AN ABSTRACT OF THE THESIS OF

Wei Ding for the degree of Master of Science in Nutrition and Food Management presented on August 03, 1995. Title: Role of Ammoniagenesis in the Hypocalciuric Effect of Phosphorus in Young Men.

Abstract approved: _____
Florian L. Cerklewski

The present study sought to define a possible role of phosphorus-stimulated ammoniagenesis, previously observed in vitro and in experimental animals, in the hypocalciuric effect of phosphorus in young men. It was hypothesized that titrating some of the hydrogen ions destined for excretion with ammonia would be beneficial to kidney calcium reabsorption because ammonium ion unlike hydrogen ion does not increase urinary calcium loss. To test this hypothesis, seven young men (22 - 31 years old and average weight of 70 kg) were fed a single menu providing 800 mg phosphorus, 1200 mg calcium, 11.7 MJ (~ 2800 kcal) and 14.5 g nitrogen for 10 days. In a subsequent 10-day period, dietary phosphorus was doubled to 1600 mg by the addition of cheddar cheese and milk to the menu. Dietary nitrogen, calcium and vitamin D were equalized for the two periods. Three 24-hour urine samples and a fasting blood sample were collected at the end of each experimental

period. Comparison of period 2 with 1 showed urinary phosphorus excretion + 82%, urinary calcium excretion – 38%, serum phosphorus + 6.2%, serum calcium – 3.8%, urinary sulfate and creatinine excretion no change. Urinary ammonia nitrogen excretion increased in all seven subjects with an average increase of 13%. Furthermore each individual increase in ammonia nitrogen excretion was found to be directly correlated with urinary phosphorus excretion ($r = 0.76$, $P < 0.05$). Urinary urea nitrogen excretion was found to vary inversely with urinary ammonia nitrogen excretion with an average decrease of 12% and urinary total nitrogen excretion decreased 14% for the entire group. Results obtained therefore offer a mechanism by which phosphorus directly improves the non-parathyroid hormone portion of tubular calcium reabsorption. The magnitude of the effects seen however suggest that phosphorus-stimulated ammoniagenesis only plays a small role in the mechanism of the hypocalciuric effect of phosphorus in young men.

Role of Ammoniagenesis in the Hypocalciuric Effect of Phosphorus in Young Men

by

Wei Ding

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed August 03, 1995
Commencement June 1996

Master of Science thesis of Wei Ding presented on August 03, 1995

APPROVED:

Major Professor, representing Nutrition and Food Management

Chair of Department of Nutrition and Food Management

Dean of Graduate School

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.

Wei Ding, Author

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to Dr. Florian L. Cerklewski, my major professor, for giving me the opportunity, knowledge and encouragement during my studies at Oregon State University. Without his guidance and incredible patience, I would never have completed my work.

Special appreciation is also extended to Dr. Lorraine T. Miller, Dr. Michael H. Penner and Dr. Chaur-Fong Chen, Graduate Council Representative, for being on my defense committee. Their constructive suggestions and comments have been very helpful in writing this thesis. I would also like to thank Dr. Jim Leklem for his advice and help with the conduct of the metabolic study.

A heartful thanks is owed to Irene Cerklewski for her help in preparation of bread and mashed potatoes. Many thanks to Karin Hardin for her help in collecting blood samples. I would like to thank Dr. Jim Ridlington for his help in the laboratory. I would also like to thank Marla Parrish for her help in obtaining cookware and dinnerware needed to conduct the metabolic study.

I would like to thank Brian Bizik for his help in conducting the analysis of urinary total nitrogen excretion, for his cooperation during the whole study and for his friendship. Thanks also to my fellow graduate students for their help, friendship and support in many ways.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF TABLES

LIST OF APPENDICES

ROLE OF AMMONIAGENESIS IN THE HYPOCALCIURIC EFFECT OF PHOSPHORUS IN YOUNG MEN

INTRODUCTION

The macro-mineral elements calcium and phosphorus account for a major portion of the inorganic phase of bone. Calcium is the most abundant divalent cation in the human (Arnaud & Sanchez, 1990). More than 99% of body calcium and 85% of body phosphorus are present in the skeleton. To achieve peak bone density prior to age 35, it is generally believed that it is necessary to have a dietary calcium : phosphorus ratio of 1:1. For example, recommended intake of calcium and phosphorus for both men and women aged 19 - 24 years old is 1200 mg/day (Committee on Dietary Allowances, 1989). Regulation of the absorption and utilization of calcium and phosphorus is tied together in common systems with 1, 25-dihydroxycholecalciferol [1, 25$(OH)_2D_3$], parathyroid hormone (PTH) and calcitonin (CT) as key hormonal factors (Arnaud & Sanchez, 1990).

Recommended intake of calcium and phosphorus is set at a high level in an attempt to reduce the frequency of occurrence and severity of the bone-losing disease osteoporosis. Osteoporosis is a disease characterized by an absolute decrease in bone mass that results in an increased susceptibility to fracture especially at the wrist, spine, and hip.

Osteoporotic fractures, although more common in women, are increasingly recognized as a significant health problem in men. Each year in the United States 1.5 million fractures involving the hip, distal forearm, spinal vertebrae, and other sites are attributable to osteoporosis, resulting in an estimated annual cost of $7-10 billion (Mitlak & Nussbaum, 1993).

One dietary factor that opposes the attainment of peak bone density is protein, because a high protein intake, typical of many Western diets, has been shown to cause increased urinary calcium excretion (hypercalciuria) and negative calcium balance. This effect of protein is largely due to the fact that when protein is catabolized as an energy source, it produces both endogenous acid and sulfate waste. Both of these factors have been implicated in the observed decrease in renal reabsorption of calcium (Yuen et al., 1984). Fortunately this protein effect is largely opposed by the coincident high dietary intake of phosphorus because phosphorus decreases urinary excretion of calcium.

The hypocalciuric effect of phosphorus is due to an increase in the efficiency of calcium reabsorption by kidney. Part of the effect is mediated by PTH and part is independent of PTH (Lau et al., 1982). The exact mechanism for the non-PTH dependent effect of phosphorus on kidney calcium reabsorption is still unknown. Limited evidence from animal studies (Yu et al., 1976; Cerklewski, 1995), however, suggests

that one way that phosphorus could stimulate the non-PTH dependent portion of calcium reabsorption would be to stimulate ammonia nitrogen synthesis (ammoniagenesis). Increased ammonia nitrogen released into the kidney filtrate could titrate an increased amount of hydrogen ions destined for excretion beyond normal levels which would in turn decrease the known interfering effect of hydrogen ions on kidney calcium reabsorption. The present study was specifically designed to test this possibility in human volunteers.

LITERATURE REVIEW

The experimental study to be described later in this thesis was designed to add new information about the mechanism of the hypocalciuric effect of phosphorus. The following review of calcium and phosphorus bioavailability is intended to describe the background of the study. In this review, bioavailability (O' Dell, 1984) has been defined as the combination of absorption and utilization where absorption equals the transport of a nutrient across the wall of the intestinal tract (usually the small intestine) and into the blood. Utilization equals the transport, tissue uptake, biotransformation, function, and routes of internal excretion.

Calcium Bioavailability

Dietary Intake and Food Sources

According to the Nationwide Food Consumption Survey (USDA, 1984) of the late 1970s, the daily calcium intake was found to be 743 mg on average for all people. Calcium intake was generally found to be higher in males than in females. To increase calcium intake, Heaney (1993) recommended that everyone consume a diet containing calcium-rich foods such as milk and other dairy foods. More than half of the calcium intake of the United States population is from dairy products (Block et al., 1985). Other sources include some leafy green vegetables

(such as broccoli, kale, and collards), nuts, corn tortillas processed with lime, tofu precipitated with calcium, soft bones of fish (such as sardines, salmon), and tips of poultry leg bones. More recently, calcium-fortified foods such as orange juice and flour, and antacids containing calcium have become important contributors to the intake of some individuals (Allen & Wood, 1994).

Absorption

Young adults in the United States may typically absorb 20 to 40% of ingested calcium (Committee on Dietary Allowances, 1989). The percentage of ingested calcium that is absorbed varies inversely with the level of calcium intake. In normal adults, for example, a calcium intake above 800 mg/day yields a calcium absorption efficiency of approximately 15% of the amount ingested (Heaney et al., 1975).

The amount of calcium absorbed depends on its interaction with other dietary constituents, and some hormones. Vitamin D, through conversion to its hormonal form, 1, 25 - dihydroxy Vitamin D, plays an important role in calcium bioavailability by stimulating absorption in the duodenum through an active transport mechanism (Holick, 1994), especially when dietary calcium intake is low (Pansu et al., 1983). The hormonal form of vitamin D probably also enhances calcium absorption in the other segments of the small intestine, but the increase is considerably less than in the duodenum (Anderson, 1991). Most of the

calcium absorbed from foods is passively and nonsaturably moved across the gastrointestinal barrier, especially in the jejunum and ileum (Anderson, 1991). Calcium can also be absorbed by the colon, but the amount is only about 4% of dietary calcium (Allen & Wood, 1994).

Dietary protein has no demonstrable effect on calcium absorption (Allen et al., 1979; Chu et al., 1975; Kim & Linkswiler, 1979; Schuette et al., 1980; Spencer et al., 1978). Phosphates, on the other hand, have been widely believed to reduce calcium absorption as a result of the formation of insoluble calcium phosphate salts in the gut. Data from several human studies, however, do not support this conclusion (Spencer et al., 1978; Zemel & Linkswiler, 1981). Human studies indicate that a high dietary intake of phosphorus, in the form of inorganic phosphate, may actually cause an increase in apparent calcium absorption especially when calcium intake is low (< 400 mg/day). A decrease in calcium absorption in man due to high phosphorus intake has been reported when there is also a very high calcium intake (2000 mg per day or more), but not when calcium intake is 1500 mg per day or less (Hegsted et al., 1981; Zemel & Linkswiler, 1981; Zemel, 1985).

Many other dietary constituents also affect calcium absorption (Arnaud & Sanchez, 1990). Lactose, for example, has been reported to enhance passive calcium absorption, although not all studies have been able to consistently demonstrate this effect (Scrimshaw & Murray, 1988). Phytate, oxalate and the cellulose and hemicellulose components of

dietary fiber can inhibit calcium absorption to some degree. Pectin does not affect calcium absorption probably because 80% of its uronic acids are methylated and cannot bind calcium (Allen, 1982).

Blood Calcium Concentration

Serum calcium concentration is highly regulated in all healthy individuals across the life cycle. The normal range of serum calcium is 8.8 - 10.3 mg/100 mL (2.20 - 2.58 mM) (Young, 1987). Plasma calcium is about equally divided into an ionized (47.5%) and protein-bound (46%) fraction. The remaining fraction is accounted for by complexed calcium comprised of calcium citrate 1.7%, $CaHPO_4$ 1.6%, and unidentified complexes 3.2%. Only ionized calcium has biological activity, and it equilibrates rapidly with protein-bound serum calcium. This protein-bound fraction, although biologically inert, may be important because it provides a readily available reservoir for calcium in the event of a short term decrease in serum calcium concentration. The complexed calcium is ultrafiltrable (diffusible), but has little quantitative importance as a reservoir for ionized calcium (Arnaud & Sanchez, 1990).

The major plasma calcium-regulating hormones are parathyroid hormone (PTH), calcitonin (CT), and the sterol hormone, 1, 25 - dihydroxycholecalciferol [1, 25 $(OH)_2D_3$]. Several other hormones such

as estrogen and testosterone, can also affect the regulation of blood calcium at different stages of the life cycle (Eriksen et al., 1988; Horowitz, 1993; Nordin et al., 1981; Norman, 1990; Stock et al., 1985; Turner et al., 1994).

A drop in ionic plasma calcium concentration stimulates the parathyroid gland to increase PTH secretion. PTH can indirectly increase intestinal absorption (long-term regulation) through enhanced formation of 1, 25 $(OH)_2D_3$ from 25(OH)D in the kidney. As a steroid hormone, 1, 25 - dihydroxycholecalciferol stimulates the nucleus of the intestinal cells to make a calcium-binding protein. PTH together with 1, 25 $(OH)_2D_3$ can also directly stimulate bone resorption and distal nephron tubular reabsorption (short-term regulation). However, only about 1% of the bone mass can be safely mobilized, and most of kidney calcium reabsorption (98%) is not under hormonal control. Another hormone, calcitonin, is secreted by the thyroid gland when plasma calcium concentration increases above the normal range. Calcitonin inhibits the release of calcium from bone. Therefore, high plasma calcium concentration inhibits PTH secretion, and increases CT secretion, which in turn decreases both bone resorption and intestinal absorption, and increases renal excretion of calcium (Anderson, 1991). For the above reasons, plasma calcium level is little affected by a change in dietary calcium intake.

