EFFECT OF MILK PROTEIN, SODIUM CHLORIDE, AND POTASSIUM CITRATE ON ACUTE URINARY CALCIUM EXCRETION IN CHILDREN

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Tracy Duff
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ABSTRACT

Studies using adult human subjects indicate that dietary protein and sodium chloride can have a negative impact on the retention of calcium by increasing urinary calcium excretion. Conversely, alkaline potassium can improve calcium retention by decreasing urinary calcium losses. Childhood is an important time for bone growth, however, the effect of these dietary factors on calcium metabolism in children has not been addressed.

This study investigated the effects of oral potassium citrate (K-citrate), sodium chloride (NaCl), and milk protein on short-term urinary calcium excretion in 6-10 year old girls. Subjects provided a fasting urine sample then consumed a base meal and one of five supplements: 8.6 g protein (MP); 8.6 g protein + 25.7 mmol sodium chloride (MP+Na); 25.6 g protein (HP); 25.6 g protein + 25.7 mmol sodium chloride (HP+Na); or 25.6 g protein + 10.8 mmol tri-potassium citrate (HP+K). Urine was collected at 1.5 and 3.0 hours after the meal. Fourteen healthy Caucasian girls ages 6.7-10.0 years old completed all treatments, with at least 48 hours between each test. Supplemental protein was 80% casein and 20% lactalbumin, to simulate cow's milk protein, and contained 0.2 mmol phosphorus per gram. Results were statistically analyzed by Repeated Measures ANOVA and Student-Newman-Keuls test.

Urinary calcium excretion rose after the meal, peaking at 1.5 hours after all treatments. There were no significant differences in calcium excretion between treatments at any time point. HP, which did not increase urinary calcium excretion compared to MP, also did not result in an increase in either net acid or sulfate excretion at 1.5 hours. Dietary sodium chloride had no effect on urinary sodium or calcium excretion over the three hours. However, fasting calcium excretion (mmol/mmol creatinine) was correlated

with fasting sodium excretion (mmol/mmol creatinine) ($r=0.53$, $p<0.001$) for the 70 fasting samples collected during the study. Urinary potassium excretion was increased at 1.5 and 3 hours with HP+K ($p<0.001$). Also, at 1.5 hours after the HP+K treatment, sodium and phosphate excretion were increased ($p\leq0.002$) and net acid excretion was decreased ($p<0.001$).

The results of this study indicate that in children, a simultaneous increase in protein and phosphorus due to increased milk protein intake does not increase acute urinary calcium excretion. There was no effect of a sodium chloride load on urinary calcium excretion seen within the short time-frame of this model. These findings are similar to those of adult studies conducted in the same laboratory using a similar format and treatments. To study the effect of dietary sodium chloride on urinary calcium excretion, longer time periods are necessary. The observation that potassium citrate is not hypocalciuric in children differs from that for adults, who show a decrease in urinary calcium excretion in response to a load of alkaline potassium. The physiological responses of children to dietary factors are not necessarily the same as those of adults. Further research needs to be conducted to determine appropriate dietary recommendations which maximize bone mass in children.

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LIST OF ABBREVIATIONS

CT ..... Calcitonin

Cr ..... Creatinine

ECF ..... Extracellular Fluid

EFC ..... Endogenous Fecal Calcium Excretion

GFR ..... Glomerular Filtration Rate

GH ..... Growth Hormone

HP ..... High Protein

IGF-1 ..... Insulin-Like Growth Factor 1

MP ..... Moderate Protein

NAE ..... Net Acid Excretion

NUTS ..... Nutrient Analysis Software

Pi ..... Inorganic Phosphate

PTH ..... Parathyroid Hormone

RNI ..... Recommended Nutrient Intake

SD ..... Standard Deviation

SEM ..... Standard Error of the Mean

TA ..... Titratable Acid

1. LITERATURE REVIEW

1.1 Calcium Metabolism

1.1.1 Overview and Calcium Balance

Calcium is the most abundant mineral in the body. In the adult human, 99% of the body's calcium is found in the bones and teeth (Green & Kleeman, 1991). The remaining 1% of total body calcium is in the extracellular fluid and within cells. Calcium is found in very low concentration intracellularly, and its entry into the cytoplasm is associated with physiological functions such as muscle contraction and neurotransmitter release. Also, cellular responses to biologically active substances such as hormones are often mediated through changes in intracellular calcium concentration. Serum calcium concentration is tightly controlled by the complex interaction of hormones with bone, the gastrointestinal tract, and the kidney.

Calcium balance is the amount of calcium entering the body minus the calcium losses from the body. When the skeleton is fully developed and mineralized, calcium balance should be in a steady state, with no net retention or loss of calcium. During this zero calcium balance, the amount of calcium lost through urine, sweat, and endogenous gastrointestinal secretions is equal to the amount of calcium absorbed from the diet. Any factor which decreases the body's ability to absorb or retain calcium will have a negative impact on calcium balance if homeostatic mechanisms do not compensate.

Periods of growth (infancy, childhood, and adolescence) are marked by a positive calcium balance (i.e., net absorption of calcium higher than net excretion). This retention of calcium is necessary for the mineralization of

growing bone and expansion of extracellular and intracellular compartments. In a study of white girls on a self-selected diet, the calcium retention (as measured by a dual stable calcium isotope method) of 21 prepubertal girls averaged 3.3 ± 2.1 mmol/day (131 ± 83 mg/day), that of 13 early pubertal girls was 4.0 ± 2.2 mmol/day (161 ± 88 mg/day), and 17 girls in late puberty retained 1.1 ± 2.3 mmol/day (44 ± 91 mg/day) (Abrams & Stuff, 1994). Matkovic and Heaney found that calcium balance improves with increases in calcium intake up to a certain "threshold" level, after which no further increases in balance occur. They pooled the data from 34 published balance studies and derived the threshold intake and the calcium balance at that threshold intake. Their data indicate that children 2-8 years old on a calcium intake at or above the threshold of 34.7 mmol (1390 mg) per day may be able to retain an average of 6.1 mmol (246 mg) of calcium per day. Children from 9-17 years old, many of whom would be in the pubertal growth spurt, can retain an average of 9.9 ± 4.1 mmol (396 ± 164 mg) of calcium per day at or above their threshold intake of 36.9 mmol (1480 mg) of calcium per day (Matkovic & Heaney, 1992).

In prospective studies, a high calcium intake results in greater increases in bone mineral density in children. Pairs of male and female identical twins were studied over three years and there was a significantly greater increase in bone mineral density in the 22 prepubertal twins receiving a calcium supplement (as calcium citrate malate) compared to those receiving a placebo. The average calcium intake of the supplemented group was 40.2 mmol/day (1612 mg/day) and that of the placebo group was 22.7 mmol/day (908 mg/day). There was no difference in bone density between twins who were pubertal or who entered puberty during the study (Johnston, Miller, Slemenda, Reister, Hui, Christian, et al., 1992). However, another study

found a significant increase in bone density in adolescent girls receiving a calcium supplement (as calcium citrate malate) compared to a developmentally matched group receiving a placebo (Lloyd, Andon, Rollings, Martel, Landis, Demers, et al., 1993). Matkovic has postulated that these differences in rates of calcium retention and bone mineralization would lead to significant differences in peak bone density (Matkovic, Ilich, Andon, Hsieh, Tzagournis, Lagger, et al., 1995). This hypothesis is very difficult to prove conclusively and would require very long term longitudinal studies which follow children until they reach their peak bone mass.

1.1.2 Hormones Affecting Calcium Metabolism

Total serum calcium is tightly controlled and maintained within a narrow range by hormonal regulation. In adults, serum calcium is maintained at about 2.3 mmol/L, with a normal range of 2.2 to 2.5 mmol/L (Guyton, 1991). Forty-six per cent of serum calcium is bound to protein and this portion is not filterable through healthy kidneys. Forty-seven and a half per cent of serum calcium is ionized and the remaining six and a half per cent is bound to ligands such as citrate and phosphate (Allen & Wood, 1994). Total and ionized blood calcium concentration and blood phosphate concentration is slightly higher in children than in adults (Arnaud, Goldsmith, Stickler, McCall, & Arnaud, 1973).

The main hormones involved in the regulation of serum calcium are parathyroid hormone (PTH), 1,25 dihydroxyvitamin D, and calcitonin (CT). The parathyroid glands synthesize and secrete the peptide hormone PTH. When serum ionized calcium increases, the release of PTH is depressed (Guyton, 1991). Conversely, the secretion rate of PTH increases in response to even slight drops in serum ionized calcium concentration. This response

occurs within seconds of the decrease in serum ionized calcium. If the hypocalcemia persists, there is an increase in the synthesis of PTH, and parathyroid hyperplasia eventually occurs. PTH increases renal reabsorption of filtered calcium within a few minutes and in vitro bone resorption within 2-3 hours (Aurbach, Marx, & Spiegel, 1991), both of which result in increased serum calcium concentration. PTH also increases the activity of the enzyme 1-alpha hydroxylase in the renal proximal tubules, resulting in increased formation of the active form of vitamin D (1,25 dihydroxyvitamin D). This PTH-induced increase in 1,25 dihydroxyvitamin D formation occurred within 12-24 hours in a sample of patients with Paget's disease (Bilezikian, Canfield, Jacobs, Polay, D'Adamo, Eisman, et al., 1978). Little or no 1,25 dihydroxyvitamin D is formed in the absence of PTH (Guyton, 1991). PTH-induced bone resorption also releases phosphate into the circulation, however PTH decreases phosphate reabsorption from glomerular filtrate so plasma phosphate concentration does not rise as does calcium (Guyton, 1991).

In 101 British children from 2 to 15 years old, there were no significant differences in intact serum PTH concentration with age or sex (Shaw, Wheeldon, & Brocklebank, 1990). The 3rd and 97th percentiles for PTH in this population were 11.0 and 35.0 pg/mL respectively. The adult reference range for the same method is 10-65 pg/mL, indicating that children have lower levels of intact parathyroid hormone (Shaw, et al., 1990). Assays of earlier studies which measured PTH included the intact hormone and also PTH fragments. These earlier studies found that children had levels of PTH that were higher or similar to levels found in adults (Arnaud, et al., 1973).

1,25 dihydroxyvitamin D is a steroid hormone which acts on the intestine to increase gastrointestinal calcium and phosphate absorption. The amount

of 1,25 dihydroxyvitamin D formed depends on PTH levels. 1,25 dihydroxyvitamin D also acts on bone cells and in small quantities can increase bone mineralization but in large quantities increases bone resorption (Guyton, 1991). There are 1,25 dihydroxyvitamin D receptors in renal tubules although the role of these in calcium reabsorption is uncertain.

Vitamin D metabolites were measured in 104 male and 87 female Norwegian children from 8 to 18 years old (Aksnes & Aarskog, 1982). Levels of 1,25 dihydroxyvitamin D peaked around the period of maximal growth in height of the adolescent growth spurt. Similarly, alkaline phosphatase, an indicator of bone formation, peaked at about the period of maximal growth in adolescence. Fujisawa et al. measured PTH, vitamin D metabolites, and calcitonin in Japanese subjects from birth to 79 years old (Fujisawa, Kida, & Matsuda, 1984); they did not separate infants, children and adolescents. They found that serum calcium and phosphate levels declined from birth to late adulthood and that the "children" and young adults had similar serum 1,25 dihydroxyvitamin D levels (42.0 ± 1.4 pg/mL). They found no age related change in vitamin D metabolites. No sex differences were seen in any of the variables.

Calcitonin is secreted by the thyroid gland in response to elevated levels of serum calcium. Calcitonin acts on the osteoclasts to decrease bone resorption and over the long term to decrease osteoclast formation. Calcitonin results in a net increase in bone formation and a decrease in serum calcium. Calcitonin has a much weaker effect on serum calcium concentration than PTH in adults because of the low rates of bone resorption and formation (Guyton, 1991). In young children, the effects of calcitonin may be more pronounced because of higher rates of bone formation and resorption during growth (Guyton, 1991).

1.1.3 Bone and Its Role in Calcium Metabolism

Calcium and phosphorus are the most abundant minerals in bone, and in mature bone are mostly found as crystals of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$]. Serum calcium concentration is often maintained at the expense of bone calcium; however, the skeleton must maintain adequate mineralization to perform its structural function.

Approximately 97-98% of human height is due to the skeleton and the intervertebral disks (Sinclair, 1989); therefore, growth of bone length can be observed by examining growth in height. A newborn averages 38 cm in length (Sinclair, 1989). During the first two years of life, the human infant adds about 50 cm to its length. After this initial rapid rate of growth there occurs a relatively quiescent phase known as "childhood", during which the child gains about 5-6 cm in height per year. There appears to be no difference in bone growth and height between males and females before adolescence (De Schepper, Derde, Van den Broeck, Piepsz, & Jonckheer, 1991; Glastre, Braillon, David, Cochat, Meunier, & Delmas, 1990; Grimston, Morrison, Harder, & Hanley, 1992). Adolescence is the period of rapid growth and hormonal change that follows childhood. The periadolescent period has been classified into five levels of maturity, called Tanner Stages, ranging from Tanner Stage 1, the preadolescent, to Stage 5, the sexually mature or adult state. In between are stages 2, 3, and 4, which are the stages of adolescence characterized by differences in development of secondary sex characteristics.

The adolescent growth spurt begins in girls at about 10.5 to 11 years and in boys at 12.5 to 13 years. There is wide variation between individuals in timing and duration of this growth spurt (Sinclair, 1989). Over the approximately 2.5 years of the growth spurt, girls gain about 16 cm in height and boys gain about 20 cm. In North America, girls reach 98% of their final

height by about 15.5 years, boys by 17.5 years (Tanner, 1989). Even with little further increase in height (e.g. less than 2 cm per year), there may be increases in bone width and mass.

