DIET AND INDICES OF BONE BREAKDOWN AND ACID PRODUCTION IN BED REST SUBJECTS

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DEDICATION

I dedicate this work with gratitude to my husband, Sidney C. Watts, for his years of confidence, love, and unwavering devotion, and to my children, Annie and Gabriel who remind me to smile and who have endured this process by my side.

To my father, Father Paul E. Lockey, Ph.D., and mother, Annie Marlene Lockey, for their years of endless support, dedication to higher education, and many words of wisdom and encouragement.

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I offer this in honor of my grandmother Beulah M. Lockey, for serving as an inspirational role model, and for teaching me to have faith, to value life, and never give up.

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ABSTRACT

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Diet and acid-base balance can affect bone loss during simulated weightlessness. The present study evaluates the effects of acid and base components of diet on bone resorption markers before, during, and after 60-90 days of bed rest at -6° head-down tilt position. A total of eleven subjects (8M, 3F; age 26-44 y) participated in the present study. Urine samples from the subjects were analyzed for a relationship between dietary patterns and markers of bone metabolism. This study examines two procedures for estimating acid production in the body by comparing urinary net acid excretion ($NAE_{indirect}$) and by measuring the animal protein to potassium ratio, as estimated from dietary intake. It is hypothesized that both estimations would have significant correlations between markers of bone breakdown NTX (N-telopeptide) and PYD (pyridinoline). Results confirmed that bone resorption increased during bed rest as indicated by the collagen crosslinks NTX and PYD. In addition, significant correlations were recorded between $NAE_{indirect}$ and NTX ($p<0.01$), and PYD ($p<0.01$) during bed rest. However, the ratio of animal protein to potassium intake was not significantly correlated with NTX or PYD, suggesting further research on this method of approximating acid load is necessary.

Keywords: Weightlessness; bed rest; bone; bone resorption; bone markers; collagen crosslinks: NTX (N-telopeptide); PYD (pyridinoline); acid-base homeostasis; dietary acid; dietary base; urinary net acid excretion (NAE); animal to potassium ratio

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CHAPTER I

INTRODUCTION

Skeletal deterioration caused by weightlessness is a significant concern for spaceflight, and countermeasures to improve the bone health of astronauts must be developed as the duration of spaceflight is expected to increase (Smith et al., 2003; Vermeer, Wolf, Craciun, & Knapen, 1998; Zwart et al., 2009). Bed rest serves as an acceptable ground-based analogue for studying several aspects of weightlessness, with bone loss being one example (Donaldson et al., 1970; LeBlanc et al., 1995; Lueken, Arnaud, Taylor, & Baylink, 1993; Scheld et al., 2001; Smith, et al., 2003; Smith & Lane, 1999; Smith et al., 1998; Swaminathan, 2001; van der Wiel, Lips, Nauta, Netelenbos, & Hazenberg, 1991; Watanabe et al., 2004; Zerwekh, Ruml, Gottschalk, & Pak, 1998; Zwart, et al., 2009; Zwart & Smith, 2005). Although, bed rest is not exact model for weightlessness, bone breakdown occurring during bed rest parallels well with bone loss experienced by astronauts during exploration missions (Marsh, Sanchez, Michelsen, Chaffee, & Fagal, 1988; Smith, et al., 2003). In addition, the data collected during spaceflight and bed rest studies may have important implications for the general population on Earth. As osteoporosis has become a global health concern on earth, leading to greater risk of fragility, fracture, disability, and premature mortality, the study of all factors which may contribute to bone loss become of critical importance (Angus,

Sambrook, Pocock, & Eisman, 1988; Jeffcoat, 2006; New, 2003; Orwoll, 2003; Prentice, 2004; Prynne et al., 2004; Rosen, Glowacki, & Bilezikian, 1999). Because research has shown that diet plays an important role in maintaining skeletal health, the study of nutrient interactions in the form of dietary acid-base and bone health may, therefore, have important connotations for the general public and for astronauts during spaceflight (Macdonald, New, Fraser, Campbell, & Reid, 2005; Smith & Lane, 1999; Smith, Zwart, Block, Rice, & Davis-Street, 2005; Zwart et al., 2005; Zwart, Hargens, & Smith, 2004).

Diet is a modifiable and controllable factor for maintaining bone health and mass. While calcium and vitamin D have been in the forefront of bone research, many other nutrient interactions associated with bone mass also merit recognition. Research has shown that the individual nutrients of magnesium, potassium, phosphorus, and protein are also essential nutrients for bone development and maintenance. However, these nutrients are often studied independently, rather than jointly. Therefore, it is reasonable to theorize that when investigating the role of nutrition on skeletal health, the impact of specific nutrients does not only occur in isolation, but rather that dietary interactions occur synergistically, resulting in previously unidentified positive or negative effects on bone health and mass (L. Frassetto, Morris, Sellmeyer, Todd, & Sebastian, 2001; McCarthy & Frassica, 1998; Swaminathan, 2001; Viguet-Carrin, Garnero, & Delmas, 2006; Zwart & Smith, 2005).

The study of acid-base homeostasis examines the effects of nutrients interacting in unison. The two primary nutrients often studied in the context of acid-base balance are protein and potassium, with protein contributing to increases in the overall dietary acid

load and potassium salts neutralizing acid load (Cloutier, 2003; Demigne, Sabboh, Remesy, & Meneton, 2004; L. Frassetto, et al., 2001; L. A. Frassetto, Todd, Morris, & Sebastian, 1998; Lemann, Pleuss, & Gray, 1993; Macdonald, et al., 2005; New, 2003; New & Millward, 2003; Patience, 1990; Prynne, et al., 2004; Sebastian, 2005; Swaminathan, 2001; Vermeer, et al., 1998). Other nutrients which interact to a lesser degree in acid-base homeostasis include; magnesium, sodium, calcium, phosphorus and chloride. It is the combination of these nutrients, acting in unison, which may interact to increase and decrease acid and base load and therefore have an effect on total bone mass. As the bone health of astronauts during weightlessness, and the bone health of patients with bone disease such as osteoporosis, is at a compromised state, it is possible that even marginal increases in dietary acid load can have detrimental effects on total bone mass. Given the differing results regarding the exact effects of acid on bone health, additional research is required to more thoroughly examine the effects of increased acid load on overall skeletal health (Alexy, Remer, Manz, Neu, & Schoenau, 2005; Dawson-Hughes & Harris, 2002; Lanham-New, 2006; Prynne, et al., 2004; Remer, Dimitriou, & Manz, 2003; Roughead, Johnson, Lykken, & Hunt, 2003; Sebastian, 2005; Sellmeyer, Stone, Sebastian, & Cummings, 2001; Zwart, et al., 2004; Zwart & Smith, 2005).

The present study investigates the effects of acid and base components from dietary intake by comparing the two procedures: urinary net acid excretion ($NAE_{indirect}$); and the animal to protein to potassium ratio, as compared to markers of bone resorption in bed rest subjects whose bone is compromised from disuse. The primary hypothesis of this study is that there will be a correlation in urinary net acid excretion ($NAE_{indirect}$) or

poteintial renal acid load (PRAL) in the diet on either markers of bone breakdown (NTX and PYD) during bed rest. A secondary hypothesis of this study is that there will be a correlation between the animal protein to potassium ratio, and markers of bone breakdown (NTX or PYD) during bed rest. Results from this study may also serve as an analogue for spaceflight.

This study will use key terminology such as "markers of bone resorption," "regulation of acid-base homeostasis," "components of dietary acid-base," "assessments for measuring urinary net acid excretion" and "bed rest." Because one set of terms in this project directly affects the next set, this study has been organized and divided into individual sections so that each statement and concept related to the hypothesis will be more easily understood. This study will first outline and describe the general structure and mechanics of bone by detailing bone anatomy, remodeling, the breakdown of collagen, and specific markers of bone resorption. Next, this study will examine the body's ability to regulate acid-base, generally and via the intestinal tract, as well as the impact that such regulation has on skeletal health. In addition, this study will discuss the general role of diet in acid-base homeostasis, assessments for urinary net acid excretion and the effects of dietary acid and base on skeletal health. Finally, this study will conclude with the use of bed rest as an analogue for spaceflight, and examine the potential implications for spaceflight and the general public. A firm grasp of the above-listed concepts and terms is necessary to fully understand the study results and discussion that support the hypothesis of this study. It is for these reasons that such terms and concepts are clearly outlined are detailed in the sections to follow.

CHAPTER II

REVIEW OF THE LITERATURE

Bone Anatomy

The human skeleton is composed of two types of bone, cortical and cancellous. Cortical bone makes up the majority of the skeleton, and is the main component of long bones, such as the femur, tibia, fibula, radius, and ulna. Cortical bone is dense and covers the surface of all bones providing the structural support known as the cortex. Alternately, cancellous bone comprises less than thirty percent of the skeleton, and occurs at the ends of long bones and bones such as those of the pelvis, hip, and spine. Cancellous bone is highly vascular and more metabolically active than the surrounding cortical bone, and is the site of bone remodeling and calcium exchange (Arnaud, 1996; Christenson, 1997; Cummings, Bates, & Black, 2002; Viguet-Carrin, et al., 2006).

On the cellular microscopic level, bone is a living and dynamic structure which consists of specialized cells known as osteoblasts, osteocytes, and osteoclasts. Osteoblasts and osteocytes work to construct bone formation, while osteoclasts serve to degrade bone material, resulting in bone resorption. The primary function of osteoblasts is to deposit, mineralize and construct the matrix of bone known as osteoid. After the osteoid matrix is constructed, roughly fifteen percent of active osteoblasts are inserted into the newly formed bone matrix, forming cell-to-cell connections known as osteocytes further

strengthening the structure of bone (Christenson, 1997; McCarthy & Frassica, 1998). This process constructs the osteocytic membrane system that controls the calcium ion flow and electrical activities, which, in turn, maintain balanced bone remodeling.

Conversely, osteoclasts, are large multinucleate cells whose function is to digest bone, and are found on the surface of bone in resorption craters. Osteoclasts function as part of the normal bone cycle to clean away old or injured bone debris. The increase in the number of osteoclasts may occur in diseases which are characterized by high bone turnover, or in certain physical states, such as weightlessness, bed rest, or immobilization, where bone is challenged from disuse (T. Arnett, 2003; Christenson, 1997; Macdonald, et al., 2005; Orwoll, 2003).

Bone Remodeling

In healthy individuals, bone undergoes a continual finely coupled process or cycle in which old or injured bone is removed and new bone is accrued. This cycle occurs on the surface of bone and is referred to as the "cycle of bone turnover" or "bone remodeling." Bone remodeling begins with a quiescent phase and lasts for approximately two to six months, depending on the severity of damage to bone, which can be caused from injury, or from changes in bone structure and collagen strength, occurring as a natural process of aging. During the quiescent phase, osteoclasts migrate to the site of old or injured bone and erode the surface by attaching a ruffled membrane to mineralized bone matrix. After osteoclasts have dissolved away the matrix of bone, a tunnel or crater is formed depending on the type of bone. In the case of cancellous bone, craters are formed (known as a Howship lacunaes), and in the case of compact bone, tunnels are

formed (known as cutting cones) (Arnaud, 1996; Christenson, 1997; McCarthy & Frassica, 1998; Orwoll, 2003). This process of resorption lasts until the site is clean of bone debris. Once this process has ended, osteoblasts migrate to the lacunae, and work to fill the cavity, beginning from the bottom and filling to the top, with new cement line of osteoid matrix. When bone reaches the appropriate thickness, the matrix is mineralized with hydroxyapatite giving the bone strength, and the bone cycle is complete (Arnaud, 1996; T. Arnett, 2003; Christenson, 1997; Looker et al., 2000; Orwoll, 2003). During the human life span, bone formation will exceed bone resorption until the human body reaches the age of 30 to 40, after which bone formation begins to decrease (Arnaud, 1996; Orwoll, 2003).

Type 1 Collagen and Urinary Markers of Bone Metabolism

Collagen is the most abundant protein and is found throughout the body and in bone (Christenson, 1997; Orwoll, 2003; Seibel & Woitge, 1999; Viguet-Carrin, et al., 2006). The majority of the osteoid bone matrix is composed of Type 1 collagen. Type 1 collagen is also present in nonosseous locations such as the skin, tendons, blood vessels, and ligaments. However, bone has the highest quantity of this protein, as well as the highest turnover (Christenson, 1997; Orwoll, 2003). Collagen is composed of fibrillar proteins which are arranged into a triple helix with a carboxyterminal end (CTX) and an aminoterminal end (NTX) (Christenson, 1997). Collagen is synthesized through a series of intracellular and extracellular steps which form a single collagen fibril. Within each fibril, the arrangement of the collagen molecules stagger, with one collagen molecule located close to the other (Christenson, 1997; Viguet-Carrin, et al., 2006). In order to

stabilize the fibril, crosslinks are formed between the amino acids lysine and hydroxylysine on the nonhelical carboxyterminal or aminoterminal ends, connecting adjacent collagen helical molecules together (Christenson, 1997; Orwoll, 2003).

When osteoclasts resorb the collagen of bone matrix, the lysine-derived collagen crosslinks are broken apart and released into the serum, filtered through the renal glomerulus, and then excreted into the urine as intact molecules (Fig 1) (Christenson, 1997; Looker, et al., 2000; Seibel & Woitge, 1999; Swaminathan, 2001; Viguet-Carrin, et al., 2006). It is these collagen crosslinks that are considered to be biochemical markers of bone turnover and reflect bone resorption when measured in urine excretion (Beck, Sorensen, Kollerup, Jensen, & Sorensen, 1994; Christenson, 1997; Looker, et al., 2000; Seibel & Woitge, 1999; Smith, Dillon, DeKerlegand, & Davis-Street, 2004).

