retained, the serum type 1 collagen C-telopeptide concentration, bone density and bone strength, and the urinary parameters i.e. ammonia, creatinine, urea, specific gravity and osmolality were determined.

The groups on onion and potato diets had significantly higher apparent percentage calcium retained (over the balance period) than the group on control diet ($p=0.021$ and 0.008 respectively). Apparent percentage calcium retained was also significantly higher in the group on potato diet compared to the group on broccoli diet ($p=0.037$). There were no significant differences between groups for percentage phosphorus retained on ANOVA. However, the discriminant analysis (multivariate

method) showed that the group on the broccoli diet retained significantly more phosphate over the balance period compared to the other groups. The urinary ammonia excretion (over the balance period) was significantly lower in the group on broccoli diet than in the groups on base and potato diets ($p=0.040$ and 0.055 respectively). As for the urinary urea excretion over the balance period, the group on base diet had significantly higher urea excretion than the groups on onion and potato diets ($p=0.002$ and $p=0.000$ respectively). Urinary urea excretion (over the balance period) was also significantly higher in the group on broccoli diet compared to the groups on onion and potato diets ($p=0.005$ and 0.000 respectively). The differences between groups for the volume of urine produced over the balance period were also significant i.e. the group on broccoli diet produced significantly more urine than the groups on base, onion, and potato diets ($p=0.011$, $p=0.008$, and $p=0.001$ respectively). However, there were no significant differences between groups for urinary specific gravity, osmolality, and creatinine, and bone density, bone breaking strength, and serum type 1 collagen C-telopeptide concentrations on ANOVA.

In conclusion 1g of broccoli per day significantly reduced urinary ammonia excretion and increased apparent percentage phosphorus retained whereas 1g of onion or potato per day significantly increased apparent percentage calcium retained in growing male rats. The decrease in urinary ammonia excretion was most likely due to the buffering of metabolic acids by the bases present in broccoli resulting in decreased

ammonia production and secretion. Similarly the increased apparent percentage phosphorus retained (in the group on broccoli diet) may be due to the bases present in broccoli that may have buffered metabolic acids thereby reducing the need for phosphate buffering. This increased apparent percentage phosphorus retained may also be due to a high pH which is known to enhance renal phosphate uptake. The buffering of metabolic acids by the bases present in onion and potato may have reduced the need for calcium buffering resulting in higher apparent percentage calcium retained in the groups on onion and potato diets. Thus broccoli, onion, and potato intake may protect against the bone depleting effects of an acidogenic diet and may also have the potential to increase bone mineral density.

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List of Abbreviations

µg	=	Microgram
1,25 $(OH)_2D_3$	=	1,25- dihydroxy vitamin D_3 (calcitriol)
25- hydrocholecalciferol	=	Calcidiol
ANOVA	=	Analysis of Variance
ATPase	=	Adenosine Triphosphatase
Blast	=	Osteoblast
BMC	=	Bone Mineral Content
BMD	=	Bone Mineral Density
BMP	=	Bone Morphogenetic Protein
BRU	=	Bone Remodeling Unit
Ca^{2+} or Ca	=	Calcium
$Ca_{10}(PO_4)_6(OH)_2$	=	Calcium Hydroxyapatite
CAMS	=	Cell Adhesion Molecules
Cbl	=	Casitas B-lineage lymphoma
Cl	=	Chloride
Clast	=	Osteoclast
CO_2	=	Carbon Dioxide
CSF-1	=	Colony Stimulating Factor 1
CSF-2	=	Colony Stimulating Factor 2
CTX	=	C-terminal telopeptide of type- 1 collagen

DASH	=	Dietary Approaches to Stopping Hypertension
DEXA	=	Dual Energy X-ray Absorptiometry
E1	=	Estrone
E2	=	Estradiol
ECF	=	Extracellular Fluid
ECM	=	Extracellular Matrix
EDTA	=	Ethylene Diamine Tetra-Acetic Acid
ELISA	=	Enzyme-Linked Immunosorbent Assay
FGFs	=	Fibroblast Growth Factors
g	=	grams
G	=	gauge
GAGs	=	Glycosaminoglycans
Gla	=	γ- carboxyglutamate
$GluCONH_2$	=	Glutamine
GluCOO-	=	Glutamate
GPCS	=	γ-L-glutamyl-trans-S-1-propenyl-L-cysteine sulphoxide
H^+	=	Hydrogen Ion
HCl	=	Hydrochloric Acid
HCO_3^-	=	Bicarbonate
H_2CO_3	=	Carbonic Acid
HPO_4^{-2}	=	Monohydrogen Phosphate

H_2PO_4	=	Dihydrogen Phosphate
H_3PO_4	=	Phosphoric Acid
H_2SO_4	=	Sulphuric Acid
IHH	=	Indian Hedgehog
J	=	Joule
JKN	=	c- Jun N- terminal kinase
K^+	=	Potassium Ion
kg	=	Kilogram
kV	=	Kilovolt
M	=	Mole
Max	=	Maximum
M- CSF	=	Macrophage Colony Stimulating Factor
mg	=	milligram
mL	=	millilitre
mm	=	millimeter
mmol/L	=	millimoles per litre
N	=	Newton
Na or Na^+	=	Sodium
$NaHCO_3$	=	Sodium Bicarbonate
Na^+ - K^+ = ATPase	=	Sodium Potassium Adenosine Triphosphatase
Na_2SO_4	=	Sodium Sulphate
NDOs	=	Non-Digestible Oligosaccharides
NF – kB	=	Nuclear Factor kappa B

ng	=	nanogram
nm	=	nanometer
NH_3	=	Ammonia
NH_4^+	=	Ammonium Ion
NH_4Cl	=	Ammonium Chloride
OPG	=	Osteoprotegerin
P	=	Phosphorus
pH	=	Potential of Hydrogen
PO_4^{3-}	=	Phosphate
PBM	=	Peak Bone Mass
PBS	=	Phosphate Buffered Saline
PGE_2	=	Prostaglandin E_2
PTH	=	Parathyroid Hormone
PTH- rp	=	Parathyroid Hormone- related protein
RANK	=	Receptor Activator of the Nuclear Factor kappa B
RANKL	=	Receptor Activator of the Nuclear Factor kappa B ligand
rpm	=	revolutions per minute
SAPK	=	Stress Activated Protein Kinases
SE	=	Standard Error
TRAFs	=	Tumour Necrosis Factor Receptor-Associated Factors

TNF	=	Tumour Necrosis Factor
VEGF	=	Vascular Endothelial Growth Factor
WHO	=	World Health Organization
Wt	=	Weight (g)
Yrs	=	Years

THE ROLE OF VEGETABLES IN THE MAINTENANCE OF ACID-BASE BALANCE AND BONE STRUCTURE

A. General Introduction

Bones are generally viewed as inert. They perform unseen and this makes it easy for us to forget how important and valuable they actually are. However, bones are not inert as they really are dynamic, living tissues. They are continually being remodeled and rebuilt (Blair et al., 2002).

In order to live an active lifestyle, it is critical to form and maintain strong, healthy bones. The skeletal system performs the following functions:

- Support the body and protect the internal organs
- Allow movements from sitting, standing, and walking, to running, lifting and carrying
- Anchor muscles
- Store minerals e.g. calcium and phosphorus
- Form blood cells.

Problems of the skeletal system are for most of us simply a fact of life and their impact on our health, vitality and mobility can be enormous, causing great problems. Diseases of the bone, in particular osteoporosis affect about one-half of all women and one-fourth of all men who are over the age of 50 (Christenson, 1997). Osteoporosis is a complex,

2 shows the modifiable and non-modifiable factors that influence bone health. Amongst these, nutrition is an important modifiable factor in the development and maintenance of bone mass. Nutrition is also important in the prevention and treatment of osteoporosis (Ilich and Kerstetter, 2000) because many compounds present in food have biological effects within our bodies. Also, diet and disease are intimately associated. Nutrition is amenable to change as it is an exogenous factor. A nutritional approach is far more popular with osteoporosis sufferers than drug intervention because of poor long-term compliance rates associated with the latter (New, 1999).

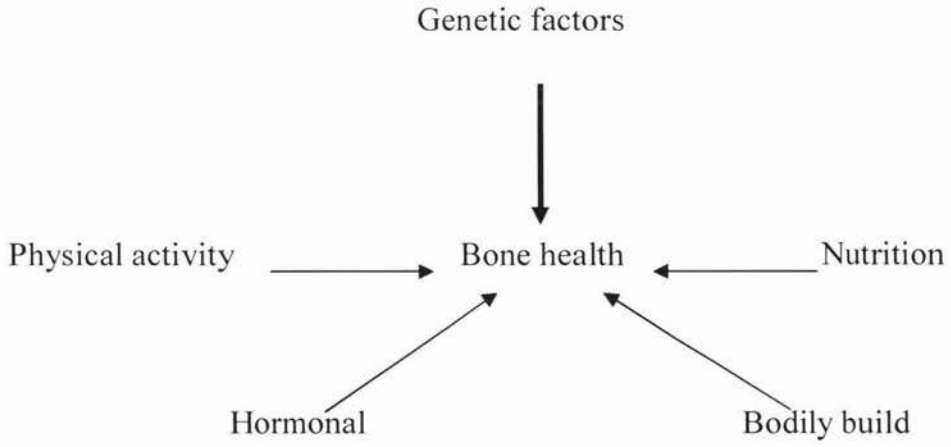


Figure 2. Modifiable v. non-modifiable factors influencing bone health. (From New, 2002).

The factors that determine post-menopausal bone mass are both peak bone mass (PBM) attained early in life and the rate of bone mass loss later in life. PBM is the mass of bony tissue present at the end of skeletal maturation and it is a major determinant of skeletal health throughout life

(Bachrach et al., 1999) i.e. the higher the maximum bone density and strength the lower will be the risk of osteoporosis. The age at which PBM is attained is not certain. However, estimates of attainment of peak total-body bone mineral density and bone mineral content range from age 18 years to the 20s (Teegarden et al., 1999). PBM is determined by gender, and as mentioned earlier by a combination of endogenous (genetic, hormonal) and environmental (nutrition, exercise) factors (Heaney, 1996).

Estrogen is required for the attainment of maximal peak bone mass in both sexes. However, the additional action of testosterone on stimulating periosteal apposition accounts for the larger size of the adult male skeleton and thicker cortical bone. Thus, men normally have greater PBM and bone density than women (Wahlqvist and Wattanapenpaiboon, 2002). Estrogen also plays a major role in maintaining bone mass and estrogen deficiency is the major cause of accelerated age-related bone loss in both sexes (Riggs et al., 2002).

In both men and women a slow loss of bone mass begins around the ages of 35 to 40 (Wahlqvist and Wattanapenpaiboon, 2002). Women however experience accelerated bone loss during the menopausal and post-menopausal period (Bord et al., 2001). This loss occurs over and above the slower rate and continues for around 5 to 10 years after menopause. Subsequently the rate of loss declines so that by age 60 it again matches that of men. The lifetime bone loss in women is up to 45-50% of bone mass

whereas in men it is 20-30%. Refer Figure 3 to view the lifetime alterations in skeletal mass in both men and women.

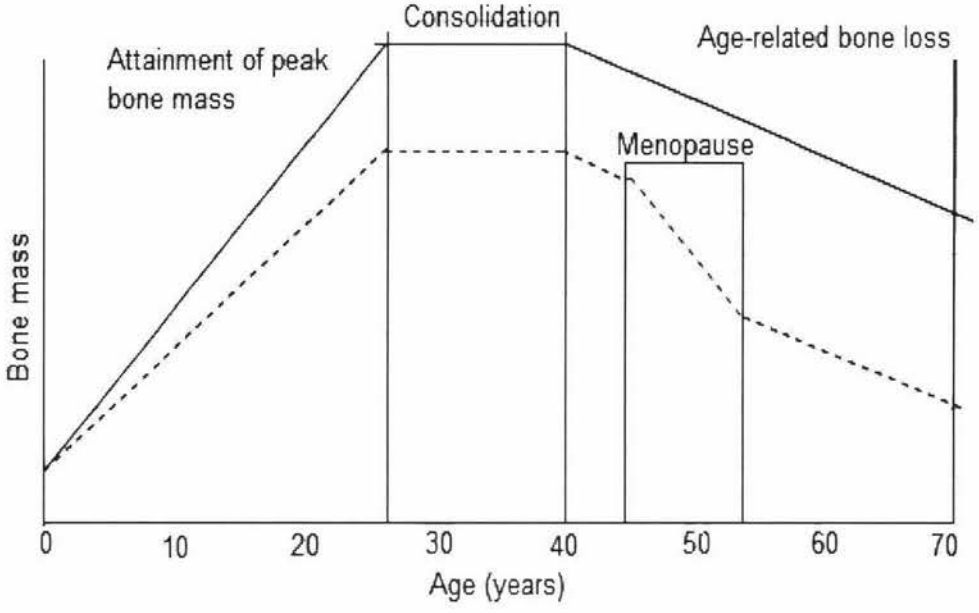


Figure 3. Alterations in skeletal mass in men (-) and women (--) throughout the life cycle. (From New, 2002).

The median age of populations is increasing. For instance, in New Zealand, during the time period 1950-52 survival to age 60 and older was about 76% and 82%, respectively for both males and females. It increased to about 84% for males and 90% for females during the time period 1990-1992, and it is projected to rise to about 89% and 94%, for both males and females respectively in 2011. In 2031, it will be about 90% for males and 95% for females (Zodzekar, 1994). As a consequence of this it is predicted that osteoporotic fractures will reach epidemic proportions (Ilich and Kerstetter, 2000). Osteoporosis is already a significant public health problem. In 1990 there were 1.66 million osteoporotic fractures

throughout the world and this figure is projected to rise to 6.26 million by 2050 (World Health Organization, 1994). The estimated lifetime risks of fracture for women with osteoporosis is >40%. In contrast, the risk of fracture for men is only 13% (Kanis and the WHO Study Group, 1994). It has been estimated that between 25% and 50% of Caucasian women and 5-10% of Caucasian men will probably suffer an osteoporosis-related fracture at some time in their lives (Melton and Riggs, 1983).

The normal Western diet is high in protein, salt and refined, processed foods. This diet when combined with a sedentary lifestyle may contribute to the increasing incidence of osteoporosis in the elderly (Maurer et al., 2003). Thus, initiating appropriate healthy behaviors such as eating enough fruits and vegetables early in life and continuing them throughout life may prevent osteoporosis and osteoporosis-related fractures (Tylavsky et al., 2004).

Our understanding of nutritional influences on bone health is limited. This is because most studies regarding the influence of nutrition on bone health have focused on the importance of dietary calcium. High fruit and vegetable intakes may also have beneficial effects on bone (Tylavsky et al., 2004; New et al., 2000; Muhlbauer and Li, 1999) as they contain amino acids and base precursors that may provide a natural source of base to buffer the acid produced by other dietary compounds such as protein-rich foods, grains and cereals (New, 2002). Another study found that there is an association between several nutrients present in fruit and vegetables (i.e.

potassium, magnesium, β-carotene and vitamin C) and bone health (New et al., 2000). Fruits and vegetables also contain antioxidants e.g. vitamin C, carotenoids, flavonoids, and other phenolics that protect the body against reactive oxygen species (Williamson and Manach, 2005). Body cells and tissues are continuously threatened by the damage caused by free radicals and reactive oxygen species. Thus, the antioxidants that are found in fruits and vegetables may positively affect bone health.

The overall renal function of humans declines as they become older and this affects their ability to excrete acid (Frassetto et al., 1996). Hence humans develop progressively increasing blood acidity as they grow older (Frassetto and Sebastian, 1996). The skeleton (alkaline bone mineral) may thus be used to buffer the acid.

A variety of population-based studies have demonstrated a beneficial effect of fruit and vegetable/potassium intake on indices of bone health (New, 2002). This beneficial effect occurs in all age groups i.e. in young boys and girls, premenopausal women, perimenopausal women, postmenopausal women, and elderly men and women (New, 2002).

B. Reason for the Study

There is evidence of a positive link between the intake of fruit and vegetables and bone health (Tucker et al., 1999; New et al., 2000). Further investigations are warranted to determine the relations found between the nutrients present in fruit and vegetables, bone mineral density (BMD), and bone turnover. Current available evidence is insufficient to generate recommendations as to the amounts of fruit and vegetables that are necessary to produce a positive effect in humans (Muhlbauer et al., 2002) mainly as it is unclear which nutrients are producing the effect.

Fruits and vegetables contain large amounts of base-producing organic acid salts (potassium and magnesium may be part of them) that are believed to buffer noncarbonic metabolic acids generated from cystine- and methionine-rich dietary proteins (Tucker et al., 1999). Potassium and magnesium are present in a variety of whole, unrefined foods, including fruits and vegetables (Remer and Manz, 1995). If these bases were absent then the metabolic acids would be buffered by bone mineral which will lead to bone dissolution (Tucker et al., 1999; New et al., 2000). However, Muhlbauer et al (2002) reported that the inhibitory effect of foodstuffs of vegetable origin on bone resorption is not mediated by their base excess but possibly by pharmacologically active compound(s). Thus, it is necessary to determine how vegetables affect bone health.

C. Vegetables included in the Study

In the present study we included onion, broccoli, and potato. Onion was included in the study as a positive control because it has been reported to have a positive effect on bone mineral content (BMC). Muhlbauer and Li (1999) found that when male rats were fed 1g dry onion per rat per day for four weeks their BMC increased (significant) by 17.7 ± 6.4% ($p<0.05$; $n=6$), mean cortical thickness increased by 14.8 ± 7.6%, and BMD increased by 13.5 ± 3.1% ($p<0.05$) relative to controls. Onions contain significant levels of potassium, calcium, phosphorus, carotenoids, and vitamin C, all of which have been positively linked to bone health.

Broccoli was included in the study as it has also been shown to significantly inhibit bone resorption in rats at a dose of 1g per rat per day (Muhlbauer and Li, 1999). Broccoli is high in carotenoids, and is a rich source of vitamin K, potassium, calcium, phosphorus, and vitamin C.

Potatoes were included as a negative control on the basis that potatoes consumed at a dose of 1g per rat per day did not significantly inhibit bone resorption in the above quoted study.

D. Objectives

The specific objective of this project was to evaluate the effect of vegetables (broccoli, onion, and potato) in male rats on:

- Bone density
- Bone strength
- Markers of bone metabolism
- Calcium balance
- Urinary buffering

Bone density is a measure of bone strength. When minerals fill the bone matrix they make it dense. Levels of serum C-terminal fragment (CTX) were measured because it is a specific marker of osteoclastic activity i.e. bones resorption. Calcium and phosphorus balance were done to determine the amount of calcium and phosphorus retained respectively. The acid load of urine was determined by measuring the urinary buffers ammonia and phosphate. The urinary ammonia excretion is increased as urine becomes more acidic.

E. Hypothesis

The regular consumption of vegetables (broccoli and onion) will increase BMD and affect acid base balance in the body.

CHAPTER 1

LITERATURE REVIEW

1.1 Bone Physiology

The physiology of bone is explained with special emphasis on bone composition and bone matrix dynamics.

1.1.1 The Composition of Bone

Bone is composed of cells, organic matrix, inorganic component (minerals), and water (Guyton, 1992). The major bone cells i.e. osteoblasts, osteocytes, and osteoclasts are described in section 1.1.1.4.

1.1.1.1 The Organic Matrix

The organic matrix is composed of proteins that are secreted by osteoblasts. Collagen (principally type 1) makes up 95% of the organic matrix. It allows bones to be flexible. The remaining 5% of the organic matrix is made up of ground substance which consists mainly of protein polysaccharides called glycosaminoglycans (GAGs). It serves as cementing substance between layers of mineralized collagen fibers (Wahlqvist and Wattanapenpaiboon, 2002).

1.1.1.2 The Inorganic Component

The bulk of the inorganic component comprises calcium phosphate, which makes bones strong, and rigid (Wahlqvist and Wattanapenpaiboon, 2002). The unique combination of the organic matrix with the minerals enables bones to withstand considerable mechanical stresses. Salts of calcium (Ca^{2+}) and phosphate (PO_4^{3-}) are deposited in the organic matrix in combination with hydroxyl ions forming crystals of hydroxyapatite, which have the general formula $Ca_{10}(PO_4)_6(OH)_2$ (Kaplan and Pesce, 1989). Other calcium precipitates e.g. brushite are present in bone as well. Besides calcium and phosphorus, bone contains sodium, magnesium, potassium, zinc, citrate, and carbonate. Chloride and fluoride are also found in bone and may be impurities. The inorganic component of bone has a role in overall Ca^{2+} and PO_4^{3-} homeostasis. Bone acts as an ion reservoir and bone minerals are available for rapid exchange with the general extracellular fluid (ECF). These minerals are lost in the obligatory buffering of excessive metabolic acids (Kaplan and Pesce, 1989).

a) Calcium Pool

99% of the body's calcium is found in bone. Bone also contains 85% of the body's phosphorus, and 66% of the magnesium. Only a small percentage of these minerals can be rapidly mobilized when there is a fall in their plasma concentration. However, this small percentage provides the initial mineral source when this happens. Trabecular bone has a large

surface area and excellent blood supply which permits a quick response to occur if there are any disturbances in plasma mineral concentrations (Kaplan and Pesce, 1989).

The calcium in bone provides an almost inexhaustible source of calcium for the blood. Calcium is borrowed and returned by the blood as needed. As such, even in a dietary deficiency state, blood calcium remains normal because calcium will be removed from the bone. There are two types of calcium in bone. One is the readily exchangeable reservoir of calcium and the other is a pool of stable calcium. The latter is a much larger pool which is only slowly exchangeable. Channels present within bone are the remnants of the vascular system that maintained the activity of osteoblasts during the phase of bone formation. Bone fluid circulates within these channels and thereby bathes the surface of the bone (Mundy, 1995).