Although growth in length of the skeleton is easily observed, the extent of mineralization and the architecture of bones is not. Young bone is generally more porous than adult bone (Rang, 1983). This porosity may help to impede lateral conduction of fractures, but can result in compression fractures which are not seen in adults (Rang, 1983). Many studies show a linear increase in bone mass of the radius and spine until puberty, when there may be larger increases in bone mass. These increases are partly due to more mineralization per unit volume of bone and partly to the overall increase in size (volume) of bone (Katzman, Bachrach, Carter, & Marcus, 1991). That the mineralization of bone increases with age is also supported by the fact that the ratio of calcium to nitrogen in bone increases from 4.9 at birth to 5.6 in adulthood (Dickerson, 1962). Many researchers are finding that bone mass at critical skeletal sites (spine, proximal femur, pelvis) reaches its peak soon after adolescence (Bonjour, Theintz, Buchs, Slosman, & Rizzoli, 1991; Matkovic, Jelic, Wardlaw, Ilich, Goel, Wright, et al., 1994). Even if these bones continue to mineralize during adulthood, the vast majority of bone mass is accumulated by the end of adolescence.

In adults, bone is continually being broken down and rebuilt. This process, known as bone remodeling, helps to repair damaged bone and therefore maintain skeletal integrity. The cells involved in bone remodeling are the osteoclasts, osteoblasts, and osteocytes. The osteoclasts are large multinucleated cells which produce acids and enzymes that resorb bone mineral and matrix. Osteoblasts, on the other hand, are known as the bone forming cells. They secrete the collagen and proteoglycans which become the

bone "matrix". Osteoblasts which become trapped in mature, mineralized bone become osteocytes. The osteoblasts and osteocytes cover the exposed surfaces of bone to form a membrane which separates the bone fluid from the extracellular fluid.

Remodeling occurs in a specific sequence in what is called the bone remodeling unit (BRU). Remodeling begins with the activation of osteoclast precursors in the BRU. The resulting osteoclasts resorb a small area of bone. After the resorption is ended, osteoblasts form new matrix which then becomes mineralized after 15 or more days (Eriksen, Axelrod, & Melsen, 1994). Therefore, in bone remodeling, resorption and formation are coupled and equal in magnitude (Eriksen, et al., 1994). Dense, compact bone, such as that found in the shaft of the long bones, is remodelled by the removal of a small core of bone and the subsequent deposition of new concentric layers of bone matrix. This type of remodelling is called Haversian remodelling and leaves an osteon with a central Haversian canal (Eriksen, et al., 1994). Trabecular bone, which is a lattice of bony spikes, is found at the ends of long bones and in the vertabrae and other bones. During remodelling of trabecular bone, resorption occurs on the surface of the spikes and then new matrix is deposited in layers.

The remodeling of existing bone also occurs in children. However, during childhood there is a net formation of new bone and the shape of the bones is constantly changing. The change in bone shape during growth is known as bone modeling. For example, the long bones (those in the arms and legs) must increase in length, which occurs at growth plates near the ends of the bone. Growing long bones also increase in diameter by the formation of new bone on the outer (periosteal) surface. The internal marrow cavity increases in width and length via bone removal from the internal (endosteal) surface.

There are several differences between bone modeling and remodeling. There is only one type of bone remodeling unit where resorption and formation are coupled. However, there are two types of bone modeling units: one which resorbs bone and one which forms new bone. Modeling occurs primarily on the periosteal and endosteal surfaces of bone while remodeling also takes place inside bone itself. Also, there are diseases which affect modeling but not remodeling (e.g., osteogenesis imperfecta, osteopetrosis, pycnodysostosis, hyperphosphatasia) and diseases which affect remodeling but not modeling (e.g. osteomalacia) (Frost, 1973).

Markers of bone formation include serum osteocalcin and serum alkaline phosphatase (Delmas, 1993). Osteocalcin, which is also called bone-Gla protein, is synthesized by the osteoblasts and incorporated into the bone matrix. A small amount of the osteocalcin synthesized is released into the circulation and can be measured in the blood. Osteocalcin is specific for bone and dentin. Alkaline phosphatase is secreted by the osteoblasts when they are forming bone and may be an indicator of mineralization (Eriksen, Axelrod, & Melsen, 1994). Total alkaline phosphatase is not specific, as the liver also produces alkaline phosphatase, but the bone specific isoenzyme of alkaline phosphatase can be measured. There are many different types of collagen found in the body, but type I is virtually the only one found in bone. In addition, 85-90% of total bone protein is collagen I (Termine, 1993). Aminoterminal or carboxyterminal fragments cleaved during the processing of collagen I are released into the circulation and can also be used as a measure of bone formation. Collagen is also found in tissues other than bone and these fragments are therefore not specific for bone formation.

One easily assayed indicator of bone resorption is fasting urinary calcium excretion corrected for creatinine excretion (Delmas, 1993). Other markers of

bone resorption include urine hydroxyproline and hydroxylysine excretion. After collagen is formed, some of the proline and lysine residues are hydroxylated post-translationally. Collagen degradation results in the liberation of hydroxyproline and hydroxylysine. However urine hydroxyproline is not a specific or sensitive indicator of bone resorption, and hydroxylysine may be a more sensitive indicator (Delmas, 1993). Plasma tartrate resistant acid phosphatase, which is released from osteoclasts, is also used as an indicator of bone resorption (Delmas, 1993). Pyridinoline and deoxypyridinoline are involved in linking strands of collagen and deoxypyridinoline is mainly found in bone matrix while pyridinoline is also found in cartilage. Both of these can be measured in the urine.

The rate of bone turnover in children is higher than in adults. This high rate of turnover during growth is necessary for the constant change of bone shape. Blumsohn et al. measured several markers of bone formation and bone resorption in girls from 11.6 to 15.5 years old. As indicated by these markers, the prepubertal girls (Tanner stage 1) had higher rates of formation and resorption than those who were classified as Tanner stage 5 (i.e. postadolescent) (Blumsohn, Hannon, Wrate, Barton, Al-Dehaimi, Colwell, et al., 1994). They found greater changes in bone specific alkaline phosphatase, osteocalcin and urinary deoxypyridinoline than other markers which may indicate that these are more sensitive measurements of bone metabolism during puberty. Also, stable isotope studies of calcium kinetics indicate that children between 3 and 16 years old have a higher calcium accretion rate into bone per kilogram body weight than adults (Abrams, Esteban, Vieira, Sidbury, Specker, & Yergey, 1992). This study also found that children have a larger total exchangeable calcium pool per kilogram which may indicate that a larger percentage of young bone is metabolically active. In agreement

with this, young bone has higher water content and is more chemically reactive (Green & Kleeman, 1991).

Many endogenous factors affect the growth of bone. Genetic factors influence bone growth and may largely determine an individual's peak bone mass. Bone growth is controlled by the endocrine system and is related to pubertal status (Bonjour, et al., 1991; Slemenda, Reister, Hui, Miller, Christian, & Johnston, 1994). Growth hormone, an important hormone in children, affects skeletal metabolism. Growth hormone acts via intermediates, primarily insulin-like growth factor one (IGF-1), to increase skeletal growth (Guyton, 1991). In 450 children 6 to 19 years old, serum IGF-1 was correlated with serum osteocalcin (Johansen, Giwercman, Hartwell, Nielsen, Price, Christiansen, et al., 1988). Also, there is a positive association between serum IGF-1 concentration and height for age (Blum, Albertsson-Wikland, Rosberg, & Ranke, 1993). Growth hormone secretion is not the only determinant of IGF-1 secretion, as other factors such as protein and energy intake also effect IGF-1 levels (Thissen, Ketelslegers, & Underwood, 1994). Other hormones such as estrogen also effect bone growth (Dhuper, Warren, Brooks-Gunn, & Fox, 1990). Exogenous factors such as diet (Matkovic, et al., 1995) and physical activity (Slemenda, et al., 1994) may also modulate bone growth.

The rat has frequently been used as a model for human skeletal metabolism. Frost and Jee (1992) state that "in growing rats, exactly the same biological mechanisms increase bone mass as in children." The growing rat would therefore be a suitable model for the skeletal metabolism of children. The use of mice and rats as models for adult human skeletal metabolism has been questioned because these animals have little or no Haversian remodeling in cortical bone.

> "While large-boned mammals possess both modeling and remodeling during growth, small-boned mammals do not have remodeling in significant amounts and exhibit primarily or exclusively the modeling activity alone. Such mammals would include mice, shrews, rats, gerbils and hamsters, and that lack makes them inappropriate to use as experimental models of diseases of human BMU-based remodeling." (Frost, 1973).

Also, while the growth plates of the long bones in humans fuse and become mineralized, the rat does not experience this epiphyseal closure. However, rats do exhibit remodeling on bone surfaces next to marrow, and it is this type of remodeling which seems to be responsible for the development of human osteoporosis. Frost and Jee (1992) have subsequently concluded that "the principal biologic mechanisms responsible for bone gains and losses during normal growth and adult life, and in osteoporoses (sic) too, are the same in the human and rat. ... The rat skeleton should provide a good model of human osteopenias."

1.1.4 Gastrointestinal Calcium Absorption

Calcium absorption occurs via two different mechanisms in the gastrointestinal tract. Calcium is passively absorbed throughout the small intestine by paracellular diffusion. In addition, calcium is also absorbed by a saturable, transcellular mechanism which is most prevalent in the duodenum and proximal jejunum. This saturable mechanism is 1,25 dihydroxyvitamin D-dependent and is mediated by calcium-binding proteins. A small amount of calcium (about 0.2 mmol/day) is also absorbed in the colon (Allen & Wood, 1994).

It is thought that young children may absorb calcium more efficiently than adults. In agreement with this, Staun et al. found that pediatric gastrointestinal patients (1.3-10.5 years old, n=10) had higher intestinal

calcium binding protein per gram of total soluble protein than adult patients (20-89 years old, n=94) (Staun, Boesby, Daugaard, & Jarnum, 1988; Staun, Paerregaard, & Krasilnikoff, 1991). The youngest children had the highest calcium binding protein levels. In the adults, calcium binding protein was positively correlated with serum 1,25 dihydroxyvitamin D. Serum 1,25 dihydroxyvitamin D was not measured in the children, and because of the small number of children involved, it is difficult to compare them to the adults. The data indicate that 1,25 dihydroxyvitamin D-dependent proteins are present in the small intestine by 15 months of age. Since children may require similar amounts of calcium as adults and have a smaller intestinal surface area, this could account for higher levels of calcium binding protein.

Dual stable isotope studies of calcium absorption indicate similar percent absorption of an oral calcium dose between adolescents and young adults (Miller, Smith, Flora, Slemenda, Jiang, & Johnston, 1988; Smith, Heaney, Flora, & Hinders, 1987). The studies were similar in design and numbers. Miller et al. gave 6 male and 6 female adolescents (10-17 y) a dose of 6.2 mmol (250 mg) of calcium as calcium citrate malate (CCM) and found a mean ± SEM fractional absorption of 36.2 ± 2.7%. In 10 adult females (21 to 30 y) Smith et al. found calcium absorption from CCM to be 37.3 ± 2.0% of the 6.2 mmol dose. Also, absorption of calcium (%) as $CaCO_3$ was 29.6 ± 1.7 in the adults and 26.4 ± 2.2 in the adolescents. A stable isotope study of black and white children 9 to 18 years old found no racial differences in calcium absorption. However, the boys had a higher fractional calcium absorption of 0.007 mmol (0.3 mg) of $CaCl_2$/ kg, with eleven boys having 62 ± 4%, and fifteen girls having 39 ± 2% absorption (Bell, Yergey, Vieira, Oexmann, & Shary, 1993). Pubertal status and ages of girls compared to boys was not given.

1.1.5 Fecal Loss of Endogenous Calcium

Endogenous fecal calcium excretion (EFC) is the portion of endogenous calcium which has been secreted into the gastrointestinal tract and not reabsorbed. Secretion of calcium into the gastrointestinal tract can only be estimated (Charles, Eriksen, Hasling, Sondergard, & Mosekilde, 1991) but EFC can be measured by injecting a stable or radioactive isotope of calcium into the blood and measuring the amount of the isotope lost through the feces (Abrams, Sidbury, Muenzer, Esteban, Vieira, & Yergey, 1991; Charles, et al., 1991). Endogenous fecal calcium ranged from 2.0 to 5.6 mmol/day with a mean of 3.4 mmol/day in 17 normal adults, with no difference between sexes (Charles, et al., 1991). Another study found an endogenous fecal calcium excretion of 2.6 ± 0.7 mmol/d in 168 women (36-45 y) (Heaney & Recker, 1982). This study also found that body size may partly determine endogenous gastrointestinal secretion of calcium. The above studies do not give subject weights, but other studies in adults have found EFC to be about 0.05 mmol/kg/day (Abrams, et al., 1991). A study of 5 children from 3-14 years old found endogenous fecal calcium to be 0.03 mmol/kg/day (Abrams, et al., 1991).

1.1.6 Dermal Loss of Calcium

Calcium is lost dermally mostly through sweat, and losses would therefore increase in response to heavy exercise or high temperatures. In adults, average dermal calcium losses have been estimated by radioactive calcium kinetics and found to be weakly correlated to body surface area. In 77 adults, average daily dermal calcium loss was 1.5 mmol per 1.73 m^2 of body surface area (Charles, Taagehoj Jensen, Mosekilde, & Hvid Hansen, 1983). Because children have a smaller surface area, they would be expected to have lower 'resting' dermal calcium losses than adults.

1.1.7 Urinary Loss of Calcium

Urine is a major route of calcium excretion; in adults, 50% or more of absorbed dietary calcium is lost through the urine. Prolonged elevated urinary losses of calcium may have a negative impact on bone mass if net calcium absorption is not correspondingly increased. In a recent study of 370 white girls in pubertal stage 2 based on breast or pubic hair development, a three-day food record, blood sample, bone density measurements, and a 24-hour urine sample were obtained. This study found an inverse relationship between urinary calcium excretion and bone density, indicating that excess urinary calcium loss can have a negative impact on bone (Matkovic, et al., 1995).

Calcium is not secreted in the renal tubule (Peacock, 1988), so urinary calcium is the amount of filtered calcium which is not reabsorbed. Children may be better able to reabsorb calcium filtered in the kidney as indicated by the theoretical variable "maximum tubular calcium reabsorption" (TmCa). TmCa is calculated by the formula $UFCa\text{-}CaE/[1\text{-}0.08\log_e(UFCa/CaE)]$ where UFCa is ultrafilterable calcium and CaE is the calcium excretion index (urinary calcium to creatinine ratio multiplied by plasma creatinine). One-hundred and ten children (2 to 14 years old, measured prior to surgery) had a 95% range in TmCa of 1.87 to 3.39 mmol per litre of glomerular filtrate (Shaw, Wheeldon, & Brocklebank, 1992). The TmCa of 130 adults had a 95% range of 1.75 to 2.61 mmol/L filtrate (Need, Guerin, Pain, Hartley, & Nordin, 1985). The children had a wider range of TmCa but the values did not appear to vary with age or sex.

