AN ABSTRACT OF THE THESIS OF

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In the present study we hypothesized that a high intake of dietary phosphorus would be compatible with bone health if accompanied by a high calcium intake obtained primarily from milk and cheese. The study consisted of two 10-day periods in which seven male subjects (22-31 years and an average weight of 70 kg) were fed diets made up of common foods. In diet period 1, all subjects were fed diets providing 800 mg P, 1200 mg Ca, 11.7 MJ (2800 kcal) and 14.5 g nitrogen. In diet period 2, dietary phosphorus intake was doubled to 1600 mg by the addition of cheese and milk. Dietary nitrogen and calcium were equalized between both diet periods through the addition of spray-dried egg white and calcium carbonate to the bread and beef patty of diet period 1. Three 24-hour urine samples and one blood sample were collected at the end of each 10-day diet period. Comparison of the two diet periods revealed that in diet period 2, as compared with period 1, urinary phosphorus increased 82%, urinary calcium decreased 38%, serum phosphorus increased 6%, serum calcium decreased 4% while urinary sulfate remained relatively constant. Although an increase in intact parathyroid hormone levels of 12.5%, would suggest an increase in bone turnover, this possibility was not supported by the

sensitive urinary bone resorption marker deoxypryidinoline (DPD) which decreased by 14% during period 2. A high phosphorus intake is not likely to be deleterious to bone health if the dietary ratio of calcium to phosphorus is less than 1:1.5.

High Dietary Phosphorus is not Detrimental to Bone Health of Young Men, Assessed by Urinary Deoxypyridinoline, when Calcium Intake is at the Recommended Level

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Brian Bizik, Author

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TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

High Dietary Phosphorus is not Detrimental to Bone Health of Young Men, Assessed by Urinary Deoxypyridinoline, when Calcium Intake is at the Recommended Level

Introduction

Osteoporosis is a major public health problem in many countries around the world and is being given greater scientific attention every year. It is estimated that there are 1.5 million fractures per year in the United States alone which can be directly related to osteoporosis. Health care costs associated with osteoporotic fractures reaches 7 to 10 billion dollars a year (Marchant, 1994). Studies have shown that by age 65 years, 50% of the women in the United States have bone density at a level called the fracture threshold, where breaks are common. By age 85 years, nearly 100% of American women are at the threshold (Perez, 1994). One of the most common injuries is a fracture of the hip. It is so common that one out of three persons living to the age of 90 will experience a hip fracture (Perez, 1994).

While women are more affected by osteoporosis than men, it is not a gender-specific ailment. In 1990, 30% of the 1.66 million hip fractures worldwide occurred in men (Seeman, 1993). As more research is done, the impact, treatment, and prevention of osteoporosis in men will be better understood.

Factors that have been associated with osteoporosis include diet, exercise, sex, race, age and genetic make-up. With regard to diet, many studies have addressed the relationship between calcium, the major bone mineral, and bone health. More recently, however, researchers have examined the role of phosphorus on bone health as well. Calvo

and others (1988, 1990, 1993) have shown that high intakes of phosphorus with a low to moderately-low calcium intake can have a detrimental effect on bone. Short term ingestion of a high phosphorus, low calcium-containing diet for example, had possible negative effects on bone health when compared to a diet that provided calcium and phosphorus levels near the Recommended Dietary Allowance (RDA) (Calvo et al., 1988). The negative effect of the high phosphorus containing diet was hypothesized to be due to an increase in the parathyroid hormone (PTH) secretion. Greater secretion of PTH can be expected when consuming a high phosphorus containing diet because PTH promotes urinary phosphorus excretion thus keeping internal phosphorus in balance. Too great of an increase in serum PTH, however, would be expected to mediate an increase in bone resorption. With a low intake of calcium and a high intake of phosphorus, a kind of 'double negative' effect on bone health would be seen. It is unclear, however whether the combination of high phosphorus plus low calcium intake is of sufficient magnitude to negatively impact bone health because specific biomarkers of bone health have not been tested under such conditions. It also is unclear whether the proposed negative effect of phosphorus can be prevented by also having a high calcium intake. This latter question is the cornerstone of the present project.

Many methods have recently become available to measure the effect of a dietary treatment on the bone health of subjects. These biomarkers of bone formation and resorption are far more specific than older measures such as urinary hydroxyproline excretion. This latter measurement for example has been used in the past as an indicator of bone turnover, but hydroxyproline is not specific to bone. In the present study the plan was to assess dietary treatment effects on bone resorption by using a recently developed

immunoassay for a peptide fragment of bone resorption found in the urine. The protein fragment, called deoxypyridinoline (DPD), is excreted in the urine daily and provides a sensitive and specific biomarker of bone resorption.

The aim of this thesis was specifically to determine if a high phosphorus diet would be detrimental to the bone health of young men when dietary calcium was at the recommended level. From a practical application standpoint, failure to find a detrimental effect of a high phosphorus containing diet could suggest that the way to deal with this typical dietary situation is simply to supplement the diet with calcium rather than attempting to reduce phosphorus intake. Reducing phosphorus intake is likely to be impractical because it is found in all the major food groups. The following section reviews the background, theory and rationale that led to the experimental plan.

Literature Review

Metabolic Fate of Phosphorus

Dietary intake

All living cells contain phosphorus. Because of this, it is found in all food groups and nearly all foods. Meat, poultry, fish and eggs are major contributors to the phosphorus intake of Americans providing approximately 20-40% of the daily supply. Milk and milk products provide 20-35% while grains provide approximately 12% (Greger and Krystofiak, 1982). The remaining 10-30%, depending on dietary patterns, comes from processed foods or other sources (RDA, 1989).

Recommended Dietary Intake of Phosphorus

A new Recommended Dietary Allowance for phosphorus was published in 1997 (Dietary Reference Intakes Committee, 1997). During infancy, an adequate intake of phosphorus was specified based upon that obtainable from human breast milk. For children 1-3 years of age the RDA is 460 mg/day; and for 4-8 years it is 500 mg. The allowance jumps to 1250 mg/day for both men and women from 9 to 18 years and drops back to 700 mg/day from 19 and above years of age. An intake of 700 mg/day is advised during pregnancy and lactation. The recommendations represent a considerable reduction for most age groups compared to that published previously (RDA, 1989).

Phosphorus Intake Patterns

The phosphorus intake of Americans has been studied in many national surveys and reveals that the amount of ingested phosphorus has been relatively stable since the beginning of this century. These studies have consistently estimated phosphorus intake to be between 800 and 1500 mg/day. Regardless of the source of protein, carbohydrates, or fat, American diets provide adequate phosphorus (Berner and Shike, 1988). In 1986, a study by the US Department of Health and Human Services reported that the phosphorus intake of Americans averaged 136% of the recommended intake. Similarly, a 1987-88 Department of Agriculture Food Consumption Survey showed a phosphorus intake of 132% of that recommended (Anderson and Barrett, 1994).

There are two concerns arising from the data on phosphorus intake. The first concern is that current estimates of consumption could be low, at least partly due to the fact that phosphorus from food additives is not included in phosphorus intake estimates from food surveys (Calvo, 1996). One example is a study by Oenning et al. in 1988. In this study, analysis of food, when compared to data from nutrient composition tables, showed that the tables consistently underestimated the phosphorus content. The difference in daily amounts were from 250 to 350 mg, or approximately 20%. Estimated values were closer to actual analysis if the diet contained less processed or convenience foods leading researchers to hypothesize that processed foods could be adding more phosphorus to the diet than is generally believed. Another study estimated that phosphorus intake could reach as high as 3000 mg/day for individuals with unusually high intake of processed foods (Bell et al., 1977).

A second concern is that phosphorus from the processed foods category may be on the increase in the American population, especially in the youth of America. In 1982, Greger and Krystofiak estimated that the United States Food Supply provided approximately 400 mg/person/day of phosphorus. New data however, has suggested that this figure is rising. In 1992, the Food Additive Council suggested that phosphorus intake in the form of phosphate additives in processed foods were approaching 500 mg/person/day (Anderson and Barrett, 1994). One age group in particular, persons from 19 to 44 years of age, are particularly likely to have high intakes of phosphorus due to a high consumption of soft drinks (Calvo, 1996).

The most recent RDA, published in 1997 suggests that intake patterns of phosphorus are probably of little consequence if the calcium intake follows suit. This question is the cornerstone of the current research: are high phosphorus levels detrimental to health if accompanied with adequate calcium?

Phosphorus Bioavailability

Absorption of Phosphorus

Although the jejunum is the most active site for phosphorus uptake into the blood, phosphorus can be absorbed along the entire intestinal tract through three different methods: (1) calcium-coupled, vitamin D-dependent; (2) noncalcium-coupled, vitamin D-dependent; and (3) noncalcium coupled, vitamin D-independent (Dietary Reference Intakes Committee, 1997). Absorption rates for adults vary between 50 and 70% and rise

slightly in infants at 65-70% (Anderson and Barrett, 1994 and Kowarski and Schachter, 1969).

Internal homeostasis of phosphate is closely regulated by the interaction of two protein hormones, parathyroid hormone (PTH) and calcitonin (CT), and the most biologically active form of vitamin D, 1,25-dihydroxycholecalciferol (1,25DHCC) (Arnaud and Sanchez, 1996). Post absorptively, rather than by regulating intestinal absorption, this doubly hydroxylated form of vitamin D functions as a steroid hormone.

Regulation of Serum Phosphorus

Serum phosphorus values range from 0.8-1.6 mmol/L and are regulated by the kidney. Because phosphorus is a threshold substance the body will try to keep levels above 0.8 mmol/L (Guyton, 1991). Levels below this threshold level can have serious health consequences. Above this level, increases in phosphorus intakes are quickly followed in the same manner by serum levels and bring a quick response by the kidney to restore blood levels (Guyton, 1991). In humans, the kidney excretes phosphate in an amount equal to the phosphorus absorption in the digestive tract. By regulating excretion in this manner there is a zero balance of phosphorus. Urinary losses average 600-800 mg/day (Berner and Shike, 1988).

Phosphorus Distribution

Phosphorus is distributed in the body in roughly the following manner. In the body as a whole, 14% is found in the soft tissues, 1% is in the blood and the rest or 85% is

found in bone. The 1% found in the blood can further be classified as 30% organic and 70% inorganic. Of the 30% inorganic phosphorus, 83% is found free while 17% is found bound to protein. As the sixth most abundant element in the body, phosphorus constitutes approximately 1% of the weight of the body (Guyton, 1991). In soft tissue, the roles of phosphorus include acting as a structural component of cell membranes, and intracellular buffer, a role in intermediate energy metabolism, and as an acid buffer (Berner and Shike, 1988). The role of phosphorus in bone will be described with calcium in a later section.

Metabolic Fate of Calcium

Dietary Intake

The dairy group provides approximately 55% of the daily intake of calcium for the US population. Items like yogurt (1 cup = 480 mg), milk (1 cup = 325 mg), and cheese (1oz = 220 mg) are popular and provide the bulk of the calcium from the milk group (Whitney and Rolfes, 1993 and Block et al. 1985). The most commonly consumed non-milk foods that supply calcium include soy beans (1 cup = 180 mg) and broccoli (1 cup = 80 mg). Recently, there has been a popular media push for the use of supplements as a source of calcium. Products like Tums Chewable antacids or similar sources of calcium carbonate are using their calcium content as strong selling points.