Two independent homeostatic systems influence the calcium in bone. Plasma calcium is regulated by one system which is able to move 500mmol of calcium into and out of the readily exchangeable pool in the bone per day. The other system is concerned with bone remodeling and is able to exchange around 7.5mmol of calcium per day between plasma and the stable pool of bone calcium (Ganong, 2003).

b) Phosphate Pool

Phosphorus is found in the body as inorganic phosphate or as phosphate esters. The bulk of the inorganic phosphate is in bone where it forms part of the mineralized extracellular matrix. However, this phosphate pool is only accessible in a somewhat limited fashion. About 300mg of phosphate enters and exits bone tissue each day (Ganong, 2003).

1.1.1.3 Water

Water makes up about 25% of the weight of living bone and it is found in the organic matrix, bone canals, and cavities that house cells. Water in bone is either 'bound' to the organic phase (collagen and cement substance) or it is free (bulk water) (Fernández-Seara et al., 2002).

Bulk (free) water fills the pores of the calcified matrix, which forms a network of interconnecting channels. This network consists of the lacunocanalicular system and this system communicates the Haversian canals (described in section 1.1.2) with the osteocytes. The function of this communicating network is to transport nutrients, waste products, and signaling molecules from the vascular system to the osteocyte and vice versa. The water channels also function as transport pathways for calcium and phosphorus ions that flow in and out of the bone tissue (Fernández-Seara et al., 2002).

early development from the mesenchyme is the same as that of fibroblasts (Lorenzo, 2000). Osteoblasts are found along surfaces of both cortical and trabecular bone.

Osteoblasts arise from bone marrow stromal stem cells of mesenchymal origin (Lorenzo, 2000). The exact number and sequence of steps leading to formation of mature osteoblasts is not known. However, the regulating process involves fibroblast growth factors (FGFs), bone morphogenic protein (BMP) and parathyroid hormone-related protein (PTH-rp) (Blair et al., 2002). Eventually, the osteoblasts become surrounded by the bone matrix that they form. At this stage, they are known as osteocytes.

b) Osteocytes (Osteoblasts)

They are bone cells that are derived from osteoblasts. The osteocyte lacunae and canaliculi are holes within the bone matrix that contains osteocytes and their processes. The osteocyte sits within the extracellular fluid inside the lacunae. Canaliculi are small tunnels which connect the lacunae. Canalicular processes starting at osteocytes travel through the osteocyte canaliculi to connect osteocytes. It is believed that these interconnections provide a pathway through which osteocytes can communicate information about deformation states (Robins and New, 1997). Thus, these cells may play an important role in detecting imperfections or microfractures and initiating a remodeling cycle. However, as they age they lose their bone forming ability.

c) Osteoclasts

Osteoclasts are large multinucleated cells that resorb previously formed bone. They are only found when bone is being resorbed and may be seen in small depressions on the bone surface (Masi and Brandi, 2001). They arise from haemopoietic cells in the monocyte-macrophage lineage. A range of cytokines and peptide regulators are required for the formation of mature osteoclasts from their precursor cells (Blair et al., 2002). They include colony stimulating factors (M-CSF or CSF-1, GM-CSF or CSF-2), the receptor activator of the nuclear factor kappa B ligand (RANKL), interleukins (IL-1 and IL-6), tumour necrosis factor alpha (TNF-α), prostaglandin-E2 and the active metabolite of vitamin D_3 (1,25 $[OH]_2$ D_3) (Blair et al., 2002).

1.1.2 Compact and Spongy Bone

The human skeleton comprises two different forms of bones viz. the cortical (compact) bone and trabecular (spongy) bone. Figure 5 shows compact and spongy bone.

spread throughout the compact bone. The Haversian canals contain blood vessels. Collagen is arranged in concentric layers around each Haversian canal forming cylinders called osteons or Haversian systems.

The trabecular bone 'is enclosed by' the cortical bone. It constitutes the remaining 20% of the bone in the body. It is made up of a honeycomb of vertical and horizontal bars called trabeculae. Trabecular bone contains many cells that sit on the surface of the trabeculae or bars. It is supplied with blood vessels, and it has a greater surface area to mass ratio relative to cortical bone. In addition, trabecular bone is more metabolically active because of its proximity to and extensive interface with bone marrow. It is also sensitive to hormones (described in detail in section 1.1.5.1) that influence daily deposits and withdrawals of calcium. Thus, trabecular bone is lost more rapidly than the compact bone in bone remodeling. The distal forearm, vertebral body, and hip are common sites for fractures as these areas have a high content of trabecular bone (Ganong, 2003).

Both the cortical and trabecular bone provide strength to bone. The cortical bone provides a sturdy outer wall whereas the trabecular bone provides support along the lines of stress (Wahlqvist and Wattanapenpaiboon, 2002).

Bone marrow lies within the internal cavities of the bones. In healthy adults, bone marrow produces blood cells and platelets and this

process (haemopoiesis) continues throughout life, replacing aged blood and immune cells (Ganong, 2003).

1.1.3 Bone Growth

Most of the fetal bones are modeled in cartilage which subsequently undergoes ossification i.e. transformed into bone. This is called endochondral (within the cartilage) or primary bone formation. However in a few bones such as the flat bones of the skull, clavicles, and mandible the mesenchymal cells differentiate directly into osteoblasts which lay down a matrix that is rich in type 1 collagen in a process called intramembranous bone formation (Kronenberg et al., 1998).

In endochondral bone formation, chondrogenesis (formation of cartilage) begins with the recruitment of chondrogenic mesenchymal cells into condensations. These cells then differentiate into chondrocytes, which produce cartilage specific extracellular matrix (ECM) proteins including type 11 collagen and the proteoglycan aggrecan. Chondrocytes then exit the cell cycle, become prehypertrophic and then hypertrophic. The hypertrophic chondrocytes are also master regulatory cells as they direct the mineralization of their surrounding matrix, and attract blood vessels through the production of vascular endothelial growth factor (VEGF) and other factors. They also direct adjacent perichondrial cells to become osteoblasts which secrete a characteristic matrix. A bone collar is thus formed. The hypertrophic chondrocytes subsequently undergo apoptosis

(programmed cell death) and the cartilage matrix left behind provides scaffolding for the osteoblasts. The blood vessels invade the cartilage and in association with vascular invasion, the osteoblasts replace the cartilage with bone (Kronenberg et al., 1998).

During growth, there are specialized areas called epiphyses which are present at the end of each long bone. These epiphyses are separated from the shaft of the bone by the epiphysial plate which is a plate of actively proliferating cartilage. This plate lays down new bone on the end of the shaft with resultant increase in the length of the bone which can go on as long as the epiphyses are separated from the shaft of the bone. However, such growth stops after the epiphyses unite with the shaft and this stage is called epiphysial closure. The cartilage cells (chondroblasts) stop proliferating. They then become hypertrophic and secrete vascular endothelial growth factor (VEGF) which leads to vascularization and ossification.

1.1.4 Bone Turnover (remodeling)

The skeleton is not metabolically inert as it undergoes considerable alterations throughout an individual's life (New, 2002). In bone remodeling, the skeletal matrix undergoes continuous site specific degradation that is balanced by new bone synthesis (Blair et al., 2002). Bone is continually remodeled in order to:

- Support a growing body
- Adapt to changes in applied force with growth that lead to different

stresses and strains

- Maintain calcium levels that are appropriate in extracellular fluid
- Repair microscopic and 'macroscopic' fractures that may be present over time (Blair et al., 2002).

This process of continual remodeling is normally maintained in a tightly linked balance between resorption (removal) of old or injured bone and formation of new bone (Christenson, 1997). Bone remodeling is mainly a local process. It is carried out in small areas by populations of cells that are known as bone-remodeling units (BRU). As bone remodeling occurs in discrete areas of the bone the site of remodeling is thus most likely under the control of localized regulatory factors. Bone remodeling is a continuous process in all living bone and about 5% of the bone mass is being remodeled at any given time by about 2 million remodeling units (Ganong, 2003).

Bone remodeling process involves the action of highly specialized cells called osteoclasts and osteoblasts (Christenson, 1997). Figure 6 shows the bone remodeling cycle for a single lacuna or cavity. The actual overall bone remodeling, however represents a sum of many such units.

between stromal cells and osteoclast progenitors. In addition, CAM's also appear to play a role in the interaction among osteoclast progenitors (Kurachi et al., 1993). CAM's are complimentary molecules that are present on cell surfaces. These molecules allow cells to interact with each other. Osteoclasts are carried by the circulation to the bone. This occurs in response to stimuli that are chemical and/or electrical (Blair et al. 2002). Osteoclasts are attached to bone via integrins in a membrane extension which is called the sealing zone. An isolated area is thus created between the bone and a portion of the osteoclast. This is then followed by the movement of proton pumps (H^+-dependent ATPases) from endosomes into the cell membrane apposed to the isolated area. The massive expression of vacuolar-like H^+-ATPase at the surface of the osteoclast distinguishes it from other giant cells. Osteoclasts acidify the area to approximately pH 4.0 by secreting hydrochloric acid (HCl) which dissolves hydroxyapatite crystals. Osteoclasts also secrete acid proteases which dissolve collagen causing shallow depressions in the bone. The digestion products are then endocytosed. These products move across the osteoclast into the interstitial fluid by transcytosis (Blair et al., 2002).

During bone remodeling, the osteoclasts resorb bone for about 2-3 weeks and this is followed by their programmed cell death (apoptosis). The osteoclast apoptosis is in turn followed by a series of sequential changes in cells of the osteoblast lineage which includes osteoblast chemotaxis, proliferation and differentiation (Masi and Brandi, 2001).

The initial event during bone formation is the secretion of collagen rich ground substance by osteoblasts and this substance is essential for later mineralization of hydroxyapatite and other crystals. Collagen fibers pack together forming collagen fibrils. Collagen fibrils are composed of molecules of tropocollagen in linear arrays. Tropocollagen is the forerunner for collagen. It is a subunit from which collagen fibrils self-assemble. Each fiber is separated from the fibers before and after it by a distance of 68nm. However, the distance between each fiber from the fibers on either side is only 1.5nm. The collagen strands form osteoids which are actually spiral fibers of precalcified bone matrix. There are distinct gaps within the packing of the collagen fibers and these gaps are sometimes called hole zones. The mineral crystals are formed within these holes between the fiber rows. The crystals grow over time and eventually expand out of the holes thereby forming continuous sheets throughout the fiber. Thus, the fiber spacing is decreased from 1.5nm to 1.1nm. These crystals grow parallel to each other within a specific collagen fibril in line with the orientation of the fibril channels. Osteoblasts cause calcium salts and phosphorus to precipitate from the blood. These minerals bond with the newly formed osteoid to mineralize the bone tissue.

The final form of the mineral crystal is believed to be dahllite which is a carbonate apatite mineral. It may initially resemble an octacalcium crystal which naturally forms in plates. These plates are packed into the type 1 collagen fibrils (Weiner and Traub, 1992). The

mineralized collagen fibril is the basic building block of bone. The orientation of the mineral crystals is determined by the orientation of the collagen fibrils. Osteoblastic-mediated bone formation is a slower process compared to osteoclastic-mediated bone resorption as it takes 2-3 months to complete (Kanis, 1994). The bone formation process is followed by the stoppage of osteoblast activity due to osteoblast apoptosis which is the final phase of the formation process (Masi and Brandi, 2001).

Cytokines (cell intercellular messengers) generated in the microenvironment of the bone resorbing pockets control the cellular events which make up the remodeling sequence. These cytokines are secreted by marrow mononuclear cells or by bone cells themselves. Cytokines can also be incorporated in the bone matrix and released in biologically active form as bone resorbs. The role of cytokines is described in greater detail in section 1.1.5.2. This remodeling sequence is a continuous phenomenon which is same on cancellous bone surfaces as it is within the Haversian systems of cortical bone (Mundy, 1992).

The renewal rate of compact bone is 4% per year and it begins with osteoclasts tunneling into the bone. On the other hand, the renewal rate of trabecular bone is 20% per year and remodeling occurs on the surfaces of trabeculae (Ganong, 2003).

1.1.4.1 Factors Affecting Bone Remodeling

Both general and local factors affect bone health.

a) General Factors

The general factors that affect bone health include age, nutrition, renal function, alcohol consumption, smoking, exercise, and estrogen.

With increasing age osteoblasts gradually become less active while osteoclasts continue working. This results in loss of bone strength and density. Calcium intake may also be low in old age. Also the kidneys do not activate vitamin D as well with increasing age. Many older people spend little or no time outdoors in the sunshine which affects vitamin D production. Some hormones that regulate bone remodeling e.g. estrogen also change (decreases) with age and this leads to accelerated bone mineral withdrawal.

Bone requires many nutrients such as calcium, phosphorus, magnesium, vitamins D, C, K, and A, and protein to develop and remain healthy (Heaney, 1996). Poor nutrition may result in lower peak bone mass. A diet high in meat products and cereals contributes to the acid load in the body.

Both alcohol and smoking are associated with increased risk of fracture. High blood alcohol levels may be directly toxic to osteoblasts (Heaney, 1996).

b) Local Factors

Exercise is one of the local factors that influence bone health. As a living and dynamic tissue, bone responds to exercise. Exercise has been shown to increase bone mineral density (BMD) in all age groups (Beck and Snow, 2003). The feet and legs bear the impact of the body's weight against the force of gravity during exercises such as walking, jogging, jumping, climbing stairs, or playing tennis (Beck and Snow, 2003).

Astronauts and bed ridden individuals show significant reduction in their bone density because they are not subjected to the impact of their body weight against gravity (Wronski, 1998). Weight bearing exercises appear to have a positive impact on bone density as they place stress on specific areas of the bone. This leads to remodeling of bone at these sites which results in stronger bones.

Exercise affects several aspects of bone metabolism as described below:

- Exercise increases cardiopulmonary efficiency which leads to increases in the partial pressure of oxygen in the circulation. Oxygen in turn is necessary for the energy deriving pathways in bone. Increased cardiopulmonary efficiency also increases the blood flow to the bone i.e. there will be an increase in the amount of nutrients supplied and waste products removed by the blood. This improves bone health (Loré, 1989).

- Individuals who exercise regularly have lower levels of corticosteroids. Glucocorticoid excess (seen in Cushing's syndrome either due to genetic disorder or glucocorticoid drug therapy) has been shown to suppress osteoblastogenesis in bone marrow. In addition, there is simultaneous inhibition of osteoprotegerin (OPG) and up-regulation of receptor activator of nuclear factor kappa B ligand (RANKL) expression which is required for osteoclastogenesis. There is also initial promotion of osteoclast survival. However, osteoclasts, osteoblasts and osteocytes subsequently undergo increased rates of apoptosis (Manolagas, 2000).
- Active individuals have increased muscle strength and coordination which in turn makes them less likely to fall and fracture their bones (Loré, 1989). The muscles are attached to bone by tendons that tug against the bones during muscle contraction. This tugging stimulates the bones to develop more trabeculae and grow denser. Thus, stronger muscles will provide greater stimulation (Vuori, 1996). In turn, stronger bones and muscles provide better protection against osteoporosis.

Obesity also influences bone mass. In one study of older women, those in the lower weight quartiles had significantly more bone loss than did those who were heavier (Hannan et al., 2000). Having extra body weight means that most movement is weight bearing. Estrogen is also produced by adipose tissue. Estrone (E1) is an estrogen that is synthesized from either adrenal androstenedione via aromatization in peripheral tissue e.g. adipose tissue, or from estradiol (E2) via a reversible reaction. Enzyme

aromatase is also present in the adipose tissue and it (aromatase) catalyses the conversion of androstenedione to estrone (Smith and Dowsett, 2003). Most of the estrogen in males and in post-menopausal women comes from adipose tissue. Estrogen in turn plays a positive role in bone health (refer section 1.1.5.1d). Thus, obesity also influences bone mass.

1.1.5 Regulation of Bone Turnover

Skeletal tissue is known to contain various growth factors that are believed to regulate bone turnover in growing and mature skeletal tissue. Growth factors may also participate in the anabolic processes involved in fracture repair. The cellular events that comprise the bone remodeling sequence are controlled by cytokines which are generated in the micro-environment of the bone resorbing pockets (Blair et al., 2002). However, the endocrines exert a primary control over the whole bone remodeling process.

1.1.5.1 General Regulation of Bone Turnover—The Role of the Endocrine Glands

Endocrine glands secrete hormones. The hormones that control the whole bone remodeling process are described below:

reabsorption of calcium in the distal tubules. Besides increasing the plasma calcium and depressing the plasma phosphate, PTH increases phosphate excretion in the urine. This action is called phosphaturic action and is due to a decrease in reabsorption of phosphate in the proximal tubule of the kidney. The increase in the formation of 1,25-dihydroxycholecalciferol in turn increases calcium absorption from the intestines (Kaplan and Pesce, 1989). In addition to the PTH secretion being increased when the levels of serum calcium are low, calcium is also released from the calcium pool in the bone fluid. The long term action of PTH on both osteoblasts and osteoclasts is stimulatory. However, the net effect varies. If the plasma PTH levels are mildly elevated then the effect is usually anabolic. Higher levels of PTH stimulate osteoclastic activity to increase bone resorption and mobilize calcium (Mannstadt et al., 1999). Thus, the amount of calcium filtered by the glomeruli is increased and so is the amount reabsorbed. However, the overall effect of PTH produces hypercalciuria.

Although PTH also plays an important role in the control of phosphate excretion, changes in phosphate concentration do not affect secretion of the hormone (Jilka et al., 1999). In conclusion, PTH acts on the bone, kidney, and indirectly on the intestine to increase plasma calcium concentration. PTH elevates the phosphorus concentration by its action on bone and indirectly the intestine. However, the PTH effect of increased renal phosphorus excretion is more than its other effects. Consequently, there is resultant decreased serum phosphorus concentration.

b) The Role of Calcitriol

With the help of sunlight, the body can synthesize vitamin D from a precursor that it makes from cholesterol. The action of ultraviolet light on 7-dehydrocholesterol which is present in the skin results in the formation of most of the cholecalciferol (vitamin D_3). This is the form in which vitamin D is found in animal tissues, especially the liver.

Vitamins D_2 and D_3 are inactive and must be metabolized to the active form. Vitamin D_3 is converted to 25-hydroxycholecalciferol by successive hydroxylation (on the 25-carbon) in the liver and this hydroxylation is catalyzed by enzyme 25-hydroxylase. 25-hydrocholecalciferol is the main circulating form of vitamin D and its rate of formation is affected by the supply of cholecalciferol derived from skin and intestine. Further hydroxylation of 25-hydrocholecalciferol in the cells of the proximal tubules of the kidney (on the 1α-hydroxy position) results in the formation of the more active metabolite 1,25-dihydroxycholecalciferol. This active metabolite is also called calcitriol or $1,25(OH)_2D_3$ which is the active vitamin D hormone (DeLuca and Zierold, 1998). Enzyme 1α-hydroxylase catalyses the hydroxylation of 25-hydrocholecalciferol to calcitriol. The activity of this enzyme and consequently the production of calcitriol may be stimulated by a low plasma phosphate concentration, an increase in the plasma PTH concentration and other hormones such as estrogens, prolactin and growth hormone. Estrogens, prolactin and growth hormone also increase the

production of calcitriol which leads to an increase in calcium absorption, during pregnancy, lactation and growth respectively. The primary stimuli for calcitriol synthesis are decreased serum calcium concentration, increased PTH secretion, and decreased intracellular phosphorus concentration. PTH is the major stimulus for the formation of calcitriol and it may act by stimulating enzyme 1-hydroxylase which hydroxylates calcidiol at the C-1 position and indirectly by lowering the intracellular concentrations of phosphorus (Ganong, 2003).

Calcitriol stimulates the absorption of dietary calcium and phosphate in the gut. This mechanism involves the synthesis of a calcium-binding protein (calbindin) in enterocytes which facilitates calcium movement across the intestine. Calcitriol also promotes mineralization in bone. The actions of calcitriol are closely interrelated to the actions of PTH (Kaplan and Pesce, 1989). Both act on the intestine, bone and kidney to increase serum calcium concentration. Figure 7 depicts the interrelationship of serum calcium concentrations and PTH, and calcitriol. Besides increasing calcium absorption from the intestine and facilitating calcium reabsorption in the kidneys, calcitriol also increases the synthetic activity of osteoblasts and is necessary for normal calcification of bone matrix (White et al., 1998). However, calcitriol stimulates osteoclastic bone resorption when it is present at high concentrations. This action releases calcium and phosphate into the extra cellular fluid (ECF) (Marshall, 1992).

Thus, calcitriol acts on three major target organs and they are the intestine, bone, and kidney. In the intestine, calcitriol facilitates the absorption of both calcium and phosphate. By working cooperatively with PTH, calcitriol increases bone resorption by increasing osteoclastic activity. The resultant net effect of the action of calcitriol at bone and intestine is to raise the concentrations of available blood calcium and phosphorus. This in turn facilitates the mineralization of newly formed bone matrix. In the kidney, calcitriol increases the renal absorption of both calcium and phosphorus. However, the effect of plasma calcitriol concentrations on renal calcium reabsorption is small because most of the filtered calcium is normally reabsorbed anyway.

