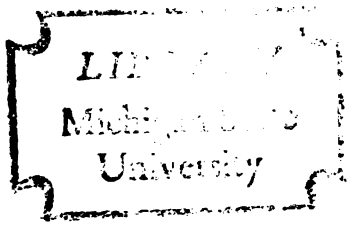
EFFECTS OF DIETARY PROTEIN AND MAGNESIUM LEVELS ON MINERAL METABOLISM IN THE RAT

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY
ANNE MARIE ROGEL

1976



ABSTRACT

EFFECTS OF DIETARY PROTEIN AND MAGNESIUM LEVELS ON MINERAL METABOLISM IN THE RAT

By

Anne Marie Rogel

Many reports have indicated that increasing the protein levels in the diet will lead to an increase in urinary calcium excretion in both humans and experimental animals. Whether this calciuria is caused by an increase in the intestinal absorption or by enhanced bone resorption has not been demonstrated conclusively, and conflicting results among studies might be accredited to differences in experimental design.

This investigation was designed primarily to determine whether high protein diets cause increased urinary calcium excretion when the mineral composition of the diet is maintained at a constant and adequate level.

In experiment 1, thirty-two, 200 g, male, Sprague-Dawley rats were divided into six groups. Two protein sources (beef and soy concentrate) were evaluated at two levels of intake (20% and 40%) and two levels of calcium and magnesium (Ca:P:Mg = 2.0:1.0:0.6 or 1.0:1.0:0.3). Because of the high magnesium content of soy, adjusting minerals in the diets to constant levels resulted in excessive intakes of magnesium. After 27 days of feeding ad libitum,

a 7-day collection of urine and feces was made for analysis of calcium, phosphorus and magnesium. When the animals were sacrificed, bones and soft tissues were removed for mineral analysis.

With Ca:P = 2.0, calcium balances were near zero (-17.9 to 8.6 mg/day) irrespective of source or level of protein. Positive calcium balances (37.0 and 22.1 mg/day) were observed, however, with Ca:P = 1.0 at 20% protein intakes. Increasing the protein levels led to calciuria in the soy-fed rats but not in those fed meat. Phosphorus balances were not affected by protein level, but were more positive with Ca:P = 1.0 when soy protein was used. In all cases, fecal phosphorus losses were greater with soy than with meat, and urinary losses were less. Magnesium balances were less positive at 40% than 20% protein ingestion for both protein sources. No changes in mineral composition of either bone or soft tissues were observed.

A second experiment was conducted to determine the effects of high magnesium intakes as well as high protein intakes on mineral metabolism. Four groups of rats (10 per group) were fed diets containing 20% or 40% protein from casein. Calcium and phosphorus levels were equivalent in all diets (1.0 and 0.5%, respectively) and magnesium levels were set at either 0.05 or 0.26%. Mineral balances were determined at two separate times during the 57-day experiment from 48-hour collections. Total carcass analyses were also conducted.

In this experiment, weight gains were lower for those animals consuming high protein diets. Since a difference in weight gain was not observed in experiment 1, the effect may have been related to protein source. Calcium balances were positive for all four groups

(37.1 to 79.9 mg/day). Increasing the protein intake enhanced calcium retention when the magnesium levels were low, but decreased retention when they were high. Both phosphorus and magnesium balances were more positive with high protein intakes due to increased apparent % absorption in the gut. A more dramatic increase in magnesium balance was observed when magnesium intakes were increased, however. Increasing the protein level of the diet led to an increase in urinary calcium. An even greater increase in urinary calcium along with a decrease in urinary phosphorus was observed when dietary magnesium levels were high regardless of protein level. In both experiments 1 and 2, calcium balances reflected fecal losses. Compared with total calcium excretion, urinary calcium losses were small. The changes in fecal calcium which accompanied the calciuria were not significant. Although changes in carcass calcium and phosphorus were not observed, there was an increase in total carcass magnesium (mg/g fat-free dry weight) with high magnesium intakes.

Two experiments were designed to study the mechanism for calciuria from high protein intakes. In experiment 3, rats were given either a 20% protein diet with a 5% sucrose solution in place of drinking water or a 40% protein diet. Urinary calcium losses in these groups were compared with those observed during a control period when rats were given a 20% protein diet. Despite greatly increased urine volumes, calcium losses during sucrose loading were not increased indicating that calciuria from high protein intakes is probably not caused by a diuresis. Likewise, no differences were observed in serum ultrafiltrable calcium levels in animals fed either 20% or 40% protein diets. Large variations among animals, however, were observed in both groups.

EFFECTS OF DIETARY PROTEIN AND MAGNESIUM LEVELS ON MINERAL METABOLISM IN THE RAT

By

Anne Marie Rogel

A THESIS

Submitted to

Michigan State University

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

Department of Food Science and Human Nutrition

1976

ACKNOWLEDGEMENTS

Sincere thanks are extended to the following persons for their guidance and assistance throughout this study: Dr. Wanda L. Chenoweth, Dr. Maurice R. Bennink, Dr. Olaf Mickelsen, Dr. Duane E. Ullrey, Dr. Pao K. Ku, Dr. Jenny T. Johnson, and Dr. John L. Gill.