Recommended Intake of Calcium

The optimal intake of calcium is one of the most commonly debated topics in the field of nutrition. Calcium intake recommendations worldwide range from a low of 400 mg/day for women in Thailand to a high of 1000 mg/day for persons over 75 years in the Netherlands (Schaafsma, 1992 and RDA, 1989). Despite the fact that low calcium intake is implicated in hypertension and colon cancer, most authorities agree that the calcium requirements set by the government should reflect the amount needed for optimal bone development and maintenance of bone health (Schaafsma, 1992). While this amount is still uncertain, some feel that current AI levels are too low. Supporting this contention are two recent studies showing that growing children receiving supplemental calcium exhibited significantly enhanced gain in bone mass relative to non-supplemented children. In both cases the supplemented amount was significantly higher than the current RDA (Johnson et al., 1992 and Lloyd et al., 1993). In addition to current concerns regarding the RDA, strong research has shown that calcium intake prior to puberty plays a more crucial role in total bone mineral density than calcium consumed after (Anderson, 1996). Because of these concerns, this topic was in fact addressed by new recommendations for calcium intake in 1997 (Dietary Reference Intakes Committee, 1997). Although a new RDA was not proposed in the 1997 update of the recommended intakes, a recommendation called an adequate intake for calcium was given of 1300 mg for ages 9-18 years for both sexes. For ages 19-50 years, an adequate calcium intake of 1000 mg

was recommended. To reduce risk of bone loss for those over 50 years, the adequate intake recommendation increased to 1200 mg.

Usual Intake and Pattern of Intake

The most striking fact related to calcium that came out of the 1977-78 Nationwide Food Consumption Survey was that no group of women above the age of 35 in the US had average intakes above the 800 mg/day (Calvo et al., 1990). Recent data on intake patterns reveal that this pattern is continuing and possibly affecting young women (16 to 25 years) even more than in the past as items like soda pop replace milk as popular beverages (Calvo, 1993 and Matkovic, 1992).

The food survey reported an average intake of 743 mg/day for all Americans. The values ranged from 530 mg/day for women age 35 to 50 years to 1179 mg/day for men 12 to 18 years. Recent urban studies have found typical intakes ranging from 627 mg per day in Pittsburgh to 772 mg per day in Minneapolis (Cumming et al., 1997).

Calcium Bioavailibility

Calcium Absorption

Like many bivalent cations, calcium is generally poorly absorbed from the intestinal tract (Guyton, 1991). In humans, one of the greatest factors influencing absorption is the physiological requirement of calcium. Children, with high calcium needs, may absorb up to 75% of ingested calcium while adult absorption ranges from 10-40% (Heaney et al.,

1975). Above 800 mg/day, calcium absorption falls to approximately 15% in healthy adults (Heaney et al., 1975). The approximate daily turnover rates for calcium are shown in Table 1 (Guyton, 1991).

Table 1
Daily Turnover rates (in mg) for Calcium in the Adult

Intake	1000
Intestinal Absorption	350
Secretion of gastrointestinal juices	250
Net absorption over secretion	100
Loss in the feces	900
Excretion in the urine	100

There are two methods of calcium absorption in the human intestinal tract. The first is a vitamin D dependent active process located in the duodenum. This method accounts for the majority of calcium absorbed and is most affected by dietary intakes (Charles, 1992). The second, a passive component located in the ileum, is fairly consistent and may be facilitated by the milk sugar lactose (Guyton, 1991; Arnaud and Sanchez, 1996). This passive process is not saturable whereas the active component can reach a threshold maximum (Charles, 1992). Because the absorption of calcium is tied to the hormonal events in question in this thesis, this item will be discussed in a later section.

Calcium Regulation

Calcium is regulated by a complex system in much the same way as phosphorus in that the hormones PTH, CT and $1,25(OH)_2D_3$ are involved. The normal range for serum calcium is quite narrow at 2.2-2.5 mmol/L. Values above 2.5 mmol/L or below 2.2 mmol/L reflect conditions of hypercalcemia and hypocalcemia respectively (Arnaud and Sanchez, 1996).

Another concern in calcium regulation is the protein level of the diet. The typical high protein diet of many Americans has been shown to cause an increase in urinary calcium excretion or hypercalciuria (Yuen et al., 1984).

Calcium Distribution

Approximately 98% of the calcium in the body is stored in the bone with an additional 1% found in the teeth. The remaining 1% of the calcium is located in the soft tissues of the body. A portion of this, the plasma calcium, is found in three forms: ionized, protein bound, and complexed (Arnaud and Sanchez, 1996). The ionized fraction is biologically active and constitutes just less than half of one percent of total calcium. Protein bound calcium is similar in amount to the ionized fraction, and because it obeys the law of mass action can dissociate from its protein complex if plasma calcium levels fall. In this capacity, protein bound calcium serves as an important first line of defense against low calcium levels in the blood (Arnaud and Sanchez, 1996). The complexed portion is small, less than one tenth of total calcium, and probably plays a small role in maintaining plasma levels.

Phosphorus and Calcium Interrelationships

Regulation of Phosphorus and Calcium

The highly complex system used in the human to maintain plasma phosphorus and calcium levels relies on PTH, CT, and 1,25DHCC. These three hormones must respond quickly to changing serum levels of phosphorus and calcium or serious problems will arise. The following sections will examine the human body's response to changing levels of these important minerals.

Falling Serum Calcium Levels

As plasma ionized calcium level falls, the parathyroid gland increases its secretion of PTH. PTH increases the kidney conversion of 25-hydroxycholecalciferol to 1,25 dihydroxycholecalciferol (1,25DHCC). This active form of vitamin D, in concert with PTH, will increase calcium reabsorption in the kidney tubules to reduce calcium losses to the urine, together with PTH it will stimulate mobilization of calcium from bone, and most importantly will stimulate intestinal calcium absorption (Charles, 1992; Guyton, 1991). The combination of all these events serves to increase plasma calcium concentration. Acting as a hormone in this active and saturable process, 1,25DHCC will induce the proliferation of calcium binding protein in duodenal epithelial cells. This protein will then become part of the cell surface in the lumen of the gut, increasing the calcium binding ability of the cell (Charles, 1992).

A second bodily response to decreasing calcium levels is to decrease the production of CT by the thyroid gland. This action decreases calcium uptake into bone which further allows plasma calcium to rise.

Increasing Plasma Calcium Concentrations

As plasma calcium rises the body responds by reducing the formation of PTH and increasing CT production. With PTH levels falling 1,25DHCC levels also fall and intestinal absorption efficiency drops (Charles, 1992).

CT production increases in the thyroid gland and mediates two effects. First, the hormone will decrease the bone resorptive effects of osteoclast cells. Second, and a longer lasting effect, will be to decrease the production of new osteoclasts (Guyton, 1991). Both of these events will help lower serum calcium levels and maintain healthy bone.

The Hypocalciuric Effect of Phosphorus

If dietary levels of phosphorus rise in proportion to calcium, the human body will try to restore the balance through the increased secretion of PTH (Anderson, 1996). In this scenario, an increase in PTH secretion along with an increase in 1,25DHCC, will increase the efficiency of kidney calcium reabsorption, thus creating a hypocalciuric condition and raising blood calcium to meet the rising phosphorus levels (Hegsted et al., 1981 and Yuen, 1984). As PTH levels increase in the plasma, calcium absorption will also

increase in the small intestine due to an increase in the production of 1,25DHCC. PTH will also act to increase urinary loss of phosphorus (Guyton, 1991).

Calcium reabsorption is also mediated by phosphorus in a non-PTH dependent manner. While the mechanism of this process is unclear, recent evidence in rats has indicated that the hypocalciuria could be tied to an increase in ammoniagenesis (Cerklewski, 1995).

Effect of Phosphorus on Calcium Absorption

For many years it was believed that increasing the level of phosphorus in the diet would result in a decrease in the amount of calcium absorbed in the small intestine. The reason given was that insoluble salts of phosphorus and calcium formed and thus reduced the amount of calcium available for absorption (Zemel and Linkswiler, 1981 and Hegsted et al, 1981). Many studies however, have shown that the effect is minimal, and in one case, phosphorus in the form of an orthophosphate supplement actually improved calcium absorption (Zemel and Linkswiler, 1981). Spencer et al. also found no reduction in calcium absorption (1978). In these studies, large phosphorus supplements were given with no significant effect on calcium absorption.

Skeletal Concerns

Bone Structure

Bone is a specialized form of connective tissue. What distinguishes bone from other tissues, and allows it to support the human structure, is the mineralization of the intercellular matrix (Ross and Romrell, 1989). This matrix is composed of two crystal forms. The first is known as hydroxyapatite crystals. These long, flat plate like crystals salts are comprised of calcium and phosphorus in the general formula of $Ca_{10}(PO_4)_6(OH)_2$. The ratio of calcium to phosphorus (Ca/P) in hydroxyapatite crystals can vary under different nutritional conditions (Guyton, 1991). The second form is an amorphous calcium phosphate. This latter portion is present in higher quantities in younger bone and in areas of active bone formation (Arnaud and Sanchez, 1996). The two major bone types are cortical bone, which is densely packed mineralized collagen predominately found in the peripheral skeleton (long bones for example) and trabecular bone (Guyton, 1991 and Mosekilde, 1992).

Bone Modeling and Remodeling

Bone is resorbed and formed continuously throughout life. It is this process that allows for adjustment to physical stress, repair and growth. The relative amount of resorption and formation is controlled by both hormonal and dietary factors. In some nutritional cases, pathology can cause one of the two processes dominate. Because of this, it is important to understand the processes of modeling and remodeling.

Three types of cells are involved in bone shaping. Osteoblasts are the bone forming cells. They are actively involved in the synthesis of primary collagen and play a role in the movement of calcium and phosphorus into the bone mineralization process (Arnaud and Sanchez, 1996). Osteoclasts on the other hand are the giant multinucleated cells responsible for bone resorption. These cells contain many lysosomes which hold the digestive enzymes, including collagenase, which 'eat' away at exposed surfaces of bone (Ross and Romrell, 1989). Once the enzymes have made the matrix soluble, the calcium and phosphorus can be released into the extracellular fluid and ultimately the blood. The osteocyte is the third type of bone cell and is actually a mature, transformed osteoblast. Osteocytes are responsible for maintaining the bone matrix. To a limited extent they have the ability to synthesize and resorb bone matrix, thus playing a role in plasma calcium homeostasis (Guyton, 1991; Ross and Romrell, 1989).

When speaking of bone, the term modeling refers to the process of the formation of the macroscopic skeleton which ceases by age 20 in most individuals (Arnaud and Sanchez, 1996). Bone mass gains start to slow soon after the cessation of modeling but continue slowly until the early to mid thirties in most persons (Mosekilde, 1992). At this point, peak bone mass is reached and no new net bone mass will be added. This mass, which is 25% higher for males than females, has been the topic of much research. Reaching an optimal peak bone mass is currently believed to be the best preventative measure against bone diseases like osteoporosis (Matkovic, 1992).

Bone remodeling signifies the continued process of renewal of old bone with new bone. This process takes place in both cortical and trabecular bone. Remodeling is continuous throughout life and is typically not associated with major changes in bone

mass. The objective is to renew old osteocytes, repair fractures, and to adjust the skeleton to mechanical strain (Mosekilde, 1992 and Matkovic, 1992).

The process of remodeling is quite complex and will only be discussed briefly here. The remodeling process starts with osteoclasts which resorb bone. Following this 4-6 week phase, osteoblasts will invade the area and form new bone for a period of 3-4 months (Mosekilde, 1992). This remodeling, much like home remodeling, allows for new "better" bone to replace older bone. This new bone is stronger and free of fractures (Anderson, 1996).