Children have a lower glomerular filtration rate (GFR) than adults (Spitzer & Schwartz, 1992) and lower GFR would presumably reduce calcium losses in the urine. However, if GFR is expressed per body surface area, adult values are reached in children after about 2 years of age. When expressed per total

body water, GFR is higher in children than adults because children have a higher body water percentage and decreased total water volume (Spitzer & Schwartz, 1992).

The urinary calcium to creatinine ratio in fasting urine has been used to diagnose hypercalciuria and to indicate bone calcium loss (Peacock, 1988). Creatinine is an endogenously produced byproduct of muscle metabolism. It is filtered through the glomerulus but there is no net secretion or reabsorption in the tubule. Creatinine is assumed to be produced at a constant rate and varies positively with muscle mass in adults (Bingham & Cummings, 1985). Correcting for creatinine therefore corrects for lean body mass and also for time (Peacock, 1988). The upper normal limit (mean + 2 SD) for fasting urinary calcium/creatinine ratio in a sample of 60 British children was 0.74 mmol/mmol (Ghazali & Barratt, 1974). In 101 British children from 2 to 15 years old, fasting urinary calcium excretion did not vary with age or sex; the 90th percentile for urinary calcium excretion (mmol/mmol creatinine) was 0.49, and the 97th percentile was 0.69 mmol/mmol creatinine (Shaw, et al., 1990).

1.1.8 Effect of Calcium Intake on Calcium Metabolism

Calcium absorption varies with requirement for calcium and with intake. At low calcium intakes, increases in parathyroid hormone stimulate 1,25 dihydroxyvitamin D production and thereby increase gastrointestinal calcium absorption. Also, with increased PTH secretion, retention of calcium improves as renal reabsorption of calcium increases. At high intakes, greater passive absorption of calcium will depress PTH secretion. Therefore, the body can compensate to a limited extent for changes in calcium intake. However, if

absorption is not sufficient to cover obligatory calcium losses a negative calcium balance ensues.

Extra calcium is required during periods of growth for mineralization of bone. In adults, the greater the calcium intake, the greater the urinary calcium excretion. In children, however, there is little relationship between calcium intake and urinary calcium excretion, except at very high calcium intakes above the threshold at which calcium retention is maximal (Matkovic, Fontana, Tominac, Goel, & Chestnut, 1990; Matkovic, et al., 1995).

1.2 Dietary Factors Affecting Urinary Calcium Excretion

1.2.1 Effect of Protein on 24-Hour Urinary Calcium Excretion

Many studies in adults have shown an increase in urinary calcium excretion in response to an increase in dietary protein when other nutrients are held constant. Doubling the level of dietary protein results in a 50% increase in urinary calcium excretion (Heaney & Recker, 1982). Protein-induced increases in urinary calcium excretion are maintained over long periods of high protein intake and result in negative calcium balance (Hegsted & Linkswiler, 1981; Hegsted, Schuette, Zemel, & Linkswiler, 1981). Like adult humans, rats show an increase in urinary calcium excretion in response to increases in protein intake (Whiting & Draper, 1980). The protein-induced increase in urinary calcium excretion can be seen postprandially in adults (Holl & Allen, 1988).

Protein metabolism increases glomerular filtration rate (GFR) (Hegsted, et al., 1981). This increase in GFR would account for a small increase in urinary calcium excretion. The major effect of protein on calcium excretion may result from decreased renal reabsorption of filtered calcium (Holl & Allen, 1988; Licata, 1981). This decrease in calcium reabsorption is thought to be

mostly due to the acid load produced by protein metabolism (Licata, 1981). Alternatively, high protein intakes may increase the dissolution of bone, which contains a large store of alkali, to buffer the acid generated from protein metabolism (Barzel, 1995). This increase in bone dissolution would increase plasma calcium concentration and therefore urinary calcium excretion.

Sulfur-containing amino acids are especially acid-generating, as their catabolism results in the production of two moles of hydrogen ions for every mole of sulfur amino acid (Trilok & Draper, 1989). North American diets may generate 100 mEq of acid per day, a large part of which is due to the catabolism of sulfur amino acids (Barzel, 1995). Proteins with different sulfur amino acid contents may vary in the degree of calciuria they produce. Animal protein, which typically has a high sulfur-amino acid content compared to vegetable protein, has been implicated in causing greater urinary calcium loss. Lacto-ovo vegetarian women between the ages of 50 and 87 were found to have significantly higher bone mineral density than paired omnivores; however, there were no significant differences in bone mineral density between younger vegetarian and omnivore women (Marsh, Sanchez, Michelson, Chaffee, & Fagal, 1988).

Some studies indicate a link between high dietary protein intake and bone mass or incidence of osteoporosis. It should be noted that high protein intakes in free-living populations are generally accompanied by high phosphorus intakes (Spencer, Kramer, & Osis, 1988). Eskimos, who consume a meat-based diet, were found to have lower bone mineral content than a white, U.S. population; however, this difference only developed after the age of 40 years (Mazess & Mather, 1974). An epidemiological study in free-living populations has shown a positive relationship between per capita animal protein consumption and rate of hip fracture (Abelow, Holford, & Insogna, 1992).

Hu et al. obtained 3-day weighed food intakes and collected overnight urine samples from 764 Chinese women. They found that animal-protein intake was positively correlated with urinary titratable acid, ammonia, and sulfate. Calcium excretion was negatively correlated with total protein and animal protein (Hu, Zhao, Parpia, & Campbell, 1993).

A two-year study of healthy adolescent boys initially 13-14 years old found a significant increase in urinary calcium excretion with 93 g protein/day compared to 43 g protein/day. Whether phosphorus content was equalized is unclear. They found no significant effect of protein on calcium balance or apparent absorption (Schwartz, Woodcock, Blakely, & MacKellar, 1973). Another study in adolescents with chronic renal insufficiency also found an increase in urinary calcium excretion in response to an increase in protein intake as meat, eggs, and dairy. However, the high protein diet contained more than twice the amount of calcium and phosphorus (Nakano, Alon, Jennings, & Chan, 1989).

Schofield et al. studied 6.7 to 10.0 year old preadolescent girls in four studies from 1954 to 1958 (Technical Committee, 1959; Schofield & Morrell, 1960). The studies were carried out in a metabolic unit and the experimental period lasted between 24 days and 64 days. Protein intake ranged from about 1 g/kg per day to 3 g/kg per day. Calcium and phosphorus intake were maintained fairly constant between all treatments. Subjects were grouped into 3 categories of protein intake. The subjects on the lowest protein intake had a significantly lower urinary calcium excretion (and higher urinary phosphate excretion) than the other groups. The retention of calcium was 0.188 g/day with lowest protein intake, 0.162 g/day with the intermediate protein intake, and 0.156 g/day with the highest protein intake but there were no significant differences in retention of calcium.

In a study of 27 preterm infants, Hillman et al. found that increasing the level of protein from 2.2 to 2.7 to 3 g protein per 100 kcal of formula resulted in decreases in urinary calcium excretion (Hillman, Salmons, Erickson, Hansen, Hillman, & Chesney, 1994). The formulas were identical in calcium and phosphorus content. The authors concluded that the infants had a high protein requirement and the lower protein formulas resulted in underutilization of calcium.

1.2.2 Effect of Protein on Post-Prandial Urinary Calcium Excretion

Studies in adults indicate that the effect of protein on urinary calcium excretion can be studied using a short-term unacclimatized acute model (Fagnou, 1995; Holl & Allen, 1988). In a randomized cross-over study, investigators fed 12 fasting adults liquid meals containing an extra 30 g of protein, sucrose, or starch and collected urine every 30 minutes after the ingestion of the meal (Holl & Allen, 1988). All other nutrients, including phosphorus, were held constant. The rate of urinary calcium excretion was greatest with the protein load by 2.5 hours after the meal. By 3 hours after the meal, 60% more calcium had been excreted after the high protein meal than after any other treatment. The acute post-prandial response of urinary calcium to variations in protein intake has not been studied in children.

A number of studies using adult subjects have been performed to investigate the acute effect of milk protein on urinary calcium excretion. In adult women who were fasted overnight, a high milk protein meal did not increase acute urinary calcium excretion compared to a moderate milk protein meal (Fagnou, 1995). Phosphate was not equalized between treatments, and the higher protein treatment had a naturally higher phosphate content. In adult men given similar treatments but with the

phosphate content equalized by adding NaH_2PO_4 to the moderate protein treatment, high milk protein did result in increased urinary calcium excretion (Anderson, 1995).

1.2.3 Effect of Phosphorus on Urinary Calcium Excretion

In adults, an increase in phosphorus intake decreases urinary calcium excretion associated with high protein feeding (Hegsted, et al., 1981; Zemel, Schuette, Hegsted, & Linkswiler, 1981) and may improve the negative calcium balance associated with high protein intake. Food sources of protein, such as meat, are also high in phosphate. Therefore, in free-living populations an increase in protein intake is also accompanied by an increase in phosphate intake. An epidemiological study in free-living populations has shown a positive relationship between animal protein consumption and rate of fracture (Abelow, Holford, & Insogna, 1992). Also, Hu et al. found that animal protein intake was positively correlated with urinary calcium excretion in Chinese women (Hu, et al., 1993). Heaney and Recker found that an increase in phosphorus intake resulted in a slight decrease in urinary calcium excretion and a slight increase in fecal calcium, so that there was no net effect on calcium balance (Heaney & Recker, 1982). However, other studies have found no change in urinary calcium or calcium balance with an increase in protein as meat (Hunt, Gallagher, Johnson, & Lykken, 1995; Spencer, Kramer, DeBartolo, Norris, & Osis, 1983).

Although phosphate has been found to decrease urinary calcium excretion, very high phosphate to calcium ratios in the diet may be detrimental to bone. In many animals, high phosphate, low calcium diets lead to secondary hyperparathyroidism and osteopenia, however, this did not occur in a study using monkeys (Anderson, Hunt, Griffiths, McIntyre, & Zimmerman, 1977).

Some studies in humans show an increase in PTH levels with high phosphorus low calcium intakes (Calvo, Kumar, & Heath, 1990).

1.2.4 Effect of Sodium Chloride on Urinary Calcium Excretion

Sodium chloride intake influences urinary calcium excretion. An increase of 100 mmol urinary sodium excretion per day resulted in an increase of 1.5 mmol urinary calcium excretion per day in normal adults (Sabto, Powell, Breidahl, & Gurr, 1984). Like adult humans, rats show an increase in urinary calcium excretion in response to sodium chloride intake (Whiting, 1992). Sodium chloride supplementation in young women (Goulding & Lim, 1983) and in postmenopausal women raises urinary excretion of hydroxyproline, an indicator of bone resorption (McParland, Goulding, & Campbell, 1989). Sodium chloride is thought to affect urinary calcium primarily via a renal mechanism. Increasing luminal concentrations of sodium were found to decrease distal tubular reabsorption of calcium (Brunette, Mailloux, & Lajeunesse, 1992). Although it is the sodium ion which is emphasized in most studies, the accompanying anion is also important. For example, while sodium chloride increases urinary calcium excretion, sodium bicarbonate does not (Lemann, Pleuss, Gray, & Hoffmann, 1991).

In children aged 2-14 years, Shaw et al. observed an inverse correlation between log TmCa and the log of the sodium excretion index (Shaw, et al., 1992), so that as urinary sodium excretion increased, calcium reabsorption decreased. In a study of 381 white females aged 8-13 years, all of whom were classified as Tanner Stage 2, Matkovic found a positive association between urinary sodium and urinary calcium excretion and an inverse relationship between urinary calcium excretion and whole body and radius bone mass (Matkovic, et al., 1995). These studies indicate that sodium chloride intake

affects urinary calcium excretion in children, and may also affect bone development.

1.2.5 Effect of Alkaline Potassium on Urinary Calcium Excretion

Potassium bicarbonate administration results in a sustained decrease in urinary calcium excretion (Green & Whiting, 1994; Lemann, et al., 1991) and improves calcium balance in adult men (Lemann, Gray, & Pluess, 1989) and post-menopausal women (Sebastian, Harris, Ottaway, Todd, & Morris Jr., 1994). Potassium citrate has a similar effect to potassium bicarbonate on calcium excretion, as citrate is metabolized to bicarbonate in vivo (Sakhaee, Alpern, Jacobson, & Pak, 1991). However, potassium chloride administration has no effect on 24-hour urinary calcium excretion while $NaHCO_3$ causes only a transient decrease in urinary calcium excretion (Lemann, et al., 1991).

Bicarbonate or citrate potassium salt generates a base load and results in less net acid excretion. This decrease in urinary net acid may be at least partly responsible for the decrease in urinary calcium excretion seen with alkaline potassium administration. Hu et al. found a negative correlation between urinary calcium excretion and intake of plant foods, and hypothesized that this may be due to the alkaline nature of these foods (Hu, et al., 1993). Potassium itself may decrease urinary calcium excretion. Lemann has postulated that potassium may increase phosphate retention thereby decreasing 1,25 dihydroxyvitamin D synthesis and decreasing gastrointestinal calcium absorption (Lemann, Pleuss, & Gray, 1993). However, supplemental potassium bicarbonate given to postmenopausal women resulted in positive calcium balance (Sebastian, Harris, Ottaway, Todd, & Morris Jr., 1994). Another study found that increasing the concentration of potassium in the distal tubular fluid was found to increase

calcium reabsorption (Brunette, et al., 1992).

In contrast to adult humans, rats do not show a decrease in urinary calcium excretion with the addition of potassium bicarbonate to their diet (Whiting, 1992; Greger, Kaup, & Behling, 1991).