TABLE OF CONTENTS

LIST OF TABLES	iv
LIST OF FIGURES	vi
REVIEW OF LITERATURE	1
MATERIALS AND METHODS	21
EXPERIMENT 1	21
EXPERIMENT 2	27
EXPERIMENT 3	31
EXPERIMENT 4	32
RESULTS	35
EXPERIMENT 1	35
EXPERIMENT 2	42
EXPERIMENT 3	62
EXPERIMENT 4	62
DISCUSSION	69
SUMMARY AND CONCLUSIONS	77
RECOMMENDATIONS	81
APPENDIX	84
PHOSPHORUS ANALYSIS	84
SERUM DEPROTEINATION	84
STATISTICAL EVALUATION	85
BIBLIOGRAPHY	92

LIST OF TABLES

1. Mineral composition of freeze-dried, fat-free meat (beef) and soy concentrate. 23

2. Design of experimental diets and actual mineral analyses of the prepared diets, experiment 1. 24

3. Ingredients added to each diet (%), experiment 1. 25

4. Design of experimental diets and actual mineral analyses of the prepared diets, experiment 2. 28

5. Ingredients added to each diet (%), experiment 2. 29

6. Percentage dietary composition, experiment 3. 33

7. Weight gain, food intake and feed efficiency, experiment 1. 36

8. Urine volumes and fecal dry weights, experiment 1. 37

9. Urinary and fecal excretion of calcium and calcium balance, experiment 1. 38

10. Urinary and fecal excretion of phosphorus and phosphorus balance, experiment 1. 40

11. Urinary and fecal excretion of magnesium and magnesium balance, experiment 1. 41

12. Calcium content of soft tissues, experiment 1. 43

13. Phosphorus content of soft tissues, experiment 1. 44

14. Magnesium content of soft tissues, experiment 1. 45

15. Femur fat-free dry weights and ash, experiment 1. 46

16. Mineral content of femurs, experiment 1. 47

17. Kidney, percent body weight, experiment 1. 48

18. Weight gain, food intake and feed efficiency, experiment 2. 50

19. Food intake, fecal dry weights, urinary volumes and urinary pH during balance periods, experiment 2. 51

20. Effect of dietary protein and magnesium levels on calcium balance, experiment 2. 53

21. Effect of dietary protein and magnesium levels on phosphorus balance, experiment 2. 54

22. Effect of dietary protein and magnesium levels on magnesium balance, experiment 2. 55

23. Total carcass analysis, mineral composition, experiment 2. 63

24. Lipid and water content of carcasses, experiment 2. 64

25. Urine volume and urinary calcium excretion, experiment 3. 65

26. Weight gain, food intake and feed efficiency, experiment 4. 66

27. Total serum calcium and serum ultrafiltrable calcium, experiment 4. 68

28. One-way analysis of variance. 86

29. Two-way analysis of variance for groups 1 through 4, experiment 1. (5 animals per group) 88

30. Two-way analysis of variance, experiment 2. Stage 1, comparison of balance periods a_1 and a_2. 89

31. Two-way analysis of variance, experiment 2. Stage 2. 90

LIST OF FIGURES

1. Average weight gains, experiment 2. 49

2. Effect of protein level on calcium balance and apparent % absorption of calcium at two levels of dietary magnesium, experiment 2. 56

3. Effect of protein level on fecal phosphorus excretion, phosphorus balance, and apparent % absorption of phosphorus at two levels of dietary magnesium, experiment 2. 57

4. Effect of protein level on fecal magnesium excretion and magnesium balance at two levels of dietary magnesium, experiment 2. 59

5. Effect of time on fecal magnesium excretion and apparent % absorption of magnesium at two levels of dietary magnesium, experiment 2. 60

6. Average weight gains, experiment 4. 67

REVIEW OF LITERATURE

Currently, Americans consume an average of 99 g of protein per capita per day. Approximately two-thirds of it is derived from animal sources (1). Since the Recommended Dietary Allowance for young men is 54 g per day (2), it is pertinent that studies be conducted to evaluate the effects of excess protein ingestion in the normal individual. A number of studies have been done to investigate the relationship between high protein intakes and the metabolism of calcium as well as the complications which may be presented when the ratios of calcium to phosphorus and/or magnesium in the diet are also altered.

Reports that the dietary protein level has an effect on calcium metabolism have been numerous during the past century. Low protein intakes have been shown to be detrimental to normal bone development (3). Bone, like other living tissue, is in a state of constant flux. During one's lifetime, the ratio of bone apposition to bone resorption varies. Normally, bone apposition predominates in the child. During early adulthood, a plateau is reached, and with age, bone destruction begins to occur at a more advanced rate so that older adults develop a condition known as osteoporosis (3). One can define osteoporosis as "a reduction of calcified bone mass per unit volume of anatomical bone" (4). In other words, the total bone mass is reduced, but the bone which remains is histologically normal.

The formation of new bone material begins with mesenchymally-derived cells called osteoblasts. These cells are responsible for laying down the organic bone matrix comprised primarily of collagen (90-96%) and of protein and muccopolysaccharides. When the osteoblasts no longer function in laying down the organic matrix and begin to support the crystallization of apatite ($Ca_3(PO_4)_2 \cdot CaCO_3$) between the collagen fibers, they are referred to as osteocytes. The carbonate ion of apatite can be replaced by hydroxide to form hydroxyapatite, or it can be replaced, less frequently, by citrate, chloride or fluoride. The molecule of fluoroapatite is larger than hydroxyapatite and its structure is more stable. In human bone, the ratio of calcium to phosphate is generally between 1.6 and 1.7, the variability being due to the replacement of calcium with other cations (5,6).