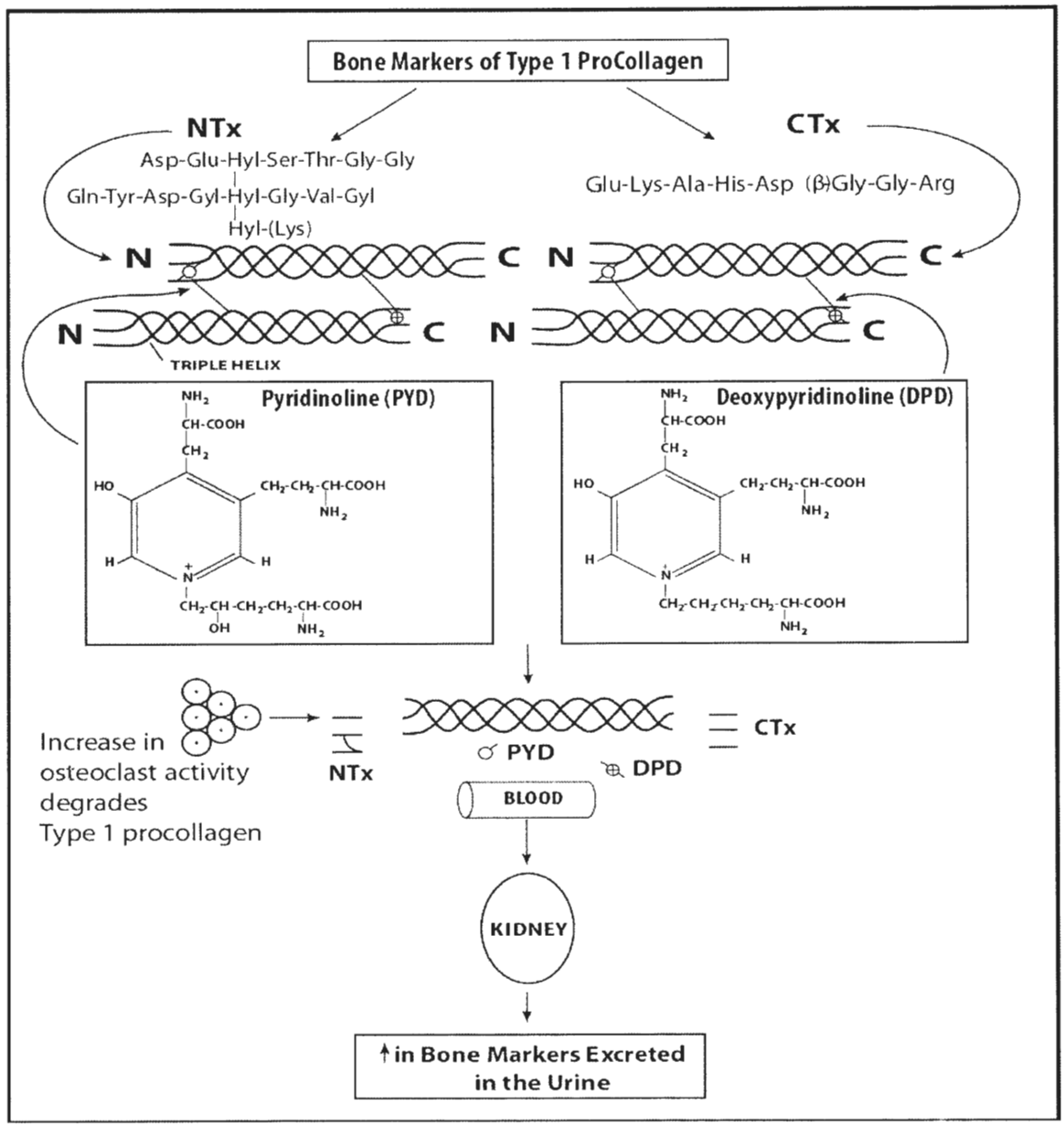

Fig.1. Representation of the effects of increased osteoclast activity and the degradation of Type 1 Procollagen. Markers of bone resorption, NTX, CTX, PYD and DPD are released into the plasma and excreted in the urine. This diagram is a variation and adaptation of figures obtained from references Christenson, (1997), Swaminathan, (2001), and Viguet-Carrin, Gamero, & Delmas, (2006).

Biochemical markers that reflect bone resorption are direct or indirect products of osteoclast activity and collagen degradation, and reflect a whole body integrated picture of the metabolic state of the skeleton. These markers include amino-terminal crosslinked telopeptide of type 1 collagen (NTX), C-telopeptide (CTX), pyridinoline (Pyd) and deoxypyridinoline (Dpd) (McCarthy & Frassica, 1998; Pagana & Pagana, 2003). Markers of bone resorption are routinely measured in the urine and show a diurnal rhythm with higher values of urinary crosslinks in the morning and lower values in the afternoon. When frozen, collagen crosslinks may retain stability for decades (Ralston & Kleerekoper, 2002; Smith, et al., 1998).

The use of markers of bone resorption has been criticized for high degree of both within and between subject variability (Ralston & Kleerekoper, 2002; Seibel & Woitge, 1999; Smith, et al., 1998). However, the use of 24-hour urine collections, pooling samples, and control of subjects' activity and dietary intake, have been shown to mitigate subject variability, addressing such criticism by obtaining a higher degree of consistency between urine samples (Arnaud, 1996; Baecker et al., 2003; Christenson, 1997; Inoue et al., 2000; Looker, et al., 2000; Ralston & Kleerekoper, 2002; Seibel & Woitge, 1999; Smith, et al., 2004; Swaminathan, 2001; van der Wiel, et al., 1991). In the bed rest model, biochemical markers of bone turnover are sensitive, and have proven to be early indicators of bone breakdown. Heer and colleagues found that the presence of bone resorption markers in urine may increase in bed rest subjects in as little as 24-48 h (Baecker, et al., 2003; Heer, Baecker, Mika, Boese, & Gerzer, 2005).

Despite some criticism, the use of collagen crosslinks are a noninvasive cost effective method for assessing and monitoring bone turnover as contrasted to more expensive and invasive methods such as Reamer-Irrigator-Aspirator (RIA) or dual-energy x-ray absorptiometry (DEXA) (Ralston & Kleerekoper, 2002; Seibel & Woitge, 1999). Comparatively, crosslink fragments can be measured routinely in a laboratory, by high-performance liquid chromatography (HPLC), or using the commercially available enzyme-linked immunosorbent assay (ELISA) (McCarthy & Frassica, 1998; Ralston & Kleerekoper, 2002; Seibel & Woitge, 1999; Smith, et al., 1998). To date, the use the use of crosslink fragments can be considered an accurate, convenient, and valuable tool for measuring and assessing bone turnover.

Regulation of Acid-Base Balance in the Human Body

The human body maintains acid-base homeostasis by preserving a constant equilibrium of the intracellular and extracellular hydrogen ion (H^+) concentrations. Equilibrium of the H^+ is achieved when the intake or production and the net removal of H^+ is equal to zero, or in favor of the bicarbonate (HCO_3^-). Almost all enzyme systems within the body are influenced by H+ ion concentrations, and it is customary to express H^+ concentrations as pH, which is equal to –log[H+]. Change in blood pH reflects variations in the H^+ ion and can alter the structural configuration of both cells and body functions. The normal level of arterial blood pH is 7.4. If pH falls below 7.4 a person is said to be in a state of acidosis, or to have an excess addition of H+ in the body. On the contrary, if pH rises above 7.4 a person is in a state of alkalosis (note that the terms alkali and base can be used interchangeably) or to have an excess removal of H^+ from body

fluids (T. Arnett, 2003; T. R. Arnett, 2010; Barzel, 1995; Gluck, 1998; Guyton & Hall, 2006; Madias, Adrogue, Horowitz, Cohen, & Schwartz, 1979; New, 2003; Patience, 1990; Remer, 2000, 2001b). The three primary interrelated adaptive mechanisms by which the body regulates acid-base status, or H^+ balance, are: through the chemical acid-base buffer systems of body fluids; exhalation of CO_2 through respiratory exchange; and by through kidney function which excretes acid or alkali in the urine (T. R. Arnett, 2010; Gluck, 1998; Guyton & Hall, 2006; Madias, et al., 1979; New, 2003; Patience, 1990; Remer, 2000).

The chemical acid-base buffer system of the body fluids is the first line of defense in the regulation of acid-base homeostasis. Large amounts of hydrogen is either ingested or produced each day by normal metabolic function (e.g. from the metabolism of sulfur, nitrogen, and phosphorus containing molecules) in the body (Florin, Neale, Gibson, Christl, & Cummings, 1991; Guyton & Hall, 2006; Remer, 2000). In order to mitigate the changes that this process could cause in the human body by reducing pH and bicarbonate (HCO_3^-), these substances are buffered by reversibly binding the H+ ion of an acid or base, so that the total concentration of H^+ is minimized. There are several buffer systems in the human body that act to control both extracellular and intracellular fluid. These systems act according to the isohydric principle which implies that when one of the buffer systems changes so will the other buffer system, thereby shifting H^+ back and forth between the systems to keep homeostatic balance with extracellular and intracellular fluid (Gluck, 1998; Guyton & Hall, 2006; New, 2003; Patience, 1990).

The second level of defense against acid-base disturbances occurs in the respiratory center. Such disturbances are regulated by the removal of CO_2, and through the quantitative dissociation of carbonic acid (H_2CO_3), to H^+, and HCO_3^-, from extracellular fluids. Increases in pulmonary ventilation releases CO_2 from the extracellular fluid, to the aveoli of the lungs, and then into the atmosphere, therefore decreasing H^+ concentrations in the extracellular fluid. Conversely, decreases in pulmonary ventilation, increases CO_2 in the extracellular fluid, and thereby increases total H+ concentrations. A decrease in pulmonary carbon dioxide (pCO_2) will cause a decrease in pH and HCO_3^-, causing a shift towards acidosis. An increase in pCO_2 will cause an increase in HCO_3^-, thereby causing pH to increase and acid-base balance to shift towards alkalosis (T. R. Arnett, 2010; Gluck, 1998; Guyton & Hall, 2006; New, 2003; Patience, 1990). However, even though the lungs maintain or cause changes in pH, the lungs do not function in the creation of new bicarbonate, which is one of the significant roles of the kidneys (Guyton & Hall, 2006; Remer, 2000).

The third and most powerful regulatory system of acid base homeostasis is the kidneys. The kidney is the primary mechanism for the excess removal of fixed acids form the body. By excreting acid or alkali in the urine, the kidney is able to readjust the extracellular fluid H^+ concentration toward normal pH levels. If more H^+ is present in the blood than HCO_3^- as the blood is filtered through the kidney, there will be a net removal of acid from the extracellular fluid into the urine. Conversely, if more HCO_3^- than H^+ is present in the blood as the blood is filtered through the kidney, there will be a net

removal of base from the extracellular fluid into the urine (Barzel, 1995; Gluck, 1998; Guyton & Hall, 2006; New, 2003; Patience, 1990).

Everyday metabolism produces as much as 80 milliequivalents of nonvolatile acid from the breakdown of amino acids in the human body. These acids are considered nonvolatile (also termed fixed acids) because they cannot be excreted by the lungs, as they are not carbonic acid, and therefore increases in H^+ within the blood must be expelled by the kidney. As much as it is the job of the kidney to dispel H^+, it is the job of the kidney to reabsorb HCO_3^- back into the blood stream in order to buffer H^+ in the extracellular fluid. Under normal conditions, for each HCO_3^- reabsorbed, there is also a renal tubular secretion of H^+ (Gluck, 1998; Guyton & Hall, 2006; New, 2003; Patience, 1990). However, when the body is in a state of acidosis, nonvolatile acid accumulates, or in other words HCO_3^- is lost at rate which causes the net retention of H^+. This net accumulation of the H^+ in extracellular fluid causes three adaptive physiological responses from the kidney to attempt to adjust the body back to normal homeostasis: the kidney works to secrete more H^+ into the urine and rid the body of accumulated H^+, the kidney reabsorbs existing HCO_3^- from the filtered blood to buffer H^+ in the extracellular fluid, and/or the kidney generates new HCO_3^-, in order to buffer the increased H^+ concentrations in the extracellular fluid (Gluck, 1998; Guyton & Hall, 2006; New, 2003; Patience, 1990; Vormann & Daniel, 2001). It is this final function, the ability of the kidney to function in the creation of new HCO_3^- that makes it a particularly unique organ in the body's ability to regulate acid-base homeostasis.

The kidney can function in the generation of new bicarbonate in the tubular epithelial cells from non-bicarbonate buffers in order to decrease H^+ concentrations. This allows for either H^+ to be released back into in the urine, or for the newly produced HCO_3^- to be released back into the extracellular fluid of the blood in order to buffer increases in H^+. The two most important of these non-bicarbonate buffers involved in the generation of new HCO_3^- are the phosphate and ammonia.

The body generates new HCO_3^- using the phosphate buffer system. In the state of acidosis, once all the excess HCO_3^- is resorbed to the blood, HCO_3^- is no longer available to combine with H^+ to be excreted in the urine, so in order to be emitted it must combine with other tubular lumen buffers such as phosphate (HPO_4^-). This forms to $H_2PO_4^-$ which will combine with sodium and be released into the urine as sodium salt ($NaH_2PO_4^-$). It is by this means that $NaH_2PO_4^-$ takes with it the excess H^+ and re-establishes acid-base balance (Gluck, 1998; Guyton & Hall, 2006; Susan A. New, et al., 2003; Patience, 1990).

Another buffer system, which is quantitatively more important than phosphate buffer system, is the ammonia (NH_3) and ammonium ion (NH_4^+) buffer system. Ammonia is synthesized from the deamination of glutamine. Glutamine comes from the metabolism of the amino acids in the liver and is transported to the epithelial cells of the ascending limb of the loop of Henle and the distal tubules of the kidney. Inside the epithelial cells each molecule of glutamine is metabolized to two $NH_4^+$and two HCO_3^-. For each molecule of glutamine metabolized in the proximal tubule two NH_4^+ are created and excreted into the urine and two HCO_3^- are reabsorbed into the blood. The new HCO_3^-

created in this process constitutes the generation of new bicarbonate (Gluck, 1998; Guyton & Hall, 2006; New, 2003; Patience, 1990).

It is by these general mechanisms that the buffer systems of body fluids, lungs, and the kidney operate in the regulation of the hydrogen ion. As the kidney serves the body as the primary remover of fixed acids, monitoring renal urinary excretion of organic acids can provide useful insight into the body's ability to maintain acid-base homeostasis (T. R. Arnett, 2010; Gluck, 1998; Guyton & Hall, 2006; New, 2003; Patience, 1990).

The Intestine, Liver, and Pancreas in Acid-Base Regulation

The intestines, liver, and pancreas organ systems also functions in the maintenance of acid-base homeostasis (Florin, et al., 1991; Remer, 2000, 2001b). The role of these organs in acid-base metabolism has been outlined in detail by Remer et al., and is briefly restated here (Remer, 2000, 2001b).

The intestine is the initial organ which has an impact on acid-base metabolism from dietary intake, and has three primary functions. First, the intestine determines the specific rate at which each individual nutrient is absorbed. This is determined by the bioavailability of the nutrients ingested. Nutrients can be absorbed at comparable rates or with one nutrient in excess of another (Remer, 2000, 2001). If the nutrient absorbed is in excess of acidic anions then the pancreas will secrete sodium bicarbonate into the intestines and thereby buffer the increase in acid load. Traditional acid-ash theorist taught that if dietary anions were consumed in equal amounts to dietary cations there would be no net effect on acid-base metabolism. However this is not likely, because foods consumed and ingested contribute to both acid and alkali, and since intestinal

absorption rates vary, the net result will always be in excess of either acid or base equivalents (Oh, 2000; Remer, 2000, 2001b; Remer & Manz, 1995).

Second, the role of the intestines is to contribute to blood bicarbonate by adjusting the amount of alkali which is created and reabsorbed. In this way the intestines does not directly contribute to H^+, but instead contributes to the regulations of arterial bicarbonate. This is in part, again due to the role of the pancreas, and to the metabolically active tissue of the intestinal tract, which functions to secrete large quantities of bicarbonate ions in order to neutralize acidity and increase the bicarbonate pool (Guyton & Hall, 2006; Remer, 2000, 2001b) .