Tissue Distribution

Calcium is the most abundant divalent cation in the human body (Arnaud & Sanchez, 1990) exemplified by the fact that approximately 2 percent of the body weight of an adult is calcium (Committee on Dietary Allowances, 1989). More than 99% of body calcium is present in the bone (98%) and teeth (1%), and the rest is in the extracellular fluids and soft tissues.

Functions

Because more than 99% of body calcium is present in the skeleton, calcium becomes one of the essential nutrients affecting adult bone health in addition to phosphorus, vitamins C and D (Heaney, 1993; McBean et al., 1994). The chemical structure in fully mature bone and teeth is a hydroxyapatite-like crystal, with the hypothesized formula $Ca_{10}(PO_4)_6(OH)_2$. Bone contains some other calcium salts, especially as the carbonate, but these forms decline as bone matures. In the adult, bone turns over every 5 to 6 years (Allen & Wood, 1994).

Failure to achieve peak bone density at skeletal maturity can have serious consequences later in life. Osteoporosis, in particular, is a reduction in bone mass that renders an individual susceptible to fracture with a moderate degree of trauma. In osteoporosis, matrix of bone mineral and protein is lost which results in less overall bone (Krall & Dawson-Hughes, 1994; Sowers, 1990). The more bone mass that is

available before the period of age-related bone loss, the less likely it will decrease to a level at which fracture will occur (Marcus, 1982; Parfitt, 1983). In an international investigation (Nordin, 1966) of calcium intake and osteoporotic fractures, Japanese women (average calcium intake was 400 mg/day) had the highest fracture frequency, whereas Finnish women (calcium intake 1300 mg/day) had the lowest risk. In a study by Matkovic et al. (1979), humans (all ages and both sexes) in a high calcium intake (1100 mg/day) community, had greater bone mass than those living in a lower calcium intake (500 mg/day) community. Life-long calcium intake therefore can apparently play an important role in achieving peak bone mass at maturity.

Calcium in soft tissues and extracellular fluids supports a number of vital functions (Arnaud & Sanchez, 1990; Allen & Wood, 1994). Calcium is required for transmission of nerve impulses. It is also necessary for muscle contraction because calcium ion activates adenosine triphosphatase (ATPase) that hydrolyzes ATP to make energy available for muscle contraction. Furthermore, some calcium is found to be essential for the integrity of cell membranes and to regulate cell membrane permeability. Calcium is also required in the blood clotting mechanism.

Routes of Internal Excretion

Calcium is excreted mainly by way of the feces, which includes unabsorbed dietary calcium and secreted calcium that is not reabsorbed. Approximately 3.75 mmol/day (150 mg/day) of calcium enter the intestinal lumen in secretions such as succus entericus and bile, but only about 30% of this calcium is reabsorbed so that the minimum endogenous secretion of calcium is usually about 2.5 mmol/day (100 mg/day). Calcium loss from the skin is only about 0.4 mmol/day (15 mg/day) (Allen & Wood, 1994).

A typical filtered load of calcium by the kidney, calculated as ionized calcium in plasma (4.75 mg/100 mL) x glomerular filtration rate (125 mL/minute) x 1440 minute/day, is 8550 mg per day. Nearly all of the filtered load of calcium is reabsorbed (98%) (Allen & Wood, 1994). Renal calcium transport is similar to that in the intestine. Passive transport, by which most reabsorption occurs, takes place in the proximal tubular (60%), the thick ascending limb of the loop of Henle (20 - 25%), and the descending loop of Henle (10%). Active transport, on the other hand, is found predominately in the distal convoluted tubule (5 - 10%). Urinary calcium excretion is 2.5 to 6 mmol/day (100 to 240 mg/day) and varies greatly among normal individuals. Approximately 50% of urinary calcium is in the ionized form, the remainder is

complexed with sulfate, phosphate, citrate, and oxalate (Allen & Wood, 1994).

The fraction of filtered kidney calcium that is actually reabsorbed can be significantly influenced by the dietary intake of protein and phosphorus. High protein diets, for example, have been found to cause a hypercalciuria (Yuen et al., 1984) whereas a high phosphorus diet causes a hypocalciuria (Hegsted et al., 1981). Endogenous acid production, generated from the catabolism of excess dietary protein, and sulfate resulting from the oxidation of the sulfur amino acids, methionine and cysteine, are thought to be the main cause of protein-induced hypercalciuria. Increased renal excretion of acid was found to directly decrease renal tubular reabsorption of calcium (Whiting et al., 1981; Yuen et al., 1984). The work of Brosnan and coworkers (1978) on rats and Adams et al. (1979) on humans showed that both a high protein diet and an administered acid load as ammonium chloride were associated with a negative calcium balance.

Fortunately, the hypercalciuric effect of protein is largely opposed by the coincident high dietary intake of phosphorus because phosphorus decreases urinary excretion of calcium. In contrast to the hypercalciuric effect caused by purified proteins (Johnson et al., 1970; Linkswiler et al., 1974; Margen et al., 1974; Schuette et al., 1980), high meat intake showed the lack of change of urinary calcium (Calvo et al., 1982; Spencer

et al., 1978). The lack of significant calciuria on the high-meat-protein diet was attributed to the simultaneous increase in phosphorus intake. Phosphorus has been shown to decrease urinary calcium excretion in many studies (Goldsmith & Ingbar, 1966; Goldsmith et al., 1967; Hegsted et al., 1981; Schuette & Linkswiler, 1982; Spencer et al., 1965; Spencer et al., 1978). For example, Hegsted et al. (1981) measured calcium balance in young adult males at two levels of protein (50 and 150g) and phosphorus (1010 and 2525 mg) intake, and phosphorus came from both foods and mineral supplements. At both levels of protein intake, phosphorus reduced urinary calcium losses by approximately 40%.

It is generally accepted that the hypocalciuric effect of phosphorus is largely mediated by an increase in PTH secretion. PTH increases the efficiency of calcium reabsorption in the distal region of the kidney nephron as discussed earlier. This effect is aided by the action of the active form of vitamin D, 1, 25 - dihydroxycholecalciferol.

Recommended Intake

The recommended calcium intake for adults is based on the amount of dietary calcium required to replace losses in endogenous intestinal secretions, urine, and sweat. The efficiency of intestinal absorption is estimated at 20 - 40%. The daily Recommended Dietary Allowances (RDA) for 11 to 24 years of age, in both males and females, is

30 mmol (1200 mg), and 26 mmol (800 mg) for ages older than 24 years (Committee on Dietary Allowances, 1989).

Phosphorus Bioavailability

Dietary Intake and Food Sources

Unlike calcium, phosphorus intake is almost always above recommended levels because this mineral element is available in a large variety of foods. In the United States, the average daily intake of phosphorus is about 1500 mg for males and 1000 mg for females (Allen & Wood, 1994). About 60% of total phosphorus intake is from milk, meat, poultry, fish and eggs, 20% from cereals and legumes, and 10% from fruits and fruit juices. Some beverages such as coffee, tea, and cola beverages provide 3% of daily phosphorus intake (Block, 1985).

Phosphorus is also found in some food additives (Allen & Wood, 1994). Use of highly processed foods such as soft cheeses, refrigerated breads and pastries, processed meats, cola beverages and snack foods can significantly raise total phosphorus intake. Polyphosphate (any substance of formula $HO\text{-}[PO(OH)\text{-}O\text{-}]_n\text{-}H$ or its derived anions and esters) food additives are useful because they retard food spoilage, reduce staling and prolong product shelf-life. Phosphoric acid is commonly used to enhance the flavor of cola drinks by contributing a rapidly perceptible sharpness. Phosphoric acid is also frequently used in

processed foods to enhance the effectiveness of the antioxidants that prevent rancidity and browning.

Absorption

About 60 to 80% of dietary phosphorus is absorbed (Allen & Wood, 1994). Intestinal phosphorus absorption is accomplished by two mechanisms (Berner & Shike, 1988). One is a saturable, sodium-dependent active transport process which occurs mainly in the proximal intestine. The other route is nonsaturable and linearly related to the phosphorus concentration in the intestinal lumen. It operates mainly in the jejunum and ileum. Because intracellular phosphorus levels are high and the cell interior is electronegative, it is probable that the active transport process is the predominant pathway to get phosphorus into the cell, but that phosphorus may exit the cell by diffusion.

The intestinal absorption of phosphorus is influenced by the endocrine system (Arnaud & Sanchez, 1990; Berner & Shike, 1988). Although parathyroid hormone does not appear to play a direct role in regulating the absorption of phosphorus across the intestine, it does exert an indirect enhancing effect on intestinal phosphate absorption by stimulating kidney synthesis of the active metabolite of vitamin D, 1, 25 $(OH)_2D_3$ (Anderson, 1991). The active transport of phosphorus can be enhanced by either 1, 25 $(OH)_2D_3$, or generally by all vitamin D metabolites, but 1, 25 $(OH)_2D_3$ seems to be the most active one in the

enhancement of phosphorus intestinal absorption (Boyle et al., 1973; Chen et al., 1974; Kabakoff et al., 1982; Kowarski & Schachter, 1969).

The intestinal absorption of phosphorus is in part dependent upon the phosphate source (Schuette & Linkswiler, 1982; Zemel & Linkswiler, 1981; Zemel, 1985). Inorganic phosphate is rapidly absorbed which causes an immediate rise in circulating phosphorus levels, whereas ingested polyphosphates must be hydrolyzed by an intestinal alkaline phosphatase (Ivey & Shaver, 1977). A possible consequence of incomplete hydrolysis of polyphosphates is that calcium can form insoluble complexes with polyphosphate in the intestinal lumen, thus decreasing the bioavailability of both calcium and phosphate (Clark, 1969).

Blood Phosphorus Concentration

About 70% of blood phosphate is in the organic form as a constituent of phospholipids. The remaining 30% is inorganic phosphate. In contrast to plasma calcium, only 15% of plasma phosphate is bound to proteins. At physiological pH, the ultrafiltrable portion consists mainly of HPO_4^{2-} (50%), 10% as $H_2PO_4^{1-}$, 40% as a component of sodium, calcium, and magnesium salt, and less than 0.01% as PO_4^{3-} (Berner & Shike, 1988). Plasma phosphate concentration has a wide range of normal values at 2.5 - 5.0 mg/100 mL (0.8 - 1.6 mM) (Young, 1987).

Plasma phosphorus reflects the net rate of phosphorus flux between intestine, kidney, bone, and soft tissue. Its level is primarily regulated by altering the rate of phosphorus reabsorption of the filtered phosphorus load in the kidney (Arnaud & Sanchez, 1990). When plasma phosphorus concentration is lower than the normal range, the kidney must increase renal phosphorus reabsorption to minimize urinary phosphorus losses, the intestine must increase phosphorus absorption, and the bone can also redistribute phosphorus to the blood and soft tissues (Berner & Shike, 1988).

Plasma phosphorus concentration is also regulated hormonally (Calvo, 1993; Berner & Shike, 1988). High plasma phosphorus concentration stimulates parathyroid gland to secrete PTH. PTH then decreases phosphorus reabsorption by the kidney which causes increased renal phosphate excretion (Allen & Wood, 1994). A vitamin D metabolite, 24, 25$(OH)_2D_3$ is also thought to increase bone mineralization which removes phosphate from the plasma into the bone to decrease phosphorus concentration in plasma (DeLuca, 1973; Ornoy, 1978).

Tissue Distribution

Phosphorus is the sixth most abundant element in the human body after oxygen, hydrogen, carbon, nitrogen, and calcium (Berner & Shike, 1988). Of the 700 g phosphorus found in the adult male body

(Committee on Dietary Allowances, 1989), approximately 85% is in bone, 14% in cells in soft tissues, and 1% in extracellular fluids.

Functions

Like calcium, phosphorus plays a prime structural role in the inorganic salts that contribute rigidity to bones and teeth. The ratio of phosphorus to calcium in the bone is normally 1 : 2 (Arnaud & Sanchez, 1990).