Due to the impermeability of the inorganic bone matrix, osteocytes are dependent upon an extensive capillary network for proper nourishment. No cell in bone is more than a few millimeters from the blood supply. In addition, there is an extensive canal system derived from the cytoplasmic extensions of the osteoblasts which connect the mature bone cells. Perhaps it is the nutritional status of the osteocytes which determines whether or not the polynucleate osteoclasts will be formed by the coalescence of neighboring osteocytes. Their presence results in the destruction of hydroxyapatite. At present, their mode of action is speculative, but two possibilities have recently been postulated. First, degradation of bone matrix may be the result of collagenases or other proteolytic enzymes released from the lysosomes. Secondly, intracellular citric acid or other Kreb's cycle intermediates may contribute to the resorption of bone by weakening the calcium to

phosphorus bonding (7). Jowsey and Nguyen (8) showed that in dogs infusion of citric acid into the fluid circulating the bone could, indeed, enhance bone resorption.

Protein-calorie malnutrition may interfere with normal bone development in children. Although the bones are nearly normal in size and ossification occurs at a normal period of development, the marrow cavities are enlarged and cranial bones are thinner, indicating enhanced bone resorption at the endosteal surface (3). Protein deficiency in the diets of these children, however, may be but a single factor in the etiology of bone loss.

Animal experimentation has shown that starvation will lead to a condition known as matrix osteoporosis in which there is a reduction in bone matrix deposition--that is, a reduction in osteoblast activity. Mineral osteoporosis, in contrast to matrix osteoporosis, is associated with the ingestion of diets which contain adequate or high levels of protein but low levels of minerals, especially calcium. Although the deposition of new bone matrix is normal, the rate of destruction of old bone is increased to maintain the normal serum levels of calcium (9).

In pigs, Platt and Stewart (10) observed a reduction in the growth rate of long bones, skull and vertebrae from malnutrition. Although there was a low ash content, the ratio of ash to bone matrix was not altered by the experimental diets. Replacing part of the carbohydrate in these diets with protein, however, improved the rate of bone growth. In their studies with both young and old rats, El-Maraghi et al. (9) found that low protein diets initiated a matrix osteoporosis. This condition could be corrected even in old rats by increasing the

protein level of the diet to adequate levels. Mineral osteoporosis could be cured by the addition of calcium. However, the addition of extra protein to a low calcium diet served only to intensify the bone rarefaction.

Experiments have been conducted in both humans and experimental animals to determine the effects of high protein intakes on calcium metabolism. Many investigators have favored short-term studies of several weeks or months utilizing balance techniques, and many have used the results to predict gains or losses of minerals from the body which might be observed after several years of similar dietary regimens. Recently, Heroux and Peter (11) have challenged this practice. They concluded from their work that results from extended balance studies, as well as from short-term studies, may be misleading and probably do not accurately reflect the net retention or loss of minerals from the body. By determining total body content of calcium and magnesium in rats in conjunction with a number of balance studies, they found errors predicting excess mineral retention with positive balances and excess loss with negative balances. Nevertheless, the balance study can be a useful tool for studying intestinal and renal handling of minerals and has been used extensively during the past few decades to investigate the effect of high protein intakes on the absorption and retention of calcium.

As early as 1920, it was found that dietary protein has a calciuric effect (12). Some data suggest that the apparent absorption of calcium in the gut is concurrently enhanced (13-15), but the calcium retention may be dependent upon a number of factors aside from dietary protein levels.

A series of experiments (14, 16-18) were conducted in young adolescent males receiving three different levels of protein (47, 95 and 142 g) and three levels of calcium (500, 800 and 1400 mg/day). During each 15-day experimental period, the youths were given typical, varied diets. When they consumed 47 g of protein, all subjects were in positive calcium balance, and the amount of calcium retained was not dependent on the calcium intake. None of the subjects attained calcium balance when 500 mg of calcium and 95 g of protein were ingested, but balance was achieved at this protein level when the calcium intake was increased. With the highest level of protein, only 3 out of 15 subjects were in calcium balance, and then only at the highest level of calcium ingestion. The data indicate that high levels of protein are detrimental to calcium balance regardless of the calcium intakes. However, with the addition of calcium to the diet, a partial reversal of these adverse effects can be observed. Calcium loss appeared to be caused by a greatly increased urinary calcium output without a concomitant increase in calcium absorption in the intestine.

The manner in which high protein intakes affect the intestinal absorption of calcium has been a matter of controversy (19-21). Perhaps this is related to the specific effects of different protein sources on the absorption and utilization of calcium. Several amino acids are known to increase the solubility of calcium salts (15). Conversely, calcium is utilized poorly from diets which are deficient in one or more amino acids. Wasserman et al. (22) have indicated that lysine and arginine are the most effective in promoting the appearance of radioactive strontium and calcium into femurs when the radioisotopes are administered by stomach tube to fasted rats. The

effect of lysine appears to be associated primarily with the rate of absorption of calcium in the intestinal mucosa. Tryptophan, leucine and aspartate are also effective in promoting calcium absorption.

In some of the early studies, high protein diets were achieved by replacing other foods such as vegetables with meat (12, 23). That the altered calcium metabolism was due to meat per se in these subjects seemed unlikely when subsequent experimentation showed that the administration of large quantities of individual amino acids or high-protein, semipurified diets also induced calciuria.