There are three important concepts in determining the effect of remodeling on overall bone health and pathology. The first concept is the remodeling process rate of activation. The frequency of this activation denotes the number of times with which a given bone site is remodeled. Because newly formed bone is stronger, any process which causes a marked reduction in the activation frequency will induce the accumulation of older and more fragile bone (Mosekilde, 1992). A second important concept in remodeling is the remodeling space. The remodeling space is the amount of bone that is in the active process of remodeling. This term is important because any portion of bone between the start of bone resorption of the osteoclast, and the termination of bone formation via the osteoblast, will have less bone structure and be weaker. Typically, 4-10% of total bone is in the remodeling space at a given time. Any trigger which alters this percentage could have an effect on bone health (Mosekilde, 1992). The final concept is the final resorption depth. It can be envisioned like digging a hole in the ground. If you dig 10 feet down and then replace the dirt into the hole to a depth of only nine feet, you

are left with a one foot hole. In bone, if the final resorption depth is less than the initial bone surface, weaker skeletal conditions will ensue.

Mechanisms for Bone Loss

The three processes described above can all lead to healthy functional bone. They can also lead to weaker more brittle bones. There are many factors that can effect the direction of bone health including exercise, smoking history, genetic make-up and diet to name a few (Heaney, 1992). It is the diet portion which is the focus of the research described later in this thesis.

Reversible Bone Loss

Reversible bone loss is the temporary loss in bone due to an increase in the remodeling space (Mosekilde, 1992). This is controlled by the activation frequency. As the activation frequency increases, the amount of bone actively involved in remodeling increases thus total bone mineralization decreases.

Irreversible Bone Loss

Irreversible loss of bone occurs when the final resorption depth does not equal the starting resorption depth. If this is the case, each remodeling cycle will reduce total bone content (Mosekilde, 1992). It is unlikely that losses of bone in this process will ever be made up. This is the primary cause of the crippling disease osteoporosis.

Dietary and Hormonal Effects on Bone Health

One physiological condition that could increase activation frequency can be described as secondary hyperparathyroidism (SH). SH has been shown to occur in both dogs and rats when dietary intakes of calcium are low and intakes of phosphorus are high (Krook et al., 1975 and Draper et al., 1972). Similar results have been found in humans but have not been as pronounced. Calvo and Park, in 1996, showed that an increase in parathyroid hormone was found in young men and women consuming a high phosphorus containing diet. The calcium level in this diet was kept very low. It was postulated by Calvo that the combination of a high phosphorus and low calcium diet would raise plasma PTH levels high enough to have a negative effect on bone health by way of PTH mediated increase in bone turnover. Later studies supported that theory. Metz et al., 1993, showed reduced bone density in women consuming a high phosphorus diet. It was thought that this was again due to an increase in PTH production and an increase in bone demineralization.

In the two situations described above, the body is trying to stabilize the relationship between plasma calcium and phosphorus. In theory, the increased rate of remodeling will lead to higher plasma calcium levels (Arnaud and Sanchez, 1996). While these changes are to be considered transient, any reduction in total bone mass could have a negative health impact. It has been postulated that while changes in PTH secretion may be small and within the range of normal values, sustained over time the changes could negatively impact skeletal health (Calvo, 1988).

In summary, bone loss as it pertains to the current research theorizes that low dietary intake of calcium, either with high phosphorus or normal phosphorus, will stimulate endogenous PTH secretion, leading to a decrease in bone mass through increases in bone remodeling and incomplete bone formation. It is the goal of this research to define the role that phosphorus levels play in the complex interaction between phosphorus, calcium, hormones, and bone health.

Methods of Assessment

To accurately assess the role of phosphorus and calcium in bone health sensitive indicators are needed. These indicators, listed below allow researchers to peek into the world of bone formation and resorption to monitor the effect of diet on bone health.

Bone Formation Markers

Total Alkaline Phosphatase

Total alkaline phosphatase in serum is the most commonly used indicator of bone health. The phosphatase itself is actually a group of similar enzymes that are closely related and originate in various parts of the body including the skeleton and liver. The largest drawback of total body alkaline phosphatase is the lack of specificity.

While total alkaline phosphatase may be the most common assay, a more specific assay is skeletal alkaline phosphatase (sALP). This is an enzyme found in the membrane of osteoblast cells (Garnero and Delmas, 1993). It is believed to either increase the local

concentration of inorganic phosphate or possibly to activate collagen fibers to increase mineralization (Guyton, 1991). During bone formation, some of the enzyme is released into the blood and can be assessed to indicate bone formation.

Recently an immunoradiometric assay (IRMA) was developed for sALP. This breakthrough test offered by Hybritech (San Diego, CA) increases the sensitivity and the specificity of the assay as the assay only recognizes the bone isoenzyme and ignores other alkaline phosphatases.

Osteocalcin

Osteocalcin is a small noncollagenous protein that is specific to bone and dentin (Delmas, 1993). While the function of osteocalcin is unclear, it is known to be synthesized by osteoblast cells and incorporated into the matrix of bone. Some of the protein is released into circulation where it can be measured by radioimmunoassay (RIA) (Riis, 1993). While this test is generally regarded as an accurate assessment of bone formation, especially when coupled with markers of resorption, some questions about consistency remain. Some indications are that a sandwich RIA assay is needed to increase accuracy. In this assay, only intact osteocalcin would be recognized by using two separate antibodies to sandwich the protein and hold it for the assay (Delmas, 1993).

Bone Resorption Markers

Hydroxyproline

Hydroxyproline is an amino acid that is specific to collagen. When collagen is resorbed, either from bone or other tissues, the amino acid is excreted into the urine. Because half of the collagen in the body is located in the bone, and this tissue is turned over more rapidly than other collagens, hydroxyproline is regarded as a measure of bone resorption (Delmas, 1993). While this assay is inexpensive, the clear problem of tissue origin has never been resolved. Because of this, urinary hydroxyproline is considered an ambiguous test. The assay can indicate large changes in bone resorbtion rates, but it lacks the sensitivity needed in short term dietary modification studies. Therefore, the amino acid would be poorly correlated with bone health in the present study, and will probably remain so since there is currently no way to distinguish bone hydroxyproline from any other source.

Urinary Pyridinium Crosslinks

Pyridinoline (PYD) and deoxypyridinoline (DPD) are found mainly in Type 1 collagen of bone. After the collagen is formed, the enzymatic action of lysyl oxidase on the amino acids lysine and hydroxylysine cause the formation of PYD and DPD (Delmas, 1993). When bone is resorbed, both PYD and DPD are released into the blood and cleared to the urine by the kidney. Tissue concentrations of PYD and DPD vary greatly. The amount of PYD in bone is low, but is still a significant source. DPD however, is

found almost exclusively in bone and dentin (Siebel et al., 1992). Because the turnover in most collagenous tissues is low, both are good indicators of bone health. The reason for this is that during bone resorption, both are released into the blood and research indicates that in the blood they are left intact (Delmas, 1993). Following this they are filtered into the urine and excreted in both the free and protein bound forms.

Studies have shown that the assays for DPD and PYD are accurate for assessing bone resorption. A 1991 study showed that both DPD and PYD levels were increased by 50-100% in women at the time of menopause, but returned to normal following hormone replacement therapy (Uebelhart et al., 1992) Another study examined PYD levels in the elderly and in patients with Paget's disease, a bone disease of unknown etiology characterized by soft and weakened bone. In older persons, the levels of PYD increased with age and were marked by an average increase of 37% following menopause in women. the Paget's disease patients showed high levels of PYD that were reduced by 71% after three days of treatment (Delmas et al., 1993).

Recently developed assays for DPD and PYD have proven to be simpler and as accurate as those done by High Performance Liquid Chromatography (HPLC). For both PYD and DPD, competitive enzyme immunoassays have been developed and are approved for research use. The simple assay kits produced y Metra Biosystems (Palo Alto, CA) are available for purchase and can be used with I^{125} radio actively labeled antibody and measured using a gamma counter. Because of the ease of use and high degree of accuracy, the assay for DPD as a measure of bone resorption was chosen for the present research project.

Public Health Implications

Because osteoporosis is a major public health problem, greater scientific attention every year is being focused on prevention. It is estimated that there are 1.5 million fractures per year in the United States alone which can be directly related to osteoporosis. As stated previously, the expense of osteoporosis is reaching 10 billion dollars a year (Marchant, 1994).

One of the most common injuries a fracture of the hip. It is so common that one out of three persons living to the age of 90 will experience a hip fracture (Perez, 1994). A 50 year old Caucasian woman today faces a 17% chance that she will have a hip fracture in her lifetime (Meunier, 1993).

With these figures in mind, there is little doubt that the emphasis placed on bone health is well deserved. As pointed out in this thesis, the ratio of calcium to phosphorus in the diet could be of real importance to men and women later in life. In one scenario, young women could replace two glasses of milk with two cans of diet soda. This seemingly harmless event will lower calcium intake by 650 mg and increase phosphorus intake by 100 mg for a net change of 750 mg. This could very well be harmless for a few weeks or months but dangerous for extended periods of time.

As the average age of Americans gets older, the implications of bone disease will grow larger and take a bigger piece of the health care dollar. Research done today on nutritional trends could have a large impact on the health care of the future.

The goal of this research is to determine if ingestion of common foods containing high phosphorus levels will have a short term negative impact on bone health if calcium

intake is near the recommended level. This study, along with others, will help determine if changes in the RDA need to be made to best ensure the bone health of Americans in years to come. By using sensitive bone turnover indicators, it can be determined if adequate dietary calcium can serve as a prophylactic agent in the prevention of possible high dietary phosphorus-mediated bone damage.

Materials and Methods

This study was part of a larger project which sought to determine a mechanism for the non-PTH dependent portion of the hypocalciuric effect of phosphorus. The present description of methods emphasizes those concerned with the effect of high dietary phosphorus on bone health. Therefore, analysis of some of the samples will not appear in this report.

Recruitment of Subjects

Poster Recruitment

Recruitment of subjects started in late June and early July 1994. The first stage was to prepare and distribute recruitment posters around campus (Appendix 1). Of three posters that were initially designed, two were decided on for use. Approximately 45 posters were placed around the Oregon State University campus and in some of the local stores and restaurants. Nearly every building on campus had a poster. A second set of posters was distributed (18 in all) three weeks later after some of the initial respondents either dropped out of consideration for the study or failed to meet the basic study requirements. The poster used during the second round of postings was a modified version of the one used previously. The modification was to add tear-off strips to the bottom of the poster (Appendix 2). We found this poster type to be quite effective. We

also found that graduate students and undergraduate students with a significant summer course load were most likely to make a commitment to participate.

Initial Phone Interview

Initial response was good and immediate. Within a week, 13 calls had been received in our office in Milam Hall. All calls were logged at the office and phone calls were returned within two working days. Phone calls continued to filter into the office up to the start of the study.

The returned phone calls required potential subjects to give some general information to complete a preliminary phone questionnaire to determine if potential subjects would meet the study requirements (Appendix 3). If the subject was interested in participation and met the initial requirements for the study an interview time was arranged for the potential subject to meet with researchers and to view the metabolic unit where the study was to take place (Appendix 4). From this group of persons, a main and alternate list of desirable subjects was selected for a personal interview.

Personal Interviews

All desirable subjects completed a personal interview. The purpose of the interview was to review the study in detail, and to get important health/dietary information from the potential subjects (Appendix 5). If subjects were still interested in participation and met the study requirements, they were told that they would be contacted for a second personal meeting prior to the start of the study.

The second personal meeting was intended to include the entire group of potential subjects and was designed to provide additional information, answer any questions, and to sign an informed consent form (Appendix 6). Because some potential subjects were not able to attend this group meeting, individual informed consent signing times were set up to complete this process for all study participants. Either at the final meeting or at the time of consent signing, a study calendar and study sample collection schedule were given to the subjects.

Facilities

The three meals consumed each day and most of the sample analysis occurred in the metabolic unit in Milam Hall on the campus of Oregon State University in Corvallis, Oregon. Otherwise the subjects lived at home and continued their normal routine. Subjects, however, were asked not to do any strenuous exercise during the study so as not to create a caloric intake problem.