In soft tissues, phosphorus plays different roles (Allen & Wood, 1994; Berner & Shike, 1988). Phosphorus is an essential part of the nucleic acids in deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). The main energy source for metabolic processes and muscle contraction comes from the release of high energy phosphate by hydrolysis of adenosine triphosphate (ATP). In the mitochondrion, phosphate-containing proteins play essential roles in the electron transport system. Cyclic adenosine monophosphate (cAMP) is an important secondary messager to mediate the intracellular effects of polypeptide hormones. Intracellular phosphorus, along with proteins, ties-up (buffers) hydrogen ion generated in metabolism to maintain acid-base balance. Phosphorus functions in the phosphate group to activate substances, such as glucose-6-phosphate, necessary for a variety of metabolic processes. Phospholipids are a constituent of cellular membranes and are used as lipoprotein transport agents for lipids.

Routes of Internal Excretion

A typical filtered load of phosphate, calculated as plasma phosphate (3.0 mg/100 mL) x 85% filtrable (15% is protein-bound) x glomerular filtration rate (125 mL/minute) x 1440 minute/day], is 4590 mg phosphate/day. More than 80% of filtered phosphorus is reabsorbed. Phosphorus reabsorption occurs primarily in the proximal tubule (60 - 70%). The remaining 20 - 30% of filtered phosphorus is reabsorbed from the distal tubule (Allen & Wood, 1994). The primary regulator of the rate of renal phosphorus reabsorption is the plasma phosphorus concentration.

The kidney is the main regulatory organ for maintenance of phosphate balance. In the healthy human adult, the kidney excretes phosphate in an amount equal to the net phosphate absorption in the gut. Therefore, the amount of phosphorus excreted into the urine is highly dependent on dietary intake. Under usual conditions, urinary phosphorus excretion is 600 - 1000 mg/day. Endogenous fecal losses of phosphorus, which contain unabsorbed dietary phosphorus and secreted phosphorus that is not reabsorbed, vary from 0.03 to 0.14 mmol/kg body weight (0.9 to 4 mg/kg body weight) per day (Allen & Wood, 1994).

Recommended Intake

The actual requirement for phosphorus is unknown. The RDA for phosphorus intake has been set to equal the RDA for calcium. The daily

RDA for both males and females ages 11 - 24 years, for example, is 39 mmol (1200 mg), and 26 mmol (800 mg) for 25 years of age and older (Committee on Dietary Allowances, 1989).

Negative Effect of High Phosphorus and Low Calcium Intake

To achieve peak bone density at skeletal maturity, it is generally believed that it is necessary to have a recommended dietary calcium : phosphorus intake ratio of 1:1. For example, the recommended intake of calcium and phosphorus for both men and women aged 19 - 24 years old is 1200 mg/day (Committee on Dietary Allowances, 1989). However, the ratio of calcium to phosphorus actually consumed has been found to be quite different than recommended. Data from the National Health and Nutrition Examination Survey II, 1976 - 1980 (NHANES II), for example, indicated that calcium : phosphorus ratios at the 50th percentile were 1:1.6 for both men and women for all age groups.

Many studies have been done to investigate the possible negative effects of high phosphorus-containing diets on bone health when calcium intake is low. To assess the physiological effects of a diet rich in foods containing phosphate additives, Bell et al. (1977) fed young adults a control diet (phosphorus 1.0 g, calcium 0.7 g) for 4 weeks followed by 4 weeks of consuming a high phosphorus-containing diet (2.1 g). High phosphorus intake increased serum and urinary phosphorus, and decreased serum and urinary calcium. An observed increase of

hydroxyproline and cAMP excretion in urine was concluded to be due to enhanced parathyroid activity (secondary hyperparathyroidism). Hydroxyproline is a crude marker of bone resorption (Civitelli, 1993), because it also reflects liver metabolism and other non-skeletal sources plus the small contribution made by dietary hydroxyproline (Mitlak & Nussbaum, 1993). Calvo et al. (1988) tested the effect of an even wider calcium : phosphorus ratio (1700 mg phosphorus/day and 400 mg calcium/day) on bone health indicator in adults. They found an increase of serum phosphorus, immunoreactive PTH, plasma 1, 25$(OH)_2D_3$, urinary hydroxyproline and cAMP excretion in both sexes and a decrease of serum ionized and total calcium levels in women only. Although the observed increase in serum PTH was within the normal range, it was found to be sustainable over time (Calvo et al., 1990). Elevated serum PTH levels have also been seen when phosphorus was given as a dose (Berner & Shike, 1988).

Hypocalciuric Effect of Phosphorus

The hypocalciuric effect of phosphorus is due to an increase in the efficiency of calcium reabsorption by kidney. Part of the effect is mediated by PTH and part is independent of PTH (Lau et al., 1982). To evaluate the mode and tubular sites of action of phosphate administration on renal calcium reabsorption, Lau et al. (1982) performed micropuncture and clearance experiments on acutely

parathyroidectomized rats. Significant calcium reabsorption was observed beyond the superficial late distal tubule only during phosphate infusion, suggesting that phosphate infusion increased calcium reabsorption independently of PTH.

The exact mechanism for the non-PTH dependent effect of phosphorus on kidney calcium reabsorption is still unknown. Several observations grouped together, however, suggest that a stimulation of kidney ammonia nitrogen synthesis (ammoniagenesis) by increased phosphorus could play a role. First of all the bulk of renal ammonia is formed metabolically in the kidney by deamination of glutamine (Guder et al., 1987). One of the enzymes responsible for renal ammonia formation, glutaminase, is known to be stimulated by increased phosphate concentration in vitro (O'Donovan & Lotspeich, 1966). On the other hand, phosphate has been shown to inhibit enzymatic activity of glutamine synthetase (Tate et al., 1972), thus decreasing the amount of ammonia used for glutamine synthesis by kidney. The net effect of these two phosphate effects would be to increase the total amount of ammonia in kidney tubules. Any extra ammonia nitrogen released into the kidney filtrate could titrate an increased amount of hydrogen ions destined for excretion beyond normal levels, which would in turn decrease the known interfering effect of hydrogen ions on kidney calcium reabsorption. Kidney calcium reabsorption is unaffected by the ammonium ion (NH_4^+).

Limited evidence for phosphate-stimulated ammoniagenesis has been reported in both rabbits (Yu et al., 1976) and in rats (Cerklewski, 1995). In the rabbit study, it was found that infusion of sodium phosphate (pH 7.4) at a rate of 130 μmol/minute could increase ammonia excretion rate about nine-fold. Ammonia production was increased significantly by phosphate in both rabbit renal cortex and in isolated renal cortex mitochondria. Use of an inhibitor of both the phosphate/hydroxyl and phosphate/dicarboxylate mitochondrial carriers to inhibit the phosphate-induced stimulation of ammoniagenesis in isolated mitochondria, indicated that phosphate must enter the mitochondrion for stimulation of ammonia production. The study in rats demonstrated that urinary ammonia nitrogen excretion and calcium excretion were inversely related. Urinary ammonia nitrogen excretion was highly correlated with dietary phosphorus intake ($r = 0.88$, $P < 0.001$). Doubling diet phosphorus intake, doubled urinary ammonia nitrogen excretion. A direct relationship ($r = 0.73$, $P < 0.01$) between kidney glutaminase activity and urinary ammonia nitrogen was also reported, suggesting that phosphate-stimulated deamination of glutamine was the probable source of ammonia nitrogen.

Research Hypothesis

The present study was designed to test the possible role of phosphorus-stimulated ammoniagenesis in the hypocalciuric effect of

phosphorus in human volunteers. Based upon results from animal studies, it is likely that phosphorus-stimulated ammoniagenesis will be highly correlated with increased urinary phosphorus excretion in men. Increased formation of ammonium ion would be beneficial to calcium reabsorption because ammonium ion does not interfere with calcium reabsorption as does hydrogen ion. Results could therefore offer a mechanism by which phosphorus directly improves the parathyroid hormone-independent portion of tubular calcium reabsorption.

METHODS

This study was part of a larger project which sought to test the effect of a high phosphorus-containing diet on bone health. Analyses of some of the samples collected will therefore appear in another report by others.

Recruitment of Subjects

Subjects were recruited from the campus community beginning in late June using recruitment posters (Appendix 1). Posters were placed in campus buildings and in local stores and restaurants. About three weeks later, additional posters (Appendix 2) with tear-off contact strips were posted after some of the first respondents either lost interest or failed to meet the requirements. An attempt was also made to recruit subjects attending summer school (example: General Chemistry).

All telephone calls were logged in a notebook and responded to the same day. During the initial contact, general requirements of the study were discussed (Appendix 3). If subjects were interested and met the initial requirements, they were interviewed in person to go over the study in detail (Appendix 4) and to give important personal health/dietary information (Appendix 5). If subjects were still interested and met the requirements, a final interview was scheduled.

The final interview was scheduled as a group meeting in the metabolic laboratory of Milam Hall where the study was to be done. Study schedules and the informed consent document (Appendix 6) were given to the subjects, the study was briefly reviewed and questions were answered. Subjects were then asked to signify their commitment to complete the study by signing the informed consent form.

Human Subjects Approval

The research project was approved by the Oregon State University Institutional Review Board (IRB) for the protection of human subjects. The application included a description of the significance of this project, methods and procedures to be used, the benefits and risks to the subjects, a description of the subject population and the method of obtaining informed consent.

Facilities

The study was conducted in the Milam Hall metabolic unit on the Oregon State University campus. The kitchen provided a fully-equipped food preparation area including cooktops, 4 ovens, sinks, refrigerators, freezers, and a generous array of storage cabinets. The dining area in the unit can comfortably seat about 20 subjects to consume their meals. Adjoining laboratory space was available to store urine samples as they were turned in, to collect blood and to analyze samples. During the

period of study all meals were consumed in Milam Hall. Otherwise the subjects lived at home and continued their normal life. Subjects were instructed not to engage in unusual exercise such as running 5 miles/day or bicycling 15 - 20 miles/day because diets for this study only provided 11.7 MJ (about 2800 kcal). Access to the study site was facilitated by providing an outside door key and security pass to each subject. Each subject recorded his own body weight with light clothing and no shoes before breakfast (Scale Health Meter, Continental Scale Corp., Chicago, Illinois) to insure that individual caloric needs were met.

Diet Composition

The study consisted of two 10-day periods. In period 1, the diet supplied about 800 mg phosphorus (Table 1), whereas in period 2, the diet supplied about 1600 mg phosphorus (Table 2). Phosphorus content of menu 2 was achieved by the addition of cheddar cheese and 2% milk. Diets were constructed from ordinary foods purchased in local grocery stores. No polyphosphate food additives were used to adjust diet phosphorus level. Levels of nitrogen (14.5 g/day as set by menu 2 composition), kcal (~2800 kcal), calcium (~1200 mg), and other nutrients met or exceeded recommended intakes (Committee on Dietary Allowances, 1989).