The design of some of the early animal studies utilizing diets consisting primarily of meat has been criticized by investigators who suggested that the detrimental effects of these diets were due to a mineral imbalance. Since muscle meat is a poor source of calcium and magnesium, an unsupplemented meat diet could result in deficiencies which could cause severe bone disorders. Tal and Guggenheim (24) prevented bone loss in weanling rats by giving a calcium supplement along with the meat diets. Bone abnormalities could also be prevented by the addition of a copper-manganese supplement, but the effects of calcium supplementation were superior.

In similar experiments, Havivi et al. (25) found that weanling mice fed high meat diets for six weeks had lower femur concentrations of calcium and phosphorus and lower total bone ash than the controls which were given semipurified diets containing adequate protein levels. The changes in bone metabolism were prevented by the addition of calcium or copper to the meat diets. To see whether the bone abnormalities were related to a mineral imbalance or to some property of the meat, they prepared similar high-protein diets substituting fish (which is

also low in calcium) or casein for the meat. Neither of these two subsequent diets produced a decrease in bone ash. After further experimentation, they concluded that the bone rarefaction was due to a quality of beef rather than an excess of protein or a deficiency of calcium.

From an epidemiological study in Eskimos, Mazess and Mather (26) have concluded that the high meat consumption by this group of people is the most probable explanation for the finding that bone loss occurs at an earlier age in them than in whites.

Bone loss accompanying the consumption of a high meat diet could be related to the role of bone in the maintenance of acid-base balance in the body in conjunction with the lungs and kidneys. Early studies have shown that omnivores and carnivores generally excrete an acidic urine while herbivores excrete an alkaline urine. Bernard (27), for example, observed that rabbits which were fed a meat diet or which were starved excreted an acidic urine rather than the normal alkaline urine excreted by this herbivorous species. Meat is described as an acid-ash food, one in which anions predominate or are formed during metabolism in greater quantities than cations. Those foods which produce an abundance of cations are, conversely, said to have an alkaline ash. The presence of cations or anions in the body can initiate a buffer response whether they are acquired by the ingestion of free ions or nutrients which yield ions during metabolism, or whether they are released during soft tissue growth, skeletal mineralization or the production of new body fluid (28).

The urinary pH reflects the urinary loss of fixed acids (non-volatile endogenously produced acids) and exogenous acids which are

not metabolized further. In the steady state, one can define urinary acidity as the urine titrable acidity plus ammonium ions minus bicarbonate ions. There are three major sources of endogenous fixed acids: 1) from the oxidation of sulfur-containing amino acids, $S \rightarrow SO_4^-$, 2) by the formation of unmetabolizeable organic acids, and 3) from the hydrolysis of phosphate esters of protein (29). Relman et al. (29) calculated the amount of endogenous acid which would be expected for a given whole food diet in human subjects and found this to be directly related to the renal acid excretion.

Because of its generous supply of basic salts, bone could be easily implicated as an acid-base homeostatic control. The apatite crystals of bone serve as a storehouse for phosphorus. In fact, some evolutionists have proposed that bone originated for this purpose, and only later did it acquire the function of support (30). Within the cell, phosphate is thought to be the major buffer (pK for $HPO_4^=/H_2PO_4^-$ = 6.8), while the bicarbonate/carbonic acid system predominates in extracellular fluid.

Wachman and Bernstein (31) have proposed that osteoporosis may be the result of a lifetime utilization of the buffering capacity of bone. If as little as 2 meq of calcium were lost each day through the buffering process, one could expect a 15% loss in inorganic bone mass within a decade.

A number of studies have been done to determine whether acidosis will affect the balance of various minerals. Reidenberg et al. (32), for example, found that six obese women who were fasted for $5\frac{1}{2}$ weeks went into negative calcium balance as a result of the ketosis. A partial reversal of the negative balance was achieved by the

administration of $NaHCO_3$. A marked increase in urinary calcium was observed as the acidosis developed, and a decrease in urinary calcium occurred with the administration of a basic salt.

Several animal studies have shown that acidosis specifically affects bone. Nichols and Nichols (33) found that there is a measurable loss of sodium from the bone during ketoacidosis. Irving and Chute (34) observed that the consumption of HCl by rats and guinea pigs caused a reduction in bone CO_2 and calcium and, in some cases, phosphorus, while no CO_2 was lost from muscle tissue. Apparently, bone bicarbonate stores are more labile than phosphorus.

During acidosis, the production of ammonium by the kidney is also an important mechanism for the neutralization of acid. The extent to which the kidney buffer is utilized compared to that of the bone may depend on the length of time to which the animal is exposed to an acid insult, but it may also be species specific. During fasting, the increase in calcium excretion by the rabbit is much greater than the loss of calcium which would be expected from soft tissue reserves (35). In the cat, however, acidosis results in a greatly increased ammonium ion production rather than a loss of calcium (36). Humans represent an intermediate species in this respect, since both ammonium ion excretion and urinary calcium excretion increase during fasting (37).