The final function of the intestines is to regulate the amount of sulfur containing amino acids (AA-SH) and alkali salts, which are metabolized to organic acid (OA^-) and then transported to the liver. It is, then, the job of the liver to oxidize the absorbed AA-SH and OA^- and contribute these as hydrogen ions and bicarbonate ions to the arterial pool. After entering the blood, the sulfuric acid from hepatic AA-SH is buffered by arterial bicarbonate (Remer, 2000, 2001).

If the blood stream is lacking in bicarbonate, bone may then be utilized, and broken down to add the needed bicarbonate to buffer the increases in H^+ (L. A. Frassetto, Todd, Morris, & Sebastian, 2000; Guyton & Hall, 2006). During this process increased calcium stored in bone may also be excreted in the urine. Consequently, the ingested nutrients from dietary components may directly affect acid-base balance resulting in direct effects on bone metabolism and health (Lemann, Bushinsky, & Hamm, 2003). A schematic outlining these processes is demonstrated in figure 2.

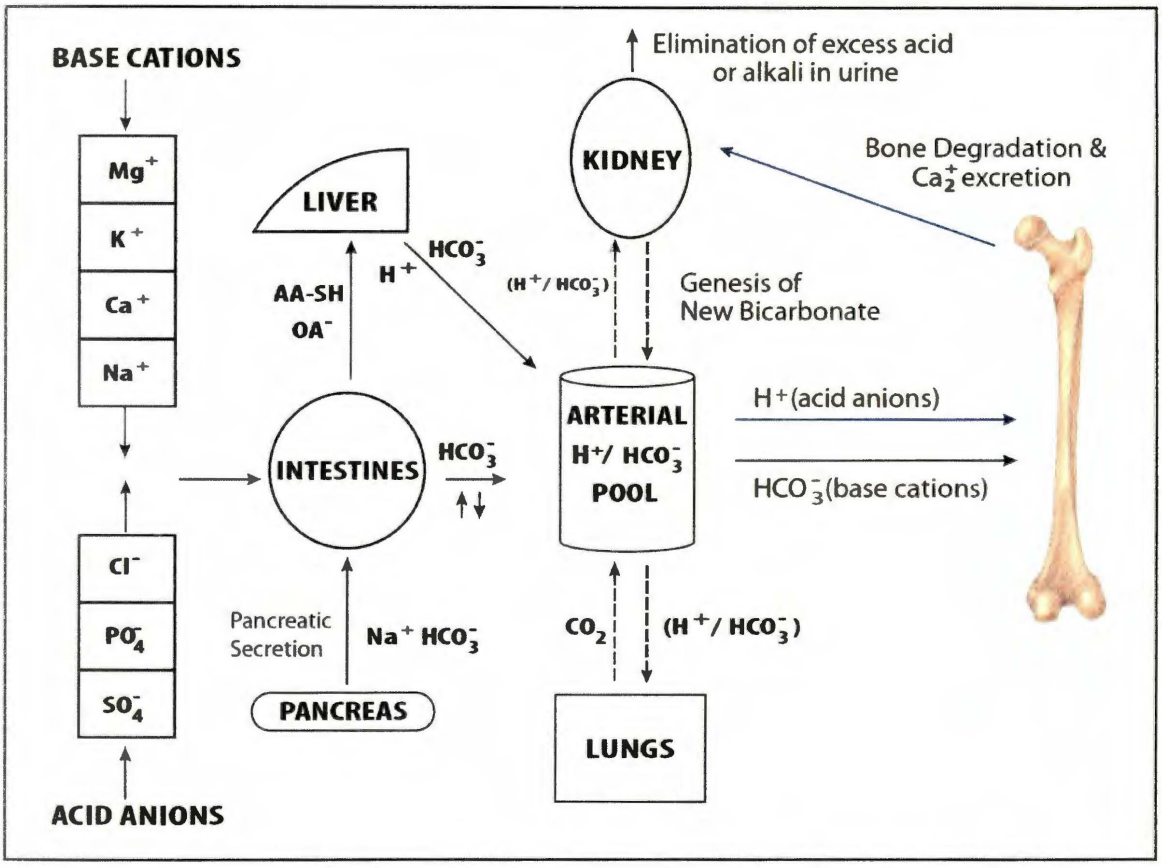

Fig. 2. The figure represents the interactions of diet and organs involved in acid-base regulation and the impact on bone (H+, hydrogen ions, HCO3- encompases bicarbonate ions and alkali load. AA-SH, sulfur containing amino acids, OA- alkali salts of organic acids). Bone serves as a giant ion exchange column and increases in acid cause increased bone degradation and clacium excretion (Buclin et al., 2001; Remer, 2000; Zwart & Smith, 2005). This diagram is a variation and compilation of figures obtained from references Remer (2000; 2001), and Zwart & Smith (2005).

The Role of Diet in Acid-Base Homeostasis

The effect of food on the acid load in the human body has been researched for several decades (Manz, 2001; Patience, 1990). Yet, it is only recently that attention has been given to the transformation of diet on an evolutionary time scale, from that of pre-agriculture homo-sapiens, consisting of a diet richer in basic components, to that of

contemporary man, consisting of a diet richer in acid-producing components (Cordain et al., 2005; L. Frassetto, et al., 2001; Manz, 2001; New, 2003; Remer, 2000; Sebastian, Frassetto, Sellmeyer, Merriam, & Morris, 2002).

From the period of our pre-agricultural ancestors living in the Upper Paleolithic period, to date, the dietary intake of humans has changed. The daily caloric intake of our hunter-gatherer ancestors was from a menu consisting of uncultivated plants, and wild animals (L. Frassetto, et al., 2001). This diet was rich in base production, and through millions of years of natural selection created the genetic metabolic mechanisms by which our bodies function today (Cordain, et al., 2005). In recent time, however, with a decline in the local farming industry, and an increase in industrialization, diets have altered and become suboptimal. Modern diets contain lower intakes of fiber, calcium, magnesium, and potassium, and overabundant intakes of grains, fat, simple sugars, sodium, and animal proteins (Cordain, et al., 2005; Rylander, Remer, Berkemeyer, & Vormann, 2006; Sebastian, et al., 2002). Present day food processing and preparation has only compounded the problem with increases in empty calorie foods further displacing the consumption of fruits and vegetables (Remer & Manz, 2003b). It has been suggested that with the elimination of only a few base rich foods in our daily caloric intakes, diets may rapidly become net acid forming and base deprived (Cordain, et al., 2005; L. Frassetto, et al., 2001; Manz, 2001; New, 2003; Remer, 2000; Rylander, et al., 2006).

Certain foods can increase acid load in the body because the products of their metabolism are acids, such as sulfuric acid and phosphoric acid. One way to estimate the acid load of a food is to calculate the potential renal acid load (PRAL), which is based on

the sulfur, phosphorus, calcium, magnesium, and potassium content of foods (the higher the PRAL, the higher the acid load that is generated in the body as a result of acidic food consumption) (Barzel, 1995; Prynne, et al., 2004; Sebastian, Sellmeyer, Stone, & Cummings, 2001). Some foods which are considered to be more acidogenic than others are those of animal origin, and foods such nuts, cereals, cheese or milk products (Barzel, 1995; New, 2003; Rylander, et al., 2006; Sabboh et al., 2005). Remer and colleagues have calculated PRAL values for a number of different foods. For example, milk and yogurt yield about 1 mEq per of acid 100 g serving, whereas, fish, poultry, cheese, and even some grain products potentially yield 7 mEq or more acid per 100 g serving (Remer, 2001b). Animal protein is not the sole source of acid load in the diet as other foods may also contribute to higher concentrations of sulfur-containing amino acids or phosphorus (T. Arnett, 2003; Cloutier, 2003; Sebastian, et al., 2001). It should be emphasized that all foods, in addition to animal protein, have the potential to contribute to either acid or alkaline by products or both, and sulfuric acid yields may increase from vegetable protein intake as well as animal protein intake (T. Arnett, 2003; Remer & Manz, 1995).

Alternatively to acid forming foods, fruits and vegetables tend to lower PRAL values because these foods tend to be rich in base precursors. Fruits and vegetables are higher in organic salts of potassium and bicarbonate precursors that are metabolized to base, and thus provide the needed alkalinity to neutralize dietary acid loads (L. Frassetto, et al., 2001; Macdonald, et al., 2005; Remer, 2000; Sebastian, et al., 2001). And, in regards to the diet of Western cultures, not only is protein intake below the evolutionary norm, but so too is the intake fruits and vegetables. So, it is not exclusively animal

protein intake that is the reason for elevated levels of acidity in the diet. It is a combination of increases in the intake of animal protein, and foods which are converted to acid precursors, along with the absence of base forming precursors needed to buffer higher levels of in acid in the human body (Lanham-New, 2006; Prynne et al., 2006; Sebastian, et al., 2001; Tucker et al., 1999). It can therefore be suggested that eating higher levels of fruits and vegetables should provide the necessary levels of bicarbonate needed to neutralize acid ash (L. Frassetto, et al., 2001; Prynne, et al., 2006).

In the struggle to obtain and improve bone health, the impact of acidity on the human skeleton must also be considered. The effects of prolonged consumption of acid-forming foods can be seen throughout the body and markedly on bone which acts as a reservoir for base to neutralize the acid load (L. Frassetto, et al., 2001; Remer, 2000; Sebastian, et al., 2001). A diet containing high levels of acidic precursors may give rise to a mild, subclinical metabolic acidosis, which may alter bone metabolism. Metabolic acidosis induces the release of calcium and carbonate from bone. Thus, long term periods of low-grade metabolic acidosis will lead lower bone mineral density (BMD) and bone dissolution resulting in higher skeletal fracture risk (L. Frassetto, et al., 2001; L. A. Frassetto, et al., 1998; New, 2003; Prynne, et al., 2004; Sabboh, et al., 2005; Swaminathan, 2001; Tucker, Hannan, & Kiel, 2001). It is believed by lowering acidic foods which contribute to higher potential renal acid load (PRAL) values, and by increasing intakes of fruits and vegetables, overall bone mineral density will improve in both men and women (Barzel, 1995; Lanham-New, 2006; Macdonald, et al., 2005; Prynne, et al., 2004; Swaminathan, 2001).

Therefore, it can be put forward, that with consistent intakes of diets which yield higher acid content, the human body will develop into a state of chronic low-grade metabolic acidosis, and over time as this state persists, the body may adapt by breaking down bone to buffer the increases in net acid load. By continuing to examine the skeletal system and the effects of foods rich in acid precursors, foods rich in base precursors, and the interactions of acid and base in unison, we can begin to develop the understanding of how each these components contributes to PRAL values and in turn how this impacts bone homeostasis and health.

Procedures for Estimating Acid Load in the Diet

Early 20th century researchers attempted to develop various calculation models to estimate individual nutrients from the diet involved in the function of acid or alkali loads, but were unsuccessful in determining appropriate computations (Remer, 2001b). In great part this was because the role of the intestines and liver as an acid or base forming organ being directly involved in acid-base generation for the human body has only recently been given attention in the scientific field (Manz, 2001; Remer, 2000). More recent analytical techniques have since been developed to quantify a dietary acid load directly from the urine or from the diet. These assessments include the measurement of net acid excretion directly ($NAE_{analyzed}$) from titratable acids and bases in the urine, or calculating urinary NAE indirectly ($NAE_{indirect}$) by using dietary intake of acid and base precursors in the diet. Another approximation for acid load based on dietary intake is to determine the ratio of animal protein to potassium intake (L. Frassetto, et al., 2001; Manz, 2001; Remer,

2000, 2001b; Remer, et al., 2003; Whiting & Draper, 1981). This study focuses on the latter two as methods to evaluate dietary acid-base status.

$NAE_{analyzed}$ is a direct laboratory measurement of urine, and is used to determine the body's renal contribution to acid-base equilibrium. The components which make up the $NAE_{analyzed}$ calculation can be measured individually, by various lab techniques, or in summation through a method of titration. Titratable acid is excreted in the form of phosphates, sulfates, and other proton acceptors. The titration analysis method of $NAE_{analyzed}$ enables the determination of titratable acid (TA), bicarbonate, and ammonium in 1 ml of urine. The calculation of net acid excretion is defined as: the sum of excretion rates of ammonium, plus titratable acid (TA), minus bicarbonate (Net hydrogen ions = $TA + NH_4^+ - HCO_3^-$) (Chan, 1972; Luthy, Moser, & Oetliker, 1977; Oh, 2000; Patience, 1990; Whiting & Muirhead, 2005). Values determining $NAE_{analyzed}$ can be replicated only when urine is frozen and thawed once; repeated freeze-thaw has been shown to skew results (Chan, 1972; Oh, 2000).

Another approach for estimating net acid load is to calculate $NAE_{indirect}$ from the diet. $NAE_{indirect}$ is a systematic computation that corrects for intestinal absorption of minerals and sulfur containing protein. It is involved in the formation of acid or alkali loads, and assumes a rate of urinary excretion of organic acids (OA_{anthro}). The calculation for $NAE_{indirect}$ includes adding the potential renal acid load (PRAL) to the sum of organic acids, and is determined by the formula ($NAE_{indirect} = PRAL + OA_{anthro}$) (Macdonald, et al., 2005; Remer, 2000, 2001b; Remer & Manz, 2003a; Sebastian, et al., 2001).

As an established method for estimating acid loads, PRAL is also diet-based, and is an estimate of noncarbonic anions in excess of the level of mineral cations eliminated in the urine. PRAL is determined by the formula [(Cl +P + SO_4 + organic acid) – (Na + K+ Ca + Mg)] (Remer, 2000, 2001b; Remer, et al., 2003). In order to fully understand the dietary components which contribute to the acid load in the PRAL equation the term organic acid must also be understood. Organic acid anions are comprised of a spectrum of single analytes including citric acid, oxaclic acid, malic acid, succinic acid, and lactic acid, as well as glutamic and aspartic acid. Organic acid anions can be measured directly from 24-hour urine samples (24h-OA_{urine}) by titration method, or can be estimated, either from the diet (OA_{diet}) or by an anthropometrics estimate. In this study instead of using dietary unmeasured anions components twice, organic acids were measured anthropometrically as according to according to Remer, et al., (2003). Organic acids estimated anthropometrically have been shown to encompass basal energy requirements, are highly correlated to body surface area, and is relatively constant for each individual (Remer, 2000, 2001b; Remer, et al., 2003).

Dietary sulfate and phosphate are the main contributing components to acid load and the estimate of PRAL as nonbicarbonate anions. In this study, dietary sulfur (from sulfur containing amino acids methionine and cysteine) was used to more accurately reflect sulfate, instead of estimating sulfur load from total protein. The dietary components which contribute as mineral cations to the base load of the estimate of PRAL are potassium, magnesium, and calcium (L. Frassetto, et al., 2001; New, 2003; Prynne, et al., 2004; Remer, 2001b; Remer & Manz, 2003b). As conducted in previous research

studies, the mutually canceling terms of sodium (Na^+) and (Cl^-) were omitted from the calculation in this study (L. A. Frassetto, et al., 1998; Prynne, et al., 2004). This is accepted in certain clinical circumstances, where clear minimal day-to-day variations in nutrient intake can be determined (Remer, 2000). It can therefore be understood that with the exception of OA's, the components which make up the equation of urinary NAE (nonbicrbonate anions – cations), are primarily influenced by nutritional intake alone (Remer, et al., 2003).