PTH is secreted when the concentration of plasma calcium is low and it (PTH) then acutely restores plasma calcium concentration to normal (Figure 7). This is accomplished by stimulating osteoclasts to resorb bone. Within hours the calcitriol synthesis is increased which leads to increased intestinal calcium absorption. Subsequently, the plasma calcium concentration and indirectly the PTH concentration are restored to normal. If, on the other hand, the plasma calcium concentration is elevated then there will be an opposite effect i.e. a reduction in both plasma PTH concentration and in the production of calcitriol (Kaplan and Pesce, 1989).

secretion is decreased parallel to an acute drop in plasma calcium (Marshall, 1992).

d) The Role of Estrogen

The roles of estrogen are multifunctional as it influences growth, differentiation, and functions in many tissues (Oursler et al., 1991). Estrogen receptors have been identified in bone (Komm et al., 1988; Oursler et al., 1991) and this indicates that the hormone likely has a direct effect on bone cells.

The action of estrogen is to terminate bone growth in females at puberty and it also plays an important role in the maintenance of adult bone mass (Nilsson et al., 2001). However, the fundamental processes that are involved in bone formation and resorption do not require estrogen (Blair et al, 2002).

Deficiency of estrogen leads to increased osteoclastic activity (Riggs et al., 2002). The mechanisms by which estrogen deficiency brings about bone resorption remain controversial.

1.1.5.2 Local Regulation of Bone Turnover--The Role of Cytokines

Cytokines are polypeptides secreted by specific cells in response to a stimulus. They act as cell intercellular messengers. Cytokines bind receptors on target cells and then evoke changes in them. This happens through a series of signal transduction events that mostly lead to changes in

gene expression. Cytokines exhibit three modes of action and they are autocrine, paracrine, and endocrine (Lazar-Molnar et al., 2000). They are described below:

- Autocrine action. In this case the cytokine influences the same cell that produces it. Some cells have receptors for the cytokines that they produce.
- Paracrine action. The cytokine, in this instance, acts on neighbouring cells.
- Endocrine action. In this case, the cytokine is released into the circulation and acts in an endocrine manner. It may travel to other areas in the body before reacting with appropriate receptors.

Cytokines play an important role not just in physiological bone remodeling, but also in common diseases of bone remodeling such as osteoporosis, osteopetrosis and Paget's disease (Hofbauer and Heufelder, 2000). Cytokines regulate the underlying cell differentiation of osteoblasts and osteoclasts.

Cytokines such as fibroblast growth factor (FGF), bone morphogenetic protein (BMP), parathyroid hormone-related protein (PTH-rp) and Indian hedgehog (IHH) are involved in the process of change of the mesenchymal stem cells from chondrocyte to osteoblast differentiation (Kronenberg et al., 1998). Osteoblasts in turn produce important regulatory proteins such as the tumour necrosis factor (TNF), family protein receptor

activator of nuclear factor kappa B ligand (RANKL), that plays a role in increasing the number of osteoclasts (Blair et al., 2002).

The main sources of RANKL production in the bone/bone marrow microenvironment are bone marrow stromal cells, osteoblast lineage cells (bone forming), and the T lymphocytes. RANKL is expressed as a molecule on their surface (Ganong, 2003).

The final mediator of the regulation of bone resorption is most likely the RANK ligand (RANKL) RANK/osteoprotegerin (OPG) system. RANKL binds to its receptor RANK on the surface of the bone resorbing or osteoclast precursor cells (monocytes) in the bone marrow. RANKL, along with another molecule called Macrophage Colony Stimulating Factor (M-CSF) that is made by the pre-osteoblasts are both necessary and sufficient to develop osteoclasts. M-CSF binds to a receptor (c-fins) on the monocytes. The binding of RANKL to RANK on hematopoietic progenitors in the presence of M-CSF influences the differentiation of osteoclasts. This occurs through a signal transduction cascade that involves specific signaling molecules called Tumour Necrosis Factor Receptor-Associated Factors (TRAFs). The cytoplasmic tail of RANK has proteins c-Src and Cbl that send stimulatory signals to downstream second messenger pathways that include NF-κB and JKN/SAPK. These second messenger pathways regulate bone resorption as well as the activation, survival and differentiation of osteoclasts. The binding of RANKL to RANK also increases mature osteoclast activity. The pre-osteoblastic cells also make a

soluble decoy receptor called osteoprotegerin (OPG). OPG binds and neutralizes RANKL i.e. it competes with RANK for binding of RANKL. (Kartsogiannis et al., 1999). The binding of RANKL to osteoprotegerin (OPG) inhibits the formation of osteoclasts and also the activation of mature osteoclasts (Theill et al., 2002). Thus, changes in the RANKL to OPG ratio determine, in the end, how many osteoclasts are formed in the bone marrow. This in turn determines the amount of bone that will be resorbed.

1.1.6 Mis-regulation of Bone Metabolism

The most common condition that results from mis-regulation of bone turnover in adults is osteopenia and osteoporosis.

Osteopenia refers to the presence of less than normal amount of bone i.e. decreased calcification or density of bone which is not as severe as in osteoporosis. If not treated osteopenia may result in osteoporosis. The risk factors for osteopenia are similar to osteoporosis. They include genetic predispositions, sedentary lifestyle, low dietary intake of calcium, and vitamin D, petite stature and small bone structure. Factors such as long term use of steroids or heparin, cigarette smoking, and declining levels of estrogen after menopause are also risk factors for osteopenia (Croarkin, 1999).

Osteoporosis, on the other hand, is a systemic skeletal disease characterized by low bone mass in association with micro architectural

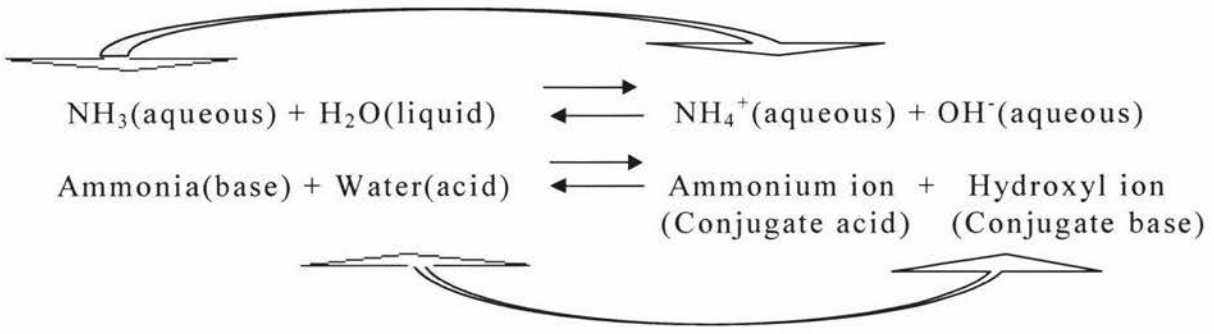
deterioration of bone tissue. In osteoporosis, the bone mass is so reduced that the skeleton loses its integrity and becomes unable to perform its supportive function. Also, there is an increased risk of fracture (Khastgir et al., 2001).

1.2 Acid-Base Homeostasis

Acid-base homeostasis is critical to health. The pH of extracellular fluid remains between 7.35 and 7.45 (New, 2002). pH is the reciprocal of the log of the hydrogen ion concentration. The H^+ concentrations must be kept between narrow limits as this is essential to survival (Green and Kleeman, 1991). At higher concentrations, H^+ can bind to negatively charged proteins, including enzymes, with resultant impairment of their function. Even mild metabolic acidosis may over time contribute to the origin and development of the physiological disturbances and degenerative diseases typical of aging such as osteoporosis (Frassetto et al., 1996).

1.2.1 Acids and Bases

An acid is a substance that generates protons or hydrogen ions (H^+). A base, on the other hand, is a substance that accepts protons or H^+. Every acid has a conjugate base. Like-wise, every base has a conjugate acid. An example is shown below:



Conjugate pairs are acids and bases that differ only in the number of protons (H^+) i.e. an acid or base related by loss or gain of proton as shown in the equation above. Bases which are soluble in water are called alkali. Ammonia (NH_3) is an important base. It forms an alkaline solution in water (refer to the above equation).

Carbonic acid (H_2CO_3) and bicarbonate (HCO_3^-) are physiological examples of a weak acid and its conjugate base respectively. The equilibrium reaction is as follows:

$$[H_2CO_3] \rightleftharpoons [H^+] + [HCO_3^-]$$

Two types of acids are generated during metabolism i.e. fixed acids and the volatile acids. Fixed acids are either nongaseous or organic acids. Some examples of nongaseous acids are phosphoric acid (H_3PO_4) and sulphuric acid (H_2SO_4). Acids such as lactic acid, acetoacetic acid, and beta-hydroxybutyric acid are organic acids. Carbonic acid (H_2CO_3), on the other hand, is a physiologically important volatile acid and its volatility is due to its ability to dissociate into water (H_2O) and carbon dioxide (CO_2).

This CO_2 can be released as a gas. Refer to the following equation for the complete reaction scheme for carbonic acid.

$$[CO_2](gas) \rightleftharpoons [CO_2](dissolved) \overset{+H_2O}{\underset{-H_2O}{\rightleftharpoons}} [H_2CO_3] \rightleftharpoons [H^+] + [HCO_3^-]$$

Carbon dioxide is at one end of the equilibrium and it can be regarded as the anhydrous (without water) form of H_2CO_3. At the other end of the equilibrium is HCO_3^- which is the conjugate base of H_2CO_3. The enzyme carbonic anhydrase catalyzes the reaction of CO_2 and H_2O to form H_2CO_3 *in vivo*.

Organic acids are generated by oxidation reactions during the metabolism of carbohydrates, lipids and proteins. These acids must be neutralized.

Carbohydrates are metabolized to lactic acid under anaerobic conditions that occur during respiratory distress or strenuous exercise. The acid tends to accumulate until normal oxygenation is restored. Normal oxygenation leads to the resumption of aerobic metabolism of these acids i.e. they can be metabolized to CO_2 which is the final oxidation product.

Triglycerides are metabolized to fatty acids. Under anaerobic conditions, these fatty acids can be further metabolized to ketone bodies such as acetoacetic acid and beta-hydroxybutyric acids. Further oxidation of the ketone bodies leads to the formation of CO_2.

The CO_2 that is produced during metabolism of carbohydrates, proteins, and fats is excreted by the lungs. However, proteins that are

composed of sulfur-containing amino acids such as methionine, cysteine and cystine are catabolized by oxidation in part to sulphuric acid. The phosphorus containing lipids (phospholipids) and nucleoproteins are oxidized to phosphoric acid (Kaplan and Pesce, 1989). These nonvolatile acids are first buffered by the bicarbonate/carbonic acid system. The reaction with sulphuric acid is shown below:

$[H_2SO_4] + [2NaHCO_3] \rightleftharpoons [Na_2SO_4] + [2H_2CO_3] \rightleftharpoons [2H_2O] + [CO_2]$

Sulphuric acid + Sodium bicarbonate $\rightleftharpoons$ Sodium sulphate + Carbonic acid $\rightleftharpoons$ Water + Carbon dioxide

In this instance, the net result is that H_2SO_4 is buffered by 2 molecules of HCO_3^- and a weak acid H_2CO_3 is produced. Thus, the change in pH is minimized. The CO_2 produced is excreted by the lungs. In order to prevent the progressive loss of HCO_3^- in metabolic acidosis the kidney replaces the consumed HCO_3^-. This is principally done by the simultaneous secretion of H^+ in the collecting duct and HCO_3^- into the plasma (Kaplan and Pesce, 1989).

1.2.2 pH, Hydrogen Ion, and Buffers

pH can be defined as the negative logarithm of the concentration of H^+. The pH of arterial blood generally lies between 7.35 to 7.45 whereas the pH of the venous blood is expected to be lower because of the waste (CO_2) produced during metabolism in the body i.e. the concentration of CO_2 in the blood that leaves the tissues is higher than it is in the blood that

leaves the lungs. This higher concentration of CO_2 in the venous blood stimulates the production of HCO_3. Thus, the venous pH will tend to be lower than the arterial pH. This is how the arteriovenous difference in the ratio of HCO_3^-:CO_2, and thus the pH is kept relatively constant. In a healthy individual, the pH value (7.40) usually varies less than ±0.05 pH unit. The buffering capacity of the body fluids stabilizes the body pH (Zilva et al., 1988).

Normal human whole blood is buffered at the pH range of 7.35 to 7.45. At this range it is slightly alkaline (Kaplan and Pesce, 1989).

Buffers are weak acids or bases that can minimize changes in pH by taking up or releasing H^+. In this way, the pH of the solution is kept relatively constant even when considerable quantities of acid or base are added (Kaplan and Pesce, 1989). Thus, buffers act as first line defence to blunt the changes in pH.

1.2.3 Law of Mass Action

This law states that the speed (velocity) of any given chemical reaction is commensurable to the product of the reactant concentrations.

$$[H_2CO_3]\,(\text{acid}) \rightleftharpoons [H^+] + [HCO_3^-]\,(\text{base})$$

$$\text{Carbonic acid} \rightleftharpoons \text{Hydrogen ion} + \text{Bicarbonate}$$

The Law of Mass Action is universal. Thus, this law can be applied under any circumstances. When the reversible reaction has reached equilibrium at

a given temperature, the reaction quotient remains constant (Ganong, 2003).

Diffusion is a process by which a gas or a substance in solution expands and this expansion is due to the motion of its particles that try to fill all of the available volume. The particles (molecules or atoms) of a substance that are dissolved in a solvent are moving continuously in a random manner. Thus, a given particle is just as likely to move into or out of an area in which it is present in high concentration. However, there will be a net flux of solute particles from areas of high to areas of low concentration because there are more particles in the area of high concentration. The magnitude of the diffusing tendency from one area to another is directly proportional to the cross-sectional area across which diffusion is taking place and the concentration gradient, or chemical gradient (Ganong, 2003). We can use the bicarbonate pair as an example. Carbonic acid (H_2CO_3) dissociates into H^+ and HCO_3^- and this will go on until equilibrium is reached (in this instance it strongly favours H_2CO_3). The ratio of the two forms will be constant (dissociation constant, K) when equilibrium is reached. Thus, we can write:

$$K\ [H_2CO_3] = [H^+] \times [HCO_3^-]$$

(which means that at equilibrium the concentration of H_2CO_3 is K multiplied by the product of $[H^+]$ and $[HCO_3^-]$). The square brackets "[ ]" that are placed around the chemical species depict their concentrations.

Interchanging $$[H^+] = K \frac{[H_2CO_3]}{[HCO_3^-]}$$

Thus, by the law of mass action, the product of the concentrations of the products in a chemical reaction divided by the product of the concentration of the reactants at equilibrium is a constant (Zilva et al., 1988).

1.2.4 Physiological Buffers

The physiologically important buffers in the body are hemoglobin, bicarbonate, phosphate, and proteins (Kaplan and Pesce, 1989). They help to maintain the narrow pH range.

1.2.5 Acid-Base Regulatory Mechanisms

There are two acid-base regulatory mechanisms viz. systemic regulation (buffering) and the eliminative regulation (removal of H^+).

1.2.5.1 Systemic Regulation

The systemic regulatory mechanism deals with the buffering of acids which could be either rapid or delayed.

a) Rapid Buffering

The acid load is first distributed in the extracellular fliud volume. This occurs rapidly and in this way a great deal of acid can be

neutralized. However, the acid has not been excreted. The carbonic acid-bicarbonate buffer system, plasma proteins, and phosphate rapidly buffer acids.

The carbonic acid-bicarbonate system is an important buffer system in the body and it takes place in the extracellular fluid. Carbonic acid dissociates to hydrogen ion and bicarbonate and this process is reversible as shown in the following equation:

$$[H_2CO_3] \rightleftharpoons [H^+] + [HCO_3^-]$$

$$\text{Carbonic Acid} \rightleftharpoons \text{Hydrogen Ion} + \text{Bicarbonate}$$

When H^+ is added to the blood the HCO_3^- will decline as more H_2CO_3 is formed as a result of the Law of Mass Action. In the body H_2CO_3 is in equilibrium with CO_2.

$$[H_2CO_3] \rightleftharpoons [CO_2] + [H_2O]$$

The extra H_2CO_3 is converted to carbon dioxide (CO_2) and water (H_2O). The CO_2 is excreted by the lungs which means that the concentration of dissolved CO_2 is controlled by respiration. Thus, all the extra H_2CO_3 that is formed is removed. The HCO_3^- that is consumed during this reaction is regenerated by the kidney.

In chronic metabolic acidosis, intracellular buffers such as hemoglobin and bone may be more important than HCO_3^- because the extracellular HCO_3^- level is low.

The plasma proteins are effective buffers in the blood and in the intracellular fluid compartment because they can dissociate both their carboxyl and amino groups as shown in the following equations:

$$RCOOH \rightleftharpoons RCOO^- + H^+$$

$$RNH_3^+ \rightleftharpoons RNH_2 + H^+$$

Amino acids such as lysine, arginine, histidine, glutamate, and aspartate can bind or release H^+.

Phosphate is a minor buffering component of blood. The equilibrium reaction is as follows:

$$H_2PO_4^- \rightleftharpoons H^+ + HPO_4^{-2}$$

Dihydrogen phosphate $\rightleftharpoons$ Hydrogen ion + Monohydrogen phosphate

It is not a quantitatively important buffer in the plasma because its phosphate concentration is too low. Phosphate, however, is an important buffer intracellularly and in the urine which has little protein, hemoglobin, or bicarbonate.

b) Delayed Buffering—The Role of The Skeleton in Acid-Base Imbalance

Both erythrocytes (red blood cells) and bone are involved in delayed buffering.

The buffering capacity of erythrocytes is due to the dissociation of the imidazole groups of the histidine residues of hemoglobin (Ganong,

2003). However, the mechanism is not described as it is not the main part of the study.

The skeleton can also be considered a buffer source during both acute and chronic metabolic acidosis (Barzel, 1970; Bernstein et al., 1970).

It has been shown (during acute metabolic acidosis) that serum calcium rises rapidly in nephrectomized animals that were infused with mineral acid and this means that there is dissolution of bone mineral (Kraut et al., 1984). This quick release of bone calcium is not due to cell-mediated changes in bone resorptive activity but appears to be due to changes in the physicochemical factors that govern the deposition and dissolution of bone mineral (Bushinsky et al., 1985). Another study found that calcium is released from cultured synthetic carbonated apatite discs in response to a physiologic reduction in pH (Bushinsky et al., 1994). These synthetic carbonated apatite discs can be considered to be an accurate model of bone mineral that is, however, without cells. The investigators cultured synthetic carbonated apatite discs in physiologically acidic medium and found that calcium is released from cultured discs in response to a physiologic decrement in pH. This finding supports the hypothesis that excess hydrogen ions directly bring about physicochemical calcium release from bone (Bushinsky et al., 1994).

Carbonate or phosphate in combination with calcium could be the type of bone mineral that is in equilibrium with the medium. This equilibrium can therefore be changed by the physicochemical forces with

resultant calcium release. In order to determine which of these forms were involved Bushinsky and Lechleider (1987) cultured calvariae (skullcap) in a medium where the driving forces for crystallization in relation to the solid phase of the bone mineral were modified by changing the pH of the medium. They found that there was formation of bone in a supersaturated solution in which calcium and carbonate were used. However, this was not the case where calcium and phosphate were used. Thus, it appears that bone carbonate in the form of carbonated apatite undergoes dissolution when there is a fall in medium pH with resultant release in calcium. The role of carbonate in proton mediated solubilization of bone mineral is further supported by another study which found that at a constant pH, which could be either physiologically neutral or acid, the outward flow of calcium from bone is dependent on the concentration medium bicarbonate. The efflux of calcium was greater with correspondingly lower levels of bicarbonate (Bushinsky and Sessler, 1992). Figure 8 is a schematic diagram of the mechanisms by which protons lead to the release of bone calcium and their buffering by the bone mineral during chronic metabolic acidosis

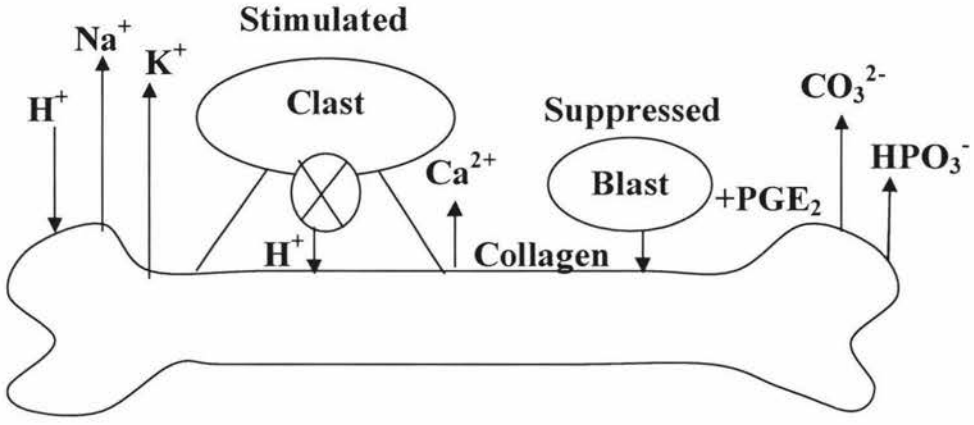


Figure 8. Metabolic acidosis. Schematic diagram of the mechanisms by which protons lead to the release of bone calcium and are buffered by the bone mineral during chronic metabolic acidosis. Clast = Osteoclast, Blast = Osteoblast, PGE_2 = Prostaglandin E_2, CO_3^{2-} = Carbonate Ion, HPO_3^- = Phosphate Ion, Ca^{2+} = Calcium Ion, H^+ = Hydrogen Ion, Na^+ = Sodium Ion, K^+ = Potassium Ion. From: Bushinsky, D.A. (2001).