More recently, Barzel and Jowsey (38) have studied the effects of chronic acid and alkali ingestion on the maintenance of the femoral bones in adult rats. They found that when rats were given a 1.5% NH_4Cl solution in place of drinking water for 330 days, their bones were lower in fat-free solid weight than those of control animals although they were equivalent in length, an effect which was independent

of the calcium levels of the diets. Microradiography indicated that the bone loss from chronic acid ingestion was due to an increase in bone resorption. When rats were given an equimolar solution of sodium and potassium bicarbonate instead, the formation of new bone material appeared to be enhanced, and the expected reduction of fat-free bone from ingestion of a low calcium diet was prevented.

Significant increases in urinary calcium and phosphorus excretion and urinary acidity have been reported after acid stress in rats. Newell and Beauchene (38) also found that serum calcium and phosphorus levels were lowered by chronic NH_4Cl ingestion, but the mineral content of the tibia was not affected.

Despite repeated indications of calcium loss from the body after high protein intakes or acid insult, few investigators have indicated that plasma calcium levels are altered. A dominant role in the maintenance of calcium levels in the extracellular fluid has been attributed to the parathyroid gland. Parathyroid hormone (PTH) is secreted by the chief cells of the parathyroids, four small glands adjacent to the thyroid, and is thought to act directly upon the kidneys and bone to increase the concentration of calcium in the blood. The release of this hormone is most probably brought about by changes in circulating calcium ion concentrations--that is, through a negative feedback loop.

Recent experiments indicate that PTH directly activates adenyl cyclase in the kidney cortex and that its effects are mediated, therefore, through the action of cyclic AMP. Chase and Aurbach (40) have shown that the injection or infusion of PTH into parathyroidectomized rats caused an increase in the urinary excretion of cyclic AMP. They also showed that adenyl cyclase in the renal cortex may

have a specific receptor site for PTH which is not present on the same enzyme isolated from any other tissues which they studied except bone (41). PTH activation of adenyl cyclase appears to be very rapid, and the increase in urinary cyclic AMP excretion precedes the phosphaturia which also results from PTH administration.

The net effect of PTH on the kidney is an increased reabsorption of calcium by the renal tubules. However, it is well known that hyperparathyroidism initiates hypercalciuria. This phenomenon can be accounted for entirely by an increase in the filtered load of calcium. Peacock et al. (42) have indicated that hyperparathyroid patients excrete much less calcium than controls when the serum calcium levels are maintained at comparable levels.

Several studies have shown that PTH stimulates bone resorption to release calcium into the blood stream. In vitro experiments with fetal rat bone cultures have shown that Ca^{45} is released from the tissue in proportion to the amount of PTH added to the medium (43). Chase and Aurbach (40), as mentioned above, found that the action of PTH on bone is mediated through cyclic AMP. At high concentrations of calcium, however, adenyl cyclase is inhibited. The effect of PTH on intestinal calcium absorption has been less extensively studied. PTH may indirectly increase the absorption of calcium in the intestine by enhancing the transcellular movement of the calcium ion (44).

The calciuric effect of high protein intakes may result from secondary hyperparathyroidism, but there is no direct evidence yet to support this hypothesis. During chronic renal failure, however, in which there is an alteration of serum electrolyte concentrations, secondary hyperparathyroidism has been observed with evidence for

increased synthesis and secretion of PTH from hyperplastic glands (45). During acute renal failure, likewise, there is a dramatic increase in the mitotic activity of the chief cells observable within 48 hours. A primary role for PTH in the maintenance of acid-base equilibrium has been proposed recently by Wills (46) in light of evolutionary data which indicate that the parathyroid gland developed about two million years after the appearance of true calcium-phosphate bone in fish. Since two homeostatic control systems for calcium were already in existence, Wills suggests that land vertebrates evolved the parathyroid gland for the elimination of endogenous acid which could no longer be excreted by simple diffusion through gills. The release of calcium would then be incidental to the activity of PTH in acid-base control.

The ratio of calcium to phosphorus in the diet may be an important factor in bone and kidney metabolism. Because calcium and phosphorus levels are inversely related in the serum, it has been suggested that a high phosphorus intake will lead to a slight decrease in serum calcium levels thus creating a secondary hyperparathyroidism. Recently, this hypothesis has been documented in the laboratory by serial blood sampling in rats (47). A dietary calcium to phosphorus ratio of 2.0 appears to be optimal for the maximum inhibition of bone resorption in the adult mouse and growing rat when the calcium level of the diet is approximately 0.8% (48). Kreshnarao and Draper (49) and Draper et al. (50) produced osteoporosis in mice and rats by feeding them high phosphorus diets. The development of bone abnormalities could not be counteracted by increasing the calcium levels in such diets, however. From

radiographic studies, it was observed that deep-labelled Ca^{45} (which had become incorporated into the bone structure) was resorbed from bone in amounts directly proportional to the dietary phosphorus levels between 0.3 and 1.2%. Further studies (51) have shown that high protein diets have no effect on bone resorption when the calcium and phosphorus intakes are adequate and balanced. The calciuria from high protein intakes appeared to be the result of a shift in the excretion of endogenous calcium from the feces to urine accompanied by an increase in absorption of dietary calcium.

Attempts to evaluate the effects of calcium to phosphorus ratios in human diets have been less extensive. Epidemiological studies by Guggenheim et al. (52) showed a greater incidence of osteoporosis in individuals from North African and Asian countries (except Israel) compared with those from North America, Europe and Israel. Random urine analyses revealed increased levels of phosphorus for those individuals from areas where osteoporosis is more prevalent. Levels of calcium, nitrogen and hydroxyproline were unrelated.