The two menus used in the study were developed with the help of the Diet Analysis Plus computer program. Chemical analysis of menu 1

Table 1

Menu 1. Normal Phosphorus-Containing (800 mg) Diet

MEAL	ITEM	QUANTITY (gram)
Breakfast	Orange juice (Minute maid calcium rich, Coca-Cola Foods, a division of the Coca-Cola Company, Houston, Texas)	120
	bread, white, 2 slice (homemade)	80
	margarine (as needed)	15
	jelly	15
	egg, whole, scrambled	50
Lunch	Ham, chopped	45
	bread, white, 2 slice (homemade)	80
	mustard	5
	margarine	5
	potato chips	20
	carrot (raw)	30
	celery (raw)	30
	apple, edible weight	140
	soda (7-Up, the Seven-Up Company, Dallas, Texas), 12 oz can	1 each
	fruit drink	240
	lettuce, iceberg	15
	mayonnaise	15
Dinner	Ground beef (cooked weight), seasoned with salt, pepper, chopped onion; bread crumbs (7.5 g) and spray-dried egg white (25 g)	110
	lettuce, iceberg	50
	peas, frozen	20
	salad dressing, French	30
	bread, white, 2 slice (homemade)	80
	fruit drink	360
	pineapple, slice	117
	margarine (as needed)	20
	catsup	10
Snack (take home)	hard candy (optional), non-dairy frozen treats, apple, plum, banana (occasional)	as needed

Table 2

Menu 2. High Phosphorus-Containing (1600 mg) Diet

MEAL	ITEM	QUANTITY (gram)
Breakfast	Orange juice, plain	120
	Canadian bacon	30
	egg, whole, scrambled	50
	cheddar cheese, slice	30
	English muffin	65
	margarine (optional)	10
Lunch	Ground beef, cooked weight, seasoned with salt, pepper, chopped onion; bread crumbs (5 g)	75
	cheddar cheese, slice	30
	roll, hamburger type	40
	lettuce, iceberg	50
	carrot (raw), shred	20
	salad dressing (Zesty Italian)	28
	pear, canned, 2 slice	154
	soda (Coca Cola, the Coca-Cola Company, Atlanta, Georgia), 12 oz can	1 each
	potato chips	30
	catsup	10
	mustard	5
Dinner	Ham, roasted (weight as sliced)	90
	pineapple, slice	78
	mashed potato	210
	cheddar cheese	30
	green beans (frozen)	90
	milk (2% fat)	240
	chocolate syrup	20
	bread, white, slice (homemade)	35
	margarine	15
Snack (take home)	Hard Candy (optional), non-dairy frozen treats, apple, plum, banana (occasional)	as needed

prior to the conduct of the study showed that it provided the desired phosphorus level at 800 mg, but it was low in both calcium and nitrogen. Initial chemical analysis of menu 2 prior to the conduct of the study showed that it provided the desired phosphorus and calcium levels (1200 mg/day each), and it provided more than adequate nitrogen (14.5 g/day). To equilibrate the calcium content of the two menus, calcium as calcium carbonate was added to the bread given in menu 1 (Table 3). Spray-dried egg white protein (Amersham Life Sc., Arlington Heights, Illinois) was added to the meat patty (Table 4) and to the bread of menu 1 to match the nitrogen content of menu 2. Other minor differences in the two menus with respect to zinc, magnesium and vitamin D were also negated by appropriate additions of reagent grade chemicals and crystalline vitamin to the bread mix to meet recommended intake (Committee on Dietary Allowances, 1989). Individual caloric intake of subjects was adjusted to maintain body weight by margarine, mayonnaise, fruit drink, fruits (such as apples, bananas, and plums), hard candy, occasional use of non-dairy frozen treats on weekends and lemon-lime soda, such as 7-Up and Sprite. These extra food items provided little phosphorus, and thus did not disturb the dietary plan.

In menu 2, mashed potato was prepared according to the following procedure: 2 teaspoons salt were added to about 3200 g peeled potato,

Table 3

Bread Preparation* in Menus

Ingredient	Menu 1 g (one loaf)	Menu 2 g (one loaf)
Bread flour (Gold Medal, General Mills Inc., Minneapolis, MN)	470	470
Calcium carbonate	6.77	0
Egg white, spray-dried	58	0
Ergocalciferol (500,000 u/g, United States Biochemical Co., Cleveland, OH)	0.0022	0.0073
$ZnCO_3$	0.0256	0
$MgCO_3$-n-hydrate	1.50	4.84
Vegetable oil (Hunt-Wesson Inc., Fullerton, CA)	13	13
Sugar	26	26
Salt (NaCl)	9	9
Dry active yeast (Fleischmann's Specialty Brands. A Division of Burns Philp Food Inc., San Francisco, CA)	8	8
Distilled water	290	290
Potato flakes	5	5

* Additions were hand-mixed into flour before proceeding, kneaded at speed 1 for 15 minutes and at speed 2 for 5 minutes by using Hobart food mixer (Model A-200 Mixer, Hobart Corporation, Troy, Ohio) with a dough hook. Dough was allowed to rise 60 minutes and then mixed 20 second to punch-down. Dough was put into the pre-sprayed (by Mazola corn oil cooking spray, Englewood Cliffs, New Jersey) 6 inch x 9 inch bread pan to rise for about 30 minutes until dough reached the top of the bread pan. Bread was baked 25 - 30 minutes at 350 °F. Expected size from earlier trials was 800 g (weigh out ~ 850 g/dough with 6 - 7% water loss). Loaves were frozen until needed.

Table 4

Ground Beef Mix Preparation* in Menus

Component	Menu 1 Gram/Patty	Menu 2 Gram/Patty
Ground beef (22% fat)	117	80.0
Egg white, spray-dried	25.0	0
Bread crumbs	7.5	5.0
Chopped onion, raw	10.0	0
Table salt	0.4	0.3
Black pepper	0.1	0.07
Distilled water	4.0	0

* Ingredients were blended with the ground beef using a Hobart food mixer. Mixing was facilitated by the addition of about 3 tablespoons distilled water. Each 164 g patty of about 5 inch diameter was wrapped in plastic, labeled, and frozen. Covered casserole dishes were used to bake the patty at 330 °F for 20 - 22 minutes to keep the product moist and edible, and broiled at Hi-temperature for 2 - 3 minutes to add a thin crust. Patties then could be held at warm temperature, or re-heated in a microwave oven for about 1 - 1.5 minutes before serving.

covered with water (about 1250 g), and boiled with occasional stirring for about 20 minutes. After draining-off some of the water, 4 tablespoons margarine and 1/4 cup 2% milk were added and mixed in a Hobart food mixer at speed 1 to blend. Cooking water was slowly added and then blended at speed 2 to fluff and blended briefly at speed 3 to give the desired consistency. Mashed potato (210 g/bowl) was wrapped with plastic wrap and stored in a refrigerator for no longer than 2 days. Refrigerator-stored mashed potato plus 2 teaspoons water were re-heated in a microwave oven at high-temperature for about 2 minutes when needed. In menu 2, frozen green beans were heated (microwave high-temperature for 1 minute) in bowls to which about 20 mg salt, 1/4 teaspoon margarine, and 1 tea spoon water had been added when needed by subjects. Also in menu 2, ham was baked in a covered casserole dish at 350 °F for about 20 minutes.

Sample Collection

Food Composites

Composites of each menu were obtained before the study began and at the end of each experimental period. Each item in a menu was weighed into a tared 1 gallon Waring blender (Model 34BL22, Waring Commercial Science Center, New Hartford, Connecticut), adjusted to a convenient volume with distilled water (total weight recorded) and blended at low speed for 15 seconds, 30 seconds at medium speed, and

again for 60 seconds at low speed. The homogenate was re-mixed for 10 seconds at low speed before weighing about 25 g in triplicate into 200 mL glass Pyrex beakers. Duplicate samples of homogenates were also saved in 4 oz polypropylene jars for nitrogen analysis to be done later, sealed with parafilm and frozen.

Urine Sample

Urine was collected on the 8th, 9th and 10th days of each experimental period using acid-washed 1-liter polypropylene bottles. A total of four bottles, each fitted with a secure top and pre-labeled (Appendix 7) were made available to collect 24-hour urine samples. As each bottle became filled throughout the collection day, subjects stored them in a refrigerator designated for that purpose and picked-up an empty bottle. Urine collections began after breakfast and ended upon arrival for breakfast the next day.

The number of urine collections was determined by the preservative required. The first set of collection bottles of each experimental period contained glacial acetic acid (3 mL/1 liter bottle) as a preservative to prevent bacterial decomposition of urea, because this sample was to be used for ammonia nitrogen and urea nitrogen analyses. The second set of collection bottles was preservative-free, because this sample was to be used for a bone turnover marker assay. The third set

of collection bottles contained 0.75 mL of 10% thymol in 2-propanol and 1 mL of mineral oil as preservatives because the sample was to be used for determination of pH.

The contents of each multiple set of bottles were mixed in a 10-liter plastic bucket using a plastic spoon and the volume was recorded with a 2-liter Pyrex glass graduated cylinder. For the second and third collections, urine was acidified with 5 mL concentrated HCl before aliquots were removed by disposable 25 mL polystyrene pipettes (Fisher Scientific, Fair Lawn, New Jersey) and stored in 5 mL freezer vials (VWR Scientific, Portland, Oregon) at - 20 °C for later analyses of calcium, phosphorus, sulfate and creatinine. Aliquots of urine were also stored in 30 mL polypropylene bottles with secure tops for total nitrogen analyses in all six urine collections. Ammonia nitrogen, urea nitrogen , and pH which were only to be measured once each experimental period were determined immediately.

Blood Sample

Fasting blood samples (2 x 10 mL) were collected from the antecubital vein by a certified medical technologist following the last urine collection in the morning (between 8:00 - 9:00 am) at the end of each experimental period. Vacutainer tubes (silicone interior, no additive, Terumo Medical, Elkton, Maryland) were used for blood collection. Blood tubes were kept sealed while blood clotted at room

temperature. Serum was collected within 2 hours by centrifugation at 2000 x g (Beckman Model TJ-6 centrifuge, Palo Alto, California). Serum aliquots were transferred to 2 mL freezer vials (VWR Scientific, Portland, Oregon), and stored at - 20 °C for later analyses of calcium and phosphorus. Additional serum samples were saved at - 80 °C (- 86 freezer Forma Scientific Incorporation, Marietta, Ohio) for analyses related to bone health biomarkers.

Analytical Methods

Most of the reagents used for analyses were purchased from either J. T. Baker, Phillipsburg, New Jersey or from Sigma Chemical Company, St. Louis, Missouri. Reagent preparation and dilutions prior to analyses were made with deionized water. Freshly made, glass distilled water was used for urea nitrogen and ammonia nitrogen determinations to prevent ammonia contamination. Glassware used was Pyrex that had been previously soaked in a 5% nitric acid bath.

Phosphorus

Principle of the Method Used

Phosphorus was analyzed by a colorimetric procedure (Fiske & SubbaRow, 1925). A trichloroacetic acid filtrate of either urine or serum was treated with ammonium molybdate in an acid solution to form phosphomolybdate. Fiske & SubbaRow Solution, consisting of sodium

bisulfite, sodium sulfite and 1-amino-2-naphthol-4-sulfonic acid reduces the phosphomolybdate to form a phosphomolybdenum blue complex. The intensity of the color was proportional to the phosphate concentration as measured at 660 nm.

Reagents

Acid Molybdate Solution. Fourteen mL of concentrated H_2SO_4 was carefully added to about 150 mL deionized water. After cooling, 2.5 g ammonium molybdate-$4H_2O$ was added and the solution was transferred to a 200 mL volumetric flask and made to the mark. The solution was stored in a clean, polypropylene bottle at room temperature.

Fiske & SubbaRow Solution. 1-amino-2-naphthol-4-sulfonic acid (0.10 g), sodium bisulfite (5.85 g), and sodium sulfite (0.20 g) were dissolved in 40 mL deionized water under low heat (Model S 7225 Magnetic Stirrer, Thermolyne Corporation, Dubuque, Iowa). The solution was stored in a brown glass bottle at room temperature.

Phosphorus Standard Solution (Stock Standard contained 1 mg phosphorus/mL). KH_2PO_4 was dried for two hours at 105 °C (Mechanical Convection Oven, GCA Corporation, Chicago, Illinois) before using. This pre-dried KH_2PO_4 (4.3942 g) was dissolved in deionized water, 3 mL concentrated H_2SO_4 was added as a preservative, and diluted to 1 L.

Phosphorus Working Standard Solution (contained 20 µg phosphorus/mL). Phosphorus Stock Standard Solution was diluted (on the day of use) 1 : 50 with deionized water.

20% Trichloroacetic Acid (TCA). TCA (20.0 g) was dissolved and made to 100 mL with deionized water. Solution was stored under refrigeration.

Analytical Method

Urine and Serum

A 0.5 mL of either serum or an appropriately diluted urine sample was pipetted into a centrifuge tube containing 2.5 mL deionized water and 2.0 mL of 20% (w/v) trichloroacetic acid, mixed thoroughly by vortexing (Vortex Genie 2, Model G-560, VWR Scientific, Portland, Oregon), allowed to stand 10 minutes on ice, and then centrifuged at 4 °C (Beckman Model TJ-6 Centrifuge, Palo Alto, California) until clear. A blank contained 2.0 mL 20% (w/v) trichloroacetic acid and 3.0 mL water. Each sample test was identical to the blank except that it contained 2 mL of the supernatant fluid from step 2 instead of the trichloroacetic acid solution. Into each tube 1.0 mL acid molybdate solution was added and mixed by vortexing, then 0.25 mL of Fiske & SubbaRow Solution was pipetted into each tube, the solution was mixed by vortexing and allowed to stand 10 minutes for color development. The final solution was aspirated into the flow-through cell of a Beckman DU-40

Spectrophotometer (Irvine, California, sipper setting: 30). Absorbance of each sample was read and recorded using the blank as reference at 660 nm. Absorbance readings were completed within 10 minutes after color development. Standards were prepared from the Phosphorus Working Standard ranging from 5 to 25 μg phosphorus (Into six labeled test tubes, 0.00, 0.25, 0.50, 0.75, 1.00, 1.25 mL of the Phosphorus Working Standard Solution was pipetted, respectively. Then 5.00, 4.75, 4.50, 4.25, 4.00, 3.75 mL deionized water was added to each tube.). Color development was found to be unaffected by TCA.