Our metabolic unit features four gas powered stoves with ranges, two refrigerator-freezer units, four sinks, one restaurant style dish washer and ample counter space. The unit is well lit, and includes six large windows which provide excellent views of the campus and make the unit an extremely pleasant place to eat. The unit also contained dry-erase boards that were used to give messages to the subjects during the study.

During the study, easy-listening and classical style music, as well as a daily newspaper were provided for the enjoyment of the students. Outside door keys were

issued to allow subjects access to Milam Hall for meals and to deposit urine collections as needed, especially on weekends. Each subject recorded his body weight without shoes before breakfast to ensure individual calorie intake was adequate to maintain weight throughout the study (Health Scale, Continental Scale Corp., Chicago, IL).

The laboratory used for analysis of most of the samples was located across the hall from the metabolic unit. Also located across the hall was a storage room which contained a refrigerator that was used for storage of the subject's urine. This room was accessible at all times to the subjects for the duration of the study and was used for periodic urine storage. A nearby room contained a phone which allowed subjects to make and receive free local calls.

Diet Composition

Diet Compilation and Computer Analysis

The metabolic study provided subjects with two distinct diets, each lasting 10 days. In both 10-day periods, the diets were composed of common foods (Tables 2 and 3) and with the help of a diet analysis computer program (Diet Analysis Plus, ESHA Research, Salem, OR), were designed to provide equal amounts of nitrogen (14.5g), calcium (~1200 mg), and kilocalories (~2800). The diets were designed so that diet #1 and diet #2 would provide 800 and 1600 mg of phosphorus respectively.

Table 2
Menu #1 Normal Phosphorus Diet. (800 mg/day)

Meal	Food	Quantity (grams)
Breakfast	Orange Juice (Minute Maid, with calcium, Coca-Cola Food Corp. Houston, Texas.	120
	Homemade white bread (2 slices)	80
	Margarine, ad lib	~15
	Jelly	15
	Whole egg, scrambled	50
Lunch	Chopped ham	45
	Homemade white bread (2 slices)	80
	Mustard	5
	Lettuce, iceberg	15
	Mayonnaise	15
	Margarine	5
	Potato chips	20
	Carrots	30
	Celery	30
	Apple	140
	Soft Drink (7-up, the Seven-Up Company, Dallas, Texas)	355
	Fruit drink	240
Dinner	Ground Beef Patty, see recipe	110 (cooked weight)
	Lettuce, iceberg	50
	Peas	20
	French style salad dressing	30
	Homemade white bread (2 slices)	80
	Fruit Drink	360
	Pineapple	117
	Margarine, ad lib	~20
	Catsup	10

Snack (take home) Hard candy, nondairy frozen treats, apples, plums and bananas. All were taken home as needed for satiety.

Table 3
Menu #2 High Phosphorus Diet. (1600 mg/day)

Meal	Food	Quantity (grams)
Breakfast	Orange Juice	120
	Canadian bacon	30
	Margarine, ad lib	~10
	Cheddar cheese slice	30
	English muffin	65
	Whole egg, scrambled	50
Lunch	Ground beef patty, seasoned with salt, pepper, chopped onion and 5 g of bread crumbs.	75 (cooked weight)
	Cheddar cheese slice	30
	Hamburger bun	40
	Catsup	10
	Mustard	5
	Lettuce, iceberg	50
	Potato chips	30
	Zesty Italian type salad dressing	28
	Pear slices	154
	Carrot, shredded	20
	Soft Drink (Coke, the Coca-Cola Company, Dallas, Texas)	355
Dinner	Roasted ham	90 (sliced weight)
	Pineapple	78
	Mashed potatoes	210
	Cheddar cheese	30
	Homemade white bread (1 slice)	35
	Green Beans, from frozen	90
	Milk, 2% fat	240
	Chocolate syrup	20
	Margarine	15
Snack (take home)	Hard candy, nondairy frozen treats, apples, plums and bananas. All were consumed as needed for satiety.	

Dietary Analysis and Nutrient Equalization

Chemical analysis in our laboratory revealed that diet #2 met our goals of 1600 mg of phosphorus, contained the desired amount of calcium, and had adequate nitrogen. Analysis of diet #1 showed that it also contained the required 800 mg of phosphorus, but was low in both calcium and protein. To balance the amount of calcium in the two diets, calcium was added to the bread made for diet #1, in the form of calcium carbonate (Table 4). Because diet #2 contained more protein than diet #1, spray dried egg white (Amersham Life Science, Arlington Heights, IL) was added to both the bread and the meat patty in diet #1 (Tables 4, 5)

Table 4
Bread Ingredients and Preparation

Ingredient	Diet #1 (g per loaf)	Diet #2 (g per loaf)
Bread flour (Gold Medal, General Mills Inc., Minn., MN)	470	470
Calcium Carbonate	6.77	0
Egg white, spray dried	58	0
Ergocalciferol (500,000 μ/g, United States Biochemical Co., Cleveland, OH)	0.0022	0.0073
Zinc Carbonate	0.0256	0
Magnesium Carbonate-n-hydrate	1.50	4.84
Vegetable oil (Hunt-Wesson Inc., Fullerton, CA)	13	13
Sugar	26	26
Sodium Chloride	9	9
Dry active yeast (Fleischmann's Specialty Brands. A division of Burns Philip Food Inc., San Francisco, CA)	8	8
Distilled water	290	290
Potato flakes	5	5

Table 4 (Continued)

All additions were hand mixed into the flour. The flour was then kneaded for 15 minutes at speed level #1 followed by 15 minutes at speed level #2 using a Hobart food mixer (Model A-200, Hobart Corporation, Troy, OH) with an attached dough hook. The dough was then allowed to rise for 60 minutes and then mixed for 20 seconds to punch down the dough. The mixed dough was placed on a 6 x 9 inch bread pan which had been sprayed with corn oil (Mazola cooking spray, Englewood Cliffs, NJ). The dough was allowed to rise to the top of the pan and then baked for 25-30 minutes at 350 degrees F. The expected size from earlier trials was 800 grams per loaf. Loaves were frozen until needed.

Table 5
Ground Beef Patty Ingredients and Preparation

Ingredient	Diet #1 (g per patty)	Diet #2 (g per patty)
Ground beef (22% fat)	117	80
Egg white, spray dried	25	0
Bread crumbs	7.5	5.0
Chopped onion	10	0
Table salt	0.4	0.3
Black ground pepper	0.1	0.07
Distilled water	4.0	0

Ingredients were blended with the ground beef using a Hobart food mixer. The mixture was formed into patties weighing 164 grams. The patties were wrapped in plastic and frozen until needed. The patties were baked prior to consumption in covered baking dishes at 330 degrees F for approximately 20 minutes. To achieve the ideal color and texture, the patties were then broiled in the oven for 2-3 minutes and served hot.

With the help of the computer analysis, other micronutrients, particularly vitamin D, Zn, and Mg, were added in the appropriate amounts to the bread mix (Table 4) so that the levels between the two diets would be equal and would meet or exceed the Recommended Dietary Allowance (RDA).

Food Preparation Methods

All diet items were purchased locally and were measured to the nearest gram prior to serving. Many of the smaller foods and condiments were served in disposable one and two ounce food cups (Sweetheart Plastics obtained through Quailcrest Foods, Corvallis, OR) or plastic baggies (chips). A dry-erase marker board allowed researchers to communicate when food amounts ran low and, therefore, there were no shortages.

In diet #1, all foods, with the exception of the meat patty, were cooked or prepared using standard methods. Because of the addition of the egg white to the meat patty, frying the patty produced a rubbery and inedible product. Our second attempt was to bake the patty. This product avoided the rubbery characteristic, but it lacked the color and texture of ground beef and thus, was also unacceptable. The method we found most accepted by the subjects was to bake the patty for 22 minutes at 330 degrees F and then immediately place the patty on the top oven rack and broil for 2-4 minutes or until the desired browning had taken place. This patty, served hot and with condiments, was well liked by the subjects. In diet #2 and in diet #1, all foods, with the exception of the mashed potatoes, were prepared in a typical fashion.

Mashed potato preparation was done by the addition of 2 t. of salt to 3200 g of peeled potatoes. The potatoes were boiled until soft (~ 20 min.), drained, and blended with 4T of margarine and ¼ cup 2% milk on slow speed (Hobart food mixer). If additional moisture was needed, cooking water was added. The potatoes were then blended at higher speeds to achieve the desired texture and volume. Potatoes were then stored for up to two days in the refrigerator in plastic wrapped bowls. Potatoes were reheated using a microwave oven. Because a microwave oven was available at all times, subjects could heat up any foods they felt were too cool. Frozen green beans used were heated in plastic bowls using a microwave oven. The ham used was baked in glass dish in a conventional oven. Since the ham was precooked, baking times varied to provide a hot meal with the desired qualities. Because subject likes and dislikes surrounding food were so diverse, we made every concession possible when it came to cooking time and food appeal.

Sample Collection

Food Composites

Food composites were collected prior to the beginning of the study and again after each experimental period. For the composite, all food items were weighed and placed into a 1 gallon blender (Waring model 34BL22, New Hartford, Conn.). The total volume in the blender was brought up to a round number with distilled water then the mixture pureed for approximately two minutes. Samples were then either distributed into 25 g

allotments for immediate analysis or frozen for later use using 4 oz polypropylene jars sealed with parafilm.

Urine Collection

Twenty-four hour urine samples were collected a total of six times during the study with one each on days 8,9, and 10 of the two 10 day diet periods. Collection was done by providing the subjects with labeled, acid washed, one liter polypropylene bottles. Subjects were able to use as many bottles as needed and were given access to a rest room. Our facilities contained an easy access refrigerator for urine storage purposes. Urine collections began after breakfast each morning.

Urine bottles were collected from each subject on the mornings following the 24-hour collections and mixed together in a 10 liter plastic bucket with a plastic spoon. Total urine volume was recorded for each subject using a 2 L pyrex graduated cylinder Samples were allocated to either 5 mL freezer vials or 30 mL polypropylene bottles depending on the analysis to be performed. Urine was transferred using a disposable 25 mL polystyrene pipette fitted with a pipetting device. The 5 mL freezer vials were then frozen at -80 degrees C for later analysis.

Preservatives were used in the bottles on days 8 and 10 of each period. On day 8, 3 mL of glacial acetic acid was added to each collection bottle for the preservation of ammonia and urea nitrogen for later analysis by another researcher. On day 10, each bottle contained 1 mL of mineral oil along with 0.75 mL of 10% thymol in 2-propanol. This preservative was used for pH determination. Day 9 of each period was kept

preservative free for the analysis of the bone turnover biomarker deoxypyridinoline. Urine collection on days 9 and 10 of each period were acidified with 5 mL concentrated HCl before aliquots were removed for later analysis of Ca, P, SO_4, N, and creatinine.

Blood Collection

Fasting blood was collected twice during the study, once on day 10 of each study period. Prior to breakfast, blood was withdrawn from the antecubital vein by a certified medical technologist using two-10 milliliter Vacutainer tubes (silicone-coated interior, no additives. Terumo Medical, Elkton, MD). These tubes were used to draw approximately 8 mL of blood. The blood was then allowed to clot in the tubes at room temperature. Within two hours, the blood was centrifuged (Beckman model TJ-6, Palo Alto, CA) at 2000 x g for 10 minutes. Serum was then transferred using a serum siphon tube to the appropriate pre-labeled 2 mL freezer vial depending on the analysis to be performed. Serum to be analyzed for parathyroid hormone was stored at -80 degrees C.

Methods

Most common reagents were purchased from either Sigma Chemical Company, St. Louis MO, or J.T. Baker Co., Phillipsburg, NJ. All glassware intended for mineral analysis was made of Pyrex glass which had been acid washed in a 5% nitric acid bath, rinsed with distilled water and dried prior to use. Deionized water was used for reagent preparation and required dilutions.