1.3 Dietary Intakes of Children

1.3.1 Protein Intake of Children

As part of a prospective bone density study, multiple 24-hour recalls were obtained from participating Saskatoon children. Twenty-seven Saskatoon girls from 8 to 9 years old had a mean ± SD protein intake of 64.4 ± 14.2 grams per day, and 22 boys had an intake of 69.5 ± 19.4 grams per day (Whiting, Colleaux, & Bacchetto, 1995). In a continuing national survey in the United States, 24-hour recalls were collected between 1989 and 1991 (Tippett, Mickle, Goldman, Sykes, Cook, Sebastian, et al., 1995). The average protein intake for 533 white girls from 6 to 11 years old was 66.5 grams per day and that of 574 white boys of the same age was 69.2 grams per day.

1.3.2 Phosphorus Intake of Children

The mean ± SD phosphorus intake of 8-9 year old Saskatoon girls was 40.2 ± 10.0 mmol (1246 ± 310 mg) per day and 8-9 year old boys was 41.4 ± 12.4 mmol (1281 ± 383 mg) per day (Whiting, et al., 1995). The USDA survey found that 6 to 11 year old girls had a mean phosphorus intake of 39.7 mmol (1231 mg) per day and that of boys was 40.7 mmol (1262 mg) per day (Tippett, et al., 1995). However, actual intakes of phosphorus may be higher than indicated by nutrient database data because of the large amounts of phosphate additives presently being used in processed foods which are not accounted for in these databases (Anderson & Barrett, 1994).

1.3.3 Sodium Intake of Children

The sodium intake of Saskatoon children as estimated by 24-hour recall was 127 ± 30 mmol (2.55 ± 0.69 g) per day (girls) and 121 ± 32 mmol (2.78 ± 0.74 g) per day (boys) (Whiting, et al., 1995). The USDA survey reported the sodium intake of 6 to 11 year old girls to be 127 mmol (2.92 g) per day and that of 6 to 11 year old boys 132 mmol (3.04 g) per day (Tippett, et al., 1995). Neither of these studies attempted to include discretionary table salt use. As part of the Bogalusa heart study, sodium chloride intake was estimated by 24-hour recall including the number of "shakes" of table salt added to food (Frank, Webber, Nicklas, & Berenson, 1988). The mean ± SD sodium intake for 304 white 10-year old girls was 133 ± 71 mmol (3.05 ± 1.63 g) per day and for 305 white 10-year old boys was 164 ± 84 mmol (3.78 ± 1.93 g) per day. These data were obtained between 1973 and 1982.

Sodium intake is thought to be poorly estimated by food records or recalls because of the variable amounts of table salt added during food preparation and at the time of consumption. Matkovic et al. found that in pubertal girls sodium intake as estimated by food records had only a very weak relationship to urinary sodium excretion, the latter representing true sodium intake (Matkovic, et al., 1995).

1.3.4 Potassium Intake of Children

Saskatoon children had a mean ± SD potassium intake of 59.1 ± 15.1 mmol (2.31 ± 0.59 g) per day (girls) and 59.1 ± 17.1 mmol (2.31 ± 0.67 g) per day (boys) (Whiting, et al., 1995). The Bogalusa heart study found a potassium intake of 54.2 ± 24.0 mmol (2.12 ± 0.94 g) per day in 10-year old girls and 68.0 ± 31.2 mmol (2.66 ± 1.22 g) per day in 10-year old boys (Frank, et al., 1988). The USDA survey found that 6 to 11 year old girls had a mean potassium

intake of 60.4 mmol (2.36 g) per day and boys also averaged 60.4 mmol/day (Tippett, et al., 1995).

1.3.5 Calcium Intake of Children

Saskatoon girls from 8 to 9 years old had a mean ± SD calcium intake of 26.3 ± 8.4 mmol (1055 ± 338 mg) per day, and boys the same age had an intake of 27.3 ± 10.3 mmol (1094 ± 412 mg) per day (Whiting, et al., 1995). The USDA survey found that 6 to 11 year old girls had an average calcium intake of 23.5 mmol (942 mg) per day and boys had a mean intake of 25.0 mmol (1000 mg) per day (Tippett, et al., 1995).

1.4 Bone Growth and Risk of Osteoporosis

Studies have shown that the vast majority of bone mass at sites such as the spine, proximal femur, and hip is achieved by the late teens in women (Bonjour, et al., 1991; Matkovic, et al., 1994). The attainment of low peak bone mass in youth and/or rapid loss of bone in adulthood can lead to low bone mass in the elderly, a factor leading to osteoporotic fractures (Johnston & Melton, 1993). It is difficult to substantially increase bone mass after sexual maturity, and childhood may be the best time to ensure that the genetic potential for bone mass is achieved in order to reduce the risk of developing osteoporosis.

There is evidence that diet can affect the rate of bone growth during childhood and adolescence. Post-menopausal women who recalled having consumed milk with every meal as a child had a significantly higher bone density compared to those who reported consuming milk less frequently as a child (Sandler, Slemenda, LaPorte, Cauley, Schramm, Barresi, et al., 1985). Those who reported drinking milk with every meal as an adolescent also had a

higher bone density; however, there was no significant difference in bone density between those who reported drinking milk with every meal as an adult compared to those drinking milk less frequently as an adult. In a study comparing bone density in two regions of Yugoslavia with differing dietary habits, the region with the higher average calcium intake also had lower proximal femur fracture rates (Matkovic, Kostial, Simonovic, Buzina, Brodarec, & Nordin, 1979). The area with the high calcium intake consumed more dairy products, and also had a higher intake of protein and phosphorus.

Prospective studies also show that calcium intake affects bone density in children, presumably by affecting the rate of bone mineralization. In a three year prospective study, pairs of male and female identical twins were assigned to receive a calcium citrate malate supplement or a placebo (Johnston, et al., 1992). There was a significantly greater increase in bone mineral density in the 22 prepubertal twins receiving the calcium supplement. There was no difference in bone density between twins who were pubertal or who entered puberty during the study (Johnston, et al., 1992). However, another study found a significant increase in bone density in adolescent girls receiving a calcium citrate malate supplement compared to a developmentally matched group receiving a placebo (Lloyd, et al., 1993). In another study of adolescent girls in Tanner Stage 2, a 3-day food record was obtained, a 24-hour urine was collected, and bone density was also measured (Matkovic, et al., 1995). There was a significant positive association between calcium intake and bone density. Also, there was a significant negative association between urinary calcium excretion and bone density, with urinary sodium excretion being one of the most important predictors of urinary calcium excretion.

Although the effect of calcium intake on bone density in childhood has been

investigated, the effect of other dietary factors has not been adequately studied. Long term controlled studies of the impact of dietary factors on bone growth during childhood are needed.

2. INTRODUCTION

Low bone mass is a factor leading to osteoporotic fractures (Johnston & Melton, 1993). In the elderly, low bone mass is a result of the attainment of a low peak bone mass in youth and/or rapid loss of bone in adulthood. Many researchers have found that bone mass at critical skeletal sites (e.g. spine, proximal femur) reaches its peak by the late teens in women (Bonjour, et al., 1991; Matkovic, et al., 1994). Childhood may be the best time to ensure that the genetic potential for bone mass is achieved in order to reduce the risk of developing osteoporosis.

Urinary calcium excretion is an important determinant of calcium retention. Several dietary components, including dietary protein, sodium chloride, and alkaline potassium, are known to affect urinary calcium excretion and calcium retention in adults. Isolated protein, especially that with a high sulfur-amino acid content such as animal protein, has been shown to increase 24-hour urinary calcium excretion and have a negative impact on calcium balance in adults (Orwoll, 1992). Whether natural sources of protein increase urinary calcium excretion is less clear; the high phosphorus content of food proteins may reduce urinary calcium excretion (Orwoll, 1992). The administration of sodium chloride has been shown to increase 24-hour urinary calcium excretion (Sabto, et al., 1984) and also to increase urinary hydroxyproline (an indicator of bone resorption) in adult women (McParland, et al., 1989). Dietary potassium, administered as alkaline potassium, e.g. $KHCO_3$ or potassium citrate, is hypocalciuric in adults (Green & Whiting, 1994; Lemann, et al., 1991; Sakhaee, et al., 1991) and improves calcium

balance (Lemann, et al., 1989; Sebastian, et al., 1994). Elevated urinary calcium excretion would lead to a more negative calcium balance if absorption is not correspondingly increased. During negative calcium balance, excess urinary calcium excretion must come from bone, which is the only source of calcium over the short-term. A recent study of early pubertal girls found an inverse relationship between urinary calcium excretion and total body and radius bone mineral content and density (Matkovic, et al., 1995).

There is little information on the effect of protein, phosphorus, sodium chloride, and alkaline potassium on calcium metabolism in children. Because of the difficulties involved in using children as subjects, studies with low subject burden are desirable. We proposed to study the effects of these dietary components on urinary calcium excretion in children using a short-term model similar to that developed for testing the calciuric effect of protein (Holl & Allen, 1988). Similar acute load-tests have been performed in our laboratory using adult subjects (Anderson, 1995; Fagnou, 1995) and it is therefore possible to compare the responses between the two age groups.

It has been recommended that children from 4 to 9 years old consume 2-3 servings of milk products per day in order to build healthy bones. Some have questioned the efficiency of dairy products as a source of calcium because their high protein content may increase urinary calcium excretion (van Beresteijn, Brussaard, & van Schaik, 1990). However, the phosphorus and potassium in milk may offset protein-induced calcium losses. The high sodium chloride content of some dairy products, such as cheese, may increase urinary calcium excretion. Children eat many snack, processed, and fast foods which are high in sodium chloride and phosphate additives. It is necessary to determine the calciuric response of children to these dietary factors in order to make dietary recommendations which will result in optimal growth of bone.

Therefore, the objectives of this study were:

1) To determine the acute response of urinary calcium excretion to meals with moderate and high levels of milk protein in 7-10 year old girls.

2) To determine the acute response of urinary calcium excretion to a moderate protein meal plus a load of sodium chloride and to a high protein meal plus the same load of sodium chloride in 7-10 year old girls.

3) To determine the acute response of urinary calcium excretion to a high protein meal plus a load of potassium citrate in 7-10 year old girls.

4) To compare the diet-induced calciuric responses of children with those of adults.

The hypotheses of this study were:

1) The high protein meal will increase urinary calcium excretion compared to the moderate protein meal in children. This increase will be due primarily to increased net acid excretion caused by the oxidation of the extra sulfur amino acids.

2) The sodium chloride load will increase urinary calcium excretion compared to protein alone in children. There will be no interaction between sodium chloride and protein.

3) The high protein + potassium citrate will reduce urinary calcium excretion compared to high protein alone in children. This decrease will be accompanied by a decrease in net acid excretion.

4) Children will have similar acute diet-induced calciuric responses to adults.

3. MATERIALS AND METHODS

3.1 Experimental Design

The purpose of the study was to examine the acute effects of dietary factors on urinary calcium excretion in 6 to 10 year old girls. The study took place in Saskatoon in July and August, 1994. Study procedures were approved by the University of Saskatchewan Advisory Committee on Ethics in Human Experimentation. Appendix 7.1 contains a copy of the ethical approval form.

Test meals were fed to unacclimatized, fasting subjects to examine the effects of milk protein, sodium chloride and alkaline potassium on urinary calcium excretion at 1.5 and 3.0 hours after the meal. The treatments were moderate protein (MP), moderate protein plus sodium chloride (MP+Na), high protein (HP), high protein plus sodium chloride (HP+Na), and high protein plus potassium citrate (HP+K). The study was a repeated measures design where each subject received every treatment and acted as her own control.

3.2 Subjects

Girls between the ages of 7 and 10 years old were recruited by advertising at and around the University of Saskatchewan. Subjects were also recruited by contacting participants from a previous children's study and by sending letters to parents at a local school. Letters were sent to the parents of prospective subjects explaining the study (Appendix 7.6). Twenty subjects began the study, and fourteen subjects who met our criteria of healthy Caucasian girls completed all five treatments. Results given are from these

fourteen girls unless otherwise stated. Subject characteristics are given in Table 4.

At an initial interview before the subjects began the study, a calcium food frequency questionnaire, which has been used to assess calcium intake in adults (Fagnou, 1995), was administered to the parents regarding the child's usual calcium intake. The protocol for the day before and morning of the study was explained to the parents, as was the procedure for filling out food records. The parents filled out a health questionnaire (Appendix 7.7) including questions about factors affecting calcium metabolism and conditions which might put the children at risk if they were to participate in the study. Parents gave written consent for the girls to participate (Appendix 7.8).

3.3 Procedures

On the day prior to each study day, the parent recorded everything the child ate or drank on a food record form (Appendix 7.2). Parents also recorded, on the same form, whether the day's intake was typical and if the child took any supplements. The subjects were provided with canned fruit as a snack to prevent a high salt intake on the evening before the study. The child fasted after 8:00 p.m. except for drinking 200-250 mL Diet 7-Up® (n=11) or distilled deionized water (n=3) which we provided. On the morning of the study, the parent recorded the time that the child woke up and the time of the child's first morning void on a checklist (Appendix 7.3) which helped them follow the pre-study procedure. The child drank 250 mL of Diet 7-Up® or distilled deionized water and then came to the university between 8 and 9 a.m. The Diet 7-Up® was provided in a 600 mL bottle with marks indicating the amount the child was to drink in the evening and in the morning. The checklist and remaining Diet 7-Up® were brought to the university with the child.

On the first visit, the child received instruction about study procedures to ensure optimal urine collection. Before the administration of the first treatment, the subjects' heights and weights were measured and recorded on the subject data sheet (Appendix 7.4). If a subject did not urinate before coming to the university, her first morning urine was discarded and a fasting sample collected later.

After the child provided a fasting urine sample, she was given a meal which included the experimental supplement. The times that the child started to eat and finished her meal were recorded on a daily checklist (Appendix 7.5), and the midpoint of the meal was calculated. The children drank distilled deionized water or Diet 7-Up® throughout the morning to promote urine flow. The volume of water or Diet 7-Up® given to each subject was recorded. Each child received the same drink throughout the study, with the exception of 2 subjects. Urine was collected at 1.5 and 3 hours after the midpoint of the meal. The subjects were supervised during the study period and avoided strenuous exercise.