During acidosis, the low pH is restored toward the normal range not only by an increase in the efflux of calcium from bone but also by the apparent buffering of the additional protons by the mineral phases of bone. Acute acid loads induce the loss of sodium from bone (Bushinsky et al., 1993b) which suggests that proton is exchanged with sodium. Such acid loads also deplete bone carbonate which suggests that carbonate is consumed by protons (Bushinsky et al., 1993a).

The surface of bone is rich in sodium and potassium as compared to calcium and this has been demonstrated by examining calvariae with a high resolution scanning ion microprobe (Bushinsky et al 1993b). Fixed negative sites are present on the bone's surface and these sites can

normally complex with sodium, potassium and hydrogen ions. The sodium seems to exchange freely with the surrounding fluid. When the pH falls the additional hydrogen ions displace sodium and potassium from the mineral surface which leads to an efflux of these ions with resultant reduction of the systemic acidity. It has been demonstrated by Bushinsky et al (1993b) that there is a net influx of hydrogen ions into the bone and loss of surface sodium and potassium as compared to calcium when calvariae are incubated in an acidic medium. This finding suggests that sodium and potassium are exchanged for proton on the surface of the bone (Bushinsky et al., 1993b). This influx reduces the medium hydrogen ion concentration suggesting that bone is buffering the additional hydrogen ions.

Bone contains significant amounts of carbon dioxide (CO_2) because approximately 80% of the total body CO_2 is found there (Pasquale et al., 1980). However, nearly two-thirds of this CO_2 is found in the form of carbonate which is complexed with calcium, sodium and other cations. It is found in the lattice of the bone crystals and is therefore relatively inaccessible to the systemic circulation. The remaining third consists of bicarbonate which is found in the hydration shell of hydroxyapatite where it is readily accessible to the systemic circulation (Pasquale et al., 1980).

In chronic metabolic acidosis, the release of bone calcium appears to occur as a result of physicochemical dissolution of the bone mineral. In addition, there is also increased cell-mediated bone resorption.

Studies have also been done to determine the effects of changes in pH on cell-mediated bone resorption. Arnett and Dempster (1986) used isolated rat osteoclasts that were cultured on slices of polished bovine femur. The investigators found that bone resorption areas increased in the acidic pH medium relative to the physiologically neutral pH. It has also been shown that cell-mediated bone resorption occured when calvariae were cultured for one week in an acidic medium (Goldhaber and Rabadjija, 1987). However, this resorption was suppressed by calcitonin which is an osteoclastic inhibitor.

Metabolic acidosis increases the activity of osteoclasts. However, it decreases the activity of osteoblasts. Krieger et al (1992) reported that metabolic acidosis stimulates the release of the osteoclastic enzyme β-glucuronidase. However, metabolic acidosis inhibits the synthesis of collagen by osteoblasts, and it also inhibits alkaline phosphatase activity (Krieger et al., 1992).

Both acute acidosis and chronic acid load appear to reduce bone carbonate and thus bone carbonate may be a physiologic hydrogen ion acceptor (Burnell, 1971). The effect of chronic acidosis on bone phosphate and bicarbonate (bone hydrogen ion buffers) was studied by Bushinsky et al (1999). The authors reported that one week of mild metabolic acidosis results in a decrease in mineral phosphate and bicarbonate. This means that the excess protons are buffered by phosphate and bicarbonate in order to return the systemic pH toward normal.

1.2.5.2 Eliminative Regulation

This mechanism deals with the removal of acids in the body.

a) The Role of Kidneys in Acid-Base Homeostasis

The kidneys have to perform 2 physiological functions in order to maintain normal pH and they are as follows:

- They reclaim all the filtered HCO_3^-. It has to be stressed that any loss of HCO_3^- is equal to the addition of an equimolar amount of H^+. This function is mainly carried out by the proximal tubule.
- The next function of the kidney is to excrete the daily H^+ load. This is a function of the collecting duct. In this case the loss of H^+ is equal to the addition of an equimolar amount of HCO_3^-.

The kidneys play a vital role in compensating for chronic metabolic acidosis because they are able to replenish the buffers and also because most of the excess H^+ can only be removed from the body via the renal route (Zilva et al., 1988).

The $[HCO_3^-]$ in the extracellular fluid is controlled by two renal mechanisms:

- Bicarbonate reclamation
- Bicarbonate generation

All of the filtered bicarbonate has to be reclaimed. HCO_3^- cannot be reabsorbed directly because the luminal surfaces of the renal tubular cells are not permeable to it. It is for this reason that HCO_3^- is first

converted to CO_2 in the tubular lumen. At the same time an equimolar amount of CO_2 is converted to HCO_3^- inside the tubular cell. The reaction is shown below:

$$[HCO_3^-] + [H^+] \rightleftharpoons [H_2CO_3] \rightleftharpoons [H_2O] + [CO_2]$$

Enzyme carbonic anhydrase that is present in the brush border of the first two segments of the proximal tubule accelerates the dissolution of H_2CO_3 into H_2O and CO_2. Thus, the above reaction shifts to the right and the luminal concentration of H^+ is kept low. The CO_2 that is formed diffuses into the proximal tubular cell. Here, carbonic anhydrase combines CO_2 and H_2O to form HCO_3^- and H^+. The HCO_3^- that is formed intracellularly goes to the pericellular space and then into the circulation (Soleimani and Burnham, 2000).

This unlimiting cycle reclaims buffering capacity which would otherwise be lost to glomerular filteration. The cellular water is the source of the secreted H^+ which is then reincorporated into water in the lumen. This mechanism cannot correct an acidosis because there is no net change in hydrogen ion balance and there is also no net gain of bicarbonate. Thus it can only maintain a steady state (Zilva et al 1988).

The kidneys can generate new bicarbonate through urinary production of ammonium salts and through urinary excretion of titratable acids. The bicarbonate generation mechanism is important as it can correct acidosis due to the fact that there is net loss of H^+ as well as a net gain of HCO_3^-. It is identical to the bicarbonate reabsorption mechanism as far as

the generation of HCO_3^- is concerned. A rise in the intracellular concentration of CO_2 or a fall of HCO_3^- may stimulate the intracellular carbonate dehydratase (carbonic anhydrase) mechanism. The continued secretion of H^+ and generation of HCO_3^- depend on the presence of other filtered buffers once the luminal fluid becomes exhausted of HCO_3^-. Also, in the bicarbonate generation mechanism the H^+ are not incorporated into H_2O. Thus, there is net loss of H^+ in urine. In addition, the HCO_3^- that is formed in the cell comes from cellular CO_2 and not from luminal HCO_3^-. Therefore, there is a net gain in HCO_3^-. A mmol of HCO_3^- goes into the ECF with sodium (Na) whenever a mmol of H+ is secreted into the urine (Zilva et al., 1988).

i) Acid Excretion

The daily acid load is excreted principally through H^+ secretion by the apical H^+-ATPase in A-type intercalated cells of the collecting duct. The bicarbonate that is formed intracellularly goes back to the systemic circulation via the basolateral Cl^-/HCO_3^- exchanger called AE1 which has a gene symbol *SLC4A1*. The H^+ enters the tubular lumen through 1 of 2 apical proton pumps which is H^+-ATPase or H^+-K^+-ATPase. The H^+ secretion in these segments is affected by the reabsorption of Na^+ in the adjacent principal cells of the collecting duct. The reabsorbed Na^+ causes relative lumen negativity. This lumen negativity decreases the amount of secreted

H^+ that back-diffuses from the lumen. The weak bases ammonia or phosphate buffer the H^+ load which is then excreted in this form.

Na^+ is also extruded from the renal tubular cell into the interstitium by the Na^+-K^+-ATPase with resultant decrease in the intracellular concentration of Na^+. This causes Na^+ to enter the cell from the tubular lumen and there is concomitant extrusion of H^+ into the tubular lumen. Refer to Figure 9 to view the fate of H^+ secreted into a tubule in exchange for Na^+.

- Reactions with HPO_4^{2-} to form H_2PO_4
- Reactions with NH_3 to form NH_4^+.

Besides HCO_3^- , there are also other buffers that participate in the generation of HCO_3^- that is linked to H^+ secretion and the two most important ones are phosphate and ammonia.

ii) Excretion of Titratable Acid

The major buffer in urine is phosphate. Most of the phosphate present in the plasma at pH 7.4 is in the form of monohydrogen phosphate (HPO_4^{2-}) and therefore the same form of phosphate is also predominant in the glomerular filterate. This HPO_4^{2-} has the ability to accept the H^+ that is produced by the carbonate dehydratase mechanism to become dihydrogen phosphate ($H_2PO_4^-$). Thus, the generation of HCO_3^- can continue.

The increase in phosphate leads to an increase in buffering capacity of the glomerular filterate which in turn meets the need for increased urinary H^+ secretion. Phosphate is an important urinary buffer because its concentration increases 20 fold as water is reabsorbed from the tubular lumen. There will be an increase in the titration of HPO_4^{2-} to $H_2PO_4^-$ when there is concomitant addition of H^+ to the tubular fluid. This will go on until the pH falls below 5.5 and at this stage most of the phosphate present will be in the acid form. Luminal Na^+ is reabsorbed in exchange for cell H^+ and exits together with HCO_3^- formed in the cell across the basolateral membrane. For every H^+ secreted that titrates the

phosphate in the lumen, there is generation of one molecule of bicarbonate that enters the circulation and helps restore the buffering capacity of the body (Zilva et al., 1988).

In severe metabolic acidosis the generation of bicarbonate becomes very important, and when the pH is low the phosphate that is present in the urine cannot maintain the very important continued secretion of H^+. In addition, the amount of phosphate filtered is limited and fixed, and thus only a fraction of the secreted H^+ can be buffered by HPO_4^{2-} (Zilva et al., 1988).

Dietary phosphate can act as a urinary buffer. It will first be absorbed and then metabolized. The extra will be excreted in the urine where it can act as a buffer.

The most important form of acid excretion in the urine, however, is ammonium because the urine contains increasing amounts of ammonium ion (NH_4^+) as it becomes more acid. The ammonia that is present in the urine probably allows H^+ secretion, and hence bicarbonate formation, to go on after other buffers have been exhausted.

NH_4^+ and HCO_3^- are produced by reactions in the renal proximal tubular cells. Intracellularly, NH_4^+ is in equilibrium with NH_3 and H^+. Glutamine enters the proximal tubule cells from the peritubular capillary blood and from the filterate. Within the cell, glutamine enters the mitochondria and is deaminated by enzyme glutaminase. This reaction is enhanced by acid load. Glutaminase acts as a catalyst in the hydrolysis of

glutamine ($GluCONH_2$) to form glutamate ($GluCOO^-$) and the ammonium ion (NH_4^+) as shown in the following reaction:

$$H_2O + GluCONH_2 \xrightarrow{\text{Glutaminase}} GluCOO^- + NH_4^+$$

$$\text{Water + Glutamine} \xrightarrow{\text{Glutaminase}} \text{Glutamate + Ammonium Ion}$$

Glutaminase is abundantly present in the renal tubular cells. Glutamate is converted to α-ketoglutarate and this reaction (shown below) results in the production of more NH_4^+. Enzyme glutamic dehydrogenase catalyzes this reaction. Subsequently α-ketoglutarate is metabolized utilizing 2 H^+ and at the same time freeing $2HCO_3^-$.

$$\text{Glutamate} \xrightarrow{\text{Glutamic dehydrogenase}} \text{α-Ketoglutarate} + NH_4^+$$

The apical $Na^+/H^+(NH_4^+)$ antiporter secretes NH_4^+ into the proximal tubular lumen. NH_4^+ is then transported by the apical $Na^+/K^+(NH_4^+)2Cl^-$ cotransporter present in the thick ascending limb of the loop of Henle into the medullary interstitium. The NH_4^+ dissociates back into NH_3 and H^+ in the medullary interstitium. NH_3 is lipid-soluble and is thus able to diffuse across the cell membranes into the lumen of the collecting duct. This movement is down its concentration gradient. NH_3 is available to buffer H^+ in the lumen and by doing so it becomes NH_4^+. NH_3 is thus trapped in the lumen and subsequently excreted as neutral salts such as ammonium chloride (NH_4Cl). In essence, every H^+ that is buffered is an HCO_3^- gained to the systemic circulation. When the secretion of the H^+ in the collecting

duct is increased the following equation shifts to the right with resultant decrease in the concentration of NH_3. The decreased concentration of NH_3 in the collecting duct facilitates the continued diffusion of NH_3 from the interstitium down its concentration gradient (Law of Mass Action) thereby permitting more H^+ to be buffered (Karim et al., 2002).

$$[NH_3] + [H^+] \rightleftharpoons [NH_4^+]$$

Ammonia + Hydrogen Ion $\rightleftharpoons$ Ammonium Ion

Thus, in order to meet demand the kidney can adjust the amount of NH_3 produced. For example, in chronic metabolic acidosis, the amount of NH_4^+ excreted also increases. This occurs because more NH_3 enters the tubular urine which results in further removal of H^+ from the tubular fluid. Consequently, the secretion of H^+ is further enhanced.

The mechanism by which NH_3 is secreted into the urine and subsequently changed into NH_4^+ thereby maintaining the concentration gradient for diffusion of NH_3 is known as non-ionic diffusion. It means that the undissociated form of some weak acids and bases (e.g. NH_3) are quite soluble in cell membranes. However, this is not the case when they are present in the ionic form i.e. they cross membranes with difficulty. Thus, there will be appreciable net movement of the undissociated substance from one side of the cell membrane to the other when its molecules diffuse from one side of the membrane to the other and subsequently dissociate (Ganong, 2003).

H^+ is also produced during the formation of ammonia from glutamine i.e. during dissociation of NH_4^+. In this case, it appears that there is no advantage in buffering because as one secreted H^+ is buffered intraluminally another one is produced in the cell. The answer most probably lies in the outcome of glutamate ($GluCOO^-$) metabolism. $GluCOO^-$ is produced at the same time as the NH_4^+, and can be converted to glucose when it is further deaminated to 2-oxoglutarate by a process called gluconeogenesis. In this process an equivalent amount of H^+ is utilized as that of NH_4^+ which is yielded from glutamine. Thus, the H^+ released into the cell is most likely embodied into glucose. Figure 11 depicts the role of ammonia in renal bicarbonate generation.

The net result of this action is a gain of HCO_3^-. It has been shown that there is an increase in glutaminase activity and the rate of gluconeogenesis in acidosis (Zilva et al., 1988).

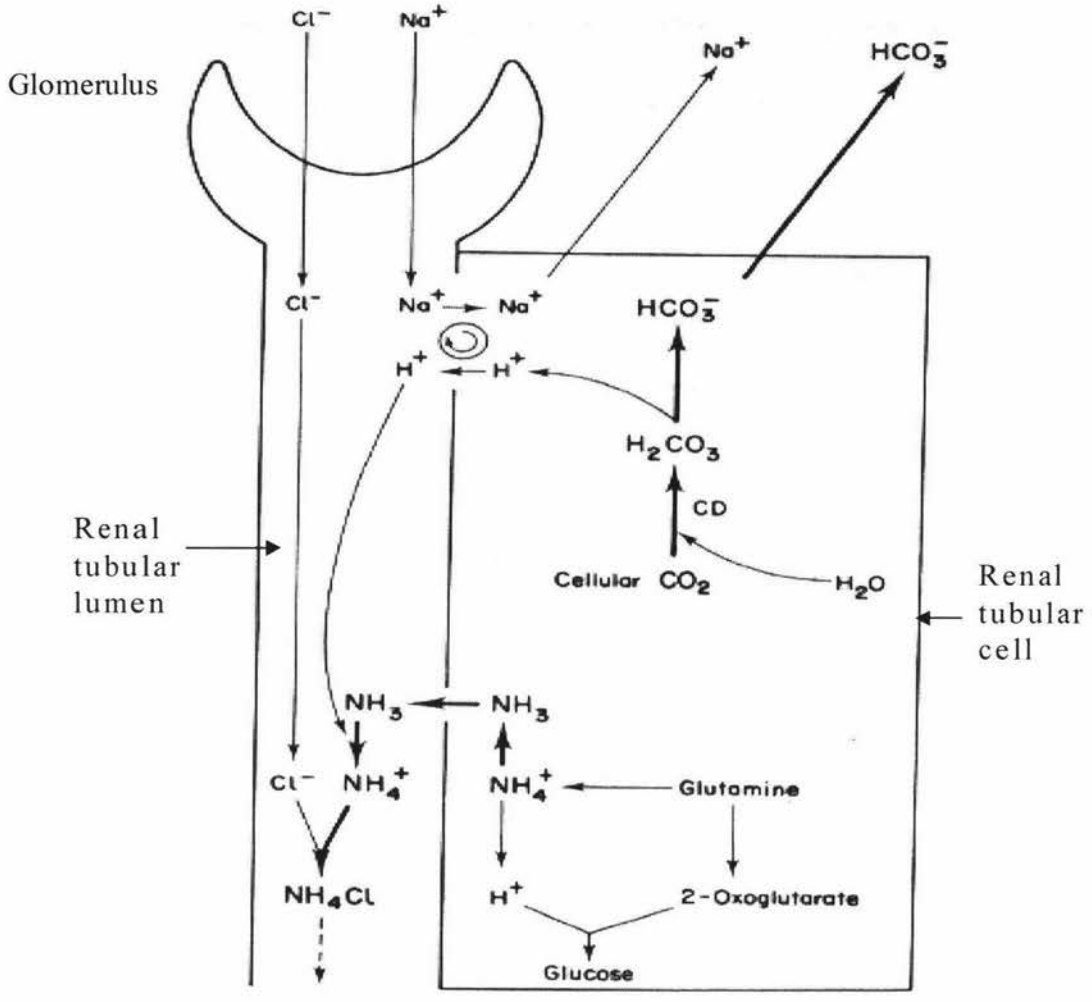


Figure 11. Role of ammonia in renal bicarbonate generation. (From: Zilva, F.J., Pannall, P.R. and Mayne, P.D. (1988). Clinical Chemistry in Diagnosis and Treatment. Fifth Edition. In: Hydrogen ion homeostasis: blood gas levels. Edward Arnold, London. p. 87).

1.2.6 Diet and Acid-Base Regulation

As mentioned earlier the typical diet of industrialized countries is rich in animal proteins, cereals, salt, and refined, processed foods. It is, however, lower in fruit and vegetables than is recommended for optimal

health (Barzel and Massey, 1998; Heaney, 1998). This diet leads to dietary net acid load (metabolic acidosis) which may be indicated by the bicarbonate and pH of the blood and may have a negative effect on calcium balance (Hu et al., 1993; Sebastian et al., 1994).

Many soft drinks contain acids such as phosphoric acid, citric acid, lactic acid, and acetic acid. The consumption of these acids may lead to a fall in the pH of the body.

Metabolic acidosis is present when there is an increase in the relative concentration of hydrogen ions caused by a reduction in the concentration of bicarbonate which is the main short term extracellular buffer (Bushinsky, 2001).

Clinical studies show that a diet that is high in protein (particularly animal protein) increases calcium excretion in the urine (Heaney, 1993; Heaney et al., 2000; Heaney, 2001). There is an association between protein consumption and osteoporotic fractures (Abelow et al., 1992; Feskanich et al., 1996; Frassetto et al., 2000). A high protein diet may adversely affect bone mineral content through effects on calcium excretion and acid base metabolism (Barzel and Massey, 1998). Such diets cause insipient aciduria which promotes urinary calcium loss with no change in blood calcium level and thus there is no change in the absorption of intestinal calcium (Heaney, 2001). The effect of protein on urinary calcium excretion has been attributed to the associated increase in acid load caused by increased sulphate production from the metabolism of sulphur

containing amino acids such as methionine and cystine. The products of their metabolism are glucose, urea, sulphate, and hydrogen ions (Heaney et al., 2000).

The progressive decline in bone mass with age (in adults) is probably due to the continued intake of acid precursors (New, 1999). Fruits and vegetables that are rich in potassium citrate metabolically generate base (Frassetto et al., 1998) which buffers the acid produced by meat diets. Citrate is oxidized to carbon dioxide, water, and bicarbonate which is an important buffer. Potassium bicarbonate is a natural base that the body produces from the metabolism of potassium citrate. It is therefore thought that fruits and vegetables protect bone (Tucker et al., 1999; New et al., 2000). Some studies have shown a difference in bone mass in vegetarians compared to omnivores and the above theory may help to explain why this is so (Hunt et al., 1989).

1.2.6.1 The Role of Vegetable Nutrients in Bone Health

A well balanced diet that supplies a full array of nutrients from all the food groups is essential to bone health. New et al (2000) reported that a diet rich in fruits and vegetables may be beneficial to bone health. Vegetables such as broccoli, onion and potato contain significant quantities of calcium and phosphorus. It has been demonstrated that bone health improves when people increase their intake of calcium-rich foods (Heaney et al., 1999). In addition, there is evidence

which shows that peak bone mass and subsequent bone loss are related to calcium intake (Smith, 1994). Phosphorus is the second most abundant mineral in the body with 85% of the body's phosphorus bound to the skeleton. It is combined with calcium in the hydroxyapatite crystals of bones and teeth. Thus, its main role is in the mineralization of bones and teeth (Ilich and Kerstetter, 2000). Phosphates are present not only in bones and teeth, but in all the cells of the body where they form part of a major buffer system which utilizes phosphoric acid and its salts (Zilva et al., 1988). Dietary phosphorus contributes to the many biological roles of phosphorus in the body such as buffering of acids.