The level of magnesium in the diet may also influence calcium metabolism. Haag and Palmer (53) recognized in 1928 that normal growth in rats is dependent upon a dietary balance between calcium, phosphorus and magnesium. Later, Colby and Frye (54) observed that high levels of calcium in the diet increased the severity of magnesium deficiency in rats and that a combination of high protein levels and high calcium intakes made the condition even more severe.

In human studies, Johnson and Linkswiler (55) found that calcium excretion is influenced by both magnesium and protein levels in the diet. When adolescent males were given high protein diets with

normal magnesium levels, they exhibited a negative calcium balance. With increased levels of both protein and magnesium in the diets, calcium balances were even more negative indicating a protein-magnesium interaction. Likewise, calcium balances were more positive when low protein diets were accompanied by low, but adequate, magnesium levels than when high magnesium levels were given.

High protein intakes appear to affect the metabolism of magnesium as well as calcium. It has been shown repeatedly that magnesium deficiency symptoms in experimental animals are enhanced when they are given rations with inadequate magnesium levels and high protein contents (54,56,57). As with calcium, urinary magnesium levels increase when the level of protein is increased even when magnesium levels in the diet are minimal (13,57,58). It is likely that the intestinal absorption and utilization of magnesium is affected, like calcium, by a number of dietary parameters which have not been consistantly controlled in different investigations. Reports concerning the effects of different protein levels on magnesium absorption and retention are conflicting. Schofield and Morrell (59), for example, observed that magnesium retentions in pre-adolescent girls were slightly reduced by increased protein intakes. Alcantara and Linkswiler (58), likewise, observed no marked changes in magnesium balance with changes in protein intakes in male adolescents. In other studies, however, reduced fecal excretion of magnesium with high protein intakes improved the magnesium retention in subjects despite increased urinary magnesium levels (13,60).

The mechanism for an increased urinary calcium and magnesium excretion with an increased protein intake has not been determined

explicitly. It is probable, however, that the kidney plays an important role. For that reason, it is necessary to consider the manner in which calcium and magnesium are handled by this organ.

Calcium is actively reabsorbed in the distal tubule of the kidney (61). Calcium clearance appears to be a function of sodium clearance; both calcium and sodium are reabsorbed in the same proportions as they are present in the plasma (62). Recent studies have shown that the excretion of calcium is primarily determined by the excretion of sodium and secondarily by the concentration of complexing anions in the urine (63,64).

Since magnesium is also a divalent cation, a number of studies have been conducted to determine whether magnesium and calcium are handled by the same mechanism in the kidneys. Chesley and Tepper (65) observed that urinary calcium as well as urinary magnesium increased when human subjects were given injections of $MgSO_4$ or magnesium acetate. Calcium excretion was increased most dramatically when the serum magnesium levels were at their highest. Womersley (66), likewise, found that calcium losses were increased when magnesium salts were infused although no alterations in serum calcium levels were observed. If magnesium is added to the diet as magnesium acetate, urinary calcium will increase, but a decrease in fecal calcium losses enable an individual to maintain a positive calcium balance (67). Finally, clearance studies in dogs have shown that urinary calcium is increased when $MgCl_2$ is infused and magnesium excretion is increased (although slightly) when $CaCl_2$ is infused (68). These studies suggest that the reabsorption of magnesium and calcium in the kidney tubules against a concentration gradient

probably occurs by a common mechanism.

Heaton et al. (69), however, argue that the absorption of calcium and magnesium both in the renal tubules and in the intestine can be explained only partially by a common transport mechanism; some absorption is accomplished by processes specific to the individual cations. In studies in patients suffering from disorders of calcium metabolism, they found that urinary magnesium levels were normal regardless of urinary calcium levels. They also found that vitamin D administration had no effect on magnesium excretion except when calcium excretion was extremely abnormal. Phosphate administration decreased urinary magnesium and calcium, however, and increased fecal excretion of both cations.

Evidence for a separate calcium transport system in the intestine was presented by Schachter et al. (70) who worked with inverted gut sacs from the proximal small intestines of rats. Transport of calcium ion from mucosal to serosal surface was observed. But neither magnesium, strontium, barium nor potassium was observed in the serosal fluid.

Polyuria has been reported to occur as a consequence of high protein intakes and may be due to an increased production of urea in the liver (51). That the calciuric effect of high protein intakes is caused simply by an increase in urine volume has not been shown. However, diuretic studies in dogs indicate that calcium is quantitatively and actively reabsorbed in the kidney tubules independently of water excretion (71). Other studies have shown that both urine volume and calcium excretion are enhanced by acid stress (39).

The filtered load of calcium is known to effect the total excretion of this ion. The form in which calcium is present in the serum will determine the amount of calcium which is filtered through the glomerulus. In the blood, calcium is present in three forms: 1) combined with protein (albumin, 81% and globulin, 19%), 2) combined with an anion such as citrate, phosphate or sulfate, and 3) in the free ionic form (72). Calcium which is not bound to protein can be recovered by filtration through a semipermeable membrane. Only the ionized form of calcium is physiologically active in the living organsim, but most of the unbound calcium in the serum is thought to be present in this form.