3.4 Base Meal

The base meal consisted of a low-protein pancake and 15 mL generic table syrup. In addition, each child received one Pringles Light® potato chip which served as a vehicle for sodium chloride administration for treatments containing added sodium chloride. All other treatments included a plain chip. One treatment (HP+K) also received 250 mL of Fresca®, as one half of the potassium citrate was dissolved in the Fresca®. For the potassium treatment, the water or Diet 7-Up® was not given with the meal as with the other treatments.

The basic pancake for the moderate protein treatments contained 25 g

flour, 1.5 g Magic® baking powder, 20 g sugar, 2 mL canola oil, and 50 mL of egg yolk mixture. The egg yolk mixture was made by adding 285 mL of distilled deionized water to an egg yolk (for a total volume of 300 mL), and 50 mL of this mixture contained 0.17 of an egg yolk. To keep the treatments isocaloric, the high protein treatments contained 2 g of Splenda® instead of the 20 g of sugar in the moderate protein treatments. Splenda® is an artificial sweetener containing maltodextrin and sucralose, the latter not being absorbed from the gastrointestinal tract. The basic pancake for the high protein treatments also contained an additional 90 mL of distilled deionized water.

3.5 Supplements

The supplements which were added to the base meal were moderate protein (MP), moderate protein plus sodium chloride (MP+Na), high protein (HP), high protein plus sodium chloride (HP+Na), and high protein plus potassium citrate (HP+K). The composition, or ingredients, of the experimental supplements is given in Table 1. The protein, calcium, phosphorus, sodium and potassium content of the MP base meal and of the MP and HP supplements is given in Table 2. The total protein, calcium, phosphorus, sodium and potassium content of each final treatment, including base meal and experimental supplement, is given in Table 3. The energy content of the moderate protein base meal (including 20 g sugar) as calculated by NUTS nutrient analysis software (Version 3.7, Quilchena Consulting Ltd., Victoria, British Columbia) was 262 kcal. The moderate protein supplements contained 35 kcal (derived from New Zealand Milk Products' data sheet), therefore the moderate protein meals contained a total of 297 kcal. The energy content of the high protein base meals (including Splenda®) was

193 kcal, and the high protein supplements contained 105 kcal. The total energy content of the high protein meals was 298 kcal, which is isocaloric with the moderate protein meals.

For the treatments containing supplemental sodium chloride, most of the salt (1.25 g) was added to the basic pancake mix. The rest of the salt was added to a chip by "painting" on 1 mL of a salt solution and then drying at low temperature in an oven. The solution consisted of 5.5 g of table salt in 9 mL of distilled deionized water and 9 mL of commercial vinegar. The total volume of the solution was 20 mL and the addition of 1 mL to a chip resulted in the addition of 0.275 g salt to each treated chip.

The potassium citrate used was tri-potassium citrate monohydrate ($C_6H_5O_7K_3 \cdot H_2O$, Wiler Fine Chemicals Ltd., London, Ontario). Half of the potassium citrate (1.7 g) was added to the pancake mix and the rest (1.8 g) was added to Fresca® to mask the bitter taste.

The dry ingredients of the basic pancakes and the treatments were weighed and placed into labeled individual plastic containers and sealed until use (except the portion of sodium chloride or potassium citrate which was added to a chip or added to Fresca®). The yolk mix was made up on the morning of the study. Leftover yolk mix was refrigerated and used within 2 days. The griddles on which the pancakes were cooked were non-stick and were lightly sprayed with Pam® before use.

The order in which treatments MP, MP+Na, HP, and HP+Na were given was random. The potassium treatment, however, was given after all other treatments had been successfully completed because children in a previous study had disliked the taste of the potassium citrate. There was one exception to this order, which was a subject who was not expected to complete all treatments. This subject received the high protein and then the potassium

treatment then the rest in random order. There was at least 48 hours between treatments for each subject.

The added protein was a mixture of 80% casein and 20% lactalbumin to simulate the protein composition of cow's milk. The casein and lactalbumin powders were from New Zealand Milk Products Inc. (Santa Rosa, California). The casein (Alacid 710), was produced by the acidification of pasteurized skim milk which resulted in the precipitation of casein curd. The curd was washed, then dried and ground. The lactalbumin powder (Alatal 812) was produced by heat precipitation and isolation of whey protein. The casein and lactalbumin proteins were analyzed by Plains Innovative Laboratory Services (University of Saskatchewan) for nitrogen, calcium, phosphorus, sodium, and potassium content, which are given in Table 2. The milk protein mixture (80% casein and 20% lactalbumin) contained 6.7 mg (0.2 mmol) of phosphorus per gram. Therefore, the high protein treatments contained higher levels of phosphorus.

The base meal contained 3.2 g of protein and the moderate protein supplement contained 8.6 g protein. Therefore the total protein content of the moderate protein treatments was 11.8 g. The high protein supplements contained 25.6 g protein, and the total protein content of the high protein treatments was 28.8 g. The RNI for girls age 7 to 9 years old is 1.03 g per kg body weight per day (Scientific Review Committee, 1990). The average body weight of the 14 subjects was 28.1 kg, therefore the daily recommended protein intake for these subjects is approximately 29 g per day. The MP treatment contains approximately one-third of the daily recommended protein intake of girls age 7 to 9 years. The total protein load of the high protein treatments is 28.8 g which is close to the daily recommended intake of protein for a 28 kg girl in this age range.

3.6 Sample Collection

For each subject, fasting urine was collected before the meal. Any urine collection after the meal but before 1.5 hours was refrigerated and the sample obtained at 1.5 hours was added to make up the total 1.5 hour collection. Similarly, any collection after 1.5 hours but before 3 hours was refrigerated and the sample obtained at 3 hours was added to make up the total 3 hour collection. Samples were collected in plastic commodes (NYCO Inc.) then transferred to Nalgene® plastic containers and refrigerated within 15 minutes. For each time period the total urine volume was measured. If the volume was less than 50 mL, distilled deionized water was added to bring the volume to 50 mL. For the fasting and 1.5 hour collections, 3.0 mL of unacidified urine was removed and added to 3.0 mL of 0.1 N HCl and frozen at -20° C until thawed and used for net acid analysis. To the remaining urine was added 1 mL of 6 N HCl for every 25 mL of urine, and the resulting volume was recorded and used for the appropriate calculations. Two samples of this acidified urine were saved: one was frozen and the other refrigerated. The refrigerated acidified urine was used for the analysis of calcium, magnesium, sodium, potassium, phosphate, and creatinine. The frozen acidified urine was thawed and analyzed for sulfate content. Urine was processed and acidified within one hour after each time period. If it was necessary to repeat a treatment, the second trial was used for analysis.

Table 1 Composition of Protein and Salt Supplements

Treatment	Casein Powder[1] (g)	Lactalbumin Powder[1] (g)	Sodium Chloride (g)	Potassium Citrate (g)
MP	7.7	1.9		
MP+Na	7.7	1.9	1.5[2]	
HP	22.8	5.7		
HP+Na	22.8	5.7	1.5	
HP+K	22.8	5.7		3.5[3]

[1] 89.8% protein (%N as analyzed by Plains Innovative Laboratory Services multiplied by 6.38)

[2] 25.7 mmol sodium chloride

[3] 32.4 mmol potassium and 10.8 mmol citrate

Table 2 Selected Nutrient Content of Base Meal, MP, and HP Supplements

Nutrient	Base Meal[1]	MP Supplement[2]	HP Supplement[2]
Protein (g)	3.2	8.6	25.6
Calcium (mmol)	0.92	0.07	0.20
Phosphorus (mmol)	3.33	2.07	6.10
Sodium (mmol)	11.05	0.02	0.05
Potassium (mmol)	1.36	0.01	0.02

[1]As analyzed by NUTS

[2]Nitrogen and mineral content analyzed by Plains Innovative Laboratory Services (PILS), Saskatoon, SK

Table 3 Selected Nutrient Content of Final Treatments[1]

Nutrient	MP	MP+Na	HP	HP+Na	HP+K
Energy (MJ)	1.81	1.81	1.81	1.81	1.81
Protein (g)	11.8	11.8	28.8	28.8	28.8
Calcium (mmol)	1.00	1.00	1.12	1.12	1.12
Phosphorus (mmol)	5.39	5.39	9.43	9.43	9.43
Sodium (mmol)	11.0	36.8	11.1	36.8	11.1
Potassium (mmol)	1.36	1.36	1.38	1.38	33.79

[1]Calculated by adding base meal analysis by NUTS plus PILS analysis of protein supplements plus weight of salts. Manufacturer's data sheets were used for the energy content of protein supplements.

3.7 Chemical Analysis

3.7.1 Calcium

Urinary calcium was analyzed using a flame atomic absorption spectrophotometer (Perkin-Elmer 4000, Perkin Elmer Corporation, Norwalk, CT). The sample is aspirated into a flame and the calcium thereby atomized. The absorption by ground state calcium atoms of a particular wavelength of light is measured. This light is from the emission spectrum of calcium and is generated in a cathode lamp by excited calcium atoms returning to the ground state. Lanthanum is added to the sample to bind with phosphate and sulfate and free the calcium for analysis (Harris, 1982).

To 50 uL of distilled deionized water, standard, or acidified urine was added 2.0 mL of the diluent. The diluent was 0.5% lanthanum in 0.5% HCl (27.14 g $LaCl_3.7H_2O$ plus 86.2 mL of concentrated HCl made up to 2 L volume). Standard solutions of 1.25, 2.50, and 3.75 mmol calcium/L were used (Sigma Diagnostics, St. Louis, MO). Samples and standards were analyzed in duplicate.

3.7.2 Sodium and Potassium

Sodium and potassium were measured in acidified urine by flame emission spectrophotometry (Corning Clinical Flame Photometer 410C, Corning Science Products, Medfield, Massachusetts). Sample is aspirated into the flame and sodium and potassium are atomized into an excited state. The spontaneous emission of a characteristic wavelength by excited atoms returning to the ground state is measured. Emission intensity is proportional to the concentration of the element in the sample (Harris, 1982).

Samples were automatically drawn up and diluted (Corning 805 Diluter). Standard solutions appropriate for urine, containing 160 mmol Na/L and 80 mmol K/L, were used to calibrate the spectrophotometer (Ciba Corning Canada Inc., Richmond Hill, ON). The photometer was first adjusted to measure sodium concentration and all samples were analyzed for sodium. Then the photometer was set to measure potassium concentration and all samples were analyzed for potassium.

3.7.3 Phosphate

Analysis of inorganic phosphate was based on the colorimetric method of Fiske and SubbaRow. Ammonium molybdate in acid solution is added to urine, resulting in the formation of colorless hexavalent ammonium phosphomolybdate $(NH_4)_3[PO_4(MoO_3)_{12}]$(Tietz, 1986). The reduction of this compound results in the conversion to the pentavalent form, which is colored, and spectrophotometric absorption is measured above 400 nm and usually around 700 nm (Tietz, 1986).

Four mL water was added to 50 uL of distilled deionized water, standard, or acidified urine, and 500 uL molybdic solution was added. The molybdic solution consisted of 1.25 g of $(NH_4)_6Mo_7O_{24}.4H_2O$ per 100 mL of 2.5 N H_2SO_4. Then 250 uL of reconstituted Fiske Subbarow Reducer (Sigma Diagnostics, St. Louis, MO), which contains 1-amino-2-napthol-4-sulfonic acid as the reducing agent, was added. Absorption at 660 nm was read 30 minutes after the addition of reducer. Standard solutions containing 0.32, 1.62, and 4.84 mmol Pi/L were used (Sigma Diagnostics, St. Louis, MO). Samples and standards were analyzed in duplicate.

3.7.4 Creatinine

The determination of creatinine was based on the method of Jaffe. Picrate ion, formed by adding base to picric acid, reacts with creatinine and results in the formation of a red-orange compound (Tietz, 1986) which is measured spectrophotometrically.

Twenty-five uL of distilled deionized water, standard, or acidified urine was pipetted into tubes. Three mL of reagent (five parts 0.6% picric acid to one part 1 N NaOH) was added at 30 second intervals. Absorbance at 500 nm was measured exactly ten minutes after addition of picrate solution (measured at 30 second intervals). Creatinine standards of 1, 2, 5, and 10 mmol/L were made from creatinine hydrochloride dissolved in 0.1 N HCl. Samples and standards were analyzed in duplicate.

3.7.5 Sulfate

The measurement of sulfate was based on the method of Ma and Chan (Ma & Chan, 1973). Urinary sulfate (SO_4^{2-}) reacts with barium chloride ($BaCl_2$) to yield barium sulfate ($BaSO_4$) and two chloride ions. The insoluble barium sulfate precipitates but is evenly suspended by dextran and is measured turbidometrically using the spectrophotometer.

Five mL of distilled deionized water was added to 100 uL of acidified urine, standard, or distilled deionized water. One mL of reagent [1% barium chloride in 10% dextran (molecular weight 71900; Sigma Chemicals)] was added. At 10 minutes after addition of the $BaCl_2$, the concentration of $BaSO_4$ was determined by spectrophotometric absorption at 650 nm. Standards of 30, 20, 10, and 5 mmol/L were made from Na_2SO_4 dissolved in distilled deionized water. Samples and standards were analyzed in duplicate. Only treatments MP, MP+Na, HP, HP+Na were analyzed for sulfate.

3.7.6 Net Acid

The measurement of net acid excretion (NAE) was based on the method of Chan (Chan, 1972). NAE is the sum of urinary titratable acid (in the absence of bicarbonate) and ammonium. In this method, titratable acid (TA) is determined by the amount of base required to titrate the urine to pH 7.40 after the removal of bicarbonate. Formaldehyde is then added to urine to convert NH_4^+ to NH_3 and H^+, which results in a drop in pH. Ammonium is determined by the amount of base required to titrate the sample to pH 7.40.

Three mL of unacidified urine or distilled deionized water was added to three mL of 0.1 N HCl which resulted in the conversion of bicarbonate (HCO_3^-) to carbonic acid (H_2CO_3). The samples were frozen at -20° C until analysis. Samples were thawed and boiled for 5 minutes to drive off the CO_2 from the conversion of H_2CO_3 to H_2O and CO_2. Samples were cooled and titrated with 0.1 N NaOH to the endpoint of pH 7.40 ± 0.02, partial pressure of carbon dioxide of zero at room temperature. Three mL of 8% formaldehyde was then added to the samples. The sample was titrated again to the same endpoint with 0.1 N NaOH. NAE is the sum of TA and NH_4^+.