Much research on bone health has focused on calcium. However, other nutrients such as vitamin K, magnesium, and potassium also help to maintain bone mineral density (Feskanich et al., 1999; Tucker et al., 1999).

The apparent protective effect of fruits and vegetables could be due directly to the nutrients. Alternatively, it is possible that the alkaline salts produced when they are digested are having an effect. They might help to counteract the acid salts that are produced by other foods such as meat. Fruit and vegetables generate alkalinity and this could prevent the need for alkaline salts to be released from bone (Barzel, 1995). Thus, bone health is maintained.

Potassium is another mineral element that seems to play a role in maintaining bone health. There is a weak but positive link between energy-adjusted intake of potassium and total body bone mass (Michaelsson et al.,

1995). Potassium bicarbonate has been shown to decrease urinary calcium excretion. This in turn improves calcium balance in healthy premenopausal (Lemann et al., 1991; Lemann et al., 1993) and postmenopausal women (Lutz, 1984). Sebastian et al (1994) reported that potassium bicarbonate can also decrease bone resorption and increase the rate of bone formation in postmenopausal women. One study reported that the administration of 60mmol/d of potassium bicarbonate to adults who were healthy decreased calcium excretion in urine by 0.9 mmol/d (Lemann et al., 1993). The authors reported that the reduction in urinary calcium during potassium administration may be due to natriuretic effects of potassium i.e. increased sodium excretion which causes a reduction in ECF volume or to phosphate retention induced by potassium. However, it could also be due to both mechanisms. The process by which such ECF-volume contraction reduces calcium excretion in the urine is not known. It can, however, be speculated that the effect of volume contraction to reduce calcium excretion during the administration of potassium could be due to trends toward hyperkalemia or raised intratubular concentrations of potassium. Potassium, in turn, has been found to directly stimulate the transfer of calcium across the distal renal tubule's luminal membrane (Lemann et al., 1993). Another study demonstrated that the administration of 90 mmol/d of potassium chloride or potassium bicarbonate to healthy men was followed by a decrease in urinary phosphate excretion, and by an increase in plasma phosphate concentrations averaging 0.12 mmol/L (Sebastian et al., 1990). These

changes were correlated with the increase in the concentration of plasma potassium and a reduction in plasma calcitriol concentrations that were inversely correlated to the increases in the concentrations of phosphate. The concentration of serum calcitriol appears to fall during the administration of potassium salt whereas the concentration of serum phosphate rises, and the concentrations of serum calcitriol appear to rise during potassium deprivation as phosphate concentration falls. Potassium increases the distal renal tubular reabsorption of filtered phosphate and as such regulates the amount of serum or plasma phosphate. The formation of calcitriol in the kidney and thus the concentrations of serum calcitriol are inversely related to the existing concentrations of serum or plasma phosphate.

Vitamin C (ascorbic acid) appears to aid bone formation although it is a mild acid. However, a lot of free vitamin C can be directly excreted because it is a weak acid. Freudenheim et al (1986) found a positive correlation between vitamin C intake and BMD in postmenopausal women.

Osteoblasts contain a specific saturable transport system for vitamin C. This system is probably Na^+ gradient dependent (Franceschi et al., 1995). Vitamin C in turn stimulates osteoblasts to lay down bone matrix (Wolf, 1996). This vitamin is required in the process of collagen hydroxylation (Peterkofsky, 1991) i.e. the hydroxylation of proline and lysine residues. Hydroxylysine is also involved in the cross-linking necessary for normal collagen fiber formation. There will be an increase in

the intracellular breakdown of collagen precursors if hydroxylation is defective with resultant perturbation in the formation of various cross-linking amino acids in collagen. The correlation between vitamin C and deoxypyridinoline is significantly negative but this is not the case with pyrinidoline. This may be due to the fact that pyrinidoline is derived from hydroxylysine within the collagen helix whereas deoxypyrinidoline involves reaction with a lysyl residue. As such, a relative deficiency of vitamin C is likely to more strongly affect deoxypyrinidoline than pyrinidoline (Tsuchiya and Bates, 1997). If the connective tissue of bone is a target for free radical damage then vitamin C may also be important because of its antioxidant effect as it is a powerful reducing agent.

Vitamin K is a cofactor that is required for the formation of γ-carboxyglutamate (Gla) residues in proteins. It is now known that osteoblasts produce at least three different Gla-containing proteins i.e. osteocalcin, matrix Gla protein, and protein S. The main part of each of these proteins remains bound to the hydroxyapatite matrix in bone. Their function remains unclear, but Ferland (1998) reported that Gla residues increase the affinity of proteins for calcium

Bone also contains magnesium. It is the fourth most abundant element in the body and it has several functions in bone metabolism, affecting remodeling, strength, and preservation. The magnesium that is present on the crystalline surface of the bone appears to influence hydroxyapatite crystals and prevent their formation. This mechanism of

limiting the size of hydroxyapatite crystals increases bone strength (Eaton-Evans, 1994). Magnesium deficiency has been shown to impair the activation of osteoblasts which in turn results in the uncoupling of bone formation and resorption (Rude et al., 1998). The investigators noticed that there is an increase in osteoclastic bone resorption associated with magnesium depletion. This resorption was seen with an inappropriate non-altered bone forming surface relative to control (% osteoid surface: low-Mg group 2.4+/-0.7 versus controls 2.6+/-0.4; % osteoid volume: low-Mg group 0.25+/-0.09 versus controls 0.38+/-0.06; number of osteoblasts per millimeter bone surface: low-Mg group 0.9+/-0.3 versus controls 1.3+/-0.3). It was found that osteoclasts occupied 38.3% ± 3.7% of bone surface in the low magnesium group as compared to 17.7% ± 2.4% in the control group ($p<0.001$). The number of osteoclasts per millimeter of bone surface was 16.9 ± 1.3 in the low magnesium group as compared to 7.8 ± 1.5 in the controls ($p<0.001$). Thus, magnesium may be necessary in maintaining the pH of the extracellular fluid (ECF) at a level which is somewhat higher than that of the blood which may result in the formation of a barrier that prevents calcium leakage from bone when the pH of blood is low. Morrison and Arnett (1998) reported that the resorptive activity in osteoclasts may be inhibited by a higher ECF pH (Morrison and Arnett, 1998). Magnesium also stimulates the production of hormone calcitonin which suppresses osteoclasts. Another study found that magnesium deficiency, especially intracellular depletion, correlates with low-serum PTH levels which may

cause symptomatic hypocalcaemia (Rude and Olerich, 1996). In such a condition, there may be growth of hydroxyapatite crystals, and there may be a decrease in the synthesis of calcitriol. The 1α-hydroxylation reaction of calcidiol is magnesium-dependent and is stimulated by PTH (Schaafsma et al., 2001). Finally, magnesium concentrations in the medium directly affect *in vitro* proliferation of osteoblast-like cells (Rude and Olerich, 1996). In rats, low levels of magnesium cause a decrease in trabecular bone volume, osteocalcin levels, and mineral content of the newly formed metaphysis (Carpenter et al., 1992).

The dietary intake of magnesium is positively correlated with forearm bone mineral density in premenopausal women (Angus et al., 1988). It has also been found that dietary intakes of magnesium are significantly reduced (Tranquilli et al., 1994) and serum magnesium is low (Reginster et al., 1989) in postmenopausal osteoporotic women. In addition, cancellous bone from osteoporotic humans is low in magnesium content (Cohen and Kitzers, 1981). Magnesium deficiency may be a cause of osteoporosis (Thomas, 1988) and is associated with low vertebral BMD in postmenopausal women (Hannan et al., 1997). It has been shown that the iliac crest and upper femur of osteoporotic patients have 10% less skeletal magnesium than that found at these sites in healthy control subjects (Cohen & Kitzers, 1981). Magnesium's effects may be explained by the impairment of a skeletal ATPase responsible for transporting potassium ions into the skeletal interstitium in exchange for hydrogen ion extrusion.

This action could result in pH imbalance and enhanced bone resorption (New et al., 2000). Magnesium supplementation has been linked to fracture prevention, increase in bone mass, and arrest of bone loss in postmenopausal women (Stendig-Lindenberg et al., 1993).

Vegetables contain a range of antioxidants such as carotenoids, vitamin C, flavonoids, and other phenolics (Vinson et al., 1998). Antioxidants are a group of vitamins, minerals and enzymes that help in protecting the body from the formation of free radicals i.e. antioxidants fight free radicals (Whitney and Rolfes, 2002). Free radicals are atoms that can cause damage to cells thereby impairing the immune system which leads to infections and various degenerative diseases (Whitney and Rolfes, 2002). The generation of oxygen-derived free radicals in cultured bone is associated with the formation of new osteoclasts and enhanced bone resorption (Garrett et al., 1990). They may also be important in the bone resorption that occurs in association with inflammatory diseases. Activated phagocytes including monocytes, macrophages and neutrophils produce oxygen derived species (Babior, 1984). These cells accumulate adjacent to bone surfaces in chronic inflammatory diseases and radical production by them could be responsible for stimulating osteoclasts for formation or activation to resorb bone (Garrett et al., 1990). Thus, the antioxidants present in vegetables may help prevent bone loss.

Summary

The skeleton is not inert. Bone has an inorganic and an organic component (osteoid). The formation and resorption of bone occurs continuously by means of cells that are influenced by mechanical, nutritional and hormonal factors. Existing bone mass is maintained as a result of bone remodeling which balances the rate of bone formation with that of resorption. This is achieved via mediation of the synthesis and apoptosis of osteoclasts and osteoblasts. Besides a mechanical function, the skeleton also has a biochemical function.

Calcium, phosphorus and magnesium are the important bone minerals. The organic collagen matrix also contains non-collagen substances which include the bone morphogenetic proteins.

Bone turnover is controlled by osteoblasts, osteoclasts and osteocytes. Osteoblasts are responsible for bone formation whereas osteoclasts are responsible for bone resorption. They respond to endocrine, nutritional and mechanical signals. Osteoblasts regulate osteoclast formation via the RANKL-RANK and the M-CSF-OPG mechanism. However, osteoclasts do not have a direct feedback on osteoblasts. Both osteoblasts and osteoclasts originate from direct ancestors (progenitors) that reside in the bone marrow; osteoblasts belong to the mesenchymal lineage of the marrow stroma, and osteoclasts to the haematopoietic lineage.

During bone remodeling, the first step is osteoclast activation which is followed by osteoclast formation, polarization of the ruffled border constituents, resorption and ultimately apoptosis. This osteoclast apoptosis is followed by a series of sequential changes in cells of the osteoblast lineage which include osteoblast chemotaxis, proliferation and differentiation. Subsequently, there is formation of mineralized bone and stoppage of osteoblast activity. This marks the final phase of the formation process. The resorption lacunae are usually repaired either completely or almost completely.

Bone remodeling is a local process occurring in all living bone that is carried out in small areas by populations of cells called bone remodeling units that are also influenced by several factors. If bone remodeling results in equal amounts of bone formation and resorption then these two processes are "coupled." The cycle starts with osteoclasts resorbing bone. This is then followed by osteoblasts laying down new bone in the same general area. Bone remodeling occurs as a result of both nonhormonal and hormonal stimuli. Most bone diseases result from alterations in coupling which could lead to either excessive bone formation or excessive resorption. Bone remodeling occurs in discrete pockets all over the skeleton and may only occur at any one site once every ten years. As such any sudden major change in the bone environment may disrupt this regulatory process.

The most important mineral in the skeleton is calcium. Calcium balance is determined by exchanges between the skeleton, the intestine and kidney. PTH, 1,25-dihydroxycholecalciferol, and calcitonin control the calcium balance. PTH acts on bone and kidneys. In bone, it stimulates osteoclastic activity which in turn releases calcium and phosphate into the plasma. Its actions in the kidneys increases calcium reabsorption but inhibit phosphate reabsorption. Thus, the plasma phosphate concentration is decreased. PTH may also increase the rate of 1α-hydroxylation of 25-hydroxycholecalciferol.

Foods such as protein-rich foods, grains, and cereals contribute to metabolic acid load. On the other hand, foods such as fruit and vegetables provide base products which neutralize the acid load. Generally, humans consume a diet that generates metabolic acids. This leads to a reduction in the concentration of systemic bicarbonate and a fall in pH. As indicated by *in vitro* experiments, this metabolic acidosis causes a release of calcium from bone. Initially, this is simply due to physico-chemical dissolution of the mineral. If metabolic acidosis persists (chronic), bone cell function is altered i.e. osteoclastic bone resorption increases and osteoblastic bone formation decreases. The dissolution and resorption of bone mineral results in the buffering of the addition protons by bone and this restores the systemic pH. This means that calcium is drawn from bones as a natural acid neutralizer when the body is too acidic.

femur) using callipers and weighed. The bones were equilibrated at 23°C in PBS for 1 hour prior to the test.

2.1.10 Statistical Analysis

Results were subject to univariate and multivariate statistical analysis. Initial exploration of data was by discriminant analysis. This method reduces the likelihood of error from multiple univariate comparisons and allows for simultaneous comparisons of the interaction of a number of variables.

All statistical analyses were conducted in Systat version 6.0 (Wilkinson, 1990). A p-value of 0.05 was taken to be significant. Univariate statistical analysis was by ANOVA with post hoc (Bonferroni) evaluation of difference between treatments. The values and graphs are shown as mean ± standard error of the mean.

2.2 Choice of the Rat as a Model for Studies Related to Bone Health

The most commonly used animal model for osteoporosis studies is the ovariectomized rat (Barlet et al., 1994). In the current study we used the growing male rat model. Rats provide a uniform population, are inexpensive, and can be used in large numbers. They can be measured under identical conditions and their diet can be artificially modified. The effect of dietary manipulation can be apparent in weeks rather than months or years.

There are a number of similarities in the physiology and responses of the skeleton and calcium metabolism in rats and humans. Rats have similar bone anatomy to humans (Mosekilde, 1995; Aufdemorte et al., 1993).

It is possible to measure bone mineral content (BMC) and bone mineral density (BMD) in rats (both *ex vivo* and *in vivo*) using DEXA with ultra-high resolution software (Grier et al., 1996). The biomechanical strength of rat bones can be readily determined (Amman et al., 1999).

There are, however, a number of disadvantages to using rats as a model. Rats are quadrupeds. Young rats have a limited naturally occurring BMU (bone modeling unit)-based remodeling (Frost and Jee, 1992; Kalu, 1991). The growth plates in male rats do not close in less than 30 months and there is lack of Haversian systems in the cortical bone of rats (Wronski and Yen, 1991). The bones of rats are more dynamically active than those of humans i.e. there are differences in bone turnover at many skeletal sites and there is also pronounced bone remodeling throughout the rat's life. Thus rat bones are more sensitive to factors that influence bone metabolic rate than human bones. The mechanical loading patterns on rat bones also differ from that which occurs on human bones. (Mosekilde, 1995).

2.3 Dual Energy X-Ray Absorptiometry

Dual Energy X-Ray Absorptiometry (DEXA) is a technique for imaging bone mineral content. This technique is based on the proportion of

a beam of photons that passes through the bone. An x-ray source that emits a stable, dual-energy photon beam is used for imaging (Formica et al., 1993). This beam is scanned across the bones and the x-ray absorption is measured at intervals. These two energy level x-rays are produced by passing them through appropriate filters. One beam is high energy (usually 70kV) while the other is low energy (usually 40kV). The use of a dual-energy photon beam allows a standardized exposure regime by a subtraction of the reference density of a pixel from that of the corresponding pixel in the high energy image.

DEXA is based on the principle that when an x-ray or photon source is set on one side of an object, the power of the beam on the other side of that object is dependent on its thickness, density, and chemical composition (Pietrobelli et al., 1996). Thus, different tissues will attenuate the x-rays of two energy levels differently because of their difference in thickness, density, and chemical composition. This means that the attenuation through bone, lean tissue, and fat will obviously differ.

DEXA is used mainly to obtain site-specific measurements of regional BMD of the lumbar spine, femur, and forearm (Lunt et al., 1997). DEXA scans were initially used to assess the amount of bone present as well as the density of that bone. More recently, DEXA has also been used to distinguish between fat tissue and other soft tissue.

Bone density measurements provide a sensitive means for diagnosing decreased bone mass and predicting the likelihood of

pathological fractures (Consensus Development Conference, 1993). It is perhaps the most useful tool in assessing bone density. Its accuracy exceeds 95% and its precision is in the range of 1% (Consensus Development Conference, 1993). DEXA can be performed within minutes at a very low x-ray dose and the results obtained almost immediately (Ellis, 2000). It is also sufficiently accurate and precise (<2%) to be used in small animal studies (Griffin et al., 1993).

There are however some limitations to DEXA which include the substantial cost of acquiring the instrument, and the need for dedicated space to place it. DEXA is also not able to assess the quality of bone i.e. the microarchitectural changes that occur at the microscopic level are not detectable by DEXA. In addition, DEXA only measures areal bone mineral density in two dimensions and this is determined by dividing the mineral content of the site of bone being assessed by the area of bone (represented by units of g/cm^2). Therefore, if a large and small bone have the same bone mineral density, the larger bone tends to have a greater bone mineral density because it has a smaller cross sectional area relative to the smaller bone when the surface area is used as a denominator (Cummings et al., 2002). In the clinical setting DEXA does not take into account the depth of the bone which is a measure of the volumetric bone density. If the depth of the bone is taken into account then DEXA measures would be less influenced by the size of the actual bone. It is thus important to be aware that subjects of short stature will have lower bone mineral density

measurements as compared to their taller counterparts (Cummings et al., 2002).

2.4 Biochemical Markers of Bone Metabolism

Markers of bone metabolism may be categorized either as indicators of bone formation, bone resorption, or overall bone turnover according to the phases of the bone cycle (Christenson, 1997). They are present as certain molecules and fragments of molecules in blood and urine.

Markers of bone formation are products of active osteoblasts and are expressed during various stages of osteoblast development. These markers mirror different aspects of osteoblast function as well as bone formation. They can be measured in either the serum or plasma in order to monitor changes in bone metabolism over shorter time periods as compared to measurements of changes in bone density. Biochemical markers such as alkaline phosphatase, procollagen 1 extension peptides and osteocalcin are some examples of markers that are used to indicate bone formation (Delmas et al., 2000).

Biochemical markers that are used to indicate bone resorption include urinary calcium, acid phosphatase, hydroxyproline, N- and C-telopeptides of type 1 collagen, pyridinoline and deoxypyridinoline crosslinks (Delmas et al., 2000). These markers may also indicate changes in bone metabolism before significant changes in bone density are observed.

Many markers such as alkaline phosphatase, osteocalcin and hydroxyproline are just not expressed in bone alone (Delmas et al., 2000). They are expressed in other tissues as well. Marker metabolism may also be altered by some drugs and diseases. In addition, there may be lack of required level of specificity in some cases regarding available assays that are used to detect markers. Usually, the changes in biochemical markers of bone turnover are not disease specific as they reflect changes in skeletal metabolism which are unrelated to the underlying cause. The usefulness and reliability of all these markers is still being assessed. To date, there is no single reliable method that can be regarded as an ideal standard for monitoring bone metabolism (Delmas et al., 2000).

2.4.1 Type-1 Collagen Cross-Links

During the bone resorption process, C-terminal (CTX) and N-terminal fragments of the collagen molecule are released into the blood following osteoclast mediated degradation of type-1 collagen. However, the osteoclasts are not active in the degradation of other tissues containing type-1 collagen. Thus, the generation of these fragments should be highly bone specific (Christenson, 1997) and the measurement of serum or urine levels of either the N- and C-terminal fragment will serve as a specific marker of bone resorption. In this study, we measured the levels of the C-terminal fragment in the plasma.

There are some limitations associated with the CTX serum assay. Theoretically, serum CTX concentration serves as a specific marker of osteoclastic activity. The serum CTX concentration, may, however, reflect total bone turnover instead of just resorption in some disease states and this claim is substantiated by the fact that it (serum CTX concentration) is not increased in Paget's disease (a disease characterized by increased resorption) but is increased in osteoporotic individuals who are on anabolic steroids (a treatment that is thought to reduce bone resorption) (Christenson, 1997). The serum CTX level only mirrors one aspect of osteoclastic action which is the breakdown of the organic matrix's type-1 collagen fibers. Also, type-1 collagen fiber synthesis may be reduced in some disease conditions resulting in decreased degradation. However, if, on the other hand, the disease condition presents an excess of collagen or unmineralized bone matrix then degradation may appear to be elevated.

2.5 Biomechanical Testing

Mechanical testing is used to measure the mechanical properties of bones. Many types of biomechanical tests are available and compression, tension, torsion, bending and fatigue tests are some examples.

In this study, a 3-point bending test was carried out using a custom-made testing machine that is suitable for measuring the mechanical properties of small bones such as rat femurs. Bones (rat femurs) were suspended horizontally between two supports with the anterior surface

upwards and then compressed at the femoral midshaft using a constant compression speed. The pressing force was directed vertically to the bone and the degree to which the bone bends was measured. Subsequently, the ultimate bone breaking point was measured.

During this test the bone passes through three stages i.e. the elastic phase, plastic phase and then the ultimate bone breaking point. The bone bends elastically when low levels of force are applied to it. During this phase the degree of deformity is proportional to the force applied. As there is no irreversible damage the bone will spring back to its original shape when the force is removed. The elastic properties of bone are mainly due to its collagen (specifically type-1) content. When the applied force exceeds the elastic limit the bone deforms plastically with resultant structural damage within the bone. Thus, the bone fails to revert to its original shape when the force is removed i.e. bone deformation is permanent. This property (plasticity) is due to both the organic and mineral composition of the bone. Further increase in the force applied lead to bone breakage (Turner and Burr, 1993). The above three phases of bone breaking are measured during biomechanical testing. Subsequently, a Load-Deformation Curve (refer Figure 13) is plotted.