The animal protein to potassium calculation proposed by Frasetto et al., estimates the net rate of endogenous noncarbonic acid production. The protein to potassium ratio corrects for intestinal absorption and is calculated as NAE = -10.2 + 54.5 x (protein/potassium) (L. A. Frassetto, et al., 1998; Remer, 2004). It is a convenient and less involved algorithm comprising of two diet constituents, dietary protein (estimated using sulfate excretion generated from methionine and cysteine) and dietary potassium contents of the diet. The animal protein to potassium ratio is essentially an estimate of the sulfur containing proteins, which increase acid load in the diet divided by dietary potassium, which is an approximation of vegetables and fruits which are rich in bicarbonate and metabolized to base (L. Frassetto, et al., 2001; L. A. Frassetto, et al., 1998). It can, therefore, be reasoned that the animal protein to potassium ratio assesses the acid-base balance challenge from a bicarbonate standpoint or from vegetable-to-acid generating potential (L. Frassetto, et al., 2001; L. A. Frassetto, et al., 1998).

It has been discussed, by researchers such as Brazel and Massey, that there is a need to develop a more convenient method for estimating urinary net acid excretion

(Remer, 2000). Because the protein to potassium ratio involves fewer components than PRAL it may be an important algorithm for the general population as an indicator for predicting acid load and the possible need for dietary counseling or supplementation (L. Frassetto, et al., 2001; Prynne, et al., 2004; Zwart, et al., 2004).

The Effects of Dietary Acid on Skeletal Health

Bone serves as the primary internal collecting pool for base in the human body, and persistent minute increases in dietary acid load have been shown to deplete bone mineral content and mass (L. Frassetto, et al., 2001; Massey, 2003; New, 2003; Patience, 1990; Prynne, et al., 2004; Sellmeyer, et al., 2001; Zwart, et al., 2004). One hypothesis to explain this is that the chronic daily low-level dietary acidosis causes increases in extracellular fluid [H+]. This in turn may increases osteoclast activity, and suppresses osteoblast activity, thereby causing the degradation of bone matrix as the body releases carbonate from the skeleton to buffer the increases in [H+]. Over prolonged time, this state of degradation to the bone matrix, may lead to the pathogenesis of clinical bone fractures and osteoporosis (L. Frassetto, et al., 2001; Sellmeyer, et al., 2001; Whiting & Draper, 1981). Therefore, it is possible, that by decreasing acid components in an individual's habitual diet, the state of dietary induced acidosis will be altered, thereby increasing osteoblast activity and resulting in bone matrix rebuilt (Demigne, et al., 2004; L. Frassetto, et al., 2001; Lanham-New, 2006; Prynne, et al., 2006; Remer, 2004).

The primary contributors to acid load in the diet include protein (in the form of methionine and cysteine), phosphorus, chloride, and organic acids of which the majority cannot be metabolized (Remer, 2000, 2004; Remer, et al., 2003). As protein and

phosphorus have biphasic roles, with both low and high intakes having adverse effects on bone, research in this field has been conflicting. Therefore, to fully understand the effects of acid on the skeleton, both dietary acidic components should be considered. Furthermore, these nutrients should be studied in unison, as it is in unanimity that the nutrient to nutrient interactions occur and create the acidic environment that gradually erodes bone (L. A. Frassetto, et al., 1998; Remer, 2000; Sebastian, 2003, 2005; Sebastian, et al., 2002).

The effect of dietary protein in acid-base metabolism on bone warrants further investigation (Ginty, 2003). Adequate protein is essential to bone health given that protein contributes to 22% of bone weight (Zwart & Smith, 2005). Research has demonstrated that there are adverse consequences to low protein intake, and that diets which fall below the Recommended Dietary Allowance (RDA) of 0.8 mg/kg body weight for protein result in decreased calcium absorption and increased bone resorption by mechanisms which are not fully understood (Kerstetter, O'Brien, & Insogna, 2003; New, 2003; Whiting & Draper, 1981; Zwart & Smith, 2005). Sufficient protein intake is necessary for the development of collagen, for enzymes needed for bone synthesis, and to stimulate the production endogenous growth factors that are necessary for bone formation (New, 2003). Low protein intake has been shown to decrease calcium absorption and induce secondary hyperparathyroidism which may lead to increases in bone loss. In certain populations such as the elderly, protein insufficiency has been shown to have greater effects on bone mineral density, with the loss of muscle coordination and strength, leading to increased risk of falls and fractures (Madias, et al., 1979; New, 2003).

It is, therefore, not suggested that dietary protein fall below the recommended current RDA, as protein is necessary to maintain adequate levels for bone homeostasis during growth, as well as in the elderly, in order to preserve healthy skeletal homeostasis (Hannan et al., 2000; Kerstetter, et al., 2003; New, 2003; Rizzoli & Bonjour, 2004).

In contrast, high protein diets consumed in the presence of a low food derived alkali loads, have been noted to adversely affect bone (Barzel & Massey, 1998; New & Millward, 2003; Patience, 1990; Remer, 2001b). Currently an upper limit (UL) for protein has not been decided upon. It has been suggested that as much as a 10 g increase above the current RDA for dietary protein increases urinary calcium by as much as 16 mg per day and lowers urine pH (Magee, Curno, Edmond, & Cummings, 2004; New, 2003; Zwart & Smith, 2005). This is due to a differentiation between the type of protein consumed, as foods containing higher levels of sulfur-containing amino acids (methionine and cysteine), which is oxidized to sulfuric acid, contribute to higher PRAL in the diet (Hamadeh & Hoffer, 2001; Zwart, et al., 2005).

Sulfate can be found in both animal (meat, fish, poultry, milk products, and eggs) and vegetable proteins (nuts, oatmeal, white rice, barley, whole wheat, and cabbage). Urine sulfate has been shown to be a reliable biomarker for total protein intake which includes animal and vegetable protein sources (Florin, Graeme, Goretski, & Cummings, 1993; Hamadeh & Hoffer, 2001; Houterman et al., 1997; Massey, 2003; Sabry, Shadarevian, Cowan, & Campbell, 1965; Zwart, et al., 2004). Substantial evidence, however, has also shown that the kidneys respond specifically to the acidifying properties of animal protein in the presence of low alkali, by increasing greater amounts of net

endogenous acid production and urinary calcium excretion. This results in greater skeletal loss (in part because vegetable protein yield higher amounts of bicarbonate as well as those of sulfuric acid) (Barzel & Massey, 1998; Cole & Evrovski, 2000; Massey, 2003; Sellmeyer, et al., 2001).

While protein intakes which fall below the RDA result in lower bone mineral density in the elderly population, detrimental effects of high protein intake on bone is also seen in this age group. This is due to decreases in glomerular filtration rate as humans age and the kidney's ability to excrete dietary acid loads is impaired, causing blood acidity to increase and plasma bicarbonate to decrease (L. Frassetto, et al., 2001; L. A. Frassetto, et al., 2000). This was noted in a study conducted by Frasetto et al., (2000) which found a cross cultural relationship between hip fracture rates and dietary protein was positively related to animal protein intake and inversely related to vegetable protein intake. It can, therefore, be determined that protein should be consumed in moderation and that high levels of dietary protein may contribute to increases in net dietary acid production. This may in turn, affect total bicarbonate levels within the body, and add to adverse effects on the overall skeletal homeostasis.

Dietary phosphorus, as an acid precursor, also merits attention. Dietary phosphorus is essential for cell functions as well as for bone augmentation and mineralization and influences the net effect of protein on bone (Brown Bowman, Russell, & International Life Sciences Institute-Nutrition Foundation., 2006; Heaney, 2004; Massey, 2003; Zwart & Smith, 2005). In the diet phosphorus is ubiquitous in its distribution, making natural deficiencies of phosphorus rare. While the kidney and small

intestine regulates the input and output of phosphorus from the plasma pool, the bone serves as the primary reservoir for phosphorus. Deficient or excessive intake of dietary phosphorus will have a direct impact on the human skeleton (Brown Bowman, et al., 2006; Calvo, 1993; Calvo & Park, 1996; Whiting, Boyle, Thompson, Mirwald, & Faulkner, 2002). Low serum levels of phosphorus may result, hypophosphatemia, hypophosphatemic rickets, and osteomalacia (Heaney, 2004). Conversely, phosphorus loading may result in hyperphosphatemia, uremic bone disease and soft tissue calcification, and in the presence of low serum ionized calcium levels will lead secondary hyperparathyroidism leading to increased bone resorption (Brown Bowman, et al., 2006; Calvo, 1993; Calvo & Park, 1996).

In the diet, sources of phosphorus include dairy, meats, fish, poultry, lentils, peas, and grain products. Phosphorous in plant foods and grains is found as phytate, which is poorly absorbed, compared to that which is found in animal foods as phosphate, bound to the amino side chains of muscle protein, and is well absorbed (Massey, 2003). Intake of dietary phosphorus for young and middle age adults in the United States is frequently in excess of the current RDA of 700 mg/d often exceeding this amount by as much as two times daily, and the overall phosphorus content of the United States food supply continues to grow as more highly processed snack and convenience foods are consumed (Calvo, 1993; Calvo & Park, 1996).

Dietary calcium and phosphorus are strongly associated and the need for achieving a balance between these two nutrients is generally recommend at a ratio of 1:1 on a mg:mg basis (Brown Bowman, et al., 2006; Calvo, 1993; Huttunen et al., 2007).

This relationship causes concern in the US as phosphorus intakes are high while at the same time calcium intakes are low (Brown Bowman, et al., 2006; Heaney & Nordin, 2002; Whiting, et al., 2002). Elevated phosphorus intake in the presence of low calcium intake has been shown to cause secondary hyperparathyroidism and bone loss in animal studies (Calvo & Park, 1996; Huttunen, et al., 2007). As phosphorus and protein are often bound to one another it is possible that the interactions of these two nutrients in the presence of low alkali may explain the impact of acidic anions on skeletal health (Massey, 2003). Further investigation of the interactions of phosphorus and protein in unison should be studied before promoting high animal protein diets as being beneficial to bone health.

As an acidic anion, chloride may also contribute to increasing total net acid excretion. As with phosphorus, chloride is abundant in food sources, and deficiencies are rare. Although processed foods contribute high amounts of chloride in Western diets, currently an upper limit for chloride has yet to be established. The role of chloride in diseases states seems to be greatly dependent on the combination of elements in which it is consumed and the rate at which chloride is absorbed intestinally (L. Frassetto, et al., 2001). This also follows through with chloride in the acid-base challenge as the contribution of chloride as an acidic anion is dependent on the rate of intestinal absorption (L. Frassetto, et al., 2001; Remer, 2000). For example, if chloride is given orally as calcium chloride ($CaCl_2$) it will contribute as an acidifying agent to the total acid pool (Remer, 2000). However, if chloride is consumed in equal amounts to that of sodium, it will contribute as neither an acid nor base (L. Frassetto, et al., 2001; New,

2003; Remer, 2000). It can therefore be recommended that the effects of chloride and sodium in the acid-base challenge should be determined according to the variation and composition of foods administered, and to the particular study in which chloride is considered.

Taken together, it stands to reason that nutrients interact together as a whole, not just individually, and that if a diet is deficient in one nutrient it will become deficient in other nutrients as well (Remer, 2004). Or equally, if the diet is in excess of one nutrient it will become in excess of other key nutrients, and that these effects can be seen throughout the body and in the case of acid generating nutrients markedly on bone (L. Frassetto, et al., 2001; Remer, 2004; Sebastian, et al., 2002). The study of acid or base forming nutrients goes beyond the study of individual nutrients. It is the total net effect of acid generating nutrients [(Cl + P + SO_4) + (OA)] minus alkali generating nutrients and takes into account the individual intestinal absorption rates (Remer, 2000, 2001b; Remer, et al., 2003; Remer & Manz, 2003b). Therefore, diets which contain excess acid loads may result in increased urinary calcium loss and reduction in total bone substance (Remer, 2000, 2001b). As bone loss is a concern for astronauts, the study of acid generating nutrients may not only have important implications for space flight, but may also have broader connotations for understanding bone loss under normal conditions on earth.

The Effects of Dietary Base on Skeletal Health

Diets abundant in fruits and vegetables have been shown to have multiple protective benefits on a variety of human diseases including those which effect bone

metabolism such as osteopenia and osteoporosis (Janet Bell & Whiting, 2004; Lanham-New, 2006; Massey, 2003; New, 2003; Prynne, et al., 2006; Tucker, et al., 1999; Vormann & Daniel, 2001). Fruits and vegetables are rich in bicarbonate [HCO_3^-] and vitamins, minerals, trace elements, phytoestrogens, and fiber, all of which contribute to the multiple health-promoting advantages of these foods (McGartland et al., 2004; New, 2003; New et al., 2000; Vormann & Daniel, 2001). Alkali rich diets have been implicated to be beneficial in the acid-base challenge as well (Janet Bell & Whiting, 2004; Lanham-New, 2006; Massey, 2003; McGartland, et al., 2004; New, 2003; Prynne, et al., 2006; Tucker, et al., 1999; Vormann & Daniel, 2001; Zwart & Smith, 2005). Fruits and vegetables are believed to have positive effects on the skeleton because these foods accept hydrogen ions, and therefore counteract increases in acid production by preserving the body's pH (Janet Bell & Whiting, 2004; McGartland, et al., 2004; Morris & Sebastian, 2002; Tucker, et al., 1999; Vormann & Daniel, 2001; Zwart & Smith, 2005). The dietary cations which contribute to base excesses of PRAL are calcium, magnesium, potassium, and sodium. These elements act in concert to benefit skeletal health by reducing osteoclast activity and increasing osteoblast activity (Janet Bell & Whiting, 2004; Lanham-New, 2006; New, et al., 2000; Prynne, et al., 2004; Tucker, et al., 1999; Vormann & Daniel, 2001).

Although some vegetable foods have been noted for contributing to increases in the sulfuric acid production, these same foods also contribute to the base pool as well. Even if the additions of base cations from these foods are small, when eaten in unison with other foods which are also rich in alkali the cumulative overall dietary pattern

appears to be beneficial to BMD (Marsh, et al., 1988; Massey, 2003; Whiting, et al., 2002). It is possible that the advantageous role of dietary alkali to bone health may not only have important implications for the general population, but also for astronauts during spaceflight, whose bone is compromised, and results in increased resorption, decreased calcium absorption, and increased calcium excretion (Zwart & Smith, 2005). Increasing fruits and vegetables in the diet may add to the needed bicarbonate load thereby acting beneficially on the skeletal mass and counterbalancing the acid load, resulting in decreased net acid excretion.