The concentration of calcium in the ultrafiltrate can be altered by a number of pathological conditions. For example, the free calcium fraction may be elevated during hyperparathyroidism and vitamin D toxicity and may be depressed during hypoparathyroidism, some bone disorders, and renal failure. Hyperphosphatemia, likewise, will cause a significant decrease in ultrafiltrable calcium with only a slight decrease in total calcium levels (73). Lemann et al. (74) have reported that serum ultrafiltrable calcium increases in humans during acidosis, but the effect of high protein levels in the diet on the ultrafiltrable calcium concentration has not been investigated.

In humans, normal ultrafiltrable calcium represents 50 to 61% of the total serum calcium (75-77). In rats 30 to 50% is protein-bound (78).

There are several factors which must be considered when the ultrafiltration procedure is used. Many investigators utilize a dialytic membrane such as cellulose which is impermeable to protein

(79-81). Serum pH and P_{CO_2} are known to affect the concentration of ionized calcium. Increasing the pH will cause an increased association of calcium with phosphate. To equilibrate the system prior to ultrafiltration, a 5% CO_2 and 95% O_2 solution is often bubbled through the serum. This procedure, in effect, establishes the pH of the serum, but it does not necessarily reproduce the conditions of the living organism. Such a procedure would be inappropriate if differences in serum pH were present between experimental groups. However, acceptable results can be obtained by anaerobic handling of the blood samples (82). Control of temperature during ultrafiltration may also be important. Toribara et al. (83) found that the percentage of ultrafiltrable calcium was considerably higher when the temperature was lowered from 37 to 10°. Favorable results have been reported by treating the samples at room temperature (82), however, many investigators carefully adjust conditions to 37° (78,84).

Although it is often assumed that the unbound calcium is the only form which is filtered through the glomerulus, the possibility that protein-bound calcium is excreted should not be ignored. This is especially pertinent when the rat is used as the animal model. Unlike humans, the adult rat normally excretes large amounts of protein in the urine (1000-3000 mg/100 ml) (85). Electrophoretic studies have shown that the protein excreted by adult rats is mainly albumin. Protein excretion is greater in male rats than in females and increases gradually with age (86). Proteinuria can be enhanced by the ingestion of high protein diets, by the injection of foreign protein, and by renin. Glycine alone, however, significantly

decreases protein excretion, though the excretion of urea and oxalate, a metabolite of glycine, increases (87). Hardy and Baumann (87) found no relationship between proteinuria and calciuria in rats with excess ingestion of $CaCl_2$. However, a similar comparison between calcium and protein excretion has not been investigated when dietary protein is elevated and calcium intakes are normal.

As early as 1926, Addis et al. (88) and Jackson and Riggs (89) observed an increase in kidney size in rats which were fed diets containing high levels of protein. These changes were not accompanied by nephrotic degeneration or other histological alterations. The increase in kidney size may be related to the increase in gluconeogenesis which is observed in this organ when rats are given high protein diets (90). Increased glucose-6-phosphatase (G-6-Pase) activity in the kidney cortex has been observed with PTH administration (91,92) and an increase in the level of calcium in the kidney tubules (93). Suzuki and Fuwa (94) found that a significant increase in G-6-Pase activity in the kidney, but not in the liver, results from feeding low calcium diets to rats. A high protein diet produces the same effect, and the effects of a high protein-low calcium diet are additive. Greater responses to mineral deficiencies led the investigators to suggest that a high protein diet increases the calcium requirement in the body and, consequently, brings about a change in the activity of G-6-Pase in the kidney cortex.

In summary, it has been known for several decades that high protein intakes initiate a calciuric effect in both humans and animals. The mechanism for this action is only now being elucidated. Studies

indicate that the mineral level and the ratio of various minerals in the diet are important factors in the control of calcium metabolism. Some of the effects of high protein intakes on mineral metabolism may be related to alterations in dietary mineral compositions which accompany changes in protein levels. Likewise, amino acids may have specific effects on calcium absorption in the intestine, and their metabolism in the cell may initiate pH changes in the body tissues.

MATERIALS AND METHODS

EXPERIMENT 1

Experiment 1 was designed to study the effects of dietary protein level and mineral composition on the metabolism of calcium, phosphorus and magnesium. With each dietary change, two protein sources, meat and soy, were compared.

Uncooked, ground beef was purchased at the Michigan State University Food Stores. It was shaped into patties about one inch thick, freeze-dried, and pulverized in a feed grinder. The lipid was extracted with petroleum ether. After excess solvent was evaporated in a drying oven[1], the freeze-dried, fat-free meat was suitable for addition to a semipurified diet.

Both processed meat and soy concentrate[2] were analyzed for mineral content before calculating the diets. Air-dried samples weighing approximately one gram were dry ashed[3], dissolved in 10 ml concentrated HCl and diluted to 50 ml[4]. Samples which were to be analyzed for calcium and magnesium were diluted further with a 1% lanthanum

---

[1]Stabil-Therm, Blue M Electric Company, Blue Island, Ill.

[2]Promosoy, Central Soya, 1825 North Laramie, Chicago, Ill.

[3]Lindberg Hevi-Duty oven, model 51441, Sola Basic Industries, 304 Hart Street, Watertown, Wisc.

[4]For all analyses in this experiment and each subsequent experiment in this study, deionized, distilled water was used for sample preparation.

solution. Calcium, magnesium, copper, potassium and sodium were determined by atomic absorption analysis, but values reported in the literature were used to estimate the phosphorus content of both meat and soy protein. These data are presented in table 1.