Phosphorus

Principle of the Method

Phosphorus was analyzed by the colormetric procedure used by Fiske and Subbarow (1925).

In this procedure, phosphomolybdate is formed when a trichloroacetic acid (TCA) supernatant of the prepared sample is treated with ammonia molybdate in an acid solution. The next step is to produce a phosphomolybdenum blue complex using a Fisk and Subbarow solution consisting of sodium bisulfite, sodium sulfite, and 1-amino-2-naphthol-4-sulfonic acid. This colorful solution can be analyzed using a spectrophotometer (Beckman DU-40, Irvine, CA) set at an absorbance of 660 nm because the intensity of color is proportional to the phosphate concentration.

Reagents

Acid Molybdate Solution. Concentrated H_2SO_4 (14 mL) was added to approximately 150 ml of deionized water and the solution allowed to cool. To this solution, 2.5 g of ammonium molybdate • $4H_20$ was added and the volume raised to 200 mL and the solution stored in a polypropylene bottle at room temperature.

Fiske and Subbarow Solution. Sodium bisulfite (5.85 g), sodium sulfite (0.20 g), and 0.10 g of 1-amino-2-napthol-4-sulfonic acid were dissolved in 40 mL of deionized water while being heated. The solution was stored in a brown glass bottle at room temperature.

Phosphorus Standard Solution (1mg/mL). KH_2PO_4 was dried for two hours at 105 degrees C prior to use. The dried KH_2PO_4 was then dissolved in deionized water and 3 mL of concentrated sulfuric acid was added as a preservative. The solution was then brought up to 1 liter total volume. A working stock (20 ug/mL) was prepared by dilution.

TCA (20%). Twenty grams of TCA was dissolved into 100 mL of deionized water and refrigerated until needed.

Analytical Method

Diet

Diet homogenates (25g) were wet ashed in reagent grade nitric acid while being heated. The ash was dissolved in 3 M HCl with gentle heat and made to 25 ml with deionized water and stored for later use. The ash was analyzed in the same manner as described below for urinary phosphorus determination.

Urine

For analysis of urinary phosphorus, 0.5 mL of pre-diluted urine was mixed with 2.5 mL deionized water and 2.0 mL of 20% (w/v) TCA. This mixture was allowed to stand for 5-10 minutes on ice and then centrifuged at 4 degrees C until clear.

For experimental analysis, 2.0 mL of TCA sample supernatant, 3.0 mL deionized water, and 1.0 mL of acid molybdate solution was added to samples, standards, and blank test tubes (blank contained 2.0 mL of 20% TCA). Following adequate mixing, 0.25 mL

of Fiske and Subarow solution was added to each tube. The mixture was mixed again and allowed to stand at room temperature for 10 minutes for color evolution. This solution was then aspirated into a Beckman DU-40 spectrophotometer (Irvine, CA) on a sipper setting of 30. Standards were prepared from the working stock ranging from 5 to 25 μg phosphorus.

Serum

Serum analysis was performed in the same manner as the urine described previously. For this experiment, 0.5 mL of serum was used in place of 0.5 mL of urine.

Calcium

Principle of the Method

Calcium was analyzed using an atomic absorption spectophotometer (Perkin-Elmer model No. 2380, Norwalk, CT). The instrument works by detecting the amount of light absorbed by atoms of calcium produced when the solution is aspirated into an air acetylene flame. Calcium atoms absorb a spectral light source at a wavelength of 422.7 nm. As the number of calcium atoms increases, the amount of light absorbed increases. Possible interference with production of free calcium atoms by phosphate is eliminated by addition of lanthanum to analysis solutions.

Reagents

Lanthanum Solution (5% Lanthanum in 1M HCl). $LaCl_3 \cdot 7H_2O$ (13.37g) was dissolved in deionized water, 33 mL of 3M HCl was added, and the solution was diluted to 100 mL with deionized water.

Blank Solution. Lanthanum solution (10 mL) was diluted to 50 mL with deionized water.

Standard Solution (5 mg/L). Calcium standard solution (1000 mg/L, EM Science, Gibbstown, NJ), 0.25 mL, and 10 mL of lanthanum solution were diluted to 50 mL with deionized water.

0.1% La in 0.06 M HCl. Lanthanum solution was diluted 1:50

Analytical Method

Diet

Calcium was determined on the wet-ashed sample previously described by the method of Willis, 1961 as described below for urine.

Urine

An appropriate dilution of sample and 1.0 mL of 5% lanthanum chloride solution were combined. This solution was diluted to 5 mL with deionized water and then analyzed using the AAS. Results were obtained by comparison to a series of calcium standard solutions (also in a 1% lanthanum chloride solution) run through the AAS.

Serum

Serum samples were diluted 1:50 using a 0.1% lanthanum diluent in 0.06% HCl. All standard solutions were made in a similar fashion and the blank used was a 0.1% lanthanum solution (Trudeau and Freier, 1967).

Creatinine

Principle of the Method

Urinary creatinine has long been recognized as an accurate measure of the completeness of urinary output (Bingham and Cummings, 1985). To normalize bone health biomarker levels it is common to report results in terms of nM of bone indicator per mM of creatinine. The assay used is based on the reaction of creatinine with an alkaline picric solution to form a red Janovinski complex. It is this complex that can be measured in terms of light absorbance at a wavelength of 520 nm (Bingham and Cummings, 1985).

Reagents

Sodium Hydroxide (10%). 10 grams of NaOH were dissolved in distilled water, diluted to 100 mL and stored in a polypropylene bottle at room temperature.

Picric Acid (1%). 1 gram of picric acid dissolved in distilled water and diluted to 100 mL.

Alkaline Picric Solution. 10 mL of 10 NaOH and 10 mL of the 1% picric acid solution were mixed and diluted to 100 mL. Solution is only prepared when needed.

Creatinine Standard Solution 1 mg/mL). The standard was made by dissolving 0.1 g of creatinine in 0.1 N HCl and made to 100 mL with 0.1 N HCl. The solution is stored under refrigeration.

Analytical Method

The method used was described in 1919 by Folin and Wu. Urine samples were pre-diluted to contain less than 40 µg of creatinine per mL. To each 0.5 mL of diluted sample, 1.5 mL of deionized water and 2.0 mL of alkaline picrate solution was added. Samples were allowed to sit at room temperature for exactly 15 minutes. Following gentle mixing, 6.0 mL of deionized water was added. Blank tubes were analyzed using 2.0 mL of the alkaline picrate solution with 8.0 mL of deionized water. Samples were analyzed by comparison of results to known creatinine solutions determined at the same time. Absorbance was read at 520 nm (Beckman DU-40 spectophotometer with sipper setting at 20).

Nitrogen

Principle of the Method

For the analysis of total nitrogen, concentrated sulfuric acid was reacted with either dietary composites or urine forming carbon dioxide and ammonium sulfate in the

general reaction $2NH_3+H_2SO_4$ ----> $(NH_4)_2SO_4$. The ammonium sulfate was then made basic by the addition of sodium hydroxide. The alkali then replaced the ammonia which was distilled into boric acid. With indicator dye added, the boric acid/nitrogen solution was titrated with 0.1230 N HCl to determine nitrogen content.

The reaction described here was modified by Scales and Harrison (1929) and has been used for decades in many laboratories. This experiment, like many that require titration and color determination, relies on the judgment of the experimenter to determine when equilibrium between acid and base has occurred.

Reagents

Concentrated Sulfuric Acid.

Potassium Sulfate.

$CuSO_4 \cdot 5H_2O$.

Fresh Mossy Zinc.

40% NaOH. NaOH (40.0g) was dissolved in deionized water and made up to 100 mL.

4.0% Boric Acid. Boric acid (4.0 g) was dissolved in deionized water and made up to 100 mL.

Standardized HCl. HCl was standardized to 0.1230 normality.

Indicator Dye. Bromcresol green (50 mg) and methyl red (10 mg) were dissolved in 60 mL of ethanol and stored at room temperature.

Analytical Method

For diet analysis, between 6.0 and 6.5 g of food composite slurry was used. Because of the variability in color determination, all samples were run in duplicate with a mean value used for statistical analysis.

Using 500 mL Kjeldahl flasks, the procedure starts by the addition of two glass beads, 10 g of potassium sulfate and 0.2 g of $CuSO_4 \cdot 5H_2O$. Next, the food sample or known standards (in this case spray dried egg white) are added to the flask. Under a hood, the next step was to add 20 mL of concentrated HCl. The Flask was then set on a Kjeldahl rack (Labconco, Lawrence MA) and allowed to boil to a clear or slightly green color. For food composite specimens, this process took from 40 to 90 minutes depending on the temperature setting. In our trials, we found an initial setting of 3-5 was best for the first 30 minutes. After this initial time period, the temperature setting was increased to maintain gray fumes. Once the solution had turned light green to colorless, the mixture was allowed to boil for an additional 30 minutes.

The flasks were then removed from the Kjeldahl rack and allowed to cool under a hood. Once cooled to room temperature, 150-200 mL of distilled water was added making sure to wash down the sides of the flask. The flasks were then allowed to re-cool after the water addition.

Next, 50 mL of 4% boric acid was placed into a 250 mL Erlenmeyer flask (one Erlenmeyer flask per Kjeldahl flask). The flask was then placed on the Kjeldahl rack under the glass tubes so that the tip of the tube was under the boric acid. The heat units of the rack were then turned on and slowly, 90 mL of the 40% NaOH was added to each flask

without mixing. Immediately, a few pieces of fresh mossy zinc which serve as a catalyst for the reaction, were dropped into the flask which was then returned to the rack.

The contents were allowed to distill into the boric acid until the level of the flask contents reached 100 mL. To this distillate, 5 drops of indicator dye were added and the mixture titrated with 0.1230 N HCl.

Sulfate

Principle of the Method

The analysis technique used was based upon the work by Ma and Chan (1973). The basic equation $BaCl_2 + SO_4^{2-} \text{----> } BaSO_4 + 2Cl^-$ is followed to yield an even suspension of the insoluble barium sulfate ($BaSO_4$). The suspension is held in a dextran solution {Dextran T70 Mw ~70,000 (light scattering), Pharmacia LKB, Biotechnology AB, Uppsala, Sweden}. The more sulfate in the urine, the more barium sulfate formed, and thus more is held in suspension. This will then in turn increase the relative cloudiness of the liquid. This turbidity can then be read with a spectrophotometer at 650 nm.

Reagents

5% TCA TCA (5.0 g) was dissolved into 100 mL of deionized water.

1% $BaCl_2$ (Barium chloride) in 10% Dextran solution. For this solution, 15.0 g of dextran was dissolved in 150 mL of deionized water. Barium chloride • $2H_2O$ (1.0 g) was then

dissolved into 100 mL of the 10% dextran solution. The mixture was centrifuged (Beckman DU Model TJ-6, Palo Alto, CA) at room temperature and 3000 times g for 10 minutes prior to use.

Na_2SO_4 (Sodium Sulfate) Stock Standard Solution (25 mEq/L). Sodium sulfate (0.1776 g) was dissolved into 100 mL of deionized water.

Analytical Method

Test tubes containing 0.5 mL of pre-diluted urine were prepared. To each, 2 mL of 5% TCA was added to precipitate any protein in the urine. The sample was then centrifuged at 3000 times g for 10 minutes at room temperature. Then, 2.0 mL of urinary supernatant was removed and to it was added 0.5 mL of 1% barium chloride in a 10% dextran solution. The solution was mixed and allowed to stand for 10 minutes prior to reading (Gilford model #252 spectrophotometer in a disposable polystyrene cuvette with a 1 cm light path) at 650 nm (slit = 0.02 mm; full aperture). The blank contained 2.0 mL of TCA and 0.5 mL of the 1% barium chloride solution in dextran. Working standards were obtained from the stock standard solution, ranging from 2 to 10 mEq/L. All of this work was done in the Milam Hall laboratory.