The role of calcium as an essential nutrient to skeletal health is unquestionable. As a basic cation, calcium serves to increase alkali load (Barzel, 1995; Buclin, et al., 2001; Dawson-Hughes & Harris, 2002; Heaney & Nordin, 2002; Kaneko et al., 1990; Miller, 2000; New, 2003; Zwart & Smith, 2005). Ninety-nine percent of total body calcium can be found in bone, and imbalances in this nutrient may lead to increased risk of osteopenia and osteoporosis (New, 2003). Osteoporosis represents an important health concern for many individuals and the aging population (Buclin, et al., 2001; Dawson-Hughes & Harris, 2002; Miller, 2000; New, 2003). Negative balance in calcium exchanges results in the release of essential minerals from bone which in turn may alter the structural integrity of the skeleton (Buclin, et al., 2001; Dawson-Hughes & Harris, 2002; New, 2003). It is proposed that in the presence of low calcium intake even the slightest degree of increase in metabolic acidosis may represents a strong stimulus for increased osteoclastic activity and decreased osteoblastic activity (Barzel, 1995; Dawson-Hughes & Harris, 2002; Hannan, et al., 2000; Heaney & Nordin, 2002; New, 2003).

Conversely, it has been suggested that high levels of calcium consumption may increase the beneficial properties of protein and phosphorus for overall bone health (J. Bell & Whiting, 2002; Dawson-Hughes & Harris, 2002; Heaney, 2002; Zwart & Smith, 2005). Although calcium has been a main area research both for the general public and for spaceflight, it is noteworthy that diets adequate in calcium are often adequate in many essential nutrients for bone health (Barzel, 1995; Buclin, et al., 2001; Dawson-Hughes & Harris, 2002; Heaney & Nordin, 2002; Marsh, et al., 1988; New, 2003; Zwart & Smith, 2005). Therefore, determining the dietary patterns and intakes of calcium is important for making food-based recommendations which result in increased skeletal health and mass.

Potassium also adds to alkali load, and it has been suggested that increases in dietary potassium may improve bone biology and mass (Barzel & Massey, 1998; Jones, Riley, & Whiting, 2001; Lemann, et al., 1993; Macdonald, et al., 2005; Massey, 2003; New, 2003; Tucker, et al., 1999; Tucker, et al., 2001; Whiting, et al., 2002). The organic salts of potassium can be found in a variety of whole, unrefined foods, including fruits and vegetables (Macdonald, et al., 2005; Tucker, et al., 1999). Green leafy vegetables contribute some of the highest amounts of this fat soluble vitamin and have been cited as having multiple benefits to the human body including a protective role against bone fracture rates (Booth, O'Brien-Morse, Dallal, Davidson, & Gundberg, 1999; Demigne, et al., 2004; New, 2003; Remer & Berkemeyer, 2005). The role of potassium in bone health may be attributed to its role as a basic cation, and the ability of this nutrient to counterbalance increases in dietary acidic loads, thereby increasing osteoblast activity

and decreasing osteoclast activity (New, 2003; Whiting & Muirhead, 2005; Zwart & Smith, 2005). Potassium is also positively associated with increased calcium retention, increased serum osteocalcin concentration, and decreased urinary calcium excretion rates (Remer & Berkemeyer, 2005; Tucker, et al., 1999). Yet, despite the well noted beneficial effects of potassium, recent studies have shown a substantial decrease in fruit and vegetable consumption, and therefore potassium intake. Currently, potassium intake has fallen below the RDA by as much as 50 mmol/d, which may result in chronic hypokalemia and have detrimental effects to bone health in the general population (Booth, et al., 1999; Demigne, et al., 2004).

Magnesium, similar to the contributing base cation of potassium, can be found in a variety of whole, unrefined foods, and fruits and vegetables (New, 2003; Tucker, et al., 1999). Magnesium has many benefits to the human body including cardiovascular, cancer, and bone health. The chief functions of this cation include the regulation of enzyme activity, control of various calcium and potassium channels, and the promotion of membrane stabilization (New, 2003; Rylander, et al., 2006). As with calcium and potassium, magnesium consumption is also well below the RDA in Western culture, indicating a decreased consumption of dietary fruits and vegetables (New, 2003).

Depletion of magnesium has been associated with a number of clinical conditions including hypocalcaemia, hypokalemia, impaired parathyroid (PTH) secretion, inhibition of calcium oxalate, hypertension, cardiac arrhythmias, and myocardial infarction, renal stones, and osteoporosis (New, 2003; Rylander, et al., 2006; Tucker, et al., 1999; Tucker, et al., 2001). There are also many important beneficial associations between intakes of

magnesium and BMD, as nearly half the human bodies magnesium quantities exist in the skeleton (New, 2003; Tucker, et al., 1999; Tucker, et al., 2001). It has been suggested that metabolic acidosis is associated with increased urinary Mg wasting, which may possibly be due the effect of H+ on distal Mg transport (New, 2003; Rylander, et al., 2006). Dietary deficiency in magnesium has been shown to have effects on decreased osteoblast activity and increased osteoclast activity, resulting in reduced skeletal growth (New, 2003; Rylander, et al., 2006; Tucker, et al., 2001). Magnesium has been shown to have important implications for spaceflight as well, as after 4 to 6 months of spaceflight urinary magnesium is decreased by 45% (Zwart, et al., 2009). The associations with magnesium and BMD suggest that long-term diets high in magnesium containing foods may be protective against skeletal losses (Tucker, et al., 1999).

Sodium, as an individual nutrient, also contributes to the acid-base challenge as a basic cation. However, the effects of dietary sodium on the human skeleton is invariably linked to the accompanying anion of chloride, as the content of sodium in foods often approximates and parallels that of chloride (Al-Bander, Nix, Katz, Korn, & Sebastian, 1988; L. A. Frassetto, et al., 1998). The combination sodium and chloride (NaCl) has been noted for inducing hypertension, hypercalciuria, and having detrimental effects on bone (Batlle et al., 1993; Li et al., 2009; New, 2003; Zwart & Smith, 2005). Elevated dietary sodium intake increases urinary calcium excretion, with as little as 100 mmol/d of NaCl increasing urinary calcium by 1 mmol/d (Martini et al., 2000; Massey & Whiting, 1996; New, 2003). Even a moderate reduction of dietary sodium could attenuate

hypercalciuria, and thereby prevent both kidney stones and osteoporosis (L. Frassetto, et al., 2001; Massey & Whiting, 1995).

As with the individual dietary anion of chloride, Na+ as an individual nutrient in the diet is affected by intestinal absorption rates. For example, this was demonstrated by Berkelhamet et al. (1988) who replaced sodium chloride with an equimolar amount of sodium acetate in patients receiving total parentral nutrition (TPN), who also had marked hypercalciuria (Barzel & Massey, 1998). Results from this study indicated that patients who received TPN with sodium acetate had decreased urinary calcium and calcium balance became established (Berkelhammer, Wood, & Sitrin, 1988). Such an example may be due to the differences in the intestinal absorption rates of sodium and chloride versus sodium and acetate (Barzel & Massey, 1998; Remer, 2001b). Therefore, in light of the effects of dietary sodium as an individual cation, and the deteriorating effects of dietary NaCl in unison on the health of bone, the effects of this nutrient in regards to the acid-base challenge should be considered in context of the study presented (Harrington et al., 2004).

Taken together, increased consumption fruits and vegetables which are abundant in calcium, potassium, and magnesium, may be advantageous to skeletal health and metabolism (Lanham-New, 2006; New, 2003; Prynne, et al., 2006; Tucker, et al., 2001). This may be due to the ability of increased dietary bicarbonate to combat the resorptive effects of increased acid on the skeleton. This also brings to light a new outlook on the aged question of the effects of acid-ash on skeletal health. Perhaps, the answer to this question does not lie so much in the detrimental effects of acid on the skeleton, but in the

beneficial effects of increased bicarbonate on the skeleton. Since diets are rarely to never equal in acidic anions and basic cations, because of varying intestinal rates, it may be possible that in order to obtain optimal of bone health, alkali containing foods should be consumed in excess of acidic containing foods (Remer, 2000). Therefore, further research may want to focus attention to the amounts of fruits and vegetables needed to obtain optimal bone health by providing the needed bicarbonate to buffer the degenerative effects of increased acid load on bone. This is not to suggest or diminish the importance of protein and phosphorus in bone metabolism, but to suggest that foods should be consumed as part of a whole diet, and that foods which are rich in fruits and vegetables add to the array of nutrients which are needed for increased skeletal metabolism and health (Janet Bell & Whiting, 2004; Lanham-New, 2006; New, 2003; Prynne, et al., 2006; Tucker, et al., 1999; Tucker, et al., 2001).

Nutrition a Countermeasure to Bone Loss in Spaceflight and Bed Rest

Microgravity results in altered bone metabolism and architecture, and is a concern for the health and safety of astronauts during exploration-class spaceflight missions (Smith et al., 2005; Vico et al., 2000). Weightlessness leads to decreased bone formation and increased bone resorption (Caillot-Augusseau et al., 1998; Vermeer, et al., 1998). Deterioration of bone during spaceflight is evident from histomorphometric analysis post spaceflight, ranging from decreased cortical and cancellous bone formation, reductions in bone strength, to osteopenia (Zerwekh, et al., 1998). As balanced nutrition and gravity related weight bearing forces are two essential and modifiable factors which increase bone metabolism on earth, these areas call for further research as countermeasures to

weightlessness-induced bone loss (Fig 3) (Caillot-Augusseau, et al., 1998; Inoue, et al., 2000; Shackelford et al., 2004; Sievanen, 2010; Smith, Zwart, et al., 2005; Vermeer, et al., 1998; Zerwekh, et al., 1998). One such model in which to study nutrition and weight bearing forces is that of extended bed rest.

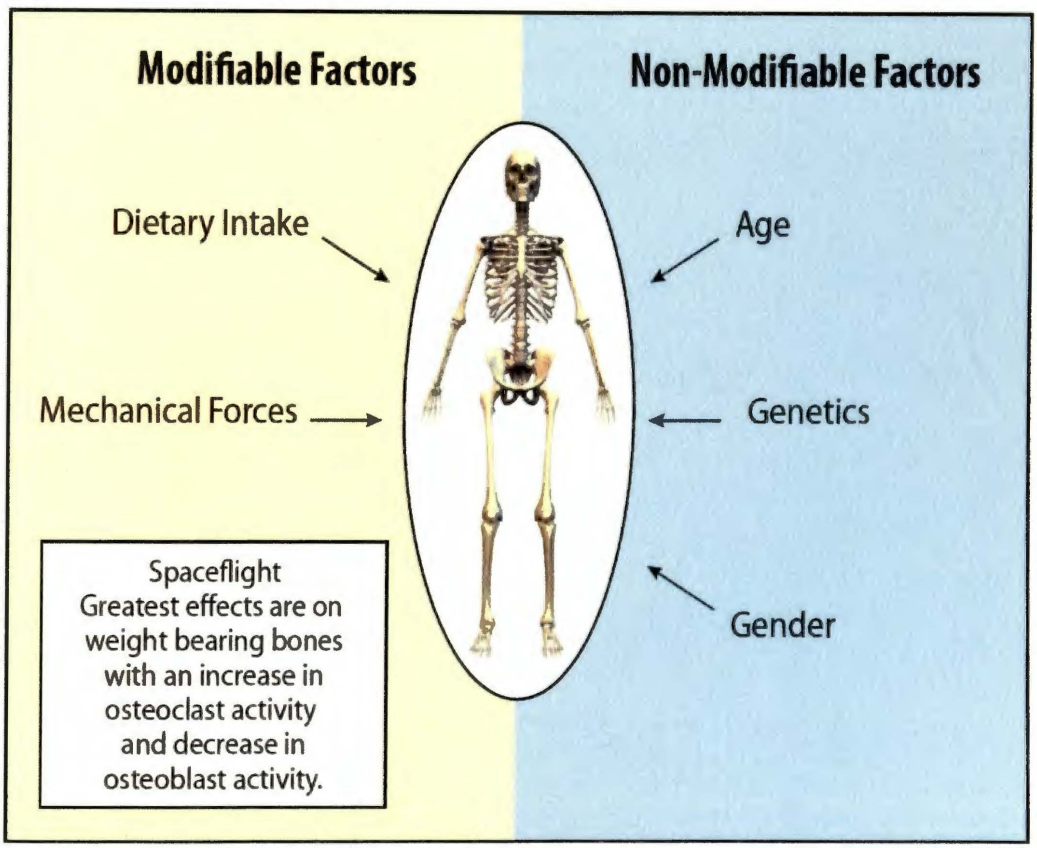

Fig. 3. A depiction of the human skeleton and critical modifiable and non-modifiable factors affecting bone turnover during spaceflight. Modifiable factors, such as dietary intake and mechanical forces (weight bearing activity and gravity on earth) are depicted in yellow. Non-modifiable factors, such as genetics, age, and gender which affect total accumulation of bone mass during spaceflight and on earth are depicted in light blue.

Bed rest is as a ground based analogue for spaceflight, and serves as a unique model where bone breakdown is occurring at increased rates to bone formation in otherwise healthy subjects (Baecker, et al., 2003; Caillot-Augusseau, et al., 1998; Heer, et

otherwise healthy subjects (Baecker, et al., 2003; Caillot-Augusseau, et al., 1998; Heer, et al., 2005; Inoue, et al., 2000; LeBlanc, et al., 1995; Lueken, et al., 1993; Scheld, et al., 2001; Smith, et al., 2004; van der Wiel, et al., 1991; Watanabe, et al., 2004; Whedon, 1984; Zerwekh, et al., 1998; Zwart, et al., 2005; Zwart, et al., 2004; Zwart & Smith, 2005). Most studies which are conducted to determine the effects of nutrients on bone are in ambulatory subjects. These models do not fully represent the effects of a weightless-induced environment, because in healthy participants bone formation should be occurring at an equal or increased rate to bone degradation. In studies using ambulatory subjects, it could take years to see the impacts of specific nutrients on bone metabolism as normal bone turnover would often overshadow these interactions. Therefore, detailed nutrient intakes provided during bed rest allows for determination of any and all dietary factors that either exacerbate or are protective against bone loss.

Understanding the physiological and metabolic functions of nutrition is essential in the development of countermeasures designed to diminish the negative effects of spaceflight and bed rest on bone loss (Whitson, Pietrzyk, & Pak, 1997). Spaceflight results in altered metabolism of some nutrients, and decrements are seen in vitamin D, the serum concentration of parathyroid hormone, calcium, magnesium, vitamin K, vitamin E, and phosphorus. Bed rest, similar to that of spaceflight, results in a diminished nutrient status and provides a distinctive representation for studying the dietary acid-base challenge as a consequence of increased bone resorption and decreased absorption of calcium (Baecker, et al., 2003; Donaldson, et al., 1970; LeBlanc, et al., 1995; van der Wiel, et al., 1991; Zerwekh, et al., 1998).

It has been suggested that responses to combating skeletal unloading observed during spaceflight could possibly be rectified with vitamin and mineral supplementation, the administration of pharmacologic agents, or resistive exercise. Yet, these trials have been unable to consistently correct for the loss of bone observed during spaceflight or bed rest alone (Smith, et al., 2003; Smith et al., 1999; Swaminathan, 2001; Zwart, et al., 2005). It thus remains the role of nutritional intake from diet to provide additional insight into the mechanisms which underlie bone loss during space flight. Further knowledge of these mechanisms may also lead to a better understanding of bone loss under normal conditions on earth as well. In light of the inconsistent results regarding the relationship between acid-base metabolism and bone, further research is warranted using bed rest as a model.