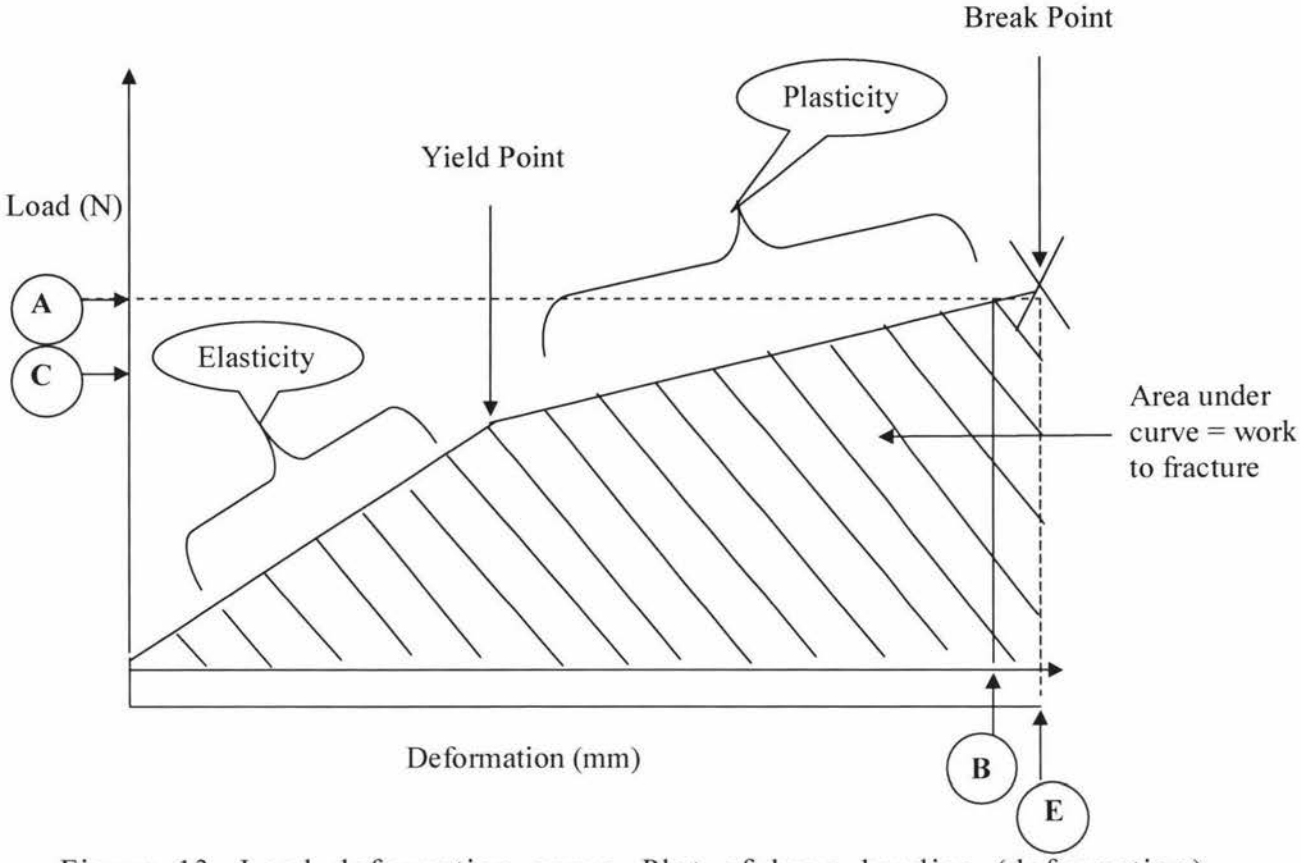


Figure 13. Load deformation curve. Plot of bone bending (deformation) against stress (load or force applied) displaying the three phases that the bone undergoes during the bone bending test – elasticity, plasticity and the ultimate break point. N = Newton

2.5.1 Definitions of Parameters Used

(A) **Maximum Load (N)** is the maximum force that the bone is able to stand up to without breaking. (**N**) is Newton. It is the unit of force which is equal to the force required to accelerate a mass of one kilogram one meter per second.

(B) **Maximum Stroke (mm)** is the maximum amount of deformation of bone which occurs before breaking.

Ⓒ **Break Load (N)** is the maximum force that is required to fracture the bone.

Break Stress (N/mm^2) is the break load expressed per unit area of the bone.

Ⓔ **Break Stroke (mm)** is the amount of deformation of bone at break point.

Break Strain (%) is the fractional change in the dimensions of the bone (degree of bending).

Elastic (N/ mm^2) is a measure of the intrinsic stiffness of the bone. It is the slope of the linear portion of the Load- Deformation curve divided by bone area.

Energy (J) is the total amount of energy the bone has to absorb in order to break i.e. the energy that is stored in the bone and is set free at the point of fracture. (J) is the unit of work or energy which is equal to the work done when the point of application of a force of one Newton is displaced one meter towards the action of the force.

Note 1) **A** and **C** are shown as the same in this diagram.

2) **B** and **E** are shown as the same in this diagram.

However in some circumstances this may not be so. For example, there may be non-elastic i.e. plastic deformation occurring before the break. This 'sudden giving' may cause the applied load to fall off before the piece actually breaks. Thus the break load is lower than the maximum

load. This effect may also show up in the relationship of the maximum stroke length (which is the amount of movement for the 'plunger' that is bearing on the bone) to the point of maximum load. Thus there will be lower length of stroke at the point of maximum load but as non-elastic. What this means is that the plastic deformation occurs and the load tends to fall off. The degree of movement will then dramatically increase so that at the point of breakage there is maximum stroke length but a lower load applied (R. Lentle, 2005; personal communication).

CHAPTER 3

RESULTS

3.1 Diet Analysis

The phosphorus (P) and calcium (Ca) concentrations of the study diets were verified by analysis and the compositions of each are shown in Table 2.

Table 2. Concentration of phosphorus (P) and calcium (Ca) in the study diets in mg/kg.

Diet Group	P (mg/kg)	Ca (mg/kg)
Base	2490	4610
Broccoli	2980	4510
Onion	2110	4080
Potato	2320	4320

3.2 Rat Weights

The baseline weights of rats and the weekly change in the weights of rats (from baseline to completion of study) are listed in Figure 14. However, there were no significant differences between food groups as shown by post hoc (Bonferroni) comparisons following ANOVA (df 3,36 $F=0.589$, $p=0.626$) for final rat weights.

by the group on broccoli diet as shown in Table 3. In the current study the rats that took less food tended to have lower body weight and this may mean that the nutrient content of the four diets were almost identical. However, this was not the main part of the study.

Table 3. Total food consumed (g) over eight weeks by the different groups of rats.

Diet Group	Total Food Consumed (g)
Base	8674.54
Broccoli	7538.39
Onion	8078.83
Potato	8162.09

3.4 Apparent Calcium Balance (Apparent Percentage Calcium Retained)

Post hoc (Bonferroni) comparisons following ANOVA (df,3,36, F=6.243, p=0.002) of the four different dietary groups for apparent percentage calcium retained (over the balance period) showed that the group that was maintained on base diet had significantly lower apparent percentage calcium retained than the groups that were on onion diet (p=0.021) and potato diet (p=0.008). Significantly lower apparent percentage calcium retained was also evident in the group on broccoli diet compared to the group on potato diet (p=0.037). There were no other significant differences between groups (Figure 15).

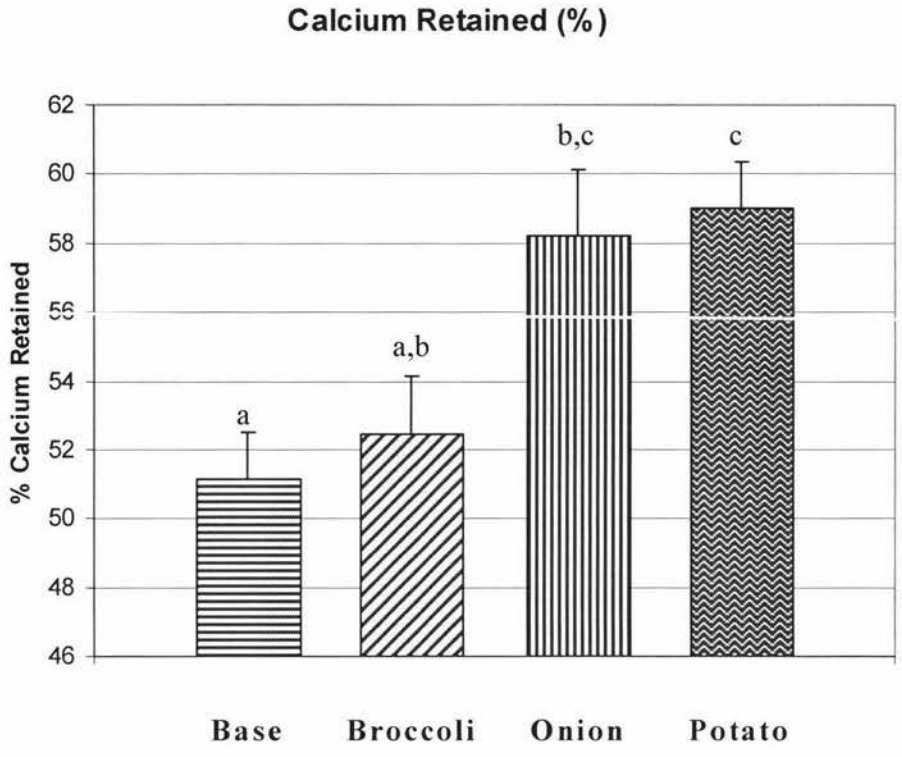


Figure 15. Apparent percentage calcium retained by the different dietary treatment groups over the balance period.
Different letters (a,b,c) denote significant difference between groups at $p<0.05$.
Standard errors are shown as vertical lines.

3.5 Apparent Phosphorus Balance (Apparent Percentage Phosphorus Retained)

There were no significant differences between the four dietary treatment groups for apparent percentage phosphorus retained over three days (balance period) as determined by post hoc (Bonferroni) comparisons following ANOVA (df,3,36. $F=1.599$, $p=0.207$). Figure 16 depicts the apparent percentage phosphorus retained by the different groups of rats.

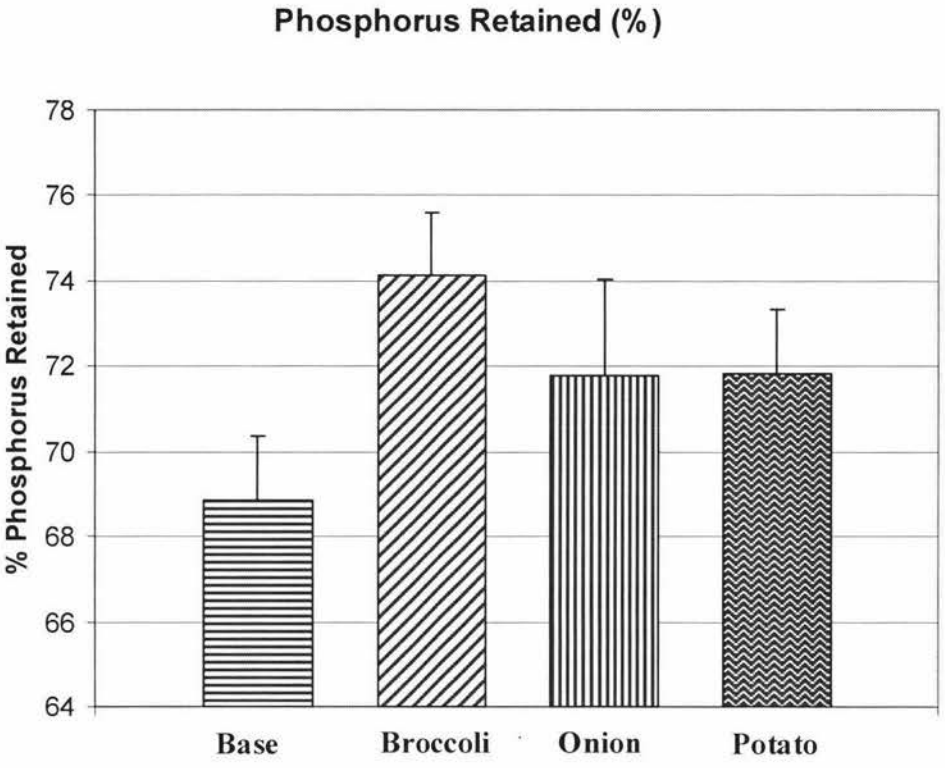


Figure 16. Apparent percentage phosphorus retained by the different groups of rats over the balance period.
Standard errors are shown as vertical lines.

3.6 Urinary Parameters

3.6.1 Urinary Ammonia

Post hoc (Bonferroni) comparisons following ANOVA (df,3,36, F=5.053, p=0.005) of the four different groups of rats for the urinary ammonia concentrations (over the balance period) showed that the group on broccoli diet had significantly lower urinary ammonia concentration than the groups on potato diet (p=0.008) and base diet (p=0.015). There were however no other significant differences between the dietary treatment groups (Table 4).

As for three day urinary ammonia excretion, post hoc (Bonferroni) comparisons following ANOVA (df,3,36, F=3.550, p=0.0238) of the four dietary treatment groups were as follows: The group on broccoli diet had significantly lower urinary ammonia excretion over three days than the groups on base diet (p=0.040) and the potato diet (p=0.055). No significant differences were apparent between any of the other groups (Table 4).

Table 4. Urinary ammonia concentrations and the three day urinary ammonia excretion of the different dietary treatment groups over the balance period.

Urinary ammonia concentration	Base Group	Broccoli Group	Onion Group	Potato Group
Mean (mmol/L)	75.07^{a}	18.78^{b}	$60.89^{a,b}$	78.49^{a}
±SE	±12.606	±5.636	±12.361	±15.881
Three day ammonia excretion				
Mean (mmol)	2.98^{a}	0.92^{b}	$2.41^{a,b}$	2.89^{a}
±SE	±0.551	±0.314	±0.522	±0.590

Different superscripts (a,b,c) denote significant differences between groups at $p<0.05$.

3.6.2 Urinary Urea

The urinary urea concentrations (over the balance period) are shown in Table 5. Post hoc (Bonferroni) comparisons following ANOVA (df,3,36, F=6.170, p=0.002) of the four different groups of rats for the urinary urea concentrations were as follows: The group on onion diet and the group on potato diet had significantly lower urinary urea concentrations than the group on base diet (p=0.004 and p=0.005 respectively). However, there were no other significant differences between the dietary treatment groups.

Statistical analysis was also done on the three day urinary urea excretion. Post hoc (Bonferroni) comparisons following ANOVA (df,3,36, F=12.588, p=0.0005) of the four dietary groups (for three day urea excretion) showed the following results: The group on onion diet and the group on potato diet had significantly lower three day urinary urea excretion than the group on base diet (p=0.002 and p=0.000 respectively) and the group on broccoli diet (p=0.005 and p=0.000 respectively). There were no other significant differences between the dietary treatment groups on ANOVA (Table 5).

Table 5. Urinary urea concentrations and the three day urinary urea excretion of the four different groups of rats over the three day balance period.

Urinary urea concentration	Base Group	Broccoli Group	Onion Group	Potato Group
Mean (mmol/L)	399.1[a]	318.6[a,b]	254.0[b]	257.1[b]
±SE	±13.73	±34.32	±34.03	±21.95
Three day urea excretion				
Mean (mmol)	15.64[a]	15.28[a]	9.40[b]	7.82[b]
±SE	±1.022	±1.506	±0.878	±1.009

Different superscripts (a,b,c) denote significant differences between groups at $p<0.05$.

3.6.3 Urinary Creatinine

Although the group on base diet had the highest concentration of creatinine (over the balance period) in the urine, there were, however, no significant differences between groups for urinary creatinine concentrations as determined by post hoc (Bonferroni) comparisons following ANOVA (df,3,36, $F=1.539$, $p=0.221$). These levels are shown in Table 6.

As for the three day creatinine excretion, post hoc (Bonferroni) comparisons following ANOVA (df,3,36, $F=1.266$, $p=0.3006$) of the four dietary treatment groups (for three day creatinine excretion) also showed that there were no significant differences between groups (Table 6).

Table 6. Urinary creatinine concentrations and the three day urinary creatinine excretion of the different dietary treatment groups over the balance period.

Creatinine concentration	Base Group	Broccoli Group	Onion Group	Potato Group
Mean (mmol/L)	4.306	3.509	4.224	4.097
±SE	±0.2114	±0.3284	±0.3506	±0.2492
Three day creatinine excretion				
Mean (mmol)	0.16	0.16	0.15	0.14
±SE	±0.007	±0.013	±0.007	±0.006

3.6.4 Specific Gravity of Urine

The specific gravity of urine samples that were collected over the balance period was measured. There were no significant differences between groups for urine specific gravity as determined by post hoc (Bonferroni) comparisons following ANOVA (df,3,36, F=2.065, p=0.1221). However, the specific gravity was slightly higher in the group on base diet than in the other groups (Table 7).

3.6.5 Osmolality of Urine

The osmolality of urine samples collected over the balance period was measured. Post hoc (Bonferroni) comparisons following ANOVA (df,3,36, F=0.890, p= 0.4556) of the different dietary groups for

osmolality however did not show any significant differences between groups (Table 7).

Table 7. Specific gravity and osmolality of urine produced by the four different groups of rats over the three day balance period.

Specific Gravity	Base Group	Broccoli Group	Onion Group	Potato Group
Mean	1.03	1.02	1.02	1.03
±SE	±0.001	±0.002	±0.002	±0.002
Osmolality				
Mean (mosmol/kg)	1.03	1.02	1.08	1.04
±SE	±0.001	±0.002	±0.048	±0.012

3.6.6 Volume of Urine

The volume of urine collected over the three day balance period was measured. Following ANOVA (df,3,36, F=7.233, p=0.0006), post hoc (Bonferroni) comparisons of the four different groups of rats showed that the group on broccoli diet had significantly higher urinary volume than the groups on base diet (p=0.011), onion diet (p=0.008), and potato diet (p=0.001). No other significant differences were apparent between the dietary treatment groups (Table 8).

Table 8. Volume of urine produced by the different dietary treatment groups over the three day balance period.

Volume of urine (mls)	Base Group	Broccoli Group	Onion Group	Potato Group
Mean	39.0^{a}	49.9^{b}	38.6^{a}	35.9^{a}
±SE	±1.71	±3.42	±1.62	±1.97

Different superscripts (a,b,c,) denote significant differences between groups at $p<0.05$.

3.7 Bone Parameters

3.7.1 Lumbar Spine Bone Mineral Content and Bone Mineral Density

The bone mineral content (BMC) and density (BMD) of lumbar spine were compared (after correcting for body weight) based on data from *ex vivo* scans at the end of the trial. There were no significant differences between the dietary treatment groups for BMC on ANOVA (Table 9). We then decided to do the discriminant analysis (multivariate method).

Table 9. Lumbar spine bone mineral content measures (*ex vivo*) of the different dietary treatment groups at the end of the trial.

BMC (g)		Base Group	Broccoli Group	Onion Group	Potato Group
	Mean	0.40	0.36	0.38	0.37
	±SE	±0.008	±0.011	±0.013	±0.011

As for the lumbar spine bone mineral density, post hoc (Bonferroni) comparisons following ANOVA (df,3,36, F=0.777, p=0.515) found little difference (not significant) between groups ((Figure 17).

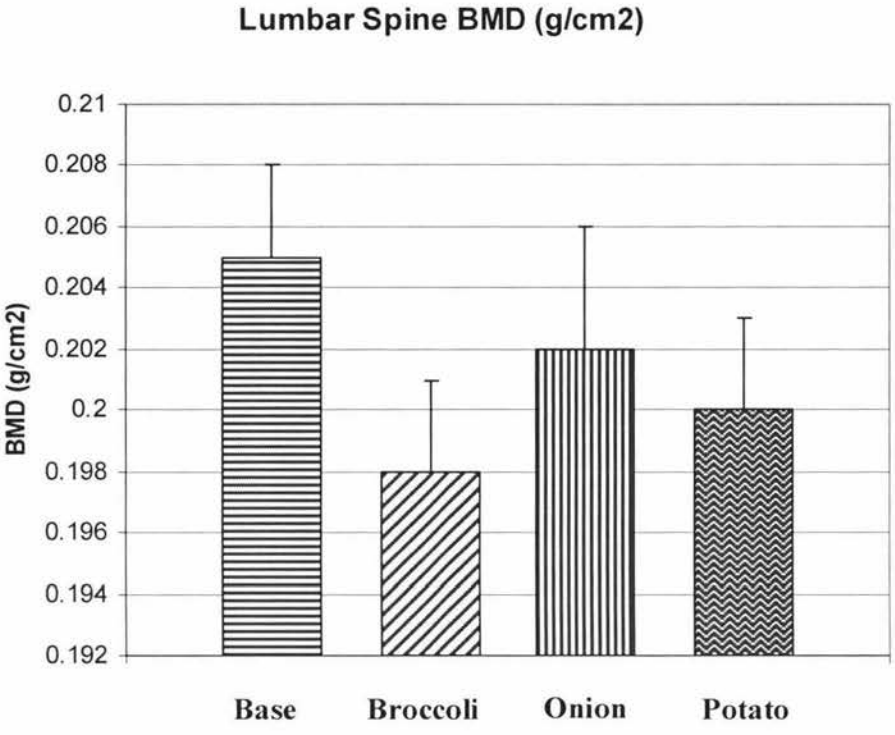


Figure 17. *Ex vivo* lumbar spine bone mineral density measures (g/cm^2) of the different groups of rats at the end of the trial.
Standard errors are shown as vertical lines.