3.8 Statistical Analysis

Results are expressed as mean ± SD for subject data and mean ± SEM for urine analysis. Urine excretion data is expressed per mmol of creatinine (except creatinine). Statistical analysis was performed using the program Primer (Glantz, 1992). At each time point, the results were analyzed by repeated measures ANOVA to detect treatment effects. If there were differences between treatments at $p \leq 0.05$, the Student-Newman-Keuls test was used to detect differences between individual treatment means. Correlations were also performed using Primer.

4. RESULTS

4.1 Subject Characteristics

The age, height, and weight of the 14 subjects are shown in Table 4. The results of the food frequency questionnaire are also given in Table 4. The average of food records from the 5 days before each study day (four days for subject 1, as one record was incomplete) indicated a lower calcium intake than the food frequency questionnaire. The range in calcium intake according to food records was 9.5 - 40.1 mmol (379-1609 mg) per day, with a mean ± SD intake of 22.3 ± 8.7 mmol (895 ± 347 mg) calcium/day. The average intake of protein, calcium, phosphorus, sodium and potassium on the pre-study days is given in Table 5. The pre-study food records indicated a significant correlation between protein and phosphorus intake ($r = 0.89$, $p<0.001$). There was also a significant correlation between calcium intake and protein intake ($r=0.75$, $p<0.001$). Subjects were healthy as determined by the responses to the health questionnaire. One subject was reported to be lactose intolerant.

4.2 Calcium Excretion

There were no significant differences in mean fasting urinary calcium excretion between treatment days. The mean fasting urinary calcium excretion for all samples was 0.25 mmol/mmol creatinine with a range of 0.06 to 0.56 (see Table 6). The median fasting urinary calcium excretion was 0.22 mmol/mmol creatinine. There was no correlation between fasting urinary calcium excretion and pre-study intake of calcium, protein, potassium, sodium or phosphorus.

There were no significant differences in 1.5 or 3.0 hour calcium excretion between treatments (Table 7). Urinary calcium excretion peaked at 1.5 hours after all treatments.

4.3 Potassium Excretion

Results for urinary potassium excretion are given in Table 8. There were no significant differences in fasting potassium excretion between treatments. The correlation between pre-study potassium intake and fasting urinary potassium excretion was not significant ($r = 0.197$, $p = 0.104$). With the potassium citrate load, which provided on average 1.2 mmol potassium per kilogram of body weight, there was a significantly higher urinary potassium excretion at 1.5 and 3 hours. The average amount of potassium excreted by 3 hours after the HP+K treatment was 16.7 mmol in excess of the amount excreted after the HP treatment. This represented 52% of the 32.4 mmol of potassium added in the HP+K treatment being excreted within 3 hours. There were no differences in potassium excretion between the other treatments.

Table 4 Subject Age, Height, Weight, and Calcium Intake

Subject	Age on July 1/94 (years)	Height (cm)	Weight (kg)	Ca-FFQ[1] (mmol/day)
1	10.0	131	29.0	20.9
2	6.7	122	20.9	26.2
3	8.8	131	30.6	33.3
4	9.3	138	25.1	32.9
5	7.7	133	33.7	12.7
6	9.1	137	28.7	32.8
7	6.8	113	18.9	19.7
8	9.0	134	30.6	32.0
9	8.6	129	27.1	28.7
10	8.0	125	24.2	19.7
11	9.3	140	33.1	30.7
12	7.8	130	27.7	35.0
13	9.0	138	33.2	55.2
14	9.3	135	31.2	22.9
Mean ± SD	8.5 ± 2.2	131 ± 7	28.1 ± 4.5	28.8 ± 10.1

[1]Usual calcium intake determined using a food frequency questionnaire (Whiting, unpublished)

Table 5 Nutrient Intakes of Subjects Calculated from Five Food Records[1]

Subject	Protein (g)	Ca (mmol)	P (mmol)	Na (mmol)	K (mmol)
1*	61 ± 30	17.4 ± 5.6	35.0 ± 10.7	107.3 ± 50.5	65.3 ± 40.2
2	39 ± 15	15.1 ± 9.0	23.0 ± 9.7	73.4 ± 25.9	35.8 ± 11.9
3	67 ± 30	26.2 ± 11.0	40.1 ± 12.2	123.3 ± 86.5	57.0 ± 16.2
4	59 ± 15	29.2 ± 11.5	44.0 ± 12.0	101.5 ± 16.6	57.5 ± 10.6
5	54 ± 12	14.9 ± 6.3	29.1 ± 3.3	71.2 ± 15.7	54.2 ± 12.6
6	62 ± 19	16.0 ± 4.1	34.9 ± 8.0	102.2 ± 24.1	46.4 ± 11.2
7	39 ± 13	16.7 ± 6.8	25.2 ± 6.7	69.6 ± 29.4	38.8 ± 6.0
8	43 ± 10	9.5 ± 7.5	22.1 ± 7.7	72.2 ± 28.6	55.2 ± 26.2
9	54 ± 14	17.8 ± 7.8	31.2 ± 9.5	93.2 ± 27.1	54.8 ± 12.6
10	55 ± 23	20.7 ± 7.2	34.1 ± 13.0	113.2 ± 36.0	66.1 ± 29.8
11	71 ± 17	29.3 ± 10.3	44.5 ± 10.6	95.9 ± 18.2	63.0 ± 11.6
12	76 ± 20	34.3 ± 16.3	52.2 ± 14.6	146.3 ± 64.4	67.7 ± 6.8
13	84 ± 25	40.1 ± 12.7	52.8 ± 13.7	120.6 ± 22.9	65.0 ± 16.3
14	63 ± 17	25.4 ± 9.0	36.2 ± 9.6	149.2 ± 15.6	43.1 ± 19.2
Mean ± SD	59 ± 13	22.3 ± 8.7	36.0 ± 9.8	102.8 ± 26.3	55.0 ± 10.4

[1]Mean ± SD

*4 records only for this subject

Table 6 Fasting Urinary Calcium Excretion (mmol/mmol Cr)

Subject	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Mean
1	0.16	0.02	0.37	0.18	0.24	0.20
2	0.03	0.05	0.41	0.05	0.03	0.11
3	0.46	0.45	0.46	0.42	0.41	0.44
4	0.16	0.15	0.16	0.12	0.12	0.14
5	0.30	0.22	0.26	0.25	0.18	0.24
6	0.31	0.30	0.69	0.33	0.36	0.40
7	0.11	0.70	0.31	0.36	0.37	0.37
8	0.22	0.13	0.07	0.26	0.23	0.18
9	0.68	0.41	0.52	0.69	0.52	0.56
10	0.06	0.15	0.11	0.07	0.02	0.08
11	0.04	0.04	0.14	0.05	0.03	0.06
12	0.39	0.30	0.18	0.18	0.38	0.28
13	0.24	0.49	0.29	0.20	0.30	0.30
14	0.07	0.05	0.03	0.10	0.06	0.06
Mean ± SD						0.25 ± 0.16

Table 7 Effect of Treatment on Urinary Calcium Excretion[1]

Treatment	Fasting Ca (mmol/mmol Cr)	1.5 Hour Ca (mmol/mmol Cr)	3.0 Hour Ca (mmol/mmol Cr)
MP	0.27 ± .06	0.38 ± .11	0.23 ± .11
MP+Na	0.26 ± .05	0.42 ± .12	0.30 ± .08
HP	0.20 ± .04	0.32 ± .06	0.19 ± .05
HP+Na	0.22 ± .04	0.32 ± .07	0.21 ± .05
HP+K	0.29 ± .05	0.40 ± .10	0.25 ± .07

[1]Mean ± SEM, n=14

Table 8 Effect of Treatment on Urinary Potassium Excretion[1]

Treatment	Fasting K (mmol/mmol Cr)	1.5 Hour K (mmol/mmol Cr)	3.0 Hour K (mmol/mmol Cr)
MP	12.54 ± 1.68	9.59 ± 0.99	9.79 ± 1.17
MP+Na	11.53 ± 1.23	9.23 ± 0.74	9.59 ± 0.83
HP	14.28 ± 1.70	9.22 ± 0.88	7.17 ± 0.53
HP+Na	12.06 ± 1.67	8.74 ± 0.93	7.21 ± 0.49
HP+K	11.98 ± 1.70	40.96 ± 4.11*	33.96 ± 2.64*

[1]Mean ± SEM, n=14

*different from all other treatment means in column ($p<0.001$)

4.4 Sodium Excretion

Results for urinary sodium excretion are given in Table 9. There were no significant differences in fasting sodium excretion between treatments and no correlation between pre-study sodium intake and fasting urinary sodium/creatinine ($r = 0.066$, $p = 0.592$). In response to the potassium treatment only, there was a significantly higher urinary sodium excretion at 1.5 hours. The average amount of sodium excreted by 3 hours after the HP+K treatment was 10.8 mmol in excess of the amount excreted after the HP treatment. There were no differences in sodium excretion between the other treatments.

The sodium from the sodium chloride loads was not excreted within the 3 hours of the study. However, we did observe a significant correlation between fasting urinary sodium (mmol/mmol creatinine) and fasting urinary calcium (mmol/mmol creatinine) ($r=0.53$, $p<0.001$), using the five fasting samples from each of the 14 subjects for a total of 70 samples.

4.5 Phosphate Excretion

Results for urinary phosphate excretion are given in Table 10. There were no significant differences in fasting phosphate excretion between treatments and there was no correlation between pre-study phosphorus intake and fasting phosphate excretion. The phosphate excretion at 1.5 hours was significantly higher with the HP+K treatment compared to the other treatments. At 3 hours, the phosphate excretion after the HP+Na treatment was significantly higher than that after the MP and MP+Na treatments.

4.6 Net Acid Excretion (NAE)

Net acid excretion results are shown in Table 11. There were no significant differences in fasting NAE between treatments. There was no correlation between fasting NAE and pre-study protein or potassium intake. There was a large drop in net acid excretion at 1.5 hours when the children (n=13) were given potassium citrate. It should be noted that the girls did not show a protein-induced increase in net acid excretion at 1.5 hours.

4.7 Sulfate Excretion

Results for urinary sulfate excretion are given in Table 12. Fasting and 1.5 hour urinary sulfate excretion was measured for treatments MP, MP+Na, HP, and HP+Na, to determine if there was any change in sulfate excretion and if the high protein treatment provided excess sulfur amino acids (i.e. greater than the requirement) for these children. There were no significant differences in fasting sulfate excretion between treatments. In the children, an increase in protein load from 11.6 g (0.41 g/kg) to 28.2 g (1.0 g/kg) did not result in increased sulfate excretion ($p=0.091$).

4.8 Urine Volume

Urine volumes are given in Table 13. There were no significant differences in fasting urine volume. The 1.5 hour urine volume for the HP+K treatment was significantly larger than that for the other treatments. At 3 hours the MP volume was significantly greater than the HP+Na volume.

4.9 Creatinine Excretion

Results for creatinine are given in Table 14. There were no significant differences in creatinine excretion between treatments at any time point. There was no significant correlation between pre-study protein intake and fasting creatinine excretion. Fasting creatinine excretion is highly variable because the fasting urine was not from a controlled period of time as were the 1.5 and 3.0 hour collections. There was a significant correlation between average 1.5 hour creatinine excretion and body weight ($r=0.81$, $p<0.001$) and between average 3.0 hour creatinine excretion and body weight ($r=0.79$, $p<0.001$).

Table 9 Effect of Treatment on Urinary Sodium Excretion[1]

Treatment	Fasting Na (mmol/mmol Cr)	1.5 Hour Na (mmol/mmol Cr)	3.0 Hour Na (mmol/mmol Cr)
MP	18.90 ± 2.62	11.90 ± 2.05	11.80 ± 3.76
MP+Na	16.11 ± 1.70	12.07 ± 2.26	13.54 ± 2.08
HP	18.35 ± 2.55	10.91 ± 1.44	10.06 ± 2.28
HP+Na	18.12 ± 2.00	12.96 ± 1.97	12.97 ± 2.33
HP+K	19.23 ± 2.60	40.65 ± 6.49*	18.32 ± 2.82

[1]Mean ± SEM, n=14

*different from all other treatment means in column ($p<0.001$)

Table 10 Effect of Treatment on Urinary Phosphate Excretion[1]

Treatment	Fasting Pi (mmol/mmol Cr)	1.5 Hour Pi (mmol/mmol Cr)	3.0 Hour Pi (mmol/mmol Cr)
MP	2.02 ± 0.23	2.62 ± 0.32	3.08 ± 0.24
MP+Na	2.03 ± 0.17	2.40 ± 0.29	3.04 ± 0.20
HP	1.79 ± 0.18	2.90 ± 0.34	3.53 ± 0.34
HP+Na	2.28 ± 0.18	3.02 ± 0.36	4.05 ± 0.48#
HP+K	2.01 ± 0.13	4.00 ± 0.43*	3.77 ± 0.36

[1]Mean ± SEM, n=14

*different from all other treatment means in column ($p=0.002$)

#different from 3 hour phosphate excretion for MP and MP+Na ($p<0.05$)

Table 11 Effect of MP, MP+Na, HP, and HP+Na Treatments on Urinary Net Acid Excretion[1]

Treatment	Fasting NAE (mmol/mmol Cr)	1.5 Hour NAE (mmol/mmol Cr)
MP	-0.70 ± 1.68	-1.33 ± 1.64
MP+Na	-0.11 ± 1.78	+0.07 ± 1.48
HP	-2.00 ± 1.79	-3.25 ± 2.36
HP+Na	-1.11 ± 1.58	-0.86 ± 1.94
HP+K	-0.24 ± 1.56	-23.62 ± 3.26*

[1]Mean ± SEM, n=13

*different from all other treatment means in column ($p<0.001$)

Table 12 Effect of MP, MP+Na, HP, and HP+Na Treatments on Urinary Sulfate Excretion[1]