Diet

The 25 g of food composite saved was wet-ashed in reagent grade nitric acid with heating (12 inch x 24 inch Hot Plate, Model HP-A2245M, Thermolyne Corporation, Dubuque, Iowa) followed by 30% hydrogen peroxide (H_2O_2). The ash was dissolved in 3 M HCl with gentle heat, made to 25 mL with reagent grade water and stored in 30 mL polypropylene bottle with a secure top. Phosphorus was analyzed by the procedure previously described for urine and serum using aliquots of the digest solution to give < 20 μg of phosphorus per tube except that the TCA step was omitted.

Calcium

Principle of the Method

Calcium was determined by atomic absorption spectrophotometry (AAS). If the right wavelength light is impinged on a free, ground state atom (the lowest energy, most stable electronic configuration of an atom), the atom will absorb the light as it enters an excited state (unstable configuration that would immediately and spontaneously return to its ground state). As the number of atoms in the light path is increased, the amount of light absorbed when it passed through a cloud of atoms is increased. Because each element absorbs light at a specific wavelength, measuring the amount of light absorbed will correspond to the amount of analyte element. Chemical compounds of calcium are separated into free atoms to produce the atom cloud required for atomic absorption measurements when the sample is aspirated into an air-acetylene flame. Phosphorus in particular can bind with calcium to form a highly refractory compound which does not dissociate in the flame, thus causing falsely low results. This interference can be eliminated by the addition of La^{3+}, which bonds preferentially with phosphorus to form a tighter complex than calcium phosphates.

Reagents

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

0.1% Lanthanum in 0.06 M HCl. Lanthanum Solution was diluted 1 : 50 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, New Jersey) 0.25 mL, and 10 mL of Lanthanum Solution were diluted to 50 mL with deionized water.

Analytical Method

The atomic absorption spectrophotometer was set-up for calcium analysis according to Standard Operating Conditions described by Perkin-Elmer (Model No.2380, Norwalk, Connecticut).

Urine and Diet

Calcium was determined in urine and acid-digested food composite by atomic absorption spectrophotometry (Willis, 1961). Into a 5 mL flask, was added an appropriate dilution of the sample and 1.0 mL 5% lanthanum chloride solution. Contents were diluted to 5 mL with deionized water and aspirated in the AAS relative to a standard calcium

solutions (5 mg/L) also containing a final concentration of 1% lanthanum.

Serum

The serum sample was diluted 1 : 50 (0.1 mL serum and 4.9 mL diluent) with 0.1% lanthanum in 0.06 M HCl. A 5 mg/L calcium standard was prepared by diluting the stock standard solution (1000 mg/L) with 0.1% lanthanum as chloride. A 0.1% lanthanum solution was used as a blank (Trudeau & Freier, 1967).

Urinary Urea Nitrogen

Principle of the Method Used

Urea nitrogen was determined by the Berthelot procedure (DiGiorgio, 1974). Urea is hydrolyzed by urease to ammonia and carbon dioxide. Ammonia then reacts with alkaline hypochlorite and phenol in the presence of a catalyst, sodium nitroprusside, to form indophenol. The concentration of ammonia is directly proportional to the absorbance of indophenol, which is measured spectrometrically at 570 nm. Ammonia nitrogen already in the urine sample is measured by reversing addition of the urease and reagents. The value obtained is subtracted from the urea analysis value.

Reagents

Phenol nitroprusside and alkaline hypochlorite reagents were prepared as described by Chaney & Marbach (1962).

Phenol Nitroprusside Solution. Phenol (5.0 g) and sodium nitroprusside dihydrate (0.028 g) were mixed and stirred to dissolve in 100 mL distilled water.

Alkaline Hypochlorite Solution. Sodium hydroxide (2.5 g) was dissolved into distilled water and 4.0 mL Clorox (contained 5.25 g NaOCl/100 mL, the Clorox Company, Oakland, California) was added, to make 100 mL solution with distilled water.

Urease Buffer. Sigma Chemical Co., St. Louis, Missouri. Cat. # 640-5.

Urea Nitrogen Standard Solution (150 mg /100 mL). Sigma Chemical Co., St. Louis, Missouri. Cat # 535-150.

Analytical Method

The method used followed the procedure outlined by Sigma Chemical Co. Kit # 640. Test tubes were set-up to contain 0.5 mL urease solution, to which either 0.01 mL water or 0.01 mL pre-diluted urine was added. Contents were mixed and then allowed to stand at room temperature for 20 minutes while urea was hydrolyzed to ammonia. Then 1.0 mL Phenol Nitroprusside Solution, 1.0 mL Alkaline Hypochlorite Solution and 5.0 mL water were added in that order. Contents were mixed after each addition. Tubes were allowed to develop color at room temperature for

20 minutes. A Beckman DU-40 Spectrophotometer (sipper setting: 20) was used to read absorbance at 570 nm. Urea nitrogen standards ranging from 15 - 75 mg/100 mL were prepared by using Urea Nitrogen Standard Solution. To 5 test tubes, 0.1, 0.2, 0.3, 0.4, 0.5 mL Standard Solution was added, then 4.9, 4.8, 4.7, 4.6, 4.5 mL distilled water was added to each tube, and mixed thoroughly by vortexing. Fifty microliters of the above diluted standard solution was pipetted into another 5 test tubes, respectively, and the blank contained 50 μL distilled water.

Urinary Ammonia Nitrogen

Principle of the Method Used

Ammonia reacts with alkaline hypochlorite and phenol in the presence of a catalyst, sodium nitroprusside, to form indophenol. The concentration of ammonia is directly proportional to the absorbance of indophenol, which is measured spectrometrically at 570 nm.

Reagents

Phenol Nitroprusside and Alkaline Hypochlorite regents were prepared as described for urinary urea nitrogen analysis.

Ammonia Standard Solution(100 μg NH_3 N/mL). $(NH_4)_2SO_4$ (0.472 g) was dissolved in distilled water. The solution was adjusted to pH 2.0 with H_2SO_4 (14 drops concentrated H_2SO_4 with transfer pipette) and diluted to 1 L (DiGiorgio, 1974).

Analytical Method

To determine ammonia nitrogen, 50 μL of diluted urine was added to a tube. Then the procedure followed the urea assay. A working ammonia nitrogen standard was prepared from the stock standard previously described. The standard ranged from 1 to 5 μg (to 6 test tubes, 0, 10, 20, 30, 40, 50 μL standard solution was added, and then 50, 40, 30, 20, 10, 0 μL distilled water was added to each tube, respectively.).

Urinary Creatinine

Principle of the Method Used

Creatinine was analyzed according to Folin & Wu (1919). Creatinine was used as an indicator of the completeness of a 24-hour urine collection and to normalize results of a bone health biomarker assay. A red tautomer of creatinine picrate is formed after addition of an alkaline picrate solution. The intensity of the color is proportional to the creatinine concentration as at 520 nm.

Reagents

10% Sodium Hydroxide. NaOH (10 g) was dissolved in deionized water and made to 100 mL. Solution was stored in a polyethylene bottle.

1% Picric Acid. Picric acid crystal (1 g) was dissolved and made to 100 mL with deionized water.

Alkaline Picric Solution. Ten mL of 10% NaOH and 10 mL of 1% picric acid were mixed and diluted to 100 mL with deionized water. This solution was prepared on the day needed.

Creatinine Standard Solution (1 mg/mL). Creatinine (0.1 g) was dissolved in 0.1N HCl and made to 100 mL with 0.1N HCl. This solution was stored under refrigeration.

Creatinine Working Standard Solution (50 μg). Creatinine Standard Solution (5 mL) was diluted to 100 mL with deionized water.

Analytical Method

Test tubes were set-up to contain 0.5 mL of pre-diluted urine to yield < 40 μg creatinine/mL. To this tube was pipetted 1.5 mL water and 2 mL of alkaline picrate solution. Tubes were allowed to stand for exactly 15 minutes. After gentle mixing, 6 mL distilled water was added. A blank was set up by diluting 2 mL of alkaline picrate mixture to 10 mL. Absorbance of samples was read at 520 nm (Beckman DU-40 Spectrophotometer with sipper setting: 20). A creatinine standard was set-up to range from 5 - 40 μg (to 6 test tubes, 0.0, 0.1, 0.2, 0.4, 0.6, 0.8 mL of the Working Standard was added, and then 2.0, 1.9, 1.8, 1.6, 1.4, 1.2 mL deionized water was added to each test tube, respectively.).

Urinary Sulfate

Principle of the Method Used

Sulfate was analyzed according to Ma & Chan's procedure (1973). In this procedure, barium chloride ($BaCl_2$, Mallinckrodt Chemical Works, St. Louis, Missouri) reacts with urinary sulfate (SO_4^{2-}) to yield barium sulfate ($BaSO_4$) and chloride ion (Cl^-). The insoluble barium sulfate is evenly suspended in the medium by utilizing a specially prepared dextran solution [Dextran T70 Mw ~ 70,000 (light scattering), Pharmacia LKB, Biotechnology AB, Uppsala, Sweden]. The turbidity associated with $BaSO_4$ formed is then read spectrophotometrically at 650 nm.

Reagents

5% Trichloroacetic Acid (TCA). TCA (5.0 g) was dissolved and made to 100 mL with deionized water. Solution was stored under refrigeration.

1% $BaCl_2$ in 10% Dextran Solution. Dextran [Dextran T70 Mw ~70,000 (light scattering), Pharmacia LKB, Biotechnology AB, Uppsala, Sweden] (15.0 g) was dissolved and made to 150 mL with deionized water. $BaCl_2 \cdot 2H_2O$ (1.0 g) was dissolved and made to 100 mL with the above 10% Dextran Solution. This solution was centrifuged (room temperature, Beckman Model TJ-6 Centrifuge, Palo Alto, California) at 3000 x g for 10 minutes before being used.

Na_2SO_4 Stock Standard Solution (25 mEq/L). Na_2SO_4 (0.1776 g) was dissolved and made to 100 mL with deionized water.

Analytical Method

Test tubes were set-up to contain 0.5 mL prediluted urinary sample. Then 2 mL of 5% trichloroacetic acid was added to precipitate any protein which may have been present. After mixing and standing for 10 minutes, the mixture was centrifuged (room temperature, Beckman Model TJ-6 Centrifuge, Palo Alto, California) at 3000 x g for 10 minutes. A 2 mL aliquot of the supernatant was pipetted into a test tube. Then 0.5 mL of 1% $BaCl_2$ in 10% dextran solution was added and mixed by vortexing. After 10 minutes, the solution was transferred into a disposable polystyrene cuvette (1 cm light path, Markson Science, Hillsboro, Oregon), and read spectrophotometrically (Gilford Model # 252 Spectrophotometer, Gilford Instrument Laboratories Incorporated, Oberlin, Ohio) at 650 nm using the blank cuvette reference (slit = 0.02 mm, full aperture). The blank consisted of 2 mL of 5% trichloroacetic acid and 0.5 mL of 1% $BaCl_2$ in 10% Dextran. The working standard solution ranged from 2.0 to 10.0 mEq/L (to six test tubes, 0.0, 0.1, 0.2, 0.3, 0.4, 0.5 mL of the Na_2SO_4 Stock Standard Solution was added, respectively, and then 0.5, 0.4, 0.3, 0.2, 0.1, 0.0 mL deionized water was added to each tube, respectively.).

Nitrogen

Principle of the Method Used

Total nitrogen was analyzed according to the method of Scales & Harrison (1929). Concentrated sulfuric acid hydrolyzes organic compounds, oxidizes the hydrogen and carbon containing compounds, and the nitrogen is converted to ammonium sulfate. The ammonia is displaced by alkali and distilled by steam into a boric acid solution. Ammonia is determined by titration with standardized acid.