Parathyroid Hormone (PTH)

Principle of the Method

The immunoassay kit used to determine PTH in serum was obtained from Nichols Diagnostics (San Juan Capistrano, CA). The procedure measures only intact molecules of PTH. It is well known that proteolytic modification of PTH leaves fragments in human blood. These fragments can alter results and give erroneous readings (Nussbaum et al., 1987). To combat this problem, the intact PTH immunoassay is a two-site immunoradiometric assay (IRMA). This process provides a good deal of accuracy as well as a high degree of sensitivity (1 pg/mL).

In the first step, one Ig molecule is attached to a glass bead. The hormone binds to this molecule and then a second radiolabeled (^{125}I) Ig molecule is added in solution to the beads. Only intact PTH molecules will bind to both the bead and the second radiolabeled Ig protein. The beads can then be washed and read in a gamma counter (Beckman Instruments, Model Gamma 8000, Fullerton ,CA).

From these readings a dose response curve of radioactivity vs. concentration of PTH is generated when compared to the results of standards which are run at the same time as the samples.

Reagents

The materials below were provided in the Nichols intact PTH kit.

PTH Antibody Coated Beads. 100 count of goat anti-human coated polystyrene beads.

^{125}I-PTH Antibody Solution. 11 mL of labeled goat anti-human PTH antibodies in phosphate buffered saline and 0.1% sodium azide.

Intact PTH Standards. A complete standard series, including a zero standard was provided in the same medium as the labeled solution.

Wash Solution Concentrate. Provided was 50 mL of a surfactant in phosphate buffered saline with 0.2% sodium azide.

Intact PTH Controls. A series of control solutions was provided in the same media as previously described for the labeled solution.

Analytical Method

Into labeled tubes, 200 µL of samples, standards, or controls were pipetted. Next, 100 µL of the labeled solution was added and the solution mixed. One antibody coated bead was then added to each tube using a bead gun supplied by Nichols Institute and then the tube was covered. Following an incubation of 22 hours +/- 2 hours at room temperature, the beads were rinsed with the wash solution twice using Nichols Institute decanting device (catalog # 39-8190) and then read in a gamma counter within one hour.

Deoxypyridinoline (DPD)

Principle of the Method

The method used to determine the amount of pyridinoline in subjects urine samples is termed an enzyme linked immunosorbant assay or ELISA. The ELISA used here was developed by Metra Biosystems (Palo Alto, CA).

The test uses highly sensitive antibodies to detect small amounts of bone resorption proteins in the urine. The test kit contains a plate with small wells aligned in strips. The bottom of these wells is coated with an immunoglobulin (Ig) or antibody from a rabbit (Seyedin et al., 1993) To obtain this antibody, mouse immunoglobulin G (IgG) proteins were injected into a rabbit. The rabbit then reacted to make antibodies against the foreign mouse protein. The assay works because the mouse IgG that was injected into the rabbit is itself an antibody that was made to react to (adhere to) human DPD. From completion of the assay, the rabbit anti-mouse antibody is affixed to the bottom of the wells. Mouse IgG is then added to the wells. This step causes the IgG to bind to the rabbit anti-mouse IgG and form a two antibody stack. This stack has the mouse IgG 'sticking out', waiting to bind to human DPD.

To quantitatively measure the amount of DPD that would bind to the mouse anti-DPD antibody, DPD is first conjugated with an enzyme that will catalyze a colormetric reaction. The enzyme used here is alkaline phosphatase. Once the DPD-enzyme conjugate is attached to the well, addition of the substrate will cause a

yellow color formation that is proportional to the amount of DPD found in the subject urine.

Reagents

The following material was provided in the DPD kit from Metra Biosystems:

Substrate Tablets. The tablets (20 mg) were dissolved in the assay buffer to provide the *p*-nitrophenyl phosphate enzyme substrate.

10X Wash Buffer. This stock solution of wash buffer is diluted 1 to 10 with deionized water to provide a working wash buffer.

Stop Solution. The solution used to stop the reaction in 1 N NaOH.

Assay Buffer.

Standards and Controls. The kit came equipped with a series of standards and controls to produce a standard curve for analysis of unknowns.

Analytical Method

To perform the assay, all samples, standards and controls were diluted 1 to 10 with assay buffer. Next, 50 µL of the diluted liquids were added to the wells. To each of the wells, 100 µL of the enzyme conjugate was then added and allowed to incubate for 2 hours in the dark and at 2-8 degrees C. To remove the enzyme solution, the next step was to wash the wells three times with diluted wash buffer. Working substrate (150 µL) was added to each well and incubation for 1 hour at room temperature followed. The last procedure was to add 100 µL of the stop solution and read the plate at an optical density

of 405 nm within 30 minutes. The microplate reader used (Titertek Multican MC, Flow Laboratories Inc., Englewood, CA) was on loan to us from the Oregon State University College of Veterinary Medicine.

Statistics

Hall (1983) describes the number of subjects needed to perform a study with the variability of the assays performed here. In this case, 6-8 subjects were needed to perform such an experiment with the hopes of finding statistically significant data. Because each subject ate from both diet periods, they served as their own control group. Significance was assigned to any P value less than 0.05. Results were to be expressed as the mean +/- SD (n=8) based upon a paired t-test (statistical reference for paired t-test).

Human Subjects Approval

This study was approved by the Oregon State University Institutional Review Board for the protection of human subjects. The application provided all necessary information including procedures, benefits and risks to the subjects. All subjects signed an informed consent form prior to the beginning of the study (Appendix 6).

Results

Subject Characteristics

Eight subjects were chosen to participate in the present study. All of the subjects came from the Oregon State University community and lived in Corvallis, Oregon during the study period. One subject dropped out near the end of the first experimental period for reasons unrelated to the study. The remaining seven subjects completed all tasks related to the study. Subject characteristics are listed in Table 6. No significant changes occurred in the subject's weight or health during the experimental period. This was largely due to our constant communication with the subjects during the study. If a particular subject felt hungry, we could add hard candy or extra mayonnaise to their diet to boost caloric consumption without changing the nutrient profile.

Table 6
Subject Characteristics

Subject	Age	Height (cm)	Weight (kg)	Nationality
1	28	173.5	69.8	Caucasian
2	23	178.3	76.4	Caucasian
3	29	170.5	56.4	French
4	31	163.5	61.8	Albanian
5	25	180.5	70.4	Spanish & French
6	26	182.5	89.3	German
7	22	176.0	71.1	Chinese
Mean + SD	**26 +/- 3**	**175.0 +/- 6.5**	**70.7 +/- 10.5**	

Analysis of Diets

With one exception, the diets provided to the subjects contained the required nutrients to support life in measures that were in agreement with current guidelines. The one exception being phosphorus which was adjusted to fit the study parameters. The goal was to provide 800 mg/day of phosphorus for diet one and 1600 mg/day for diet two. Since all other nutrient levels were controlled, the phosphorus level adjustments between the two diet periods would produce the treatment effect we sought. Chemical analysis of the diets showed that both were within 5% of the calculated values. For calcium, the study was to provide a level that never dropped below a calcium to phosphorus ratio of 0.7 (Anderson & Barrett, 1994). Upon analysis, we found that indeed, both diets provided approximately 1200 mg of calcium per day and that for diet two (high phosphorus) the calcium to phosphorus ratio was 0.75.

Both menus were to provide approximately 90 grams of protein per subject per day. This level is considered within normal values for males in the age range of the subjects. Since we had performed a pre-study questionnaire, we knew that none of the subjects were involved in vigorous weight lifting or aerobic activity that would require greater protein consumption. Using Kjeldahl nitrogen analysis, the 90 grams of protein per subject per day goal was confirmed (14.5 g nitrogen by analysis times 6.25 g protein).

Urinary Analysis

Urinary Phosphorus

Urinary phosphorus excretion was measured as dictated in previous sections of this paper. Analysis values are shown in Table 7. As shown in the table, phosphorus excretion went up in all seven subjects with an approximate doubling in many. The mean change from diet one to diet two was 17.4 mmol/day to 31.6 mg mmol/day respectively. This represents a change of 82%.

Table 7
Urinary Phosphorus Excretion (mmol/24-hour)

Subject		800mg P/day				1600mg P/day		
	Day 8	9	10	Average	18	19	20	Average
1	12.7	15.7	11.5	13.3	30.8	35.2	32.8	32.9
2	23.5	12.9	24.0	20.1	39.0	26.4	30.4	31.9
3	17.6	12.3	14.8	14.9	28.9	21.7	27.0	25.9
4	20.0	19.8	18.5	19.4	29.8	28.1	25.0	27.6
5	19.4	16.1	14.4	16.6	32.6	24.0	28.1	28.2
6	9.74	13.0	20.3	14.3	35.1	36.9	30.2	34.1
7	26.5	22.6	19.2	22.8	45.8	41.7	35.2	40.9
Overall Mean +/- SD		**17.4 +/- 4.6**						**31.6 +/- 6.0**
Overall Increase (%)				**+82**				
Overall Significance				**P < 0.001**				

Urinary Calcium

Calcium excretion decreased as was expected. Between diet one and two, urinary calcium losses decreased by 38% for a mean shift of 6.56 mmol/day to 4.06 mmol/day. Table 8 shows the values for each subject.

Table 8
Urinary Calcium Excretion (mmol/24-hour)

Subject		800mg	P/day			1600mg	P/day	
	Day 8	9	10	Average	18	19	20	Average
1	4.61	4.02	3.95	4.19	2.33	2.67	2.18	2.39
2	7.38	6.81	7.40	7.20	3.36	3.80	3.06	3.41
3	7.46	7.46	7.30	7.41	4.78	4.65	4.98	4.80
4	7.04	7.44	6.99	7.16	5.83	4.88	4.34	5.02
5	6.40	7.19	7.37	6.99	4.25	4.22	5.63	4.70
6	5.52	5.91	6.34	5.92	4.13	3.45	3.34	3.64
7	7.44	7.14	6.61	7.06	5.02	4.79	3.65	4.49
Overall Mean +/- SD		**6.56 +/- 1.13**						**4.06 +/- 1.02**
Overall Depression (%)					**-38**			
Overall Significance					**P < 0.001**			

Urinary Sulfate

To better judge the protein equality of the two diet periods, urinary sulfate was measured. The values obtained support a normal intake range (Ma & Chan, 1973) and were found to be roughly equal in both diet periods. As Table 9 demonstrates, urinary

sulfate excretion values were 52.1 mEq/day for diet 1 and 51.4 mEq/day for diet two. These findings support the study goal of providing two protein equivalent diets.

Table 9
Urinary Sulfate Excretion (mEq/24-hour)

Subject		800mg	P/day			1600mg	P/day	
	Day 8	9	10	Average	18	19	20	Average
1	45.6	48.4	50.3	48.1	46.9	50.2	51.7	49.6
2	51.6	51.5	50.9	51.3	53.4	54.4	51.6	53.1
3	53.4	56.4	55.9	55.2	51.1	53.8	51.7	52.2
4	53.5	53.9	51.9	53.1	50.6	52.6	48.5	50.6
5	53.0	56.1	54.0	54.4	54.2	57.9	56.7	56.3
6	54.3	52.6	51.0	52.6	53.0	49.6	51.9	51.5
7	48.3	51.7	49.0	49.7	46.7	46.0	46.5	46.4
Overall Mean +/- SD		**52.1 +/- 2.8**						**51.4 +/- 3.2**
Overall Significance					**NS**			

Urinary Creatinine

To better gauge the completeness of urine collection and for use with the bone resorption marker assay, urinary creatinine values were determined. Table 10 shows that creatinine levels remained remarkably constant between the two dietary periods and were within the normal values expected (Searcy, 1969).