Treatment	Fasting SO_4 (mmol/mmol Cr)	1.5 Hour SO_4 (mmol/mmol Cr)
MP	1.06 ± 0.16	1.24 ± 0.09
MP+Na	1.06 ± 0.09	1.18 ± 0.10
HP	0.92 ± 0.08	1.42 ± 0.09
HP+Na	1.04 ± 0.14	1.06 ± 0.11

[1]Mean ± SEM, n=14

Table 13 Effect of Treatment on Urine Volume[1]

Treatment	Fasting Volume (mL/mmol Cr)	1.5 Hour Volume (mL/mmol Cr)	3.0 Hour Volume (mL/mmol Cr)
MP	422.5 ± 66.8	720.3 ± 151.7	951.6 ± 161.1#
MP+Na	420.0 ± 69.6	642.4 ± 106.1	703.6 ± 156.2
HP	652.7 ± 152.6	964.9 ± 188.2	724.6 ± 123.2
HP+Na	461.0 ± 76.2	627.6 ± 125.4	432.5 ± 116.3
HP+K	496.9 ± 77.3	1476.8 ± 186.8*	764.2 ± 180.2

[1]Mean ± SEM, n=14

*different from all other treatment means in column ($p<0.001$)

#different from 3.0 hour volume for HP+Na ($p=0.021$)

Table 14 Effect of Treatment on Urinary Creatinine Excretion[1]

Treatment	Fasting Cr (mmol)	1.5 Hour Cr (mmol)	3.0 Hour Cr (mmol)
MP	0.31 ± 0.03	0.34 ± 0.03	0.32 ± 0.02
MP+Na	0.45 ± 0.13	0.35 ± 0.02	0.30 ± 0.02
HP	0.29 ± 0.06	0.38 ± 0.03	0.32 ± 0.02
HP+Na	0.38 ± 0.10	0.35 ± 0.03	0.32 ± 0.02
HP+K	0.25 ± 0.03	0.35 ± 0.03	0.29 ± 0.02

[1]Mean ± SEM, n=14

5. DISCUSSION

The use of an acute test to study the effect of dietary factors on calcium excretion in children was successfully performed in this study. Each subject was able to consume all of the treatments. Urine was adequately collected by the observation that the average 1.5 hour creatinine was similar to the 3.0 hour creatinine. If the rate of creatinine excretion was constant at the measured level, the 24 hour urinary creatinine excretion of these children would be 5.3 mmol/day, which is similar to the adult norm of 0.22 mmol/kg per day (Windholz, 1983). Subjects had normal fasting calcium to creatinine ratios. Subject 9 had a mean fasting urinary calcium excretion of 0.56 mmol/mmol creatinine, which corresponds to around the 95th percentile for fasting urinary calcium found in a group of British children aged 2-15 years old (Shaw, et al., 1990). However, the upper normal limit (mean + 2 SD) for fasting urinary calcium/creatinine ratio in another sample of 60 British children from 1 to 15 years old was 0.71 mmol/mmol (Ghazali & Barratt, 1974). Subject 9 was not excluded from the study.

In acute studies involving adults in which urine was collected for 3 hours (Fagnou, 1995) or 4.5 hours (Anderson, 1995) after a meal similar to that given to the children, urinary calcium excretion peaked at 3.0 hours and decreased by 4.5 hours. Therefore urine collection until 3.0 hours was deemed adequate in adults. In the children, urinary calcium excretion peaked at 1.5 hours and decreased by 3.0 hours. Therefore, 3.0 hours seems to be more than adequate collection time for children.

Acute studies in adults will be used to make comparisons regarding treatment effects on urinary calcium between children and adults. Ten adult women were studied during the summer of 1994 in a format similar to the children's study (Fagnou, 1995). The women had a mean ± SD age of 24.6 ± 3.7 years and mean ± SD weight of 71.1 ± 11.4 kg. The subjects received, in random order, a supplement of 17 g of milk protein, 17 g of milk protein + 23 mmol $KHCO_3$, 50 g of milk protein, or 50 g of milk protein + 70 mmol $KHCO_3$ added to the base meal. The women also received sodium chloride treatments, however the protein in these treatments consisted only of casein in order to simulate cheese. The two sodium treatments were 17 g casein + 23 mmol NaCl and 50 g casein + 70 mmol NaCl. Fasting, 1.5 hour, and 3.0 hour urine was collected. Ten adult men who were matched for weight with the women were studied using a very similar protocol (Anderson, 1995). This study included moderate protein and high protein treatments, and a moderate protein treatment supplemented with sodium phosphate so that the phosphate level was equal to that of the high protein treatment. Urine was collected at 1.5, 3.0 and 4.5 hours after the administration of the meal.

In the children, an increase in protein intake from 11.8 g (0.42 g/kg) to 28.8 g (1.0 g/kg) did not increase urinary calcium excretion. In adult women (Fagnou, 1995) an increase in protein intake from 23 g (0.32 g/kg) to 54 g (0.76 g/kg) with an increase in integral phosphate intake, did not affect urinary calcium excretion. In the adult males (Anderson, 1995) an increase in total protein from 22.6 g (0.32 g/kg) to 53.1 g (0.75 g/kg) had no effect on urinary calcium excretion in spite of an increase in sulfate and net acid excretion (which were not measured in the women). However, in the men, when the phosphorus intake between the two treatments was equalized by the addition of 8 mmol of NaH_2PO_4 to the moderate protein treatment, there

was a significant rise in urinary calcium excretion due to protein. Therefore, in the acute load test, as in long term studies (Hegsted & Linkswiler, 1981; Hegsted, et al., 1981; Orwoll, 1992), isolated protein intake, with phosphorus content held constant, increases urinary calcium excretion in adults. In adults, there was no significant change in GFR with an increase in protein intake (Fagnou, 1995). Since we did not obtain blood samples, it is not possible to quantify GFR in the children.

The hypothesis that an increase in protein intake increases acute urinary calcium excretion in children was rejected. The finding that an increase in protein intake, with an accompanying increase in phosphorus, does not increase urinary calcium excretion in children is in agreement with the finding of Matkovic et al. that protein intake did not affect 24-hour urinary calcium excretion or bone density in free-living adolescent girls (Matkovic, et al., 1995). It is possible that had phosphate content been equalized, an increase in protein would have increased urinary calcium excretion. Schofield et al. did find an increase in 24-hour urinary calcium excretion with high protein feeding in preadolescent girls, however, the phosphate content of the diet was similar between treatments (Schofield & Morrell, 1960), indicating that phosphorus may also be hypocalciuric in children.

North American diets may generate 100 mEq of acid per day, part of which is due to the catabolism of sulfur amino acids which are found in relatively large amounts in animal proteins. The adult skeleton contains a large amount of alkali, which may act as a buffer for endogenous acid production (Barzel, 1995). There is a concern that high protein intakes may increase the rate of bone loss as bone is being dissolved to buffer the acid generated from protein metabolism (Barzel, 1995). Alternatively, acid resulting from amino acid metabolism may decrease renal calcium reabsorption, resulting in

increased obligatory loss of calcium, and the resorption of bone in order to maintain plasma calcium concentration. Metabolic acidosis also results in an increase in the ionized plasma calcium fraction, which could lead to a higher filtered load of calcium and increased urinary calcium excretion.

Free-living people eating a high protein diet will also have a high phosphorus intake, as phosphorus is an integral component of protein foods. Over the short-term in fasting subjects, an increase in milk protein intake, with a concomitant increase in phosphate intake, does not increase urinary calcium, as the phosphate appears to ablate the calciuric effect of the protein. Heaney and Recker (1982) claim that although phosphorus decreases urinary calcium excretion, it increases endogenous fecal calcium losses, therefore having no effect on overall calcium balance. This hypothesis is difficult to prove and could not be examined in this study as I did not collect feces. Longer term studies in adults fed high protein and phosphorus foods, such as meat have reached differing conclusions about their effect on urinary calcium excretion and calcium retention; some investigators found no effect on urinary calcium of an increase in meat intake (Hunt, et al., 1995; Spencer, et al., 1983), while others found that added protein and phosphorus resulted in a slight increase in urinary calcium excretion and a more negative calcium balance (Hegsted, Schuette, Zemel, & Linkswiler, 1981).

A cross-sectional study indicated a positive relationship between animal protein intake and hip fracture rate (Abelow, et al., 1992). However, other factors which may accompany a typical Western high-meat diet may be wholly or partly responsible for this observation.

The girls in this did not show an increase in urine sulfate or net acid excretion in response to added dietary protein. The children appeared to utilize the sulfur amino acids provided by the high protein load suggesting that

after an overnight fast, there was a greater need for protein than reflected in the RNI. The average daily recommended protein intake for these subjects was approximately 29 g per day. The MP meal contained 11.8 g of protein, approximately one-third of the daily recommended protein intake of girls age 7 to 9 years, which is an amount that might be expected in one meal. The 28.8 g of protein of the HP treatments is close to the total daily recommended intake of protein for a 7 to 9 year old girl. The level of protein which would effect an increase in sulfate excretion in the fasting state in children is not known.

Assuming that the sulfur amino acid oxidation and resultant increase in acid production is one of the main mechanisms of protein induced calciuria, a treatment given to the girls which contained moderate protein but was equal in phosphate content to the HP treatment may not have decreased urinary calcium excretion.

Neither the children nor women (Fagnou, 1995) showed a change in acute urinary calcium excretion in response to an increase in sodium chloride intake. There was no increase in urinary sodium excretion within 3 hours of the salt load in the children or the women. From the results of this study and the adult studies conducted during the same time period, it can be seen that the short term unacclimated load test is not useful for studying the effects of sodium chloride intake on urinary calcium excretion. As there was no effect of sodium chloride on urinary calcium in our study, we did not test for an interaction between protein and sodium chloride. The hypothesis that an increase in sodium chloride intake increases acute urinary calcium excretion in children must be rejected.

The correlation between fasting urinary sodium/creatinine and fasting urinary calcium/creatinine was similar between the girls ($r=0.53$, $p<0.001$,

n=70) and the women (r=0.56, p<0.001, n=60). This finding indicates that children respond to sodium intake as do adults. Matkovic et al. found that in early pubertal girls, urinary sodium excretion was an important determinant of urinary calcium excretion, and that with a higher urinary calcium excretion, there was lower bone density (Matkovic, et al., 1995). A recently published prospective study in postmenopausal women also found a negative association between urinary sodium excretion and bone mass (Devine, Criddle, Dick, Kerr, & Prince, 1995).

In the three hours of the acute test there was no increase in urinary sodium, indicating retention of the ingested sodium. The lack of a calciuric effect of this retained sodium (and presumably chloride) suggests that it is the sodium excretion, and not the ingestion per se, which is linked to urinary calcium excretion. Extracellular fluid (ECF) volume has also been implicated in the effect of sodium chloride on urinary calcium (Lemann, 1993; Peacock, 1988). ECF expansion, as would have occurred with the sodium retention, has been thought to increase urinary calcium excretion. While ECF expansion, indicated by the retention of sodium, was probably occurring in these acute studies, there was no increase in urinary calcium excretion. This indirectly supports the idea that it may be the actual luminal sodium concentration which affects renal calcium reabsorption (Brunette, et al., 1992).

With a potassium citrate load providing 32.4 mmol of potassium (1.2 mmol/kg), there was no change in urinary calcium excretion in the children. Therefore the hypothesis that potassium citrate decreases urinary calcium excretion in children must be rejected. In adults, 50 mmol of potassium bicarbonate (0.7 mmol K/kg) (Anderson, 1995) and 70 mmol of potassium bicarbonate (1.0 mmol K/kg) (Fagnou, 1995) resulted in a decrease in urinary

calcium excretion compared to protein alone. A load of 23 mmol potassium bicarbonate (0.3 mmol K/kg) did not affect urinary calcium excretion in the women. The children excreted 52% of the 32 mmol potassium load by 3 hours, while the women excreted 38% of the 70 mmol of potassium by 3 hours. In both the children and adults, the alkaline potassium resulted in a decrease in net acid excretion. Since kidney function is similar between adults and children, perhaps the difference in response to alkaline potassium results from the action of potassium at a different site, such as bone.

In the children, urinary phosphate excretion was increased at 1.5 hours after the HP+K treatment compared to HP. This is different from the response seen in adults, where the large load of potassium resulted in a decrease (Anderson, 1995) or no change (Fagnou, 1995) in phosphate excretion. An increase in PTH secretion would result in increased phosphate excretion, but would also result in a decrease in urinary calcium excretion. Studies in young adults do not indicate a role of PTH in the decreased urinary calcium excretion seen with alkaline potassium treatments (Anderson, 1995). Lemann et al. hypothesized that the decrease in urinary calcium seen in adults with alkaline potassium treatment results from an increase in renal phosphate reabsorption with a resultant decrease in dihydroxyvitamin D synthesis and decrease in gastrointestinal calcium absorption (Lemann, et al., 1993). However, if this were the sole mechanism of potassium induced decrease in urinary calcium excretion, the effect would presumably not be seen within the short time of an acute load study and potassium would not result in long-term increases in calcium retention. Lemann et al. (1993) have alternatively postulated that alkaline potassium administration results in extracellular volume contraction leading to increased tubular calcium reabsorption.

The alkaline potassium treatment, which provided 32.4 mmol of potassium (1.2 mmol per kg), resulted in an increase in urinary sodium excretion in the children. Potassium-induced natriuresis also occurred in adults at 0.7 mmol/kg (Anderson, 1995) and 1.0 mmol/kg (Fagnou, 1995). In 10 adult women, a load of only 0.3 mmol K/kg did not affect calcium or sodium excretion. The phenomenon of potassium induced natriuresis is not well described in the literature. Lemann et al. (1991) saw an increase in sodium excretion with potassium bicarbonate administration in the first 24-hour urine collection only.

An increase in urinary sodium excretion is usually accompanied by an increase in calcium excretion, however, calcium excretion would not be affected if the sodium excretion was increased at a point distal to where sodium affects calcium handling. This implies a secretion of sodium in the distal part of the nephron with alkaline potassium loading or a disassociation between the normal sodium and calcium relationship in the more proximal parts of the nephron.