Reagents

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

4% Boric Acid. Reagent grade boric acid (4.0 g) was dissolved and made to 100 mL with deionized water.

Indicator. Methyl red (10 mg) and bromcresol green (50 mg) was dissolved in 60 mL ethanol.

Reagent Grade Chemicals. K_2SO_4, $CuSO_4 \cdot 5H_2O$, H_2SO_4, mossy zinc.

Analytical Method

Duplicate samples of either food composite homogenate (about 5 g) or urine (about 5 mL) were placed into a 500 mL Kjeldahl flask containing 10 g of K_2SO_4, 0.2 g $CuSO_4 \cdot 5H_2O$, 2 glass beads. Then in a hood, 20 mL of concentrated sulfuric acid was added to each flask. The flasks were

boiled on Kjeldahl racks (Labconco Corp., Kansas City, Missouri) until light green or colorless. The temperature was then increased to maintain gray fumes. After boiling for 30 more minutes (digestion of food samples took longer time than urine samples), the flasks were removed from burners and allowed to cool in the hood. Sides of the flask were washed down with 150 - 200 mL of distilled water and allowed to cool. Holding the kjeldahl flask at a 30 degree angle, 90 mL of 40% NaOH was slowly poured down the side of the flask without mixing. Fresh mossy zinc (J. T. Baker Incorporation, Philipsburg, New Jersey) was quickly added to the flask, and connected to the condenser. Contents were swirled to mix. Kjeldahl flask contents were distilled into 50 mL of a 4% boric acid solution contained in a 250 mL Erlenmeyer flask until there was approximately 100 mL in the Erlenmeyer flask. The distillate containing 5 drops of indicator was titrated with standardized 0.1N HCl. A sample of known nitrogen content, spray-dried egg white, was run through the procedure to serve as a standard for the procedure.

Urinary pH

Urinary pH was measured with a pH electrode (VWR Scientific, Portland, Oregon) using an Orion Ionanalyzer Model EA 940 (Orion Research Incorporated, Boston, Massachusetts).

Calculations

mg calcium/24-hour = analysis (mg/L) x dilution x urine volume (L)

mmol calcium/24-hour = mg ÷ 40 mg/mmol

All other calculations are similar as calculation for calcium.

Statistics

The number of subjects (six to eight) required in this study was estimated by the method described by Hall (1983) based upon the known biological variability associated with the analytical methods used. Treatment effects were tested by using a t-test for paired observations (Spatz, 1993). After consuming diet 1, each subject continued to consume diet 2, so that each subject served as their own control. Results were to be expressed as a mean ± SD (n = 8). Correlation coefficient was determined for urinary ammonia nitrogen excretion versus urinary phosphorus excretion if significant treatment effects were found. Effects were considered to be significant at $P < 0.05$.

RESULTS

Subject Characteristics

Although eight subjects were selected to participate in the study according to the conditions described in Methods, one subject dropped out near the end of the first experimental period for personal reasons. The characteristics of the seven men who did complete the study is shown in Table 5. Subjects readily complied with instructions. All meals were consumed as planned by each subject.

Agreement of Diet Analyses with Calculated Values

The two menus fed to subjects were designed to meet requirements for major nutrients (Committee on Dietary Allowances, 1989) except for phosphorus. The calculated value of phosphorus for menu 1 was 800 mg/day, and 1600 mg/day for menu 2. Chemical analysis showed that the actual phosphorus content was within 5% of the calculated value. Because of the two-fold difference in dietary phosphorus, we wished to have diet calcium be high enough so that the calcium : phosphorus ratio did not fall below 0.7 (Anderson & Barrett, 1994). Chemical analysis showed that menus described provided calcium at 1200 mg/day, which gave a calcium : phosphorus ratio of 0.75 for the high phosphorus (menu 2) diet. Both menus were equalized with respect to protein to provide about 90 g/day. The calculated dietary

Table 5

Subject Characteristics

Subject	Age	Height (cm)	Weight (kg) *	Origin
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**	

* No significant body weight change was observed for any subject during two experimental periods.

protein, which is typical for men in the age range listed, was confirmed by measurement of Kjeldahl nitrogen. Nitrogen was converted to protein by multiplying N x 6.25.

Urinary Analyses

Urinary Phosphorus and Calcium Excretion

The phosphorus and calcium content of 24-hour urine samples collected during each experimental period is shown in Table 6 and Table 7, respectively. The data show a wide variation in urinary phosphorus and calcium excretion among individuals fed the same diet.

As shown in Table 6, urinary phosphorus excretion increased in all 7 subjects for period 1 versus period 2 by an average of 82% ($P < 0.001$). The observed increase in urinary phosphorus was fairly consistent for each set of urine collections made at 87%, 90% and 70%.

Urinary calcium excretion decreased in all 7 subjects during the high-phosphorus experimental period (Table 7) by an average of 38% ($P < 0.001$). This hypocalciuric effect of phosphorus was consistent for the three comparisons made at 36%, 38% and 41%.

Urinary Ammonia Nitrogen, Urea Nitrogen and Total Nitrogen Excretion

As shown in Table 8, urinary ammonia nitrogen excretion increased in all 7 subjects after they consumed the higher phosphorus

Table 6

Urinary Phosphorus Excretion (mmol/24-hour)

Subject	800 mg P/day				1600 mg 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**				

Table 7

Urinary Calcium Excretion (mmol/24-hour)

Subject	800 mg P/day				1600 mg 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**				

Table 8

Urinary Ammonia Nitrogen Excretion (mmol/24-hour)

Subject	800 mg P/day	1600 mg P/day	Change (%)
1	37.9	43.5	+ 14.8
2	33.4	39.3	+ 17.7
3	28.1	29.1	+ 3.6
4	28.0	29.2	+ 4.3
5	29.3	33.8	+ 15.4
6	30.1	35.5	+ 17.9
7	52.2	59.8	+ 14.6
Mean ± SD	**34.1 ± 8.7**	**38.6 ± 10.7**	**+ 13 ± 6**
Significance		**P < 0.01**	

diet versus the lower phosphorus-containing diet. The increase ranged from 3.6% to 17.9% with a mean of 13% ($P < 0.01$). Ammonia nitrogen excretion in urine samples collected on study day 8 and day 18 was correlated with urinary phosphorus excretion ($r = 0.76$, $P < 0.05$).

Urinary urea nitrogen excretion, on the other hand, decreased in all 7 subjects on the same experimental days as conducted for ammonia nitrogen analysis (Table 9). The average decrease in urinary urea nitrogen excretion was 12% ($P < 0.001$).

Urinary total nitrogen excretion is shown in Table 10. With the exception of one subject, the average of urinary total nitrogen excretion decreased during the higher phosphorus diet period versus the lower phosphorus-containing diet period. The average decrease in urinary nitrogen excretion for the entire group was 14% ($P < 0.02$).

Other Urinary Analyses

Urinary sulfate was measured to determine if protein consumption during each period was about equal. Urinary sulfate excretion of each subject (Table 11) was found to be in the normal range stated by Ma & Chan (1973). There was no difference in urinary sulfate excretion when period 1 (800 mg phosphorus/day) was compared to period 2 (1600 mg phosphorus/day).

Urinary creatinine excretion of each subject (Table 12) was found to be in the normal range (Searcy, 1969) of 15 - 30

Table 9

Urinary Urea Nitrogen Excretion (mmol/24-hour)

Subject	800 mg P/day	1600 mg P/day
1	446	378
2	441	383
3	418	360
4	348	326
5	362	342
6	410	364
7	477	405
Mean ± SD	**414 ± 46**	**365 ± 26**
Depression (%)		**- 12**
Significance		**P < 0.001**

Table 10

Urinary Total Nitrogen Excretion (mmol/24-hour)

Subject	800 mg P/day				1600 mg P/day			
	Day 8	9	10	Average	18	19	20	Average
1	965	731	926	874	831	925	871	876
2	961	780	1085	942	894	752	708	785
3	869	791	819	826	791	674	788	751
4	831	900	793	841	856	778	644	759
5	781	872	852	835	764	581	821	722
6	921	964	939	941	779	804	771	785
7	1027	929	939	965	786	534	694	671
Overall Mean ± SD		**889 ± 90**				**764 ± 98**		
Overall Depression (%)				**– 14**				
Overall Significance				**P < 0.02**				

Table 11

Urinary Sulfate Excretion (mEq/24-hour)

Subject	800 mg P/day				1600 mg 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**				

Table 12

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

Subject	800 mg P/day				1600 mg P/day			
Day	8	9	10	Average	18	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**				

mg/kg body weight/24-hour. For the group, urinary creatinine excretion was not significantly changed over time, demonstrating the completeness of 24-hour urine collections. Subject 2, however, showed low levels of urinary creatinine excretion in study days 9, 19 and 20 compared with his other values.

Urinary pH was determined to confirm that the dietary protein consumption was about equal for the two periods (Table 13). Urinary pH was not significantly changed following ingestion the higher phosphorus diet versus the lower phosphorus-containing diet.

Serum Phosphorus and Calcium Concentration

Serum phosphorus concentration increased in all 7 subjects when period 2 (1600 mg phosphorus/day) was compared to period 1 (800 mg phosphorus/day) (Table 14). The average increase of serum phosphorus concentration was 6.2% ($P < 0.02$).

Serum calcium concentration decreased in all 7 subjects after consumption of the higher phosphorus diet versus the lower phosphorus-containing diet (Table 14). The average decrease of serum calcium concentration was 3.8% ($P < 0.05$).

Table 13

Urinary pH

Subject	800 mg P/day	1600 mg P/day
1	5.79	5.97
2	6.13	5.12
3	6.36	5.82
4	5.74	6.52
5	6.33	6.01
6	6.17	6.34
7	6.54	6.54
Mean ± SD	**6.15 ± 0.30**	**6.05 ± 0.50**
Significance	**NS**	

Table 14

Serum Phosphorus and Calcium Concentrations (mmol/L)

Subject	Phosphorus Concentration		Calcium Concentration	
	mg P/day		mg P/day	
	800	1600	800	1600
1	1.26	1.37	2.26	2.24
2	1.37	1.43	2.29	2.24
3	1.37	1.44	2.37	2.31
4	1.12	1.14	2.34	2.27
5	1.30	1.51	2.34	2.25
6	1.28	1.30	2.52	2.24
7	1.29	1.37	2.27	2.20
Mean ± SD	**1.28 ± 0.08**	**1.36 ± 0.12**	**2.34 ± 0.09**	**2.25 ± 0.03**
Change (%)	**+ 6.2**		**- 3.8**	
Significance	**P < 0.02**		**P < 0.05**	

SUMMARY AND CONCLUSIONS

In vitro data suggested over 20 years ago that phosphorus could stimulate the enzymatic activity of kidney glutaminase (O'Donovan & Lotspeich, 1966) where the amino acid glutamine is deaminated to glutamic acid and ammonia nitrogen. The importance of this observation, however, is unknown. The present study sought to define a possible application of this phosphorus effect by relating phosphorus-stimulated ammoniagenesis to the hypocalciuric effect of phosphorus in men. In this scenario, an increase beyond normal of kidney tubule ammonia nitrogen secretion (ammoniagenesis) would titrate some of the normally secreted hydrogen ions destined for urinary excretion. It was hypothesized that increased formation of ammonium ion would be beneficial to kidney calcium reabsorption, thus decreasing urinary calcium loss, because the ammonium ion does not interfere with calcium reabsorption as does hydrogen ion (Agus & Goldfarb, 1985). This effect, if it did indeed occur, could therefore offer a mechanism by which phosphorus directly improves the parathyroid hormone-independent portion of tubular calcium reabsorption (Lau et al., 1982). The results of the present study provided support for this hypothesis. Doubling dietary phosphorus intake from 800 mg to 1600 mg significantly decreased urinary calcium excretion (hypocalciuria) in all seven men as expected. This hypocalciuric effect of phosphorus was also associated with a

significant increase in urinary ammonia nitrogen excretion in all seven men studied ($P < 0.05$). Furthermore, each individual increase in ammonia nitrogen excretion was found to be directly correlated with urinary phosphorus excretion ($r = 0.76$, $P < 0.05$). If ammonia nitrogen excretion was truly increased, it should have been reflected in a decrease in the major end product of nitrogen metabolism, urea nitrogen (Lacey & Wilmore, 1990; Walser, 1991). Urinary urea nitrogen excretion was in fact found to vary inversely with urinary ammonia nitrogen excretion.