Implications for the General Population

Research gained from the study of the effects acid-base metabolism on the bone health of bed rest subjects may not only have important implications for astronauts during spaceflight, but may have important connotations for the general population as well. Results from this study may contain suggestions for certain states of paralysis such as trauma induced immobilization, and for the general aging population whose bone is at risk for increased fracture, osteopenia, and osteoporosis (Barzel & Massey, 1998; L. Frassetto, et al., 2001; Heer, et al., 2005; Ralston & Kleerekoper, 2002; Sievanen, 2010; Whedon, 1984).

Long term paralysis, or immobilization, results in increased bone resorption and decreased bone formation. If bone mass is not regained during immobilization the

outcome is deteriorated bone volume and altered bone metabolism (Heer, et al., 2005; Sievanen, 2010). Increased osteoclast activity occurs at the start of immobilization similar to that of bed rest. It is, therefore, possible that studying bone metabolism during bed rest may have beneficial connotations both for long-term exposure to microgravity and for patients whose bone is in a compromised state from paralysis (Heer, et al., 2005; Sievanen, 2010).

Bed rest may also have implications for the aging population. As the human body ages, normal cumulative organ adaptations occur (Barzel, 1995; L. Frassetto, et al., 2001). These changes which occur to the body over time can cause degenerative agitations to the skeleton, skeletal muscle, kidney and endocrine systems. Increased age-induced acidosis may also occur, resulting in losses of urinary calcium and the mobilization of bone to the bodies' own detriment (L. Frassetto, et al., 2001; L. A. Frassetto, et al., 1998; New, 2003). The progressive consequence of these losses in bone mineral content may be the decline of the bodies' skeletal support system with an outcome of increases in bone fractures, osteopenia, and osteoporosis (T. Arnett, 2003; Barzel & Massey, 1998; L. Frassetto, et al., 2001; L. A. Frassetto, et al., 1998).

Osteoporosis has become a worldwide epidemic and will affect one in every three women and one in every eight men (Orwoll, 2003; Prentice, 2004; Ralston & Kleerekoper, 2002). In a race to tilt the scale away from osteoclast activity in favor of slowing the process of bone breakdown and increasing osteoblast activity, every factor which may contribute to bone resorption becomes of critical importance. While genetics plays the greatest role in an individual's bone formation, development, and breakdown;

diet remains one modifiable factor which is in an individual's power to control, and significantly contributes to increases in bone health and maintenance as the human body ages (L. Frassetto, et al., 2001; L. A. Frassetto, et al., 1998; Prentice, 2004; Ralston & Kleerekoper, 2002).

CHAPTER III

METHODOLOGY

Background

This study is part of a larger study designed to test the efficacy of various countermeasures on physiological issues related to bed rest. This study included three separate -6° head down tilt bed rest studies which took place at the General Clinical Research Center (GCRC) at the University of Texas Medical Branch (UTMB) and was supported by the National Aeronautics and Space Administration (NASA) Flight Analog Project. The original study design included bed rest durations of 60 and 90 days.

Participants and Protocols

The bed rest study design used for this report included a 10 day ambulatory period followed by 60 days of bed rest. A total of eleven participants (8 male, 3 female; age 26-44 y) took part in the studies. All participants were recruited by the Human Test Subject Facility at the NASA Johnson Space Center. Participants were required to pass a NASA modified Air Force Class III Physical and to undergo psychological screening in order to participate in the studies. Inclusion and exclusion criteria were based on physical and psychological standards similar to those required of astronauts. Protocols for all studies were reviewed by the Institutional Review Boards at the NASA Johnson Space Center and at the University of Texas Medical Branch. Written informed consent was provided by all participants before taking part in the study.

Bed Rest Design

Participants were admitted to the GCRC 10 days prior to the start of bed rest for acclimation, diet stabilization, and baseline medical testing. During this phase, participants were ambulatory and all physical activity was recorded. Participants were observed by the nursing staff twenty four hours a day. Beginning on day one of bed rest through day 60, participants remained in bed at a -6˚head-down tilt position. All daily activities were conducted in bed. Television viewing, computer use, and reading and writing were permitted. Bathing, personal hygiene and urine collection were also conducted in bed.

Urine Collection and Determination of NTX and PYD

Urine collections for this study were performed twice before the period of bed rest and once per month during the bed rest period. Urine voids were refrigerated immediately after collection and stored at -80˚C until analysis. PYD and NTX were measured using commercially available ELISA kits (PYD kit from Quidel Corp, Santa Clara, CA, and the NTX kit from Ostex International Inc, Seattle). Both NTX and PYD kits were provided as antigen coated microwell plates. Urine specimens were allowed to undergo a maximum of three freeze/thaw cycles before analysis. Assay procedures were performed in accordance with instruction guides supplied along with the kits which included: the thawing of specimens, dilution of controls and samples, addition of an enzyme conjugate, washing with buffer reagents, incubation, and the addition of a stop reagent. Optical density, was determined on a standard curve according to standards included in the kits, and were used to calculate serum concentration of NTX and PYD.

Urine pH (both second void and 24 hour pool) was determined using a standard pH meter (Thermo Orion pH meter, Beverly, MA).

Dietary Intake

Daily energy requirements were calculated using the Harris-Benedict equation with an activity factor of 1.6 pre-bed rest and 1.3 during bed rest. Daily energy intake consisted of 55% carbohydrate, 15 % protein, and 30% fat. Meal plans were individualized to meet subject preferences, thereby allowing for variations in dietary protein and the ratio of animal to vegetable protein. All foods were measured on a Mettler balance before and after consumption. Water was provided to subjects ad libitum, and consumption of caffeine and alcohol was prohibited. The menus rotated on a seven or ten day cycle menu. Nutrient calculations were obtained by the analysis of diet data using the Nutrition Data System for Research version 4.06.34, developed by the Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN, Food and Nutrient Database.

Determination of Protein Intake, PRAL, and Net Acid Excretion

Both intakes of animal and vegetable protein were determined for this study. The dietary intakes of two sulfur containing amino acids (methionine and cysteine) were calculated in mEq SO_4 (=mmol SO_4 x 2). PRAL and net endogenous acid production were calculated as previously described (Zwart et al., 2004). NAE and PRAL were calculated using the following equation:

$$\mathbf{NAE} = \text{PRAL} + \text{OA}$$

$$\mathbf{PRAL} = 2 \times \left[\left(0.00503 \times \frac{\text{mg methioine}}{\text{day}}\right) + \left(0.0062 \times \frac{\text{mg cysteine}}{\text{day}}\right)\right] + \left(0.037 \times \frac{\text{mg phosphorus}}{\text{day}}\right) + \text{OA} - \left(0.021 \times \frac{\text{mg potassium}}{\text{day}}\right) - \left(0.026 \times \frac{\text{mg magnesium}}{\text{day}}\right) - \left(0.013 \times \frac{\text{mg calcium}}{\text{day}}\right)$$

$$\mathbf{OA} = \frac{\text{Body Suface Area } \left[\frac{\text{cm height} \times \text{kg weight}}{3600}\right] \times 41}{1.73}$$

Statistical Analysis

Results are presented as means ± standard deviations. Trends in dietary intake from the bed rest studies were analyzed using a one-way repeated measures analysis of variance with a post hoc Bonferroni. Linear associations between dietary parameters and urinary collagen crosslinks for each time point where urine was collected during bed rest is described with Pearson's correlation coefficients. All statistical analyses were performed using SigmaStat (version 2.0; Chicago, IL). An alpha of $p<0.05$ was used to define statistical significance.

CHAPTER IV

DIET AND INDICES OF BONE BREAKDOWN AND ACID PRODUCTION IN BED REST SUBJECTS

A PAPER TO BE SUBMITTED TO THE
JOURNAL OF BONE AND MINERAL RESEARCH:
THE OFFICIAL JOURNAL OF THE AMERICAN SOCIETY
FOR BONE AND MINERAL RESEARCH

SARAH M. WATTS[1], SCOTT M. SMITH[2], and SHANIL JUMA[3]

ABSTRACT

Diet and acid-base balance can affect bone loss during simulated weightlessness. The present study evaluates the effects of acid and base components of diet on bone resorption markers before, during, and after 60-90 days of bed rest at -6° head-down tilt position. A total of eleven subjects (8M, 3F; age 26-44 y) participated in the present study. Urine samples from the subjects were analyzed for a relationship between dietary patterns and markers of bone metabolism. This study examines two procedures for estimating acid production in the body by comparing urinary net acid excretion (NAE_{ind}) and by measuring the animal protein to potassium ratio, as estimated from dietary intake. It is hypothesized that both estimations would have significant correlations between markers of bone breakdown NTX (N-telopeptide) and PYD (pyridinoline). Results confirmed that bone resorption increased during bed rest as indicated by the collagen crosslinks NTX and PYD. In addition, significant correlations were recorded between NAE_{ind} and NTX ($p<0.01$), and PYD ($p<0.01$) during bed rest. However, the ratio of

animal protein to potassium intake was not significantly correlated with NTX or PYD, suggesting further research on this method of approximating dietary acid load is necessary.

Keywords: bone resorption; collagen crosslinks; dietary acid-base; urinary net acid excretion (NAE); animal to potassium ratio.

INTRODUCTION

Skeletal deterioration caused by weightlessness is a significant concern for spaceflight, and countermeasures to improve the bone health of astronauts must be developed as the duration of spaceflight is expected to increase (Smith, et al., 2003; Vermeer, et al., 1998; Zwart, et al., 2009). Bed rest serves as an acceptable ground-based analogue for studying several aspects of weightlessness, with bone loss being one example (Donaldson, et al., 1970; LeBlanc, et al., 1995; Lueken, et al., 1993; Scheld, et al., 2001; Smith, et al., 2003; Smith & Lane, 1999; Smith, et al., 1998; Swaminathan, 2001; van der Wiel, et al., 1991; Watanabe, et al., 2004; Zerwekh, et al., 1998; Zwart, et al., 2009; Zwart & Smith, 2005). Although, bed rest is not exact model for weightlessness, bone breakdown occurring during bed rest parallels well with bone loss experienced by astronauts during space exploration missions (Marsh, et al., 1988; Smith, et al., 2003). In addition, the data collected during spaceflight missions and bed rest studies may also have important implications for the bone health of the general population on Earth. As osteoporosis has become a global health concern on earth leading to greater risk of fragility, fracture, disability, and premature mortality, the study of all factors which may contribute to increases in bone loss become of critical

importance (Angus, et al., 1988; Jeffcoat, 2006; New, 2003; Orwoll, 2003; Prentice, 2004; Prynne, et al., 2004; Rosen, et al., 1999). Because research has shown that diet plays an important role in maintaining skeletal health on earth, the study of nutrient interactions in the form of dietary acid-base may have important connotations for the overall health of bone for both the general public and for astronauts during spaceflight (Macdonald, et al., 2005; Smith & Lane, 1999; Smith, Zwart, et al., 2005; Zwart, et al., 2005; Zwart, et al., 2004). This study will discuss the role of diet in acid-base homeostasis, and will review the effects the dietary acidic and basic components, such as protein and phosphorus on skeletal health. In addition this study will compare two dietary methods of urinary net acid excretion, including potential renal acid load (PRAL) and the animal protein to potassium ratio.

Diet is a modifiable and controllable factor for maintaining bone health and mass on earth. While calcium and vitamin D have been in the forefront of bone research, many other nutrient interactions associated with bone mass also merit recognition. Research has shown that the individual nutrients of magnesium, potassium, phosphorus, and protein are also essential nutrients for bone development and maintenance. However, these nutrients are often studied independently, rather than jointly. It is, therefore, reasonable to theorize that when investigating the role of nutrition on skeletal health, the impact of these nutrients does not occur in isolation, but rather that dietary interactions occur synergistically, resulting in previously unidentified positive or negative effects on bone health (L. Frassetto, et al., 2001; McCarthy & Frassica, 1998; Swaminathan, 2001; Viguet-Carrin, et al., 2006; Zwart & Smith, 2005).

The study of acid-base homeostasis examines the effects of nutrients interacting in unison. The two primary nutrients often studied in the context of acid-base balance are protein and potassium, with protein contributing to increases in the overall dietary acid load and potassium salts neutralizing the acid load (Cloutier, 2003; Demigne, et al., 2004; L. Frassetto, et al., 2001; L. A. Frassetto, et al., 1998; Lemann, et al., 1993; Macdonald, et al., 2005; New, 2003; New & Millward, 2003; Patience, 1990; Prynne, et al., 2004; Sebastian, 2005; Swaminathan, 2001; Vermeer, et al., 1998). The other nutrients which interact to a lesser degree in the acid-base homeostasis include; magnesium, sodium, calcium, phosphorus and chloride. It is the combination of these nutrients, acting in unison, which may interact to increase or decrease acid and base load and therefore have an effect on total bone mass.

As bone serves as the primary internal collecting pool for base in the human body, persistent minute increases in dietary acid load have been shown to deplete bone mineral content and mass (L. Frassetto, et al., 2001; Massey, 2003; New, 2003; Patience, 1990; Prynne, et al., 2004; Sellmeyer, et al., 2001; Zwart, et al., 2004). One hypothesis to explain this is that the chronic daily low-level dietary acidosis causes increases in extracellular fluid [H+]. This in turn may increases osteoclast activity, and suppresses osteoblast activity, thereby causing the degradation of bone matrix as the body releases carbonate from the skeleton to buffer the increases in the hydrogen ion (Fig 1). Over prolonged time, this state of degradation to the bone matrix, may lead to the pathogenesis of clinical bone fractures and osteoporosis (L. Frassetto, et al., 2001; Sellmeyer, et al., 2001; Whiting & Draper, 1981). Therefore, it is possible, that by decreasing the primary

acidic components and increasing the basic components in an individual's habitual diet, the state of dietary induced acidosis will be altered resulting in bone matrix rebuilt (Demigne, et al., 2004; L. Frassetto, et al., 2001; Lanham-New, 2006; Prynne, et al., 2006; Remer, 2004).