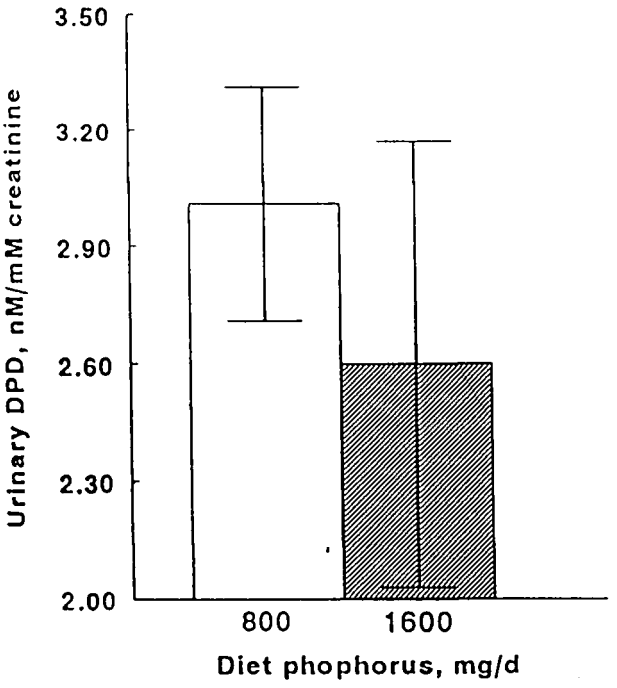
Table 10
Urinary Creatinine Excretion (mg/kg body weight/24-hour)

Subject	Day 8	800mg P/day 9	10	Average	18	1600mg P/day 19	20	Average
1	24.0	19.6	24.5	22.7	24.1	22.9	21.4	22.8
2	24.9	19.3	25.4	23.2	24.8	17.7	18.0	20.2
3	24.7	21.9	21.7	22.8	23.4	18.7	23.8	22.0
4	21.6	20.8	21.5	21.3	23.9	20.1	19.1	21.0
5	18.4	21.4	21.3	20.4	24.7	19.1	24.9	22.9
6	19.2	20.7	21.8	20.6	25.4	21.5	19.9	22.3
7	25.9	23.2	23.8	24.3	25.0	24.9	25.4	25.1
Overall Mean +/- SD		**22.2 +/- 2.2**						**22.3 +/- 2.7**
Overall Significance					**NS**			

Urinary DPD

The cornerstone of this research was to measure the change in bone resorption in response to changes in dietary conditions. It has been hypothesized that an increase in phosphorus, like the one in this study, might increase the rate of bone turnover. Figure 1 shows that in this study, DPD values actually dropped in period two. Mean values went from 3.01 nM DPD/mM Creatinine with diet 1 to 2.60 nM DPD/mM Creatinine in diet 2. While there was a slight drop, this decline was not statistically significant.

Figure 1
Urinary DPD Excretion (nM/mM creatinine)
Values are mean +/- SD, n=7



Serum Analysis

Serum Phosphorus

In all seven subjects, serum phosphorus increased between the two diet periods. As noted in Table 11, mean phosphorus values went from 1.28 mmol/L to 1.36 mmol/L in periods 1 and 2 respectively. This was a 6.2% increase.

Table 11
Serum Phosphorus Concentration (mmol/L)

Subject	mg P/day	
	800	1600
1	1.26	1.37
2	1.37	1.43
3	1.37	1.44
4	1.12	1.14
5	1.30	1.51
6	1.28	1.30
7	1.29	1.37
Mean +/- SD	**1.28 +/- 0.08**	**1.36 +/- 0.12**
Change (%)	**+ 6.2**	
Significance	**P < 0.02**	

Serum Calcium

Also measured was serum calcium. As expected, serum calcium concentrations dropped from diet one to diet two with the means shifting from 2.34 mmol/L to 2.25 mmol/L. Table 12 depicts these values for each subject.

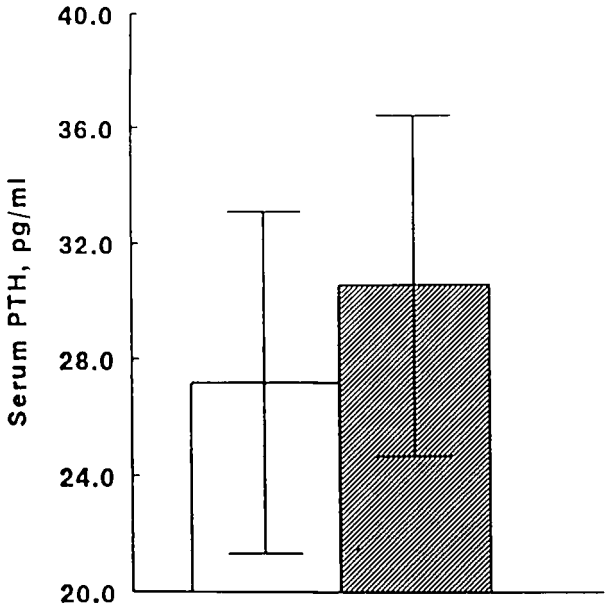
Table 12
Serum Calcium Concentration (mmol/L)

Subject	mg P/day	
	800	1600
1	2.26	2.24
2	2.29	2.24
3	2.37	2.31
4	2.34	2.27
5	2.34	2.25
6	2.52	2.24
7	2.27	2.20
Mean +/- SD	**2.34 +/- 0.09**	**2.25 +/- 0.03**
Change (%)	**- 3.8**	
Significance	**$P < 0.05$**	

Serum Parathyroid Hormone

A key indicator of bone metabolism shifting is Parathyroid hormone (PTH). In our study, as expected, PTH levels rose during the second, higher phosphate, 10-day diet period. Figure 2 shows the progression of PTH levels between the two diet periods. While there was an increase, the difference was not significant.

Figure 2
Serum PTH (ng/L)
Values are mean +/- SD, n=7



Discussion

Data strongly supporting the important role of adequate dietary calcium on bone health has existed for years. The potential adverse effects of a high phosphorus diet has also been discussed for an extended period of time. The data found in the present study support the view that while there may be adverse bone health effects of a high phosphorus diet, those effects are ameliorated or negated by a diet that is also high in calcium. We found, using sensitive bone biomarkers, that when adequate calcium was provided along with a relatively high phosphorus intake, bone resorption rates did not increase.

During our two phase experiment, where dietary phosphorus was doubled from 800 mg/day to 1600 mg/day, levels of the peptide deoxypyridinoline (DPD) were monitored. This biomarker is discarded from bone during resorption and excreted in the urine (Delmas, 1993). During times of increased bone turnover, levels of urinary DPD are also increased (Siebel et al., 1992). Our measurements found that this in fact did not happen when sufficient calcium levels were present. From diet 1 to diet 2, we found DPD levels actually dropped from an average of 3.01 nM DPD/mM creatinine to 2.60 nM DPD/mM creatinine.

This data is extremely helpful in understanding what effect the high phosphorus diet of Americans might be having on bone health (Alaimo et al., 1994). It is this high phosphorus diet, especially with low levels of calcium, that has researchers speculating that optimal bone growth and maintenance may not be occurring in many Americans (Calvo et al., 1990). Also, since the levels of phosphorus consumed may be rising and estimated consumption low, there is increasing concern that the problem may be

escalating. Some of the major sources of phosphorus in the American diet include processed foods and soft drinks (Anderson and Barrett, 1994). Since the consumption rates of these items is increasing, with no end in sight, one possible solution to the high phosphorus concern would prove to be quite valuable. Our data presented here provide one potential solution. Since we found no increase in the bone marker DPD when calcium levels were also high, the key to reducing the long term negative effect of excess phosphorus could lie in calcium intake levels. Recent additions of calcium to orange juice and supplemental calcium in antacids may be one effective strategy for increasing dietary calcium and reducing any harmful effects of excess phosphorus.

In order to monitor the single change of doubling phosphorus intake we set up two identical diets which were made up of familiar foods. It is essential that both diets contain similar amounts of other nutrients to focus in on the effects of the phosphorus. To accomplish this, diets were adjusted to equalize protein, calories, calcium, vitamin D, magnesium and zinc. Calcium levels in both diets were equalized with the addition of calcium carbonate to the bread in diet period one. In diet period two, we were careful to provide calcium and phosphorus in the from of common foods, notably, milk and cheese, rather than highly processed foods or supplements. Data supports the notion that milk consumption is correlated with bone density even though the level of phosphorus in milk is relatively high (Murphy et al., 1994) Vitamin D, magnesium and zinc were also added to the bread mix. We found making the bread ourselves gave us the freedom to add nutrients as needed and made for a pleasant way to ingest the amounts needed to secure a nutritionally equal pair of diet periods. Since the second (1600 mg P/day) diet obtained phosphorus from milk and cheese, it was essential to equalize the amount of protein in diet

one to that of diet two due to the potential hypercalciuric effect of dietary protein (Yuen et al., 1984). The addition of spray dried egg white to the beef patty and bread in diet period one accomplished this task while adding few other nutrients. This protein equalization was confirmed both by urinary sulfate excretion and creatinine values. Sulfate excretion values were found to be 52.1 mEq/day for diet one and 51.4 mEq/day for diet two. Both diets were also analyzed to confirm calcium and phosphorus levels and were found to be very close to expected values. Since any change in subject body weight would add a confounding variable, caloric levels of both diets were set at 2800 (11.7 MJ). This seemed to provide enough energy for most but single macronutrient items, like hard candy and added margarine, were available to adjust as subject hunger dictated. No significant subject weight change was observed.

Our laboratory analysis confirmed that the expected physiological changes from a high phosphorus diet were indeed occurring. In fact, all seven subjects responded unanimously in the areas of hypocalciuria ($p < 0.001$), increasing urinary phosphorus excretion ($p < 0.001$), increasing serum phosphorus ($p < 0.02$), and a small decrease in serum calcium ($p < 0.05$). These values were to be expected and support our claim that the diets were indeed as we predicted.

One important marker of this study is parathyroid hormone (PTH). As discussed earlier, it is PTH which is produced in greater quantities in response to increasing phosphorus intake (Guyton, 1991). In diet period two, when phosphorus consumption increased from 800 mg/day to 1600 mg/day, PTH levels also increased although non-significantly. This has been documented many times in the literature in both human and animal populations (Draper et al., 1972 and Reiss et al., 1970). In this study, our

group found that PTH levels increased 12.5%. The effect of this increasing PTH level on bone has been shown in the literature, most often either in association with low calcium intakes and/or with animal populations (Draper et al., 1972 and Bell et al., 1977). When PTH levels rise for any reason, calcium levels in the plasma also rise, often immediately and dramatically (Guyton, 1991). The effect of PTH goes beyond bone to increased calcium absorption in the intestine to hypocalciuria. It is the effect on the bone that is most critical for this present work. With the rise in PTH shown here, we should see an increase in the removal of bone salts from the bone matrix. This increase in bone salt removal should then again show itself by an increase in DPD levels in the urine (Delmas, 1993). Instead, we found that these bone indicators fell. Even in short term studies such as ours, an increase in PTH like the one shown here should result in increased levels of DPD in the urine. Since this was not the case, we propose that providing adequate dietary calcium was the key difference that allowed for the lack of bone loss. Calcium, at the levels we provided in both of our diet periods, provides a direct and opposite effect of the high phosphorus levels in diet two. While in the typical American diet calcium intake patterns often fall short of the RDA, our subjects consumed 1200 mg calcium per day. At this level, relatively high plasma calcium concentrations were nearly maintained and thus had a two fold anti-bone resorption effect. First, the high plasma calcium levels inhibited the formation of the active form of vitamin D while second, the calcium kept PTH levels from rising above normal levels (Guyton, 1991). This combination of effects produced a situation were plasma phosphorus was allowed to rise, followed by an increase in PTH levels but no adverse effect on bone health was seen by our biomarker survey. A decrease in DPD excretion despite an increase in PTH secretion, is in agreement with others

(Silverberg et al., 1986) who reported a reduction of urinary hydroxyproline (a measure of bone resorption) when phosphorus intake was increased. It thus is likely that the small increase of PTH, within normal levels, was part of an adjusting internal balance of phosphorus (increase in urinary PO_4)

One key factor to the success of this study was complete urine collection. Even small amounts of uncollected urine could alter DPD analysis levels as well as some nutrient calculations. For this study, we used urinary creatinine collection as a measure of completeness of urine collection. Since creatinine is a measure of muscle turnover, and is steady over time individually, it works well as a urine collection gauge. For our subjects, all were found to be within normal levels when expressed per kilogram of body weight.