Phosphorus-stimulated ammoniagenesis seen in men in the present study confirms observations made in animal studies. In rabbits (Yu et al., 1976), it was found that infusion of sodium phosphate (pH 7.4) at a rate of 130 μmol/minute could increase ammonia excretion rate about nine-fold. Ammonia production was increased significantly by phosphate in both rabbit renal cortex and in isolated renal cortex mitochondria. Use of an inhibitor of both the phosphate/hydroxyl and phosphate/dicarboxylate mitochondrial carriers to inhibit the phosphate-induced stimulation of ammoniagenesis in isolated mitochondria, indicated that phosphate must enter the mitochondrion for stimulation of ammonia production. Support for a possible role of phosphorus-stimulated ammoniagenesis in the hypocalciuric effect of phosphorus was also recently reported in rats (Cerklewski, 1995). Effects seen in men, however, were of smaller magnitude than that seen in rats. Thus

while ammonia nitrogen excretion increased in both men and rats as a result of an increase in dietary phosphorus intake, the percentage increase was only 13% in men compared to an almost linear relationship in rats. A possible explanation for this apparent species difference is that in the rat, phosphorus not only stimulates kidney glutaminase activity (increases ammonia nitrogen) but also inhibits glutamine synthetase activity (Tate et al., 1972). These combined effects of phosphorus could therefore synergistically stimulate ammoniagenesis in the rat whereas in humans, only the glutaminase reaction would be relevant because human kidney is very low in glutamine synthetase activity (Lemieux et al., 1976). Thus in men, some of the ammonia nitrogen formed may have been recycled back into glutamine and diverted to other pathways. It therefore can be concluded that phosphorus-stimulated ammoniagenesis is likely to only play a small part in the mechanism of the non-PTH dependent portion of phosphorus stimulated hypocalciuria. Other mechanisms for this well-known effect of phosphorus (Hegsted et al., 1981) apparently exist and requires more study.

In this study, completeness of urine collection was a must to have confidence in measurements made. Urinary creatinine excretion, which reflects normal muscle turnover and whose excretion is a constant over time individually, was found to be in the normal range for each subject

when expressed per kg of body weight (Searcy, 1969), indicating that each 24-hour urine collection was apparently complete. For most subjects, day to day variation in creatinine excretion was minimal indicating consistency of the collection over time except for subject 2 who showed lower levels of urinary creatinine excretion in study days 9, 19 and 20 compared with his other values. Although his lower values were still in the normal range, it likely contributed to the variability in urinary measures seen. Urinary phosphorus and total nitrogen excretion of subject 2 for example, on study days 9, 19 and 20 was lower than his values on other collection days.

An important point about the experimental design of the present study is that the extra phosphorus added to the diet to test the hypothesis was in the form of milk and cheddar cheese. Therefore, the composition of the high phosphorus diet provided a fairly high protein and calcium intake. Because it has been found that a high protein intake causes hypercalciuria (Allen et al., 1979; Yuen et al., 1984) and thus could have been a confounding variable in the present study, it was important to balance the protein content of menu 1 and menu 2. The protein selected for this purpose, spray dried egg white, not only supplied an excellent protein source, but also it did not upset balance of nutrients because it supplied little else than protein. Therefore the addition of this protein to bread and meat patty of menu 1 did not upset the desired

levels of phosphorus. The extra calcium supplied by menu 2 (the high phosphorus diet) was equalized to menu 1 primarily by addition of calcium carbonate to bread again preventing the introduction of a confounding variable. Finally, the vitamin D content of the two menus was balanced by addition of ergocalciferol (vitamin D_2) to the bread mix. This fact is relevant because of the well known effects of vitamin D on calcium and phosphorus metabolism. Failure to adjust vitamin D levels could have added to the variability of many of the measurements made.

At first glance, the observed decrease of urinary total nitrogen excretion in subjects consuming the higher phosphorus diet compared with the lower phosphorus-containing diet might suggest an imbalance in protein intake for the two menus despite assurance to the contrary. The consistency of urine pH and urinary sulfate excretion, however, argues against this possibility because both urine pH and sulfate excretion reflect catabolism of protein for energy (Guyton, 1991; Ma & Chan, 1973). The decrease of urinary total nitrogen when subjects had a high phosphorus intake is instead consistent with observations by others that there apparently is a metabolic relationship between phosphorus and nitrogen. Rudman et al. (1975), for example, have reported that phosphorus restriction impairs nitrogen retention in underweight adult men and women. The present study showed that the expected opposite effect of providing additional phosphorus is also true. The nitrogen and

phosphorus relationship although seldom mentioned in the nutrition literature dose emphasize the time-worn but valid advice to maintain a balance of nutrient intake.

One possible limitation of the present study is that it was relatively short in time. Results by others, however, have demonstrated that two 10-day test periods were adequate to test the hypothesis (Bell et al., 1977). A study of total length of more than two 10-day periods could have jeopardized the outcome of the study because of problems associated with subject compliance.

The design of present study should in no way be misconstrued as somehow recommending that extra supplemental phosphorus be consumed to improve calcium balance in humans. The human diet is already high in phosphorus primarily because it is supplied by all major food groups. Typical intake of phosphorus in the U.S. has recently been shown to be very similar to the level of phosphorus supplied in the present study (Allen & Wood, 1994). The diet was in fact designed to also supply extra calcium (milk, cheese) because high phosphorus alone with inadequate calcium intake would cause bone loss (Calvo, 1993). It is therefore of great importance to have a balanced diet with high phosphorus and high calcium as was done in the present study. The fact that serum values of phosphorus and calcium were only minimally affected by doubling phosphorus intake in the present study suggests no

harm was done. The form of phosphorus is also important as highly processed foods such as refrigerated bread dough, pastries, and processed cheese spreads, supplemented with polyphosphate additives to improve shelf life, is not a desirable source of phosphorus. Polyphosphates have been shown to be less absorbable (Zemel & Linkswiler, 1981) which makes this type of phosphorus prone to form insoluble complexes with calcium in the intestinal tract, thus decreasing absorption of dietary calcium (Clark, 1969). Phosphorus supplied by ordinary food items such as dairy products, on the other hand, does not interfere with calcium absorption (Spencer et al., 1978).

BIBLIOGRAPHY

Adams, N.D., Gray, R.W. & Lemann, J. Jr. (1979) The calciuria of increased fixed acid production in humans: evidence against a role for parathyroid hormone and 1, 25(OH)2-vitamin D. Calcified Tissue International. 28: 233 - 238.

Agus, Z.S. & Goldfarb, S. (1985) Renal regulation of calcium balance. In: The kidney: Physiology and Pathophysiology (Seldin, D.W. & Giebisch, G. editors), Vol. 2, pp. 1323-1335. Raven Press Books, Ltd. New York.

Allen, L.H. (1982) Calcium bioavailability and absorption: a review. Am. J. Clin. Nutr. 35: 783 - 808.

Allen, L.H., Oddoye, E.A. & Margen, S. (1979) Protein-induced hypercalciuria: a longer term study. Am. J. Clin. Nutr. 32: 741 - 749.

Allen, L.H. & Wood, R.J. (1994) Calcium and phosphorus. In: Modern Nutrition in Health and Disease (Shils, M.E., Olson, J.A. & Shike, M. editors), 8th edition, Vol. 1, pp. 144 - 163. Lea & Febiger Inc., Philadelphia, Pennsylvania.

Anderson, J.J.B. (1991) Nutritional biochemistry of calcium and phosphorus. J. Nutr. Biochem. 2: 300 -307.

Anderson, J.J.B. & Barrett, C.J.H. (1994) Dietary phosphorus: The benefits and the problems. Nutrition Today 29: 29 - 34.

Arnaud, C.D. & Sanchez, S.D. (1990) Calcium and phosphorus. In: Present Knowledge in Nutrition (Brown, M.L. editor), 6th. edition, pp. 212 - 223. International Life Sciences Institute-Nutrition Foundation, Washington, D.C.

Bell, R.R., Draper, H.H., Tzeng, D.Y.M., Shin, H.K. & Schmidt, G.R. (1977) Physiological responses of human adults to foods containing phosphate additives. J. Nutr. 107: 42 - 50.

Berner, Y.N. & Shike, M. (1988) Consequences of phosphate imbalance. Ann. Rev. Nutr. 8: 121 - 148.

Block, G., Dresser, C.M., Hartman, A.M. & Carroll, M.D. (1985) Nutrient sources in the American diet: Quantitative data from the NHANES II. survey. I. Vitamins and minerals. Am. J. Epidemiol. 122: 13 - 26.

Boyle, I.T., Omdahl, J.L., Gray, R.W. & Daluca, H.F. (1973) The biological activity and metabolism of 24, 25 vitamin D. J. Biol. Chem. 248: 4174 - 4180.

Brosnan, J.T., Mcphee, P., Hall, B. & Parry, D.M. (1978) Renal glutamine metabolism in rats fed high-protein diets. Am. J. Physiol. 235: E261 - E265.

Calvo, M.S. (1993) Dietary phosphorus, calcium metabolism and bone. J. Nutr. 123: 1627 - 1633.

Calvo, M.S., Kumar, R. & Heath, H.III. (1990) Persistently elevated parathyroid hormone secretion and action in young women after four weeks of ingesting high phosphorus, low calcium diets. J. Clin. Endocrinol. Metab. 70: 1334 -1340.

Calvo, M.S., Kumar, R. & Health, H.III. (1988) Elevated secretion and action of serum parathyroid hormone in young adults consuming high phosphorus, low calcium diets assembled from common foods. J. Clin. Endocrinol. Metab. 66: 823 - 829.

Calvo, M.S., Bell, R.R. & Forbes, R.M. (1982) Effect of protein-induced calciuria on calcium metabolism and bone status in adult rats. J. Nutr. 112: 1401 - 1413.

Cerklewski, F.L. (1995) Potential role of ammoniagenesis in the hypocalciuric effect of phosphorus in rats. J. Am. Coll. Nutr. 14: 66 - 70.

Chaney, A.L. & Marbach, E.P. (1962) Modified reagents for determination of urea and ammonia. Clin. Chem. 8: 130 - 132.

Chen, T.C., Castilla, L., KoryckaDahl, M. & Deluca, H.F. (1974) Role of vitamin D metabolites in phosphate transport of rat intestine. J. Nutr. 104: 1056 - 1060.

Chu, J.Y., Margen, S. & Costa, F.M. (1975) Studies on calcium metabolism. II. Effects of low calcium and variable protein intake on human calcium metabolism. Am. J. Clin. Nutr. 28: 1028 - 1035.

Civitelli, R. (1993) Biochemical markers of bone turnover. In: The Osteoporotic Syndrome: Detection, Prevention, and Treatment (Avioli, L.V., editor), 3rd. edition, pp. 39 - 61. John Wiley & Sons, Inc. New York.

Clark, I. (1969) Importance of dietary Ca : PO_4 ratios on skeletal Ca, Mg and PO_4 metabolism. Am. J. Physiol. 217: 865 - 870.

Committee on Dietary Allowances (1989) Recommended Dietary Allowances, 10th edition, pp. 174 - 187. National Academy Press, Washington, D.C.

DeLuca, H.F. (1973) The kidney as an endocrine organ for the production of 1, 25 - dihydroxyvitamin D_3, a calcium mobilizing hormone. N. Engl. J. Med. 289: 359 - 364.

DiGiorgio, J. (1974) Nonprotein nitrogenous constituents. In: Clinical Chemistry Principles and Technics (Henry, R.J., Cannon, D.C. & Winkelman, J.W. editors), 2nd edition, pp. 503 - 563. Harper & Row Publishers Inc., Hagerstown, Maryland.

Eriksen, E.F., Colvard, D.S., Berg, N.J., Graham, M.L., Mann, K.G., Spelsberg, T.C. & Riggs, B.L. (1988) Evidence of estrogen receptors in normal human osteoblast-like cells. Science. 241: 84 - 86.

Fiske, C.H. & SubbaRow, Y. (1925) The colorimetric determination of phosphorus. J. Biol. Chem. 66: 375 - 400.

Folin, O. & Wu, H. (1919) A system of blood analysis --- determination of creatinine and creatine. J. Biol. Chem. 38: 98 - 100.