3.7.2 Femur Bone Mineral Content and Bone Mineral Density

DEXA measures were obtained by scanning the right femurs (*ex vivo)* for the final bone mineral content (BMC) and bone mineral density (BMD) comparisons. Although the group on base diet had higher BMC than the other groups the differences (after correcting for body weight) between groups (for femoral BMC) were not significant (Table 10).

Table 10. *Ex vivo* femur bone mineral content (BMC) measures of the different dietary treatment groups at the end of the trial.

BMC (g)		Base Group	Broccoli Group	Onion Group	Potato Group
	Mean	0.38	0.36	0.37	0.37
	±SE	±0.010	±0.014	±0.012	±0.008

As for the BMD, post hoc (Bonferroni) comparisons following ANOVA (df,3,36, F=1.167, p=0.336) also did not show any significant differences between groups (after correcting for body weight). The group on the base diet, however, had higher BMD than all the other groups (Figure 18). .

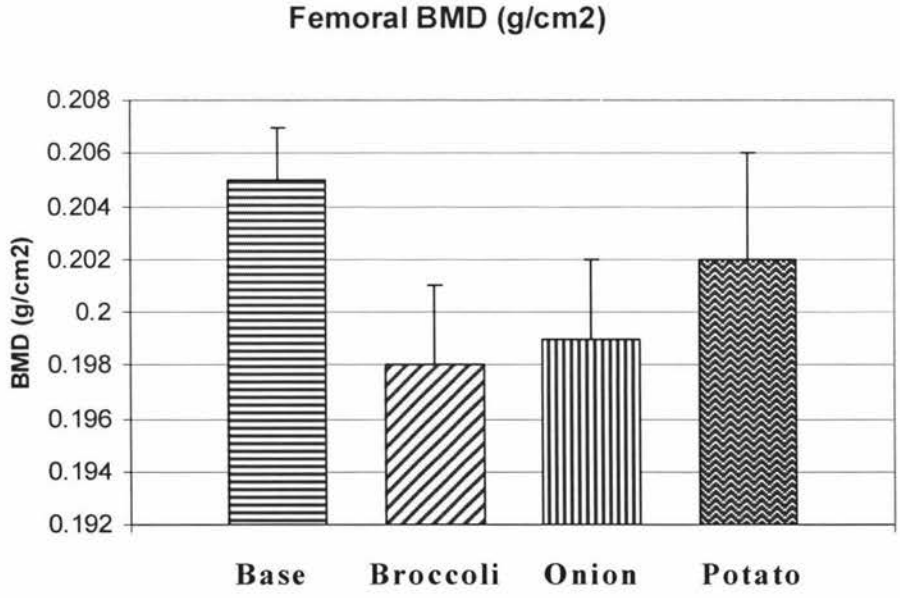


Figure 18. *Ex vivo* femoral bone mineral density measures (g/cm^2) of the different dietary groups at the end of the trial.
Standard errors are shown as vertical lines.

Regional bone mineral content (BMC) and bone mineral density (BMD) measurements were also obtained for *ex vivo* femurs. There were no significant differences between groups for regional BMC and BMD (after correcting for body weight) as determined by post hoc (Bonferroni) comparisons following ANOVA (Table 11).

Table 11. *Ex vivo* femur area bone mineral density and bone mineral content measures of the different groups of rats at the end of the trial.

		Base Group	Broccoli Group	Onion Group	Potato Group
Femoral neck BMD (g/cm^2)	Mean	0.211	0.208	0.204	0.210
	±SE	±0.002	±0.005	±0.003	±0.005
Femoral neck BMC (g)	Mean	0.097	0.089	0.089	0.096
	±SE	±0.002	±0.004	±0.003	±0.004
Shaft BMD (g/cm^2)	Mean	0.191	0.181	0.185	0.186
	±SE	±0.003	±0.003	±0.002	±0.004
Shaft BMC (g)	Mean	0.197	0.180	0.194	0.185
	±SE	±0.004	±0.006	±0.006	±0.005
Lower epiphysis BMD (g/cm^2)	Mean	0.232	0.226	0.230	0.232
	±SE	±0.008	±0.006	±0.008	±0.006
Lower epiphysis BMC (g)	Mean	0.093	0.090	0.089	0.097
	±SE	±0.006	±0.006	±0.006	±0.002

3.7.3 Lengths and Weights of Right Femurs

The length of each right femur was measured before and after drying, and the results are shown in Table 12. However, post hoc (Bonferroni) comparisons following ANOVA (df,3,36, F=0.623, p=0.6048) of the different dietary treatment groups showed that there were no significant differences between groups for dry femur lengths.

Right femur weights were taken before and after drying. There were however no significant differences between groups for dry femur weights as determined by post hoc (Bonferroni) comparisons following ANOVA (df,3,36, F=2.113, p=0.1157). Table 12 shows the femur weights before and after drying. Statistical analyses were not done on wet femur lengths and weights.

Table 12. Lengths (mm) and weights (g) of right femurs (wet and dry) of the different dietary treatment groups at the end of the trial.

		Base Group	Broccoli Group	Onion Group	Potato Group
Wet length (mm)	Mean	32.975	32.580	33.019	33.028
	±SE	±0.2569	±0.3542	±0.1657	±0.1792
Dry length (mm)	Mean	32.275	31.897	32.261	32.271
	±SE	±0.2361	±0.3365	±0.1664	±0.1597
Wet weight (g)	Mean	1.008	0.920	1.008	1.028
	±SE	±0.0285	±0.0413	±0.0144	±0.0275
Dry weight (g)	Mean	0.540	0.495	0.535	0.534
	±SE	±0.0128	±0.0204	±0.0946	±0.0116

3.7.4 Bone Breaking Strength.

The results of biomechanical tests that were done on left femurs (*ex vivo*) are shown in Table 13. There were no significant differences between groups for maximum stroke, break strain, break stroke, break energy, maximum load and elastic break strength as determined by post hoc (Bonferroni) comparisons following ANOVA (df,3,36, F=4.004, p=0.015). However, the group on potato diet required significantly lower force to break (break load) than the groups on base diet (p=0.022) and onion diet (p=0.039)

Table 13. Results of biomechanical tests on *ex vivo* left femurs of the different groups of rats done at the end of the trial.

		Base Group	Broccoli Group	Onion Group	Potato Group
Max. load (N)	Mean	162.927	157.527	162.972	151.487
	±SE	±4.754	±6.674	±7.390	±7.159
Max. stroke (mm)	Mean	1.909	1.832	1.872	1.882
	±SE	±0.043	±0.069	±0.053	±0.028
Break load (N)	Mean	163.130[a]	153.392[a,b]	161.060[a]	133.355[b]
	±SE	±5.009	±6.825	±7.272	±7.729
Break stress (N/mm^2)	Mean	72.790	76.351	74.949	63.241
	±SE	±4.015	±4.645	±3.083	±3.421
Break stroke (mm)	Mean	1.918	1.889	1.901	2.048
	±SE	±0.042	±0.076	±0.055	±0.043
Break strain (%)	Mean	17.700	16.782	17.224	18.250
	±SE	±0.583	±0.710	±0.590	±0.490
Elastic (N/mm^2)	Mean	585.322	658.729	629.233	643.503
	±SE	±58.208	±32.845	±34.670	±28.463
Energy (J)	Mean	0.177	0.176	0.178	0.191
	±SE	±0.008	±0.011	±0.009	±0.008

Different superscripts (a,b,c) denote significant differences between groups at $p<0.05$.

3.8 Plasma C- Telopeptides of Type 1 Collagen (CTX)

There were no significant differences between groups for plasma C- Telopeptides of Type-1 collagen (CTX) as determined by post hoc

(Bonferroni) comparisons following ANOVA (df,3,36, F=0.529, p=0.665). Table 14 shows the plasma CTX concentrations of the dietary treatment groups.

Table 14. Plasma type-1 collagen C-telopeptide concentrations of the dietary treatment groups at the end of the trial.

CTX	Base Group	Broccoli Group	Onion Group	Potato Group
Mean (ng/mL)	33.439	33.704	29.038	32.129
±SE	±1.3715	±5.0839	±1.6994	±2.0041

3.9 Discriminant Analysis (Multivariate Method).

In view of the number of differences of low significance shown by the post hoc (Bonferroni) bivariate comparisons between the dietary treatment groups it was decided to conduct the discriminant analysis (multivariate method).

A discriminant analysis using all of the parameters measured in this study showed that a distinction between dietary groups was possible on the basis of the first discriminant factor (refer Table 16). This factor was based on a contrast between percentage phosphorus retained and spinal bone mineral density with the percentage calcium retained and urinary ammonia levels (Table 16). The parameters that were used in this comparison are listed in Table 15.

Table 15. Group means of the various parameters used in the multivariate analysis, and the Jackknifed classification matrix. Note that the Jackknifed method was only able to distinguish the groups on base diet and broccoli diet well (70% and 80% respectively).

Parameter	Base Group	Broccoli Group	Onion Group	Potato Group
Percentage Ca retained	51.171	52.444	58.212	58.993
Percentage P retained	68.836	74.154	71.761	71.824
Urinary ammonia	75.075	18.784	60.896	78.496
Rat weight	368.516	354.452	363.563	355.211
Femur length	33.674	32.728	33.257	33.313
Spinal BMD	0.205	0.198	0.202	0.200
Jackknifed Classification Matrix				
	Base Group	Broccoli Group	Onion Group	Potato Group
% correct	70	80	10	30

The Jackknifed Classification Matrix showed that, in this analysis, the computer was able to recognize 7 rats (out of 10) as belonging to the base diet group (70% correct), 8 rats (out of 10) as belonging to the broccoli diet group (80% correct), 1 rat (out of 10) as belonging to the onion diet group (10% correct), and 3 rats (out of 10) as belonging to the potato diet group (30% correct).

Table 16. Canonical discriminant functions values—standardized by within variances. Note that the higher the functions value the more important the parameter in the contrast.

	Factor 1	Factor 2	Factor 3
Percentage (P) retained	1.579	0.088	0.251
Percentage (Ca) retained	-1.872	-0.655	-0.251
Urinary ammonia levels	-0.808	0.506	0.419
Rat weights	-0.304	0.124	-0.659
Femur length	-0.295	0.326	0.010
Spinal mineral density	0.786	0.256	-0.228

The results of the discriminant analysis indicate that the rats that were fed the broccoli diet retained significantly more phosphate than calcium than those groups that were fed potato and onion diets. Also, the group that was fed the broccoli diet had lower spinal density and lower urinary ammonia than the rats from the other two groups i.e. onion and potato.

The next most significant difference seen in the discriminant analysis was that the group which received broccoli diet tended to have greater phosphate retention compared to calcium and lower urinary ammonia levels than the base diet group. There were however no other significant differences between dietary treatment groups. Thus it appears that the broccoli diet promoted a difference between phosphate and calcium retention in the following manner: Phosphate retention was augmented. In

addition, the broccoli diet also resulted in a lower level of ammonia secretion by the renal tubular cells as there was less need for this to correct acidosis in the urine.

CHAPTER 4

DISCUSSION

Observations

The aim of this study was to test the hypothesis that broccoli and onion consumption will increase bone mineral density and affect acid-base balance in the growing rat model. The observed effects of broccoli, onion, and potato on various aspects of bone turnover that were measured in the present study are discussed and interpreted so as to determine the validity of this hypothesis.

4.1. Effects of Vegetables on Apparent Calcium Balance (Apparent Percentage Calcium Retained)

In the present study the apparent percentage calcium retained was significantly higher in the groups on onion and potato diets than in the group on base diet. The difference between the group on broccoli diet and the group on potato diet was also significant with the group on potato diet having significantly higher apparent percentage calcium retained. However, the difference between the group on onion diet and the group on broccoli diet was not significant which may mean the broccoli diet may have a slight positive effect on calcium retention. In the current study the groups that were on vegetable diets had higher apparent percentage calcium retained than the group on base diet (refer section 3.4.1) and this was evident

despite the fact that the dietary analysis showed that the base diet had higher calcium content than the vegetable diets (Table 2). Thus the vegetables used in the current study did not appear to have contributed any calcium.

The insoluble fiber present in broccoli, onion, and potato may have interfered with the bioavailability of calcium. This could be due to the fact that the charged groups on polysaccharides are usually negatively charged. What this means is that insoluble dietary fiber has the tendency to bind to cations such as calcium thereby limiting their absorption (Stipanuk, 2000). Furthermore, an increase in dietary fiber may move food through the gut faster. This gives less time for the absorption of nutrients which in turn means less is absorbed.

Alternatively, the high apparent percentage calcium retained that was seen in the groups on vegetable diets (current study) may be partly due to the presence of soluble dietary fiber i.e. nondigestible oligosaccharides (NDOs) such as inulin and fructooligosaccharides. NDOs are known to enhance the absorption of several minerals such as calcium, magnesium, iron, and zinc, and to improve mineralization of bone in rats. It is believed that the enhancing effect of some NDOs is due to their prebiotic character i.e. they act as substrates that are nondigestible but can be fermented selectively by some of the intestinal microflora. NDOs resist hydrolysis by digestive enzymes (due to the configuration of their β-osidic bond) and reach the caecum and the colon. Here, they are highly fermented by micro-

organisms (mainly bifidobacteria) into short chain fatty (carboxylic) acids i.e. acetic, propionic, and butyric acids that in turn bring about a fall in the luminal pH. As a result, the solubilization of minerals such as calcium is increased. In addition, both the passive (mucosal to-serosal calcium fluxes) and the active (cellular expression of calbindin-D9k) calcium transport routes are increased by NDOs resulting in increased calcium absorption (Scholz-Ahrens et al., 2001). It is important to note that the colon can absorb minerals and trace elements and that colonic fermentation may lead to stimulated calcium absorption (Bosscher et al., 2003).

A certain amount of calcium was also possibly retained (in part) in the present study as a result of the potassium intake from broccoli, onions and potato. These vegetables contain different amounts of calcium and potassium (Table 18). Potassium intake from fruits and vegetables has been found to be positively associated with bone mineral density (New et al., 1997; New et al., 2000). Dietary potassium has been shown to aid urinary calcium retention (Lemann et al., 1993) resulting in positive calcium balance i.e. potassium enables the body to hold on to calcium. The fall in urinary calcium may be due to the natriuretic effects of potassium, an effect which results in diuresis, or it may be due to phosphate retention (potassium-induced) and suppression of calcitriol synthesis (described in detail in Section 1.2.6.1). It may, however, be due to both mechanisms (Lemann et al., 1993).

The urinary loss of calcium is flexible. It is influenced by the dietary intake of calcium and the amount of urinary acid excretion. There is a relationship between total potassium/alkali intake and bone loss rates (Frassetto et al., 1998). These investigators reported that the protein to potassium ratio predicts net acid excretion which in turn predicts calcium excretion. As discussed earlier metabolic acidosis directly impairs the distal tubular reabsorption of calcium thereby causing hypercalciuria (Alpern and Khashayar, 1997). However, there is no change in the intestinal absorption of calcium. One of the primary buffering agents that the body uses to keep the pH of the blood stable is calcium and nearly all of the body's calcium is contained within bone and thus the increased urinary calcium seen during metabolic acidosis most likely comes from bone. It must however be emphasized that calcium release is just one feature of proton buffering by bone. Sodium and potassium are also released from bone in exchange for protons (Bushinsky et al., 1993b). In addition, bone phosphate and bicarbonate are also lost during acidosis (Bushinsky et al., 1999). The serum phosphate concentration that is lowered by metabolic acidosis may increase the synthesis of calcitriol by the kidney by activating enzyme 1α- hydrolase (Krapf, 1992). Metabolic acidosis also directly causes the dissolution of mineral alkaline salts in bone. This dissolution is due to both the stimulation of osteoclast-mediated bone resorption and inhibition of osteoblastic mediated bone formation (Krieger et al., 1992). A high dietary acid load may affect bone health

negatively by increasing both the excretion of calcium and resorption of bone (Buclin et al., 2001).

In the current study the alkalizing effect of the vegetable (broccoli, onion, and potato) potassium and other bases on the potential acidifying influence of dietary protein may have also contributed to the high apparent percentage calcium retained in the groups on vegetable diets. This claim is substantiated by the findings of the DASH (Dietary Approaches to Stopping Hypertension) study which found that increasing fruit and vegetable intake from 3.6 to 9.5 daily servings decreased the urinary excretion of calcium from 157mg/day to 110mg/day (Appel et al., 1997). The investigators reported that this was probably due to the high fiber content of the diet which impeded calcium absorption. However, Barzel (1997) hypothesized that this reduction was more likely due to a reduction in the acid load produced by the fruit and vegetable diet compared to the control diet. As discussed earlier fruits and vegetables contain base precursors that may provide a natural source of base to buffer metabolic acids. The DASH diet emphasizes fruits, vegetables, and low-fat dairy products and is reduced in red meats. Another study found that children who reported consuming ≥ 3 servings of fruit and vegetables per day had lower urinary calcium excretion ($p=0.04$) than did those children who reported consuming less than 3 servings of fruit and vegetables (Tylavsky et al., 2004).

Dietary acidity may aid calcium absorption. Most dietary calcium is absorbed in the duodenum (upper part of the small intestine) which is a region where an acidic medium prevails and this in turn makes calcium more soluble (Heaney, 2001). The absorption of calcium significantly decreases further down the intestinal tract as the pH turns more alkaline. Calcium retention was high in the groups fed the vegetable diets. Onions are slightly acidic and may have thus aided in solubilizing calcium. In turn, this may have promoted calcium absorption. In addition, as mentioned earlier, the soluble dietary fiber present in broccoli, onion, and potato may have acidified the colonic pH upon fermentation which may have enhanced the absorption of calcium in the groups on vegetable diets.

Low levels of dietary components that form insoluble complexes with calcium may promote its absorption. Broccoli is rich in calcium and is also oxalate- and phytate-free. Therefore it provides highly bioavailable calcium (Nickel et al., 1997). High levels of phytates and oxalates can impede calcium absorption by forming insoluble complexes with calcium. Thus the absorption of calcium from kale (low-oxalate) is nearly four times higher than that of spinach (high-oxalate) (Weaver et al., 1987).

The diets used in the current study clearly had different levels of calcium (refer Table 2) and these levels may have influenced the absorption of calcium. The amount of calcium absorbed also depends on the amount of calcium present in the diet. In the present study the base diet had higher levels of calcium compared to the vegetable diets. However, calcium

retention was higher in the groups on vegetable diets and this may be due to the above reasons.

In conclusion, the high apparent percentage calcium retained in the groups on vegetable diets may be due to the following: Broccoli, onion, and potato may have provided the bases for neutralizing excess dietary acid (thus sparing calcium) that in turn resulted in higher calcium retention compared to controls (note that the vegetables [broccoli, onion, and potato] did not appear to have provided any additional calcium as verified by the dietary analysis). Also, the soluble fiber present in the vegetable diets may have enhanced calcium absorption by acidifying the colonic luminal pH. Although the group on broccoli diet had higher apparent percentage calcium retained than the group on base diet the difference failed to reach significance. It is possible that longer study duration was required to produce significant differences between these two groups. However, the difference between the group on onion diet (positive control) and the group on broccoli diet was also not significant which means that onion and broccoli were not significantly different in retaining calcium. Thus it can be concluded that the provision of dietary alkali in the form of vegetables (onion, potato, and to some extent broccoli) may prevent urinary calcium loss and increase apparent percentage calcium retained.

4.2 Effects of Vegetables on Apparent Phosphorus Balance (Apparent Percentage Phosphorus Retained)

In the current study there were no significant differences between groups for apparent percentage phosphorus retained on ANOVA. However, the discriminant analysis (multivariate method) showed that percentage phosphorus retained was an important parameter in the discrimination between groups (F-to-remove value 10.22). In the analysis the group on base diet and the group on broccoli diet were well distinguished (70% and 80% respectively) relative to onion (10%) and potato (30%). Parameter percentage phosphorus retained had a Canonical discriminant functions value of 1.579 which indicates that the function value is important. Thus phosphate retention was a significant contributing factor in the discrimination of the broccoli diet from the base diet. The discriminant analysis showed that apparent percentage phosphorus retained was significantly higher in the group on broccoli diet compared to the other groups. The phosphorus content of broccoli, onion, and potato is shown in Table 17.

Table 17. Phosphorus content of broccoli, onion and potato.

Vegetable	Measure	Phosphorus (mg)
Broccoli	1 Cup	133
Onion	1 Onion	34
Potato	1 Potato (medium)	53.66

From: Phosphorus and calcium levels in NZ foods (David Coory, 2003).

Phosphate maintains the acid/base balance in the body by acting as a buffer in blood plasma and urine (Hamm and Simon, 1987). The apparent percentage phosphorus retained in the current study was higher in the groups on vegetable diets than in the group on base diet. Amongst the vegetable groups the group on broccoli diet had the highest apparent percentage phosphorus retained. It is known that titratable acid excretion is augmented during metabolic acidosis and that is due to both increased H^+ secretion and increased urinary phosphate excretion (Hamm and Simon, 1987). The bases present in broccoli, onion, and potato may have increased the medium pH which may have aided phosphate uptake by the kidney in the following manner: Increasing medium pH has been found to increase sodium-coupled phosphate transport in the kidney (Brunette et al., 1984). The investigators used isolated renal brush-border membrane vesicles and found that there is preferential transport of HPO_4^{2-} and that increasing pH results in an increase in the relative concentration of HPO_4^{2-}. The preferential transport of HPO_4^{2-} can be explained by the reasons that the brush-border membrane vesicle phosphate transport is pH sensitive and that two sodiums are transported for one phosphate at any pH value as shown by kinetic analysis (Amstutz et al., 1985). Another mechanism involved in stimulated phosphate uptake in brush-border membrane vesicles (when the pH is alkaline) is a direct effect of pH on the transporter. This direct effect is such that increased concentration of H^+ decreases the transporters affinity for sodium (Hamm and Simon, 1987).