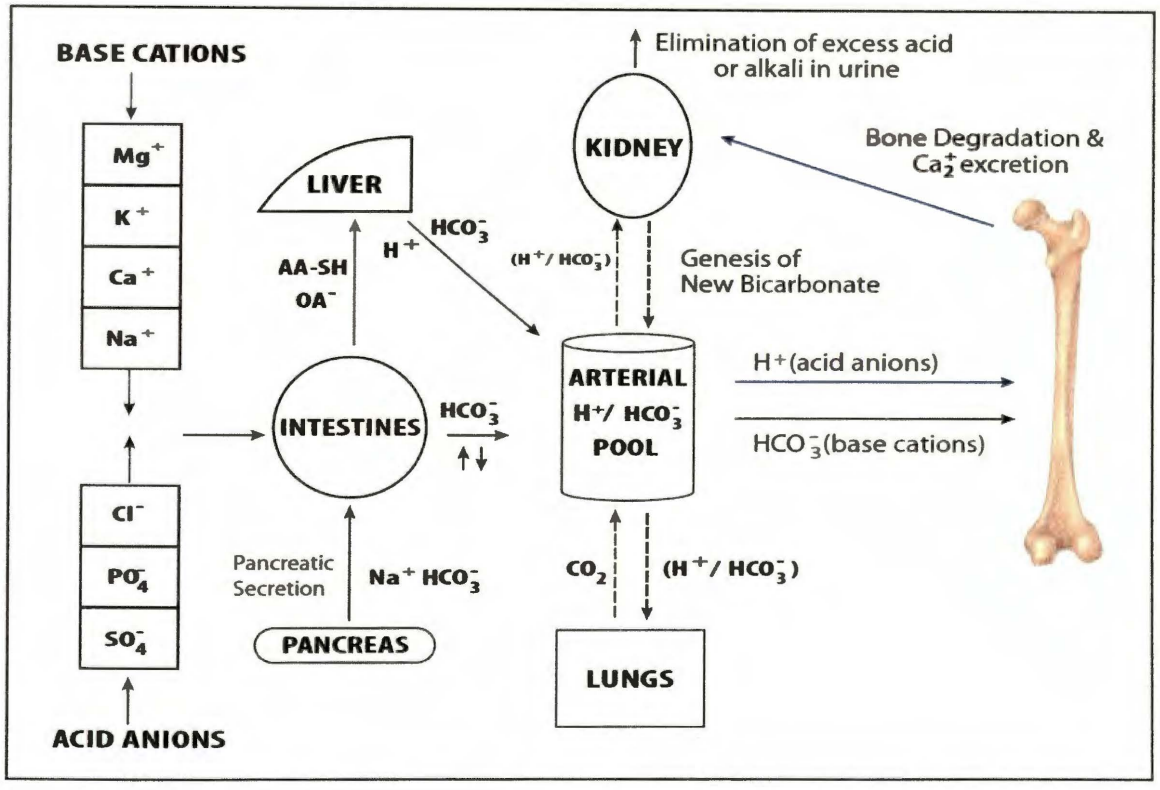

Fig. 1. The figure represents the interactions of diet and organs involved in acid-base regulation and the impact on bone (H+, hydrogen ions, HCO3- encompases bicarbonate ions and alkali load. AA-SH, sulfur containing amino acids, OA- alkali salts of organic acids). Bone serves as a giant ion exchange column and increases in acid cause increased bone degradation and clacium excretion (Buclin, et al., 2001; Remer, 2000; Zwart & Smith, 2005). This diagram is a variation and compilation of figures obtained from references Remer (2000; 2001), and Zwart & Smith (2005).

As protein (in the form of methionine and cysteine) has a biphasic role, with both low and high intakes having adverse effects on bone, research in this field has been conflicting (L. A. Frassetto, et al., 1998; Remer, 2000; Sebastian, 2003, 2005; Sebastian,

et al., 2002). High protein diets consumed in the presence of a low food derived alkali loads, have been noted to adversely affect bone (Barzel & Massey, 1998; New & Millward, 2003; Patience, 1990; Remer, 2001b). Currently an upper limit (UL) for protein has not been decided upon. It has been suggested that as much as a 10 g increase above the current RDA for dietary protein increases urinary calcium by as much as 16 mg per day and lowers urine pH (Magee, et al., 2004; New, 2003; Zwart & Smith, 2005). This is due to a differentiation between the type of protein consumed, as foods containing higher levels of sulfur-containing amino acids (methionine and cysteine), which is oxidized to sulfuric acid, contribute to their higher PRAL (Hamadeh & Hoffer, 2001; Zwart, et al., 2005).

Sulfate can be found in both animal (meat, fish, poultry, milk products, and eggs) and vegetable proteins (nuts, oatmeal, white rice, barley, whole wheat, and cabbage). Urine sulfate has been shown to be a reliable biomarker for total protein intake which includes animal and vegetable protein sources (Florin, et al., 1993; Hamadeh & Hoffer, 2001; Houterman, et al., 1997; Massey, 2003; Sabry, et al., 1965; Zwart, et al., 2004). However, substantial evidence has also shown that the kidneys respond specifically to the acidifying properties of animal protein in the presence of low alkali, by increasing greater amounts of net endogenous acid production and urinary calcium excretion resulting in greater skeletal loss (in part because vegetable protein yields higher amounts of bicarbonate as well as those of sulfuric acid) (Barzel & Massey, 1998; Cole & Evrovski, 2000; Massey, 2003; Sellmeyer, et al., 2001).

In contrast to a diet rich in acidic components, the primary dietary contributor to the base load in the diet is potassium. Diets abundant in fruits and vegetables, are also high in potassium. Potassium rich diets have been shown to have multiple protective benefits on a variety of human diseases including those which effect bone metabolism such as osteopenia and osteoporosis (Janet Bell & Whiting, 2004; Lanham-New, 2006; Massey, 2003; New, 2003; Prynne, et al., 2006; Tucker, et al., 1999; Vormann & Daniel, 2001). Fruits and vegetables are copious in bicarbonate [HCO_3^-] and vitamins, minerals, trace elements, phytoestrogens, and fiber, all of which contribute to the multiple health-promoting advantages of these foods (McGartland, et al., 2004; New, 2003; New, et al., 2000; Vormann & Daniel, 2001). Alkali rich diets have been implicated to be beneficial in the acid-base challenge as well (Janet Bell & Whiting, 2004; Lanham-New, 2006; Massey, 2003; McGartland, et al., 2004; New, 2003; Prynne, et al., 2006; Tucker, et al., 1999; Vormann & Daniel, 2001; Zwart & Smith, 2005). Fruits and vegetables are believed to have positive effects on the skeleton because these foods accept hydrogen ions, and therefore counteract increases in acid production by preserving the body's pH (Janet Bell & Whiting, 2004; McGartland, et al., 2004; Morris & Sebastian, 2002; Tucker, et al., 1999; Vormann & Daniel, 2001; Zwart & Smith, 2005). Diets which contribute to base excesses act in concert to benefit skeletal health by reducing osteoclast activity and increasing osteoblast activity (Janet Bell & Whiting, 2004; Lanham-New, 2006; New, et al., 2000; Prynne, et al., 2004; Tucker, et al., 1999; Vormann & Daniel, 2001).

Although some vegetable foods have been noted for contributing to increases in the sulfuric acid production, these same foods also contribute to the base pool as well. Even if the additions of base cations from these foods are small, when eaten in concordance with other foods which are also rich in alkali the cumulative overall dietary pattern appears to be beneficial to BMD (Marsh, et al., 1988; Massey, 2003; Whiting, et al., 2002). It is possible that the advantageous role of dietary alkali to bone health may not only have important implications for the general population, but also for astronauts during spaceflight, whose bone is compromised and results in increased resorption, decreased calcium absorption, and increased calcium excretion (Zwart & Smith, 2005). Increasing fruits and vegetables in the diet may add to the needed bicarbonate load thereby acting beneficially on the skeletal mass and counterbalancing the acid load, resulting in decreased net acid excretion.

As renal excretion is the primary remover of fixed acids from the body, monitoring urinary excretion provides useful insight into acid-base balance. Urinary net acid excretion (NAE) is a systematic computation that corrects for intestinal absorption of minerals and sulfur-containing protein. It is involved in the formation of acid or alkali loads, and assumes a rate of urinary excretion of organic acids (OA_{anthro}). The calculation for NAE_{ind} includes adding the potential renal acid load (PRAL) to the sum of organic acids and is determined by the formula ($NAE_{indirect} = PRAL + OA_{anthro}$) (Macdonald, et al., 2005; Remer, 2000, 2001b; Remer & Manz, 2003a; Sebastian, et al., 2001). As an established method for estimating acid loads, PRAL is diet-based, and is an estimate of noncarbonic anions in excess of the level of mineral cations eliminated in the urine.

PRAL is determined by the formula [(Cl +P + SO_4 + organic acid) – (Na + K+ Ca + Mg)] (Remer, 2000, 2001b; Remer, et al., 2003).

Dietary sulfate and phosphate are the main contributing components to acid load and the estimate of PRAL as nonbicarbonate anions. In this study, dietary sulfur (from sulfur containing amino acids methionine and cysteine) was used to more accurately reflect sulfate, instead of estimating sulfur load from total protein. The dietary components which contribute as mineral cations to the base load of the estimate of PRAL are potassium, magnesium, and calcium (L. Frassetto, et al., 2001; New, 2003; Prynne, et al., 2004; Remer, 2001b; Remer & Manz, 2003b). As conducted in previous research studies, the mutually canceling terms of sodium (Na^+) and (Cl^-) were omitted from the calculation in this study (L. A. Frassetto, et al., 1998; Prynne, et al., 2004). This is accepted in certain clinical circumstances, where clear minimal day-to-day variations in nutrient intake can be determined (Remer, 2000). It can therefore be understood that with the exception of OA's, the components which make up the equation of urinary NAE (nonbicrbonate anions – cations), are primarily influenced by nutritional intake alone (Remer, et al., 2003).

The animal protein to potassium calculation proposed by Frasetto et al., estimates the net rate of endogenous noncarbonic acid production. The protein to potassium ratio corrects for intestinal absorption and is calculated as NAE = -10.2 + 54.5 x (protein/potassium) (L. A. Frassetto, et al., 1998; Remer, 2004). It is a convenient and less involved algorithm comprising of two diet constituents, dietary protein (estimated using sulfate excretion generated from methionine and cysteine) and dietary potassium

contents of the diet. The animal protein to potassium ratio is essentially an estimate of the sulfur containing proteins, which increase acid load in the diet divided by dietary potassium, which is an approximation of vegetables and fruits which are rich in bicarbonate and metabolized to base (L. Frassetto, et al., 2001; L. A. Frassetto, et al., 1998). It can, therefore, be reasoned that the animal protein to potassium ratio assesses the acid-base balance challenge from a bicarbonate standpoint, or from vegetable-to-acid generating potential (L. Frassetto, et al., 2001; L. A. Frassetto, et al., 1998).

The purpose of the present study is to investigate the effects of acid and base components from dietary intake by comparing the two procedures: urinary net acid excretion (NAE_{ind}); and the animal to protein to potassium ratio, as compared to markers of bone resorption in bed rest subjects whose bone is compromised from disuse. The primary hypothesis of this study is that there will be a correlation in urinary net acid excretion (NAE_{ind}) or poteintial renal acid load (PRAL) in the diet on either markers of bone breakdown (NTX and PYD) during bed rest. A secondary hypothesis of this study is that there will be a correlation between the animal protein to potassium ratio, and markers of bone breakdown (NTX or PYD) during bed rest. Results from this study may also serve as an analogue for spaceflight.

METHODS

Background

This study is part of a larger study designed to test the efficacy of various countermeasures on physiological issues related to bed rest. This study included three separate -6˚head down tilt bed rest studies which took place at the General Clinical

Research Center (GCRC) at the University of Texas Medical Branch (UTMB) and was supported by the National Aeronautics and Space Administration (NASA) Flight Analog Project. The original study design included bed rest durations of 60 and 90 days.

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writing were permitted. Bathing, personal hygiene and urine collection were conducted in bed.

Urine Collection and Determination of NTX and PYD

Urine collections for this study were performed twice before the period of bed rest and once per month during the bed rest period. Urine voids were refrigerated immediately after collection and stored at -80°C until analysis. PYD and NTX were measured using commercially available ELISA kits (PYD kit from Quidel Corp, Santa Clara, CA, and the NTX kit from Ostex International Inc, Seattle). Both NTX and PYD kits were provided as antigen coated microwell plates. Urine specimens were allowed to undergo a maximum of three freeze/thaw cycles before analysis. Assay procedures were performed in accordance with instruction guides supplied along with the kits which included: the thawing of specimens, dilution of controls and samples, addition of an enzyme conjugate, washing with buffer reagents, incubation, and the addition of a stop reagent. Optical density, was determined on a standard curve according to standards included in the kits, and were used to calculate serum concentration of NTX and PYD. Urine pH (both second void and 24 hour pool) was determined using a standard pH meter (Thermo Orion pH meter, Beverly, MA).

Dietary Intake

Daily energy requirements were calculated using the Harris-Benedict equation with an activity factor of 1.6 pre-bed rest and 1.3 during bed rest. Daily energy intake consisted of 55% carbohydrate, 15 % protein, and 30% fat. Meal plans were individualized to meet subject preferences, thereby allowing for variations in dietary protein and the ratio of

animal to vegetable protein. All foods were measured on a Mettler balance before and after consumption. Water was provided to subjects ad libitum, and consumption of caffeine and alcohol was prohibited. The menus rotated on a seven or ten day cycle menu. Nutrient calculations were obtained by the analysis of diet data using the Nutrition Data System for Research version 4.06.34, developed by the Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN, Food and Nutrient Database.

Determination of Protein Intake, PRAL, and Net Acid Excretion

Both intakes of animal and vegetable protein were determined for this study. The dietary intakes of two sulfur containing amino acids (methionine and cysteine) were calculated in mEq SO_4 (=mmol SO_4 x 2). PRAL and net endogenous acid production were calculated as previously described (Zwart et al., 2004). NAE and PRAL were calculated using the following equation:

$$\mathbf{NAE} = \mathrm{PRAL} + \mathrm{OA}$$

$$\mathbf{PRAL} = 2 \times \left[\left(0.00503 \times \frac{\text{mg methioine}}{\text{day}}\right) + \left(0.0062 \times \frac{\text{mg cysteine}}{\text{day}}\right)\right] + \left(0.037 \times \frac{\text{mg phosphorus}}{\text{day}}\right) + \mathrm{OA} - \left(0.021 \times \frac{\text{mg potassium}}{\text{day}}\right) - \left(0.026 \times \frac{\text{mg magnesium}}{\text{day}}\right) - \left(0.013 \times \frac{\text{mg calcium}}{\text{day}}\right)$$

$$\mathbf{OA} = \frac{\text{Body Suface Area} \left[\frac{\text{cm height} \times \text{kg weight}}{3600}\right] \times 41}{1.73}$$

Statistical Analysis

Results are presented as means ± standard deviations. Trends in dietary intake from the bed rest studies were analyzed using a one-way repeated measures analysis of variance with a post hoc Bonferroni. Linear associations between dietary parameters and urinary collagen crosslinks for each time point where urine was collected during bed rest is described with Pearson's correlation coefficients. All statistical analyses were performed using SigmaStat (version 2.0; Chicago, IL). An alpha of $p<0.05$ was used to define statistical significance.

RESULTS

Dietary Intake

Mean (±SD) dietary intakes for bed rest study subjects are reported before and during bed rest (Table 1). Dietary intakes for the bed rest study for all subjects (n=11) are recorded are analyzed using one-way repeated measures ANOVA with post hoc to determine differences between pre bed rest, bed rest 28 and bed rest 60. The dietary intakes are recorded as 5-d averages; therefore, bed rest 28 data included an average of bed rest days 24-28, and bed rest day 60 data included an average of bed rest days 56-60. Energy intake was significantly lower on bed rest day 28 than in pre bed rest, and energy intake when expressed as kcal per kg body weight was significantly lower on bed rest 28 and 60 than in pre bed rest. Carbohydrate intake was significantly lower on bed rest day 28 than in pre bed rest. There were no other significant differences in nutrient intake over time, however all nutrient intake decreased during the bed rest study (Table 1).