Goldsmith, R.S. & Ingbar, S.H. (1966) Inorganic phosphate treatment of hypercalcemia of diverse etiologies. N. Engl. J. Med. 274: 1 - 7.

Goldsmith, R.S., Woodhouse, C.F., Ingbar, S.H. & Segal, O. (1967) Effect of phosphate supplements in patients with fractures. Lancet 1: 687 - 690.

Guder, W.G., Haussinger, D. & Gerok, W. (1987) Renal and hepatic nitrogen metabolism in systemic acid base regulation. J. Clin. Chem. Clin. Biochem. 25: 457 - 466.

Guyton, A.C. (1991) Regulation of acid-base balance. In: Textbook of Medical Physiology, 8th edition, pp. 338 - 339. W.B. Saunders Co. Philadelphia, Pennsylvania.

Hall, J.C. (1983) A method for the rapid assessment of sample size in dietary studies. Am. J. Clin. Nutr. 37: 473 - 477.

Heaney, R.P. (1993) Nutritional factors in osteoporosis. Ann. Rev. Nutr. 13: 287 - 316.

Heaney, R.P., Sarille, P.D. & Recker, R.R. (1975) Calcium absorption as a function of calcium intake. J. Lab. Clin. Med. 85: 881 - 890.

Hegsted, M. & Linkswiler, H.M. (1981) Long-term effect of level of protein intake on calcium metabolism in young adult women. J. Nutr. 111: 244 - 251.

Hegsted, M., Schuette, S.A., Zemel, M.B. & Linkswiler, H.M. (1981) Urinary calcium and calcium balance in young men as affected by level of protein and phosphorus intake. J. Nutr. 111: 553 - 562.

Holick, M.F. (1994) Vitamin D. In: Modern Nutrition in Health and Disease (Shils, M.E., Olson, J.A. & Shike, M. editors), 8th edition, Vol. 1, pp. 308 - 325. Lea & Febiger Inc., Philadelphia, Pennsylvania.

Horowitz, M.C. (1993) Cytokines and estrogen in bone: Anti-osteoporotic effects. Science 260: 626 - 627.

Ivey, F.J., Shaver, K. (1977) Enzymic hydrolysis of polyphosphate in the gastrointestinal tract. J. Agric. Food Chem. 25: 128 - 130.

Johnson, N.E., Alcantara, E.N. & Linkswiler, H. (1970) Effect of level of protein on urinary and fecal calcium retention of young adult males. J. Nutr. 100: 1425 - 1430.

Kabakoff, B., Kendrick, N.C., Deluca, H.F. (1982) 1, 25 - Dihydroxyvitamin D_3, stimulated active uptake of phosphate by rat jejunum. Am. J. Physiol. 243: E470 - E475.

Kim, Y. & Linkswiler, H.M. (1979) Effect of level of protein intake on calcium metabolism and on parathyroid and renal function in adult human male. J. Nutr. 109: 1399 - 1404.

Kowarski, S. & Schachter, D. (1969) Effects of vitamin D on phosphate transport and incorporsation into mucosal constituents of rat intestinal mucusa. J. Biol. Chem. 244: 211 - 217.

Krall, E.A. & Dawson-Hughes, B. (1994) Osteoporosis. In: Modern Nutrition in Health and Disease (Shils, M.E., Olson, J.A. & Shike, M. editors), 8th edition, Vol. 2, pp. 1559 - 1568. Lea & Febiger Inc., Philadelphia, Pennsylvania.

Lacey, J.M. & Wilmore, D.W. (1990) Is glutamine a conditionally essential amino acid? Nutr. Rev. 48: 297 - 309.

Lau, K., Goldfarb, S., Goldberg, M. & Agus, Z.S. (1982) Effects of phosphate administration on tubular calcium transport. J. Lab. Clin. Med. 99: 317 - 324.

Lemieux, E., Baverel, G. Vinay, P. & Wadoux, P. (1976) Glutamine synthetase and glutamyltransferase in the kidney of man, dog, and rat. Am. J. Physiol. 231: 1068 - 1073.

Linkswiler, H.M., Joyce, C.L. & Anand, C.R. (1974) Calcium retention of adult males as affected by level of protein and of calcium intake. Transactions of the New York Academy of Sciences Series II. 36: 333 - 340.

Ma, R.S.W. & Chan, J.C.M. (1973) Endogenous sulphuric acid production: a method of measurement by extrapolation. Clin. Biochem. 6: 82 - 87.

Marcus, R. (1982) The relationship of dietary calcium to the maintenance of skeletal integrity in man: an interface of endocrinology and nutrition. Metabolism 31: 93 - 102.

Margen, S., Chu, J.Y., Kaufman, N.A. & Calloway, D.H. (1974) Studies in calcium metabolism. I. The calciuretic effect of dietary protein. Am. J. Clin. Nutr. 27: 584 - 589.

Matkovic, V., Kostial, K., Simonovic, I., Buzin, R., Brodarec, A. & Nordin, B.E.C. (1979) Bone status and fracture rates in two regions of Yugoslavia. Am. J. Clin. Nutr. 32: 540 - 549.

McBean, L.D., Forgac, T. & Finn, S.C. (1994) Osteoporosis: Visions for care and prevention - A conference report. J. Am. Diet. Assoc. 94: 668 - 671.

McNecly, M.C.C. (1980) Renal function. In: Gradwohl's Clinical Laboratory Methods and Diagnosis (Sonnenwirth, A.C. & Jarett, L. editors), 8th edition, Volume 1, p. 509. The C.V. Mosby Company, St. Louis, Missouri.

Mitlak, B.H. & Nussbaum, S.R. (1993) Diagnosis and treatment of osteoporosis. Ann. Rev. Med. 44: 265 - 277.

Nordin, B.E.C. (1966) International patterns of osteoporosis. Clin. Orthop. 45: 17 - 30.

Nordin, B.E.C., Marshall, D.H., Francis, R.M. & Crilly, R.G. (1981) The effects of sex steroid and corticosteroid. J. Steroid Biochem. 15: 171 - 174.

Norman, A.W. (1990) Intestinal calcium absorption: a vitamin D-hormone-mediated adaptive response. Am. J. Clin. Nutr. 51: 290 - 300.

O' Dell, B.L. (1984) Bioavailability of trace elements. Nutr. Rev. 42: 301 - 308.

O' Donovan, D.J. & Lotspeich, W.D. (1966) Activation of kidney mitochodrial glutaminase by inorganic phosphate and organic acids. Nature. 212: 930 - 932.

Ornoy, A. (1978) 24, 25 - dihydroxyvitamin D is a metabolite of vitamin D essential for bone formation. Nature 276: 517 - 518.

Pansu, D., Bellaton, C., Roche, C. & Bronner, F. (1983) Duodenal and ileal calcium absorption in the rat and effects of vitamin D. Am. J. Physiol. 244: G696 - G700.

Parfitt, A.M. (1983) Dietary risk factors for age-related bone loss and fractures. Lancet 2: 1181 - 1185.

Rudman, D. Millikan, W.J., Richardson, T.J., Bixler, T.J.II, Stackhouse, W.J. & McGarrity, W.C. (1975) Elemental balances during intravenous hyperlimentation of underweight adult subjects. J. Clin. Invest. 55: 94 - 104.

Scales, F.M. & Harrison, A.P. (1929) Boric acid modification of Kjeldahl method for crop and soil analysis. J. Ind. Eng. Chem. 12: 350 - 352.

Schuette, S.A., Zemel, M.B. & Linkswiler, H.K. (1980) Studies on the mechanism of protein induced hypercalciuria in older men and women. J. Nutr. 110: 305 - 315.

Schuette, S.A. & Linkswiler, H.M. (1982) Effects on Ca and P metabolism in humans by adding meat, meat plus milk., or purified proteins plus Ca and P to a low protein diet. J. Nutr. 112: 338 - 349.

Scrimshaw, N.S. & Murray, E.A. (1988) The acceptability of milk and milk products in populations with high prevalence of lactose intolerance. Am. J. Clin. Nutr. 48: 342 - 390.

Searcy, R.L. (1969) Creatine and creatinine. In: Diagnostic Biochemistry, pp. 193 - 201. McGraw-Hill Book Co., New York.

Sowers, MF. (1990) Osteoporosis and osteomalacia. In: Present Knowledge in Nutrition (Brown, M.L., editor), 6th edition, pp. 371 - 376. International Life Sciences Institute-Nutrition Foundation, Washington, D.C.

Spatz, C. (1993) Hypothesis testing: two-sample tests. In: Basic Statistics, 5th edition, pp. 183 - 213. Brooks/Cole Publishing Company, Pacific Grove, California.

Spencer, H., Kramer, L., Osis, D. & Norris, C. (1978) Effect of phosphorus on the absorption of calcium and the calcium balance in man. J. Nutr. 108: 447 - 457.

Spencer, H., Menczel, J., Lewin, I. & Samachson, J. (1965) Effect of high phosphorus intake on calcium and phosphorus metabolism in man. J. Nutr. 86: 125 - 132.

Stock, J.L., Coderre, J.A. & Mallette, L.E. (1985) Effects of a short course of estrogen on mineral metabolism in postmenopausal women. J. Clin. Endocrinol. Metab. 61: 595 - 600.

Tate, S.S., Leu, F-Y. & Meister, A. (1972) Rat liver glutamine synthetase. J. Biol. Chem. 247: 5312 - 5321.

Trudeau, D.L. & Freier, E.F. (1967) Determination of calcium in urine and serum by atomic absorption spectrophotometry (AAS). Clin. Chem. 13: 101- 114.

Turner, R.T., Riggs, B.L. & Spelsberg, T.C. (1994) Skeletal effects of estrogen. Endocrine Reviews. 15: 275 - 300.

USDA (U.S. Department Of Agriculture) (1984) Nationwide Food Consumption survey. Nutrient Intakes: Individuals in 48 States, Year 1977 - 1978. Report No. I-2. p. 439. Consumer Nutrition Division, Human Nutrition Information Service, U.S. Department of Agriculture, Hyattsville, Maryland.

Walser, M. (1991) Misinterpretation of nitrogen balances when glutamine stores fall or are replenished. Am. J. Clin. Nutr. 53: 1337 - 1338.

Whiting, S.J. & Draper, H.H. (1981) Effect of chronic high protein feeding on bone composition in the adult rat. J. Nutr. 111: 178 - 183.

Willis, J.B. (1961) Determination of calcium and magnesium in urine by atomic absorption spectroscopy. Anal. Chem. 33: 556 - 559.

Young, D.S. (1987) Implementation of SI units for clinical laboratory data. Annals of Internal Medicine. 106: 114 - 129.

Yu, H.L., Giammarco, R., Goldstein, M.B., Stinebaugh, B.J. & Halperin, M.L. (1976) Stimulation of ammonia production and excretion in the rabbit by inoaganic phosphate. Study of control mechanisms. J. Clin. Invest. 58: 557 - 564.

Yuen, D.E., Draper, H.H. & Trilok, G. (1984) Effect of dietary protein on calcium metabolism in man. Nutr. Abs. Rev. 54: 447 - 459.

Zemel, M.B. (1985) Phosphates and calcium utilization in humans. In: Nutritional Bioavailability of Calcium (Kies, C. editor), pp. 29 - 39. American Chemical Society, Washington, D.C.

Zemel, M.B. & Linkswiler, H.M. (1981) Calcium metabolism in the young adult male as affected by level and form of phosphorus intake and level of calcium intake. J. Nutr. 111: 315 - 324.

APPENDICES

Appendix 1

Poster 1 Designed for Recruitment of Subjects

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

Poster 2 Designed for Recruitment of Subjects

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.

7 3 7 - 4 1 7 5 For Nutrition Study

7 3 7 - 4 1 7 5 For Nutrition Study

7 3 7 - 4 1 7 5 For Nutrition Study

7 3 7 - 4 1 7 5 For Nutrition Study

7 3 7 - 4 1 7 5 For Nutrition Study

7 3 7 - 4 1 7 5 For Nutrition Study

7 3 7 - 4 1 7 5 For Nutrition Study

Appendix 3

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.

Appendix 5

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 _____

Appendix 6

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 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 _____

Appendix 7

Example of Label for Urine Collection Bottles

Subject # 4 Bottle # 3 of 4

Dates -- from 08/16 to 08/17

Preservative Glacial Acetic Acid

KEEP IN REFRIGERATOR

CAUTION

CONTAINS ACID