The significantly higher apparent phosphorus balance in the group on broccoli diet may be partly due to the high potassium content of broccoli (Table 18). Potassium is known to decrease urinary phosphate excretion and increase plasma phosphate concentrations (refer section 1.2.7.1). The higher apparent phosphorus balance in the group on broccoli diet may also have been due to the higher amount of phosphorus present in broccoli i.e. more phosphorus was available for absorption (refer Table 17).

In conclusion broccoli, onion, and potato may have increased the systemic pH by buffering excess metabolic acids (thus acid secretion by the kidney was reduced) resulting in higher phosphate uptake by the kidney because less phosphate was needed to buffer urinary acids. This in turn may have caused higher apparent percentage phosphorus retained (not significant on ANOVA) in the groups on vegetable diets relative to the control diet and this happened even when the dietary analysis showed that the phosphorus concentrations of the onion and potato diets were lower than the base diet (Table 2). Only broccoli appeared to have contributed some phosphorus as the concentration of phosphorus was higher in the broccoli diet relative to the control and other diets. Thus onion and potato did not appear to have contributed any phosphorus but somehow they also contributed to apparent percentage phosphorus retained (not significant). The plausible mechanism regarding this effect appears to be that that was described above. The discriminant analysis gave further credence to the effect of broccoli as it showed that the group on broccoli diet had

significantly higher apparent percentage phosphorus retained relative to the other groups. Another mechanism regarding the higher apparent percentage phosphorus retained in the vegetable groups may be as follows: The fermentation of the soluble fiber present in the vegetable diets led to a lower pH of the ileal lumen, which improved mineral solubility, which in turn enhanced the availability of phosphorus for transport across the ileal epithelium. This study showed that the group on broccoli diet promoted a difference between apparent phosphorus and calcium retention in such a manner that phosphorus retention was augmented. The lack of significant differences between groups on ANOVA may have been due to the relatively short study duration (8 weeks). Thus broccoli and, to some extent, onion and potato appear to increase phosphorus retention and may have the potential to increase bone density.

4.3 Urinary Parameters

4.3.1 Urinary Ammonia

In the present study the urinary ammonia excretion (over the three day balance period and corrected for urinary volume) was significantly lower in the group on broccoli diet than in the groups on base diet and potato diet respectively. However, the difference between the group on broccoli diet and the group on onion diet was not significant which is a positive finding because onion was used as a positive control i.e.

broccoli was not significantly different from onion. The significant difference between the broccoli group and the other groups (base and potato) may be explained by the fact that broccoli is richer in base precursors such as calcium, potassium, magnesium and sodium (refer to Table 18) than potato and that they (base precursors) may have buffered metabolic acids resulting in lower production and secretion of ammonia i.e. additional ammonia was not required for urinary buffering. Ammonia is regarded as an important urinary buffer because both acute and chronic metabolic acidosis results in an increase in the urinary excretion of ammonia i.e. it is the predominant adaptive response during metabolic acidosis (Hamm and Simon, 1987). Urinary ammonia (NH_3) buffers H^+ to form NH_4^+.

Alternatively, the low urinary ammonia excretion in the group on broccoli diet may be due to the lower concentration of their urine i.e. the rats on broccoli diet may have drunk more water which resulted in significantly higher urinary volume compared to the groups on base, onion, and potato diets ($p=0.011$, $p=0.008$, and $p=0.001$ respectively). Though ammonia corrected for urine volume showed significance we unfortunately did not measure the water intake. It can therefore be concluded that the consumption of broccoli leads to a lower level of urinary ammonia buffering (ammonia is required to correct acidosis in the urine) in growing male rats.

4.3.2 Urinary Creatinine

Urinary creatinine concentration is a measure of how well the kidneys are functioning. In this study, there were no significant differences in urinary creatinine concentration (over the balance period) between the various study groups. Essentially, creatinine is a protein that is produced by muscle and released into the blood. The amount of creatinine produced daily is relatively stable. Creatinine can only be excreted by the kidneys and creatinine clearance measured in a 24 hour urine collection is the most commonly used method to estimate the glomerular filteration rate (GFR). Urinary creatinine is a marker of muscle mass and the creatinine excretion is proportional to total body creatinine. Nearly all the total body creatinine is found in the muscles (Ganong, 2003). Thus the urinary creatinine concentrations were not expected to be significantly different.

4.3.3 Urinary Urea

In the present study the urinary urea concentration over the three day balance period was significantly higher in the groups on base diet and broccoli diet compared to the other two groups i.e. potato and onion.

The amount of urinary urea is predominantly regulated by the plasma urea concentration and that the plasma urea concentration depends on the dietary intake of protein (Thornton and Wilson, 1972). Excess nitrogen is converted to urea in the urea cycle and most of the nitrogenous waste comes from the breakdown of amino acids by oxidative deamination

which results in the production of ammonia. The liver possesses a system of carrier molecules and enzymes that rapidly converts the ammonia into urea which is then transported via the bloodstream to the kidneys where it is excreted. Any defect in the enzyme systems can affect the urea cycle flux. For example, even a small change in the activity of enzyme carbamoyl phosphate synthetase I activity can cause a large change in the urea cycle flux. The synthesis of urea is also regulated by substrate availability i.e. high intakes of amino acids will result in high rates of amino acid catabolism. As a result urea production is increased (Stipanuk, 2000). It is unlikely that the rats used in this study had any defect in the enzyme system involved in the urea cycle. The significant differences between the group on base diet and the other groups (onion and potato) may be due to higher food consumption by the base diet group compared to the groups on onion and potato diets resulting in higher protein intake (refer section 3.3). The significant differences between the group on broccoli diet and the groups on onion and potato diets may be due to the protein content of these vegetables i.e. it is higher in broccoli than in onion or potato (refer Table 18).

4.3.4 Specific Gravity and Osmolality of Urine

Although the specific gravity and osmolality of urine over the three day balance period were higher in the group given the base diet than in the groups given the vegetable diets these differences failed to reach significance. The urine osmolality was lowest in the broccoli group possibly because the rats in that group drank more water. Specific gravity and osmolality of urine are measures of the solute concentration of urine. Therefore, the more concentrated the urine, the higher will be the specific gravity and osmolality which means that more solid material is dissolved in the urine. The lack of significant differences between groups could be due to the fact that all the groups drank enough water and thus none of the groups produced concentrated urine.

4.3.5 Urinary Volume

The rats in the group on broccoli diet produced significantly more urine than the other groups over the three day balance period. It is possible that the group on broccoli diet may have drunk more water compared to the other groups. Another reason could be as follows: Broccoli contains higher levels of potassium compared to onion or potato (refer Table 18) and potassium is known to produce natriuretic effect which in turn results in diuresis (refer section 1.2.7.1).

4.4 Bone Parameters

4.4.1 Bone Density

In the current study there were no significant differences between groups for bone mineral content and bone mineral density of the lumbar spine and femur on ANOVA. Perhaps a longer study period was required to produce significant differences.

Various studies on humans have shown positive associations between fruit and vegetable consumption and lumbar spine or hip bone mineral density. A previous study found that women who reported having high intakes of fruit during childhood had significantly higher femoral neck bone mineral density (measured by DEXA) than those who reported medium or low intakes ($p=0.01$) (New et al., 2000). The Framingham Osteoporosis Study showed that the dietary pattern group with the greatest average bone mineral density was the fruit, vegetables, and the cereal group (Tucker et al., 2002). The investigators measured the bone mineral densities of the proximal right femur in g/cm^2 with a Lunar dual-photon absorptiometer. New et al (1997) reported that the bone mineral density (measured by DEXA) at the lumbar spine, femoral neck, and femoral trochanter was significantly lower in women who reported a low intake of fruit in early adulthood than in those who reported a medium or high intake ($p<0.01$). McGartland et al (2004) measured bone mineral density (by DEXA) at the forearm and heel in a random sample of 12 and 15 year old boys and girls.

The investigators observed that 12 year old girls that were consuming high amounts of fruit had higher (significant) heel bone mineral density than those who consumed moderate amounts of fruit. Tylavsky et al (2004) reported that children consuming ≥3 servings of fruit and vegetables per day had more bone area of the whole body ($p=0.03$) and radius ($p=0.03$) than did those children consuming less than 3 servings of fruit and vegetables per day. The positive effects of fruits and vegetable on bone health may be due to their base excess that buffers metabolic acid (Tucker et al., 1999; New et al., 2000). Thus, the regular consumption of fruit and vegetables may be associated with greater bone mineral density.

The nutrients present in vegetables may also positively affect bone health. The potassium, magnesium, calcium, vitamin C, niacin, iron, zinc, vitamin A, vitamin K, sodium, folate, and β-carotene contents of broccoli, onion, and potato are listed in Table 18. To date the effect of nutrient intake on BMD remains largely undefined. Like any other tissue, bone needs a range of nutrients in order to develop normally and to support itself after growth. The main nutrients required are protein, calcium, phosphorus, vitamin D, vitamin C, and vitamin K (Heaney. 1996). Calcium intake is important during skeletal growth and the development of PBM (Matkovic, 1991). Phosphorus as phosphate makes up nearly half the weight of bone mineral. Calcium and phosphorus compose nearly 80% to 90% of bone mineral and they both work in tandem to strengthen and maintain bones (Ilich and Kerstetter, 2000).

Vitamin C and niacin have been shown to be associated with greater forearm bone mineral content in post-menopausal women (Freudenheim et al., 1986). Iron, magnesium, plus zinc have been linked to an increase in forearm mineral content in premenopausal women (Angus et al., 1988). Freudenheim and coworkers (1986) found that protein, phosphorus, zinc, and folate reduce bone loss in postmenopausal women. Another placebo-controlled trial involving postmenopausal women who were supplemented with calcium and the trace minerals zinc, copper, and manganese resulted in slight improvement in BMD over 2 years (Strause, 1994).

Potassium and magnesium have been linked with greater BMD in late premenopausal women (New et al., 1997; New et al., 2000). Similar associations have also been found in elderly men and women (Tucker et al., 1999). Potassium reduces blood acidity by buffering metabolic acids. It also slows calcium loss (Lemann et al., 1993) and decreases urinary phosphate excretion (Sebastian et al., 1990), and may therefore increase bone mineral density. Urinary potassium (associated with dietary potassium intake) has been positively associated with bone mass at all sites in prepubertal children (Jones et al., 2001).

Magnesium is also present in broccoli, onion, and potato (Table 18). Magnesium is required for the normal function of the parathyroid glands, and vitamin D metabolism. It is also essential to adequately sensitize target tissues to PTH and active vitamin D metabolites (Zofkova and

Kancheva, 1995). As mentioned earlier (refer section 1.2.7.1) magnesium stimulates the production of calcitonin which is a hormone that increases the levels of calcium in the bones by suppressing osteoclasts. In addition magnesium helps to reduce acid levels in the blood by acting as a buffer (Rude et al., 1998). Moderate to severe magnesium deficiency results in hypocalcemia, and the most likely cause for this appears to be impaired PTH secretion. If magnesium is administered during moderate to severe magnesium deficiency there will be a rapid rise in the concentration of serum PTH (Rude et al., 1978). As a result, serum calcium rises and PTH concentration falls to normal. When magnesium levels are low the parathyroid gland increases the secretion of PTH.

Osteocalcin and bone GLA-protein are vitamin K dependent proteins that are important constituents of bone. The vitamin K content of broccoli, onion and potato is given in Table 18 and the role of vitamin K in bone health is as follows: The bone forming cells produce at least 3 different GLA-containing proteins i.e. osteocalcin, matrix GLA-protein, and protein S. Vitamin K acts as a coenzyme for enzyme glutamate carboxylase. This enzyme mediates the conversion of glutamate to gamma carboxyglutamate, which is known as GLA-protein. During conversion, the GLA (dicarboxylic glutamyl) residues attract Ca ions which are positive resulting in the incorporation of calcium into the hydroxyapatite crystals (Ilich and Kerstetter, 2000).

Adequate amounts of vitamin A are important in the bone remodeling process. Nuclear receptors for retinoic acid are present in both osteoblasts and osteoclasts (Kindmark et al., 1993). Table 18 shows the vitamin A content of broccoli, onion, and potato.

The vegetables used in this study (broccoli, onion, and potato) also contain vitamin C (Table 18). Ascorbic acid (Vitamin C) is essential during the hydroxylation of proline and lysine residues. The crosslinking that is involved during normal collagen fiber formation requires hydroxylysine. When hydroxylation is defective the intracellular degradation of collagen precursors increases resulting in perturbed production of various cross-linking amino acids in collagen (Stipanuk, 2000).

Table 18. Calcium, magnesium, potassium, sodium, iron, zinc, protein, vitamin A, vitamin C, vitamin K, β-carotene, folate, and niacin content of broccoli, onion, and potato.

	Broccoli, raw 100g	Onion, flesh, raw 100g	Potato, flesh 100g
Calcium (mg)	42	21	4
Potassium (mg)	487	184	332
Sodium (mg)	5	2	4
Iron (mg)	1.2	0.2	0.5
Zinc (mg)	0.7	0.3	0.2
Total vitamin A equivalent (µg)	68	2	1
Vitamin C (mg)	57	7.1	9
β-carotene equivalent (µg)	410	10	7
Folate, Total (µg)	75	27	13
Total niacin equivalent (mg)	1.3	1.0	0.8
Protein (g)	4.4	1.3	2.1
Magnesium (mg)	24	12	22
Vitamin K (µg)	205	2	0.8

From: The Concise New Zealand Food Composition Tables (2003). 6th. Edition.
Magnesium From: Compositions of Foods, Agriculture Handbook No:8, United States Department of Agriculture. Reprinted October 1975.
Vitamin K From: Provisional Table on the Vitamin K Content of Foods. Revised 1994. Release 13. United States Department of Agriculture, Agriculture Research Service.

In the present study we were not able to show any positive effect of vegetables on the bone mineral content and bone mineral density of both lumbar spine and femur possibly because of the relatively short study period (8 weeks).

4.4.2 Bone Breaking Strength

The current study did not show any significant differences between groups for maximum stroke, break strain, break stroke, break energy, maximum load, and stiffness. There were, however, significant differences for break load. The bones of rats given base diet and onion diet required more (significant) force to break as compared to the rats on potato diet. This means that the onion diet may have improved bone strength (onion was used as a positive control in this study). Swiss researchers (Wetli et al., 2005) proposed that the regular consumption of onions may have a positive effect on bones and prevent osteoporosis. The investigators reported that there was significant inhibition of osteoclastic activity in osteoclasts (isolated from rats) exposed to γ-L-glutamyl-trans-S-1-propenyl-L-cysteine- sulphoxide (GPCS) than those cells that were not exposed to it (Wetli et al., 2005). They suggested that GPCS (isolated from allium cepa L. [onion]) inhibits the resorptive activity of osteoclasts and that this action is dose-dependent.

The bones of the rats given the broccoli diet also required more force to break as compared to the rats given potato diet but the difference

was not significant which means that broccoli was perhaps beginning to have an effect on bone strength and that longer study period was required to show significant differences between them. Note that there were no significant differences between the group on broccoli diet and the group on onion diet (positive control).

The lack of significant differences between groups for maximum stroke, break strain, break stroke, break energy, maximum load, and stiffness may be due to the relatively short study duration (8 weeks).

4.4.3 Serum C-Telopeptides of Type 1 Collagen

There were no significant differences between groups for serum C-Telopeptides of Type-1 collagen (CTX) in the present study. Although we were not able to show significant differences between groups for serum CTX levels previous studies have shown significant differences in markers of bone resorption. Lin et al (2003) reported the findings of the DASH-sodium trial. The investigators measured CTX levels at baseline and at the end of the trial. It was found that the DASH diet (high in fruits and vegetables) reduced serum CTX by 16-18% ($p=0.001$). This means that the DASH diet significantly reduced bone resorption. The claim that fruit and vegetables can positively affect bone health is substantiated in another study by New and coworkers (2000). The investigators calculated bone loss by measuring urinary excretion of pyridinoline and deoxypyridinoline (both are markers of bone resorption). They found that the average pyridinoline

excretion was lower with higher intakes of potassium, magnesium, and β-carotene (nutrients found in fruit and vegetables). As for the excretion of deoxypyridinoline, the average level was lower with higher intakes of potassium, magnesium, β-carotene and vitamin C (nutrients found in fruit and vegetables) (New et al., 2000).

In the current study, the lack of significant differences between the study groups for CTX could be due to the relatively short study duration i.e. 8 weeks. The above studies were done on humans but our model was the rat. Furthermore the method that was used (current study) to determine the concentration of serum CTX is relatively new. If increased resorption was present then serum CTX levels would have been raised.

4.4.4 Growing versus Postmenopausal Physiological States

Growth patterns vary from species to species, for example, rats continue to grow throughout life (although at a declining rate). Humans, on the other hand, have two periods of rapid growth, the first in infancy and second in late puberty. Between these two periods is a period of slow growth. During the periods of rapid growth the nutritional needs will also increase. The period of rapid growth requires a balanced, relatively large intake of essential nutrients and energy. For example, the growing child has rapid linear growth which involves acquisition of new skeletal tissue and muscles. As such, the growing child requires sufficient energy, protein and essential

elements such as calcium, phosphorus, potassium, and zinc (Garrow et al., 2000).

Nutritional needs of the young growing child clearly differ from those of the adult. Ageing brings about physiological changes that affect nutritional requirements. Generally, energy requirements decrease with age (because muscle mass decreases) but nutritional needs however remain relatively high. Older people tend to lose bone and muscle and gain body fat because some hormones that regulate appetite and metabolism become less active while others become more active (Morley, 1997). In addition, the immune function also declines with ageing with resultant increased need of vitamins and minerals (Buzina-Suboticanec et al., 1998). About a third of people who are over 70 have limited secretion of stomach acid. This in turn affects absorption of vitamin B_{12}, folic acid, calcium, iron, and zinc (Whitney and Rolfes, 2002). Thus there are differences between the nutritional needs and biological responses to nutrients between growing and aged animals. However these differences are not described in detail because the main aim of the current study was to test the hypothesis that broccoli and onion will increase BMD (as shown by Muhlbauer and Li, 1999) and affect acid base balance in growing male rats.

The aim of using growing rats was to determine whether broccoli and onion can increase BMD in them because building strong bones at a very young age will hopefully lessen the effect of the natural bone loss seen with aging (which includes the postmenopausal state) as there will be more bone

present at that time. Furthermore, there is very little opportunity to add additional bone mass after late adolescence. More importantly, any additions of bone mass may translate into health differences (e.g. fracture prevention). It has been shown that the BMD predicts fractures even in children (Goulding et al., 2000). Therefore, osteoporosis can be said to be a pediatric disease with geriatric implications.

CHAPTER 5

CONCLUSIONS AND FUTURE RESEARCH

5.1 Conclusions

In the current study we were not able to mimic the findings of Muhlbauer and Li (1999) who showed that male rats fed 1g of onion had increased (significant) bone mineral content and bone mineral density. They also reported that 1g of broccoli significantly inhibited bone resorption in male rats. It is likely that longer study period was required to match their findings. However, our results indicate that feeding male rats 1g of broccoli per rat per day significantly reduced urinary ammonia excretion and increased apparent percentage phosphorus retained. It is suggested that the decrease in urinary ammonia excretion was due to the bases present in broccoli that may have buffered metabolic acids resulting in decreased ammonia production and secretion. The significantly higher apparent percentage phosphorus retained may be due to the buffering of metabolic acids by the bases found in broccoli. This may have reduced acid secretion into the urine thereby reducing the need for phosphate buffering (in the urine). A high pH also increases phosphate uptake by the kidney.

The results of this study also indicate that feeding male rats 1g of onion or potato per rat per day significantly increased apparent percentage calcium retained and this increase may be due to the bases

present in onion and potato that may have buffered metabolic acids. Thus, calcium was not needed for buffering metabolic acids.

I conclude that the findings of the present study suggests that increases in onion and potato intakes may make the calcium balance more positive, and that increases in broccoli intakes may decrease urinary ammonia excretion and increase phosphate retention possibly by supplying bases needed to buffer metabolic acids. Thus we may be able to protect ourselves from the bone depleting effects of an acidogenic diet via increased intakes of broccoli, onion, and potato and other alkalizing foods. Also, broccoli, onion, and potato may have the potential to increase bone mineral density.

5.2 Implications for Future Research

The areas that have been identified as requiring further research are listed below:

- The effects of longer term broccoli, onion, and potato consumption (between 12 and 16 weeks) on bone mass.
- The quantity of broccoli, onion, and potato needed to improve bone mineral content and bone mineral density.
- The effects of different doses (low, medium, and high) of broccoli, onion, and potato on bone mineral density, and bone turnover.
- The relationship between the micronutrients found in broccoli, onion and potato, bone mineral density, and bone turnover.

- The inhibitors/pharmacologically active compound(s) present in broccoli, onion, and potato that inhibits bone resorption.
- The effect of γ-L-glutamyl-trans-S-1-propenyl-L-cysteine sulphoxide (GPCS) in human bones.

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