Some concern could be raised that our study period was too short to show changes in bone metabolism. The data does not support this concern. First, the effects of PTH on bone metabolism are immediate. Within hours, bone metabolism shifts are noticeable and dramatic. In fact, Guyton (1991) points out that within minutes of a PTH injection, bone cells (osteocytes and osteoblasts) start to pump calcium away from the bone into the extra cellular fluid. Reiss et al., (1970) found that a single 1000 mg dose of phosphorus resulted in a 60-125% increase in PTH within one hour. While our subjects were not given this large of a bolus, it is reasonable to determine that our study length was more than sufficient to provide a hyper-PTH environment, and a negative bone health effect if one were to occur. Other data supporting our research timeline is supplied by Mosekilde (1992). Here we see that 4-10% of bone is in remodeling at any one time. This amount is sufficient to show an increase in DPD excretion should one be occurring (Delmas et al., 1993). Also, it should be noted that data show that once bone remodeling

has started, DPD excretion is remarkably stable within one individual (Seyedin, 1993). Our three day collection period at the end of our two-10 day diet periods is sufficient to note any adverse bone health effects as measured by the biomarker DPD. In this case however, none was noted.

In the present research we have shown that a high phosphorus diet does indeed bring on some significant biological changes in the human. Slightly elevated PTH levels, especially over an extended period of time are a chief concern. We have shown however, that during a two-10 day dietary study, the negative effects of a high phosphorus diet are at least partly ameliorated by a diet that is also high in calcium. This data is important because of its practicality. To reduce the intake of phosphorus in Americans would be to alter the consumption of fast foods, snack foods and soft drinks, to mention just a segment of the phosphate-providing spectrum of foods high in our everyday diets. This is extremely unlikely in the foreseeable future. Because of this, an alternate plan, and one that is attainable, is of great importance. Calcium intake levels can be increased in the American population. Through awareness, education and the addition of calcium to snack foods, processed foods and soft drinks, calcium consumption could start to equal that of phosphorus. The public health implications of this are staggering. A 20% decrease in osteoporotic fractures would save an unbelievable two-billion dollars a year and free up five-million women from this disease (Lindsay, 1996). Recent evidence, using fractures as an endpoint, supports the idea that calcium and vitamin D supplementation alone could prevent almost two-million fractures in the next five years (Blank and Brockman, 1999).

There is no easy answer. We as Americans will still eat our ready-made meals and avoid many more healthful foods for those that are quick, easy and from a can. We can

however limit the bone damage caused by these choices by increasing the calcium intake and reducing any possible excess phosphate-induced bone loss.

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APPENDICES

Appendix 1

WANT TO SAVE SOME MONEY THIS SUMMER?

WOULD YOU LIKE FREE FOOD FOR 20 DAYS DURING AUGUST?

IF YOU ANSWER YES TO THESE QUESTIONS THEN THE DEPARTMENT OF NUTRITION AND FOOD MANAGEMENT WANTS TO HEAR FROM YOU TODAY.

FROM AUGUST 9th TO THE 29TH, THE DEPARTMENT WILL BE CONDUCTING A NUTRITION STUDY WHICH WILL PROVIDE ALL PARTICIPANTS WITH THREE MEALS A DAY FOR 20 DAYS. THE BEST PART IS THAT ALL THE MEALS ARE FREE!

TO QUALIFY YOU MUST BE A MALE, 19 - 30 YEARS OF AGE, AND A NON-SMOKER IN GOOD HEALTH.

CALL 737-4175 FOR MORE INFORMATION

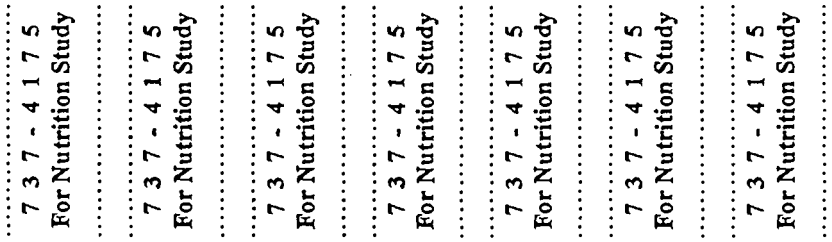
Appendix 2

FREE FOOD FOR 20 DAYS!

THE OREGON STATE UNIVERSITY DEPARTMENT OF NUTRITION AND FOOD MANAGEMENT IS CONDUCTING A NUTRITION STUDY FROM AUGUST 9th TO THE 29th. ALL PARTICIPANTS WILL RECEIVE THREE NUTRITIOUS MEALS A DAY FOR 20 DAYS ABSOLUTELY FREE!!!!!

TO QUALIFY YOU MUST BE A MALE, 19 - 30 YEARS OF AGE, AND A NON-SMOKER IN GOOD HEALTH.

CALL 737-4175 FOR MORE INFORMATION.



Preliminary Phone Questionnaire

Date of phone call _____

Name _____ Age _____

1. Do you exercise regularly? If so, what exercise, how often, and how strenuously? _____

2. Do you take any medications, or nutritional supplements? If so, which ones? _____

3. Do you typically consume 3 oz of alcohol per week or less?

_____

4. Do you smoke cigarettes or use any other form of tobacco?

_____

5. Are you allergic to any common foods? Or have lactose intolerance? _____ Are you a vegetarian? _____

6. Are you currently under the constant care of a physician? _____

7. Do you have any disorder that would interfere with absorption, metabolism, or excretion? _____ Are you anemic? _____

Common foods will be used in this study. During this study you will be asked to eat only the foods provided in Milam Hall. You will not be able to eat anything else for the entire 20 days. All three meals for 20 days must be attended for participation, there are no breaks or exceptions to the rule. While all food will be free, and all preparation and clean-up will be taken care of. urine will be collected at various points in the study, and one blood sample will be taken at the end of each 10 day diet period.

8. With these conditions in mind, are you still interested in participation? _____

What times for breakfast, lunch, and dinner are best for you?

_____

Other notes _____

Appendix 4

First Interview

First interview was scheduled between July 14 and July 20, 1994.

1. Introduction and welcome
2. Subjects were taken to Room 105 in Milam Hall, and given a quick tour to point out the clean, well lighted kitchen area, as well as the modern facilities and storage areas.
3. The health and diet questionnaire were orally discussed. It was emphasized that subjects participating in the study would not be eating at home for 20 days.
4. The menus were shown to subjects and any questions about foods were discussed.
5. Potential problems were reviewed:
   a. Not being able to party on weekends or after work.
   b. Restriction on caffeine intake (limit of one cup of coffee a day). Use of coffee candy was also discussed.
   c. Classes or work conflicts.
   d. Acceptability of meal times and possible resolution: breakfast (7:30 A.M. to 9:00 A.M.), lunch (12:00 P.M. to 1:15 P.M.), and dinner (4:45 P.M. to 6:00 P.M.)
6. Lastly, subjects asked if they have any questions and a group meeting was scheduled.

Health/Diet History

CONFIDENTIAL

Project name _____

Project dates _____

Subject name _____

Subject code # _____

Age _____ years Height _____ inches (with shoes off) Weight _____ lbs
Race _____ Marital status _____

Dietary History

a. Are you currently on a special diet? _____ yes _____ no
If yes, for what purpose? _____ weight gain or lose; control serum lipids _____; diabetes _____; allergies _____; heart condition _____; high blood pressure _____; other _____
b. Are you a vegetarian? _____ yes _____ no; type _____
c. Regular user of nutritional supplements? _____ yes _____ no.
If yes, type _____; amount _____; frequency _____
d. Any food allergies? _____ yes _____ no.
If yes, what kind of food allergy? _____
e. List foods that you will not eat (see menu):
f. List foods that you eat on a regular basis:

Habits

a. Do you smoke cigarettes? _____ yes _____ no.
If yes, how long? _____; how many each day? _____
Other tobacco products used? _____
b. Alcohol use? _____ beer _____ wine _____ spirits
how much? (include frequency) _____
c. Coffee drinker? _____ yes _____ no.
If yes, amount and frequency _____

d. Tea drinker? _____ yes _____ no.
If yes, amount and frequency _____
e. Soft-drink use (amount and frequency) _____

Exercise

a. Type of exercise activity _____
b. Frequency _____
c. Duration _____
d. Amount of food eaten to support this activity? (to determine if supplied kcal in menu would be adequate)

Medical History

a. Medical tests taken recently _____
b. Are you under the care of a physician? _____ yes _____no
c. Type of condition? (asthma, high blood pressure) _____
d. Surgical history (major operations to date) _____

Medication History

Medications taken on a regular basis? _____ yes _____ no
If yes, which one(s) _____
(Examples: sleeping aid, aspirin, cold or allergy pills, tranquilizer, diuretic, antibiotic, antacid)

Interviewer _____ Date _____

Department of Nutrition and Food Management
Oregon State University

Informed Consent

I understand that the purpose of this research is to define a mechanism by which phosphorus, an essential nutrient obtained from ordinary foods, improves the body's retention of calcium. The study will consist of two 10 day periods during which all meals will be provided for a total of 20 days.

I agree to adhere to the following rules: 1) consume only those foods and beverages prepared and served, 2) consume meals at the specified times, 3) not participate in strenuous daily physical activity such as running 5 miles/day or bicycling 15-20 miles/day, 4) record body weight daily upon arriving at breakfast-time, 5) not take medications or nutritional supplements, 6) not use tobacco, 7) give a fasting blood sample (20 mL) at the end of each of two 10 day periods, 8) collect 24-hour urine samples as instructed using the containers provided on days 8, 9 and 10 of each of the two 10 day periods (a total of 6 urine collections).

I understand that the major benefit to my participation in this study is free food for 20 days. In addition, I will be paid a single one time reward of $50 if and only if I adhere to the rules of the study and provide all of the samples requested.

All information gained from my participation in this research project will be coded and will only be seen by individuals affiliated with this project.

My participation in this research project is voluntary. I may withdraw from the study at any time. I understand, however, that I will not be paid a pro-rated reward payment if I should fail to meet all of the conditions of the study.

I understand that questions about either the research or any aspect of my participation in it can be directed to the principle investigator, Florian L. Cerklewski, telephone 737-0964. I understand that Oregon State University does not provide a research subject with compensation or medical treatment in the event that a subject is injured as a result of participation in this research project.

Persons who have ever had Hepatitis B or C, who have tested positive for HIV or any AIDS virus, or persons who have AIDS should not donate blood or any other body fluid and therefore should not participate in this study. Persons at increased risk for the AIDS virus including men who have had sexual contact with another man since 1977, persons who have used intravenous drugs, persons who have taken clotting factor concentrates for a bleeding disorder such as hemophilia, anyone who has tested positive for any AIDS virus, hepatitis B or C or any AIDS antibody, persons who have had sexual contact with a member of one of these groups, or anyone who has had sex with a male or female prostitute since 1977 also should not participate in this study.

Signed _____ Date _____

Address _____

Telephone _____

Investigator _____