Table 1
Dietary Intakes for 5-d Sessions Before and During Bed Rest as Expressed as ±SD

		Pre	Bed Rest 28	Bed Rest 60
Energy	(kcal)	2651±428	2172±322*	2292±328
	(kcal)/(kg bw)	36±3	30±3*	32±4*
Carbohydrate	(g)	378±60	298±50*	324±50
Lipid	(g)	89±15	75±12	78±10
Protein	(g)			
Total		100±16	87±12	85±14
Animal		49±16	45±16	43±15
Vegetable		35±6	29±6	29±8
Sulfur	(mEq)	53±8	47±7	45±7
Phosphorus	(mEq)	62±10	52±8	51±11
	(mg)	1689±265	1410±203	1401±299
Potassium	(mEq)	72±14	60±9	59±11
	(mg)	3505±633	2910±424	2890±562
Calcium	(mEq)	17±3	14±2	14±3
	(mg)	1410±262	1125±163	1152±229
Magnesium	(mEq)	9±2	8±2	8±3
	(mg)	393±67	321±57	318±91
Sodium	(mEq)	148±27	128±17	132±15
	(mg)	3605±651	3142±392	3180±401

• n=11
• (*) indicates statistical significance ($p<0.05$)

Urinary Biomarkers

Mean (±SD) urinary excretion of markers of bone resorption, urinary calcium, sulfate, and urine volume are reported for all subjects (n=11) before and during bed rest (Table 2). Total urine volume, NTX, PYD, urinary calcium and urinary sulfate were not statistically significant over time during bed rest. However bone resorption markers (NTX and PYD) did increase over time during bed rest. A trend was observed for an increase in NTX during bed rest, when expressed per 24 h (nmol/d), however was not statistically significant ($p<0.051$).

Table 2
Urinary Excretion Before and During Bed Rest as Expressed as ±SD

		Pre	Bed Rest	Bed Rest 60
Urine Volume	(ml/d)	2862±709	2909±753	3165±475
Urinary Creatinine	(mM/d)	14±3	14±5	15±5
NTX	(Nm/d)	457±223	794±408	780±401
NTX/Creatinine	(Nm NTX/mM creatinine/d)	38±13	51±17	57±17
PYD	(Nm/d)	310±132	456±189	469±202
PYD/Creatinine	(Nm/d)	24±8	32±11	33±16
Urinary Calcium	(Nm/d)	5±2	6±2	6±2
Urinary Sulfate	(Nm/d)	20±3	20±6	21±6

- n= 11
- (*) indicates statistical significance ($p<0.05$)

Net Acid Excretion

Net acid excretion was estimated by using calculations developed by Remer (Remer, 2000, 2001a), and was significantly correlated with NTX on bed rest day 28 ($p<0.05$), and bed rest day 60 ($p<0.01$) (Fig. 2). Net acid excretion was also correlated with PYD on bed rest day 28 ($p<0.01$) (Fig. 3).

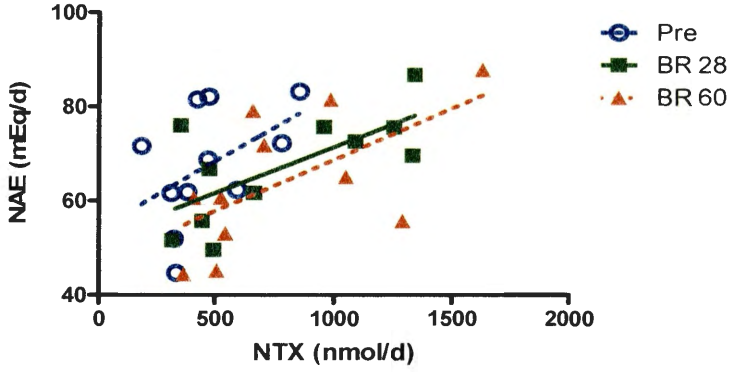

Fig. 2. Correlation between NAE and NTX. NAE composed of dietary PRAL is positively correlated with NTX on bed rest day 28 ($p<0.05$) and bed rest day 60 ($p<0.01$).

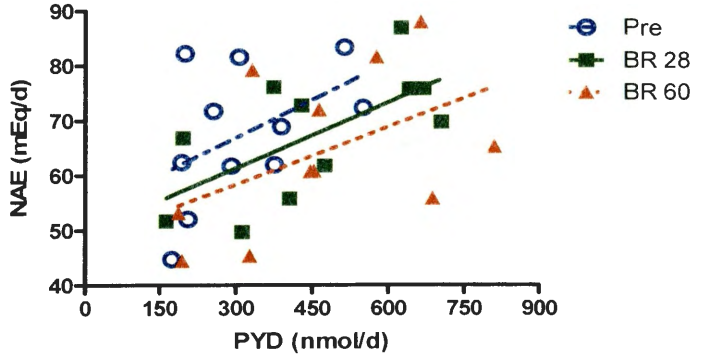

Fig. 3. Correlation between NAE and PYD. NAE composed of dietary PRAL is positively correlated with PYD on bed rest day 28 ($p<0.01$).

The Ratio of Animal Protein to Potassium

The ratio of animal protein to potassium intake was estimated by using calculations developed by Frasetto and colleagues (L. A. Frassetto, et al., 1998) and was not significantly correlated with NTX (Fig. 4) or PYD (Fig. 5) before or during bed rest.

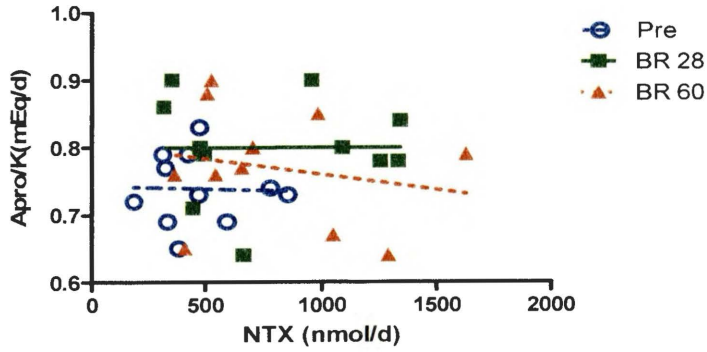

Fig. 4. A plot of the ratio of dietary animal protein to potassium and NTX. There were no significant relationships.

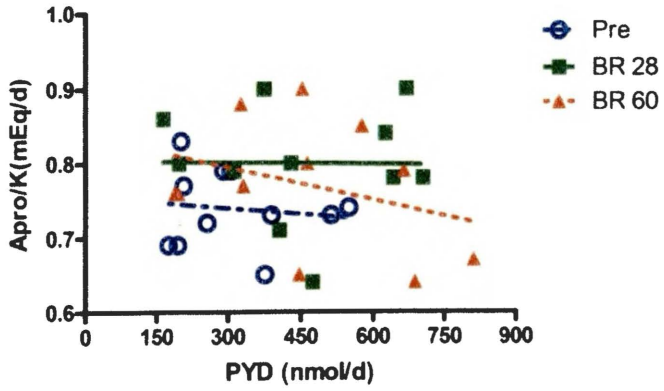

Fig. 5. A plot of the ratio of dietary animal protein to potassium and PYD. There were no significant relationships.

DISCUSSION

Diets which are not balanced in acid and base precursors may have effects on bone metabolism, and therefore may have important connotations for the skeletal health of astronauts (Zwart, et al., 2005; Zwart, et al., 2004; Zwart & Smith, 2005). The purpose of this experiment was to test two dietary methods which assess for urinary acidic levels in support of relationships to bone resorption. The hypothesis of this study was that both NAE_{ind}, and the animal protein to potassium ratio would have significant correlations with bone markers NTX and PYD in the eleven bed rest subjects reviewed. This study is part of a larger bed rest project, and results from this study should be reviewed in this context.

Results from this study showed that nutrient intake decreased throughout bed rest (Table 1). Consistent with previous studies, bone loss occurred throughout bed rest, with increases in NTX and PYD on day twenty eight and sixty (Table 2). NAE_{ind} had significant correlations with markers of bone breakdown; NTX on bed rest day twenty eight ($p<0.05$) and sixty ($p<0.01$), and PYD on bed rest day twenty eight ($p<0.01$). However the animal to potassium ratio did not have significant correlations with NTX or PYD during bed rest.

The comparison of two dietary methods for estimating acid excretion has been conducted previously by researchers such as Prynne et al., 2004. In this study Prynne and colleagues analyzed a 7 day food diary in a total of 212 male and female subjects (16-18y) for correlations between NAE_{ind} and animal to potassium ratio. The study conducted by Prynne et al., 2004, showed significant correlations between methods of

NAE_{ind} and the animal to potassium ratio ($p<0.01$). However, at higher acid levels of excretion there was less concordance between the two measurements. Prynne and colleagues were also careful to note that low NAE_{ind} does not necessarily indicate a balanced diet, but that there does appear to be a positive correlation between increased fruits and vegetables and bone health. The current study conducted differs from the former study conducted by Prynne in that the diet composition for the present study is identical, with menus being individualized to meet subjects' preferences. The present study also differs from the previous study of Prynne, because this study uses the distinctive model of bed rest in which subjects are immobilized and bone is uniquely challenged from inactivity.

Although, previous published data has shown significant correlations for either the animal to potassium ratio or NAE_{ind} to markers of bone breakdown in bed rest subjects, these methods have yet to be reviewed together in a single subject pool using bed rest subjects as a model for bone breakdown (Zwart, et al., 2005; Zwart, et al., 2004). A study conducted by Zwart, et al., 2004, for example, analyzed a relationship between protein to potassium intake to markers of bone metabolism in bed rest subjects. In this study eight pairs of identical twin males were divided and randomly assigned to either a sedentary bed rest group or a bed rest group undergoing an exercise protocol. Results from this study showed a positive correlation for the animal protein to potassium ratio and collagen crosslink excretion for the sedentary bed rest subjects. However, the relationship was not as strong for those subjects performing an exercise protocol during bed rest, concluding that the effect of diet on bone resorption may be greater for subjects

whose rate of bone breakdown is increased from immobility (Zwart, et al., 2004). The results of this former study differ from the results of the present study in that positive correlations for the animal to potassium ratio were not found.

Perhaps the link to the differences between the study conducted by Zwart, et al., 2004, and the current study, is in the levels at which the individual net acid excretion occurred for each of these subject pools. As variations in net acid excretion need only be small, with one group excreting only slightly higher amounts of acid than the next, to see changes in results from one group study to another.

One of the limitations of bed rest studies is that the subject pools used are few in number. This is because bed rest studies are costly, and finding subjects which fit the physical and mental criteria to undergo sixty days of testing confined to bed is often complex. The animal to potassium ratio is a shortened method for determining dietary NAE. It may also prove that while both methods are accurate, since the animal to potassium uses fewer components, this ratio may randomly show acidic effects in bed rest studies using small subject numbers, whereas NAE_{ind}, which takes more nutrients into consideration, may prove to be the more consistent method for calculating dietary acid excretion. So, it is possible that as the subject number increases, the animal to potassium ratio may also prove to be a consistent method for estimating acid excretion in bed rest subjects as well. This represents a further opportunity for bed rest studies to explore.

In support of the results of the current study regarding NAE_{ind}, a previous experiment conducted in by Zwart et al., 2005, showed positive results for PRAL and

markers of bone breakdown NTX and deoxypyridinoline (DPD) ($p<0.05$). This study was conducted to primarily to assess the ability of an essential amino acid supplement to mitigate loss of muscle mass and strength during bed rest. However, a secondary hypothesis reviewed in this study was that diets high in acid precursors may exacerbate bone loss. In this experiment thirteen male subjects were randomly assigned to either an amino acid supplemented group (AA), receiving 49.5 g of essential amino acids daily, or to a control group (Con) for a twenty-eight day bed rest trial. Results from this study concluded that PRAL was in fact higher in the AA group than in the Con group during bed rest ($p<0.05$). This study was particularly unique to the acid-base challenge because the AA and Con groups consumed identical diets with the only difference between the diets being the daily addition of the amino acid supplement and carbohydrate intake. It can, therefore, be deduced that the endogenous acid production resulting from diet alone was also similar for the two groups (Zwart, et al., 2005). This study concluded that the more acidic precursors in the diet the more NTX was excreted, which supports the negative effect of excess dietary acid on bone (Zwart, et al., 2005).

Finding countermeasures to combat bone loss is a critical concern for astronauts during spaceflight and for individuals with bone disease on earth as well (Baecker, et al., 2003; Donaldson, et al., 1970; Inoue, et al., 2000; LeBlanc, et al., 1995; Lueken, et al., 1993; Prentice, 2004; Rizzoli, 2008; Scheld, et al., 2001; Smith, et al., 2003; Smith, et al., 1999; van der Wiel, et al., 1991; Vermeer, et al., 1998; Watanabe, et al., 2004; Whedon, 1984; Whitson, et al., 1997; Zerwekh, et al., 1998; Zwart & Smith, 2005). In bone metabolism every factor which contributes bone health becomes of vital importance

(McCarthy & Frassica, 1998; Orwoll, 2003; Ralston & Kleerekoper, 2002; Zwart, et al., 2005; Zwart & Smith, 2005). Dietary methods used to determine acid excretion may give researchers further insight into predicting changes in bone metabolism and identifying nutrients needed for preserving and strengthening total bone mass (T. Arnett, 2003; Barzel, 1995; Barzel & Massey, 1998; Buclin, et al., 2001; Macdonald, et al., 2005; Remer, 2004; Sebastian, et al., 2001; Sellmeyer, et al., 2001).

Results from the present study confirm that dietary calculated NAE is an accurate predictor for bone loss in bed rest subjects. However, the data presented in the present study show that the dietary protein to potassium ratio alone was not as predictive of bone turnover as expected. Further research is needed to determine why this method of predicting acid load worked well as a predictor for bone resorption in one study but not another. As this project is part of a larger study, the addition of bed rest subjects may provide new data proving the protein to potassium ratio to be a more precise assessment for larger bed rest subject clusters.

Research gained from the study of the effects acid-base metabolism on bone health during bed rest may important implications for astronauts during spaceflight, and the general population as well. Results from this study may contain suggestions for certain states of paralysis, such as trauma induced immobilization where bone is compromised from disuse, and for the general aging population whose bone is at increased risk for fracture, osteopenia, and osteoporosis (Barzel & Massey, 1998; L. Frassetto, et al., 2001; Heer, et al., 2005; Ralston & Kleerekoper, 2002; Sievanen, 2010; Whedon, 1984). This study supports the need for increased basic precursors from fruits

and vegetables in the diet; as diets rich in fruits and vegetables are also high in bicarbonate which may combat the possible deteriorating effects of increased dietary acid on bone. Such dietary recommendations would not only have multiple beneficial effects to the overall health of human body, but also have considerable effects on maintaining and building bone in the human skeleton (Demigne, et al., 2004; L. A. Frassetto, et al., 1998; Lanham-New, 2006; Looker, et al., 2000; Macdonald, et al., 2005; Prynne, et al., 2006; Sebastian, et al., 2002).

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