An increase in urine volume during the HP+K treatment was observed in the children but not the adults (Fagnou, 1995). Although potassium citrate is noted to have a diuretic effect (Windholz, 1983), the increase in volume may have been due to an increase in fluid intake, which was not adequately measured, with this treatment. The hypocalciuric effect of alkaline potassium may have been countered by an increase in calcium excretion caused by the large increase in urine volume at 1.5 hours with the HP+K. However, calcium is not thought to vary with changes in free water excretion (Peacock, 1988). While rats given potassium bicarbonate had an increase in urine volume, there was no change in calcium excretion (Whiting, 1992). The large urine volume at this time period could also account for the simultaneous

increase in phosphate excretion. Studies in both humans (Whiting, 1990) and rats (Whiting, 1992) show a trend for phosphate, but not calcium, excretion to parallel urine volume.

Alkaline potassium may decrease urinary calcium in adults by neutralizing the acid produced by amino acid catabolism. The HP+K treatment may not have decreased urinary calcium excretion in the children because there was no protein induced acid to neutralize, as indicated by the lack of effect of added protein on NAE and sulfate excretion. Alternatively, children may not respond to alkaline potassium intake with a decrease in urinary calcium. Rats also do not respond to a load of alkaline potassium with a decrease in urinary calcium excretion (Greger, et al., 1991; Whiting, 1990). The differences in bone metabolism between children and adults (i.e. bone modeling vs. remodeling) may be involved in this difference in response between adults and children.

The absence of a calciuric response to alkaline potassium in children needs to be confirmed in future short and long term studies, as this is the first such study in children. Future studies could include blood samples for the determination of hormones, including calcitonin and PTH, serum creatinine for the calculation of glomerular filtration rate, and markers of bone metabolism, however blood collection may reduce the number of subjects willing to participate. Alternatively, urinary indicators of bone formation and resorption could be measured.

The hypothesis that children have similar acute diet-induced calciuric responses must be rejected since the children did not respond to the alkaline potassium as did the adults. The children and adults did respond similarly to a sodium chloride load and to an increase in protein and phosphorus intake.

The short-term format used was suitable for use with children. Similar acute load test studies could be done with different levels of protein while phosphate is held constant to determine if isolated protein is calciuric in children and if so, at what amount. For the children, the load test might work better if the subjects were fed a standard breakfast supervised throughout the morning, and then the load test was performed at lunch-time. This would give control over the pre-load meal and fluid intake could be more controlled.

Treatments giving supplementary phosphate would also determine if phosphate is hypocalciuric in children. There are difficulties with equalizing phosphate levels as this must be done by adding phosphate salts, which may alter the acidity of the diet and the anion content. The natriuretic effect of alkaline potassium may be of interest in controlling salt-sensitive hypertension and could be investigated with a load-test format. More work should be done to investigate diet-induced changes in bone in children as there may be an opportunity to maximize bone growth and prevent osteoporosis during childhood.

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7. APPENDICES

7.1 Ethical Approval

UNIVERSITY ADVISORY COMMITTEE ON ETHICS IN HUMAN EXPERIMENTATION (Medical Sciences)

NAME AND E.C.#: Susan Whiting 92-72
Nutrition & Dietetics

DATE: May 8, 1992

Your study entitled "Effect of dietary protein from daily products on urinary calcium excretion in children and adolescents"

has been approved by the University Advisory Committee on Ethics in Human Experimentation (Medical Sciences).

1(a). Therefore you are free to proceed with the project subject to the following conditions:

How will the control group be recruited and from where? At the bottom of page 1 of the child's consent form, it refers to the centre or school. What does this refer to? Under "Important Note" #1, explain what these disorders may be. How much is the honorarium?

1(b). Please submit the revisions requested in 1(a) to the Director of Research Services, Room 210, Kirk Hall.

2. Any significant changes to your protocol should be reported to the Director of Research Services, Room 210, Kirk Hall, for Committee consideration in advance of its implementation.

DR. [illegible]
Director of Research Services
University of Saskatchewan

for E.A. McKenna
Chair
University Advisory Committee on Ethics in Human Experimentation

cc: Royal University Hospital

7.2 Food Record Form

Child's Name:_____ Date:_____

Instructions: Fill out all food and drinks for the day. If necessary, include recipes on the back. Use as much detail as possible.

Meal or Activity	**Time**	**Food**	**Amount**
Child rises	____	_____	____
Breakfast	____	_____	____
	____	_____	____
	____	_____	____
	____	_____	____
Mid morning	____	_____	____
	____	_____	____
Noon Meal	____	_____	____
	____	_____	____
	____	_____	____
	____	_____	____
Afternoon	____	_____	____
	____	_____	____
Evening Meal	____	_____	____
	____	_____	____
	____	_____	____
	____	_____	____
Before Bed	____	_____	____
	____	_____	____

Please circle the correct answer.

1. Was this a usual intake for the day? yes no
   If no, please indicate the reason. (e.g. sick)
2. Did your child take any vitamin/mineral pills during this time? yes no Brand: _____

7.3 Pre-Study Checklist for Parents

Checklist for Evening Before Study and Study Day

Name:_____

Day Before Study:

_____ Child eats supper before 6:00 pm.

_____ Between 7:00 to 8:00 pm child eats snack provided.

_____ After 8:00 pm child consumes no more food.

_____ Child drinks 200-250 ml of diet beverage provided at 8:00 pm.

_____ Child goes to bed at normal time.

_____ Parent records food eaten by child during the day.

Morning of Study Date_____

_____ (time) Child rises. (RECORD TIME).

_____ (time) Child passes and discards first urine sample (RECORD TIME).

_____ Child drinks 200-250 ml of diet beverage provided (does not eat or drink anything else).

_____ Child arrives at the university (between 8:00 am and 9:00 am). Bring remaining drink.

_____ Child provides a fasting urine sample.

_____ Child is fed breakfast.

_____ Urine collected from each child for a total of 3 hours after meal.

_____ Activities, water and diet beverage provided throughout the morning.

_____ Lunch will be provided (from 12:00-1:00) and children transported to home or day care.

7.4 Subject Data Sheet

SUBJECT DATA DATE:_____

SUBJECT NAME: _____

SUBJECT CODE: _____

BIRTHDAY: (DD-MM-YYYY) _____

HEIGHT: _____

WEIGHT: _____

TREATMENT ORDER	DATE	AMOUNT EATEN
1)_____	_____	_____
2)_____	_____	_____
3)_____	_____	_____
4)_____	_____	_____
5)_____	_____	_____
6)_____	_____	_____

Comments:

7.5 Daily Checklist for Study Day

Name:_____ Date:_____

Weight: _____

Height: _____

Time of rising:_____

Time of void at home:_____

Amount of diet 7-up drunk at home:

Evening:_____ml at _____p.m.

Morning:_____ml at _____a.m.

Time of fasting void: _____a.m

Pancake Treatment: _____

Started meal at: _____a.m.

Finished meal at:_____a.m.

Time of Load: _____ (midway between start and finish)

Time of Load + 1.5 hrs:_____

Time of Load + 3.0 hrs:_____

7.6 Letter Sent to Families of Prospective Subjects

Dear Parent, June, 1994

Calcium is important for the development of strong bones during childhood, and for the prevention of osteoporosis in later life. Many factors in food affect how the body uses calcium. We are studying how components of food affect the loss of calcium through the urine in 7-10 year old girls. We performed a similar study last summer, and some of the girls from last year will be participating in this year's study.

The study will be held in July and August at the Thorvaldson building, U. of S. campus. On the mornings of the study, you would drop your child off between 8:00 and 9:00 a.m. All beverages and breakfast are supplied. We supervise and entertain the children during the morning (there will be games, crafts, etc.). Children can keep whatever they make, including hand-painted T-shirts. After the study period is completed, we provide lunch and see that each child is taken home or to a child care facility. There are also several arts and activities camps being held in the afternoons at the university, and we could provide transportation directly to these camps.

The girls will be given pancakes or a similar meal for breakfast; we then collect urine samples over the next 3 hours. Urine is collected in a plastic container that fits over the toilet. There are 6 different types of pancakes, and we would like each child to come on 6 separate days. Your child can come any 6 study days that are most convenient for you (to a maximum of 3 per week). Each type of pancake contains different amounts of things naturally found in food such as protein, sugar, and salt. Your child can discontinue the study at any time if she feels uncomfortable or does not want to continue. However, the girls who were in the study last year had a lot of fun.

The study is being supervised by Dr. Susan Whiting of the Division of Nutrition and Dietetics, University of Saskatchewan and is funded by the Dairy Bureau of Canada. The study has been approved by the Ethics Committee of the university. Consent forms are required of all participants and guardians. Other personnel working on this study are: Tracy Duff (Nutrition Graduate Student), Celeste Meyer and Virginia Guiboche (Nutrition Students). There will be at least 3 people supervising a maximum of 6 children during the study mornings.

If you would be willing to allow your child to participate in this study, or wish to obtain further information about any aspect of the study, please call Tracy Duff at 966-5823 (evening: 652-0787), or Dr. Whiting at 966-5837 (evening: 477-0214). Your child might be more comfortable having a friend participate in the study as well. I have enclosed an extra letter for any other parents of 7-10 year old girls who may be interested in the study.

Sincerely,
Tracy Duff

Example of Typical Study Evening and Day

Evening Before Study:	
5:30 pm	Child eats supper.
7:30 pm	Child eats snack provided.
after 8:00 pm	Child consumes no more food, and only drinks the diet beverage provided. Goes to bed at normal time.
Morning of Study:	
7:00 am	Child rises, passes and discards first urine sample, drinks diet beverage, dresses.
8:15 am	Child arrives at university, provides a fasting urine sample.
8:30 am	Child eats breakfast.
9:00 am-12:00 pm	Activities, water and diet pop available throughout the morning. Urine is collected from each child for a total of 3 hours after the meal.
12:00 pm	Lunch is provided.
12:45 pm	Children transported to home or day care.

7.7 Health Questionnaire

Today's Date:_____

DIETARY FACTORS AFFECTING CALCIUM METABOLISM

SUBJECT INFORMATION
(confidential)

NAME: (Child) _____

(Parent) _____

ADDRESS: _____

_____

PHONE: (H) _____ (DAY CARE) _____

(PARENT'S WORK) _____

BIRTHDAY (dd-mm-yyyy)_____

WEIGHT: _____

Please answer the following questions:

1. Is your child experiencing any unusual or abnormal digestive responses (diarrhoea, constipation)? _____ (if yes, describe)

2. Is she at risk for any disorder of the circulatory system (hypertension, cardiovascular disease) including heart and kidney? _____ (if yes, describe)

3. Is she taking any medications? _____ (if yes, please list)

_____ _____

_____ _____

4. Has she broken any bones in the last year?_____(If yes, when?)

5. Is she taking any nutritional supplements (vitamins, minerals, herbal remedies)? _____ (if yes, please list)

_____ _____

_____ _____

Would you be willing to discontinue them for the period of the study? _____

6. Is she allergic to milk, or lactose intolerant? _____

7. Does she have any other allergies? (including food and drug allergies) _____ (if yes, please list)

_____ _____

_____ _____

8. Does your child normally consume cola or chocolate beverages? _____

9. Does she have frequent headaches? _____

10. Does your child have any strong food dislikes? _____ (if yes,please list)

_____ _____

_____ _____

11. Where would you normally wish your child to be transported to after completion of study. (eg home, day care, grandparents) Please notify us if this should change.

Additional information that we should know about: (use other side of form if necessary).

7.8 Consent Form University of Saskatchewan

Division of Nutrition and Dietetics
College of Pharmacy

CONSENT FOR PARTICIPATION IN A HUMAN STUDY

Project Title: Dietary Factors Affecting Calcium Metabolism
Investigator: Susan J. Whiting, Ph.D.
Associate Professor
Division of Nutrition and Dietetics
College of Pharmacy

We invite your child to participate in a research study at the University of Saskatchewan designed to increase our understanding of risk factors of osteoporosis by investigating how dietary factors influence calcium metabolism. Taking part in the study is entirely voluntary. Personal benefit may not result from taking part in the study, but knowledge gained will be beneficial. You may withdraw your child from the study at any time. Strict anonymity/confidentiality will be maintained; we may present our findings at scientific meetings and/or publish in scientific journals but the identification of those taking part is withheld.

We are investigating whether supplementary protein, with and without salts (sodium chloride or potassium citrate), in amounts to simulate eating dairy products, affect calcium retention in children and adolescents. We will determine this by measuring fasting urinary calcium levels and levels of urinary calcium after a load of protein or protein plus salts to simulate milk or cheese. In this study we will ask you to:
-- record food and beverages consumed in the 24 hours before each study day.

-- in the evening before a study load, have the child not consume any food after 8:00 pm., although distilled water or diet beverages (provided) are permitted.

-- on the mornings of the study, your child will discard her first morning urine, drink at least 200 ml of distilled water or diet beverage (provided), but eat no food, and be dropped off at the university between 8:00 and 9:00 am.

-- upon arrival at the university, the child will provide a `fasting' urine sample.

-- a food based protein/salt supplement, which is within normal dietary amounts, will be provided to the child as pancakes or similar meal.

-- urine samples will be collected for the next 3 hours.
The child will be fed a lunch and returned to home or child care at this time.

You may also be asked to provide a food history of your child's typical food consumption prior to beginning the study. i.e. usual foods consumed, quantities, food likes and dislikes.

RISKS, POTENTIAL RISKS:
There is always the possibility of unforeseen risks in any experiment. The doses of sodium, potassium, and protein provided are not known to affect anyone with normal kidney function. Supplements are provided in amounts that would normally be consumed.

Important Note:
Do not have your child participate if she is, or suspect she is:
1) at risk for any disorder of the circulatory system including heart and kidney;
2) experiencing any unusual or abnormal digestive responses (diarrhea, constipation);
3) allergic or intolerant to cow's milk.

Retain a copy of this consent form for your records. Any questions you have will be answered by Dr. Whiting, who can be reached at 966-5837 (office) or 477-0214 (home).

Complete Item Below
I have read the explanation about this study and have been given the opportunity to discuss it and to ask questions. I hereby consent to take part in this study.

(Signature of Subject)	(Date Signed)
(Signature of Guardian)	(Date Signed)
(Signature of Investigator)	(Date Signed)
(Signature of Witness)	(Date Signed)