Table 2 summarizes the design of the experimental diets. Diets 1 and 2 (table 3) served as controls with 20% protein and a calcium to phosphorus ratio of 2.0. Protein levels were increased to 40% in diets 3 and 4, but mineral levels were unchanged. Like the controls, diets 5 and 6 contained 20% protein. However, the amount of calcium was reduced by one half to obtain a calcium to phosphorus ratio of 1.0. Magnesium levels were reduced, likewise, to preserve the calcium to magnesium ratio of diets 1 through 4. Thirty-two male, Sprague-Dawley rats weighing 200 g were divided into groups of 5 or 6. They were housed individually in hanging, wire mesh cages and given food and water ad libitum for 17 days. Constant temperature and humidity conditions and a 12-hour light-dark cycle were maintained. After a 3-day adjustment period in metabolic cages, a one-week collection of feces and urine was begun. No preservatives were added to the urine samples; they were stored under refrigeration. Upon completion of the experiment, the animals were anesthetized with ether and blood was drawn from the heart. Clotted blood samples were centrifuged for 15 minutes at 1500 g[5]. Serum was removed and frozen. After sacrificing the animals with ether, the liver, kidneys and right gastrocnemius muscle were removed, weighed and stored at 0°. Both femurs were excised and suspended in boiling water to facilitate the

---

[5] International Centrifuge, model UV, International Equipment Company, Needham, Mass.

Table 1. Mineral composition of freeze-dried, fat-free meat (beef) and soy concentrate.

Mineral	Meat	Soy
	(mg/100 g)	
Calcium	40.2	367.3
Copper	6.5	4.5
Magnesium	81.7	443.1
Phosphorus	740.0[a]	740.0[b]
Potassium	1039.6	184.7
Sodium	1089.0[c]	173.0

[a]Merkel, R. A. Inorganic constituents. in The Science of Meat and Meat Products. (ed. by J. F. Price and B. S. Schweigert) San Francisco: W. H. Freeman and Company, 1960. p. 167.

[b]Central Soya, 1825 North Laramie, Chicago, Ill.

[c]The ratio of sodium to potassium presented in the USDA Agriculture Handbood No. 8 (Watt, B. K. and Merrill. A. L. Composition of Foods. Washington, D. C.: U. S. Department of Agriculture, 1975.) is approximately 1:7. This indicates that NaCl may have been added to the meat used in this study.

Table 2. Design of experimental diets and actual mineral analyses of the prepared diets, experiment 1.

Design of experimental diets

Diet	Protein content	Protein source	Mineral ratio
	%		Ca:P:Mg
1	20	meat	2.0:1.0:0.6
2	20	soy	2.0:1.0:0.6
3	40	meat	2.0:1.0:0.6
4	40	soy	2.0:1.0:0.6
5	20	meat	1.0:1.0:0.3
6	20	soy	1.0:1.0:0.3

Mineral analyses of the prepared diets

Diet	Calcium	Phosphorus	Magnesium	Ca:P	Ca:Mg	P:Mg
		%				
1	1.06	0.47	0.28	2.26	3.78	1.68
2	1.01	0.51	0.29	1.98	3.48	1.76
3	0.87	0.45	0.26	1.93	3.35	1.73
4	0.94	0.54	0.22	1.74	4.27	2.45
5	0.56	0.42	0.16	1.33	3.50	2.63
6	0.53	0.56	0.14	0.95	3.78	4.00

Table 3. Ingredients added to each diet (%), experiment 1.

Ingredient	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Cornstarch	61.64	54.50	39.90	26.72	62.99	55.85
Cellulose	4.00	3.11	4.00	2.23	4.00	3.11
Soy concentrate[1]	-	30.60	-	61.20	-	30.60
Meat (beef)	23.00	-	46.00	-	23.00	-
Corn oil	4.00	4.00	4.00	4.00	4.00	4.00
DL-methionine	-	0.05	-	0.05	-	0.05
Choline Cl	0.30	0.30	0.30	0.30	0.30	0.30
Vitamin mix[2]	1.00	1.00	1.00	1.00	1.00	1.00
Trace mineral mix[3]	1.50	1.50	1.50	1.50	1.50	1.50
$MnSO_4 \cdot H_2O$	0.02	0.02	0.02	0.02	0.02	0.02
NaCl	0.63	1.74	0.18	1.33	0.63	1.74
$CaCO_3$	2.24	1.96	2.22	1.65	1.11	0.83
MgO	0.42	0.23	0.39	-	0.19	-
KH_2PO_4	0.62	0.50	0.25	-	0.62	0.50
$NaH_2PO_4 \cdot H_2O$	0.63	0.50	0.25	-	0.63	0.50

[1]Promosoy, Central Soya, 1825 North Laramie, Chicago, Ill.

[2]Composed of: thiamin HCl, 22 g; pyridoxine, 22 g; riboflavin, 22 g; Ca pantothenate, 66 g; p amino benzoic acid, 110 g; menadione, 50 g; inositol, 100 g; ascorbic acid, 200 g; niacin, 100 g; vitamin B_{12}, 30 g; biotin, 0.6 g; folic acid, 4 g; vitamin A, 40 g; alpha tocopherol, 100,000 IU; vitamin D_3, 200,000 IU; and cerelose to 2500 g.

[3]Composed of: KI, 98 mg; $NaSeO_3 \cdot 5H_2O$, 31.6 mg; $FeSO_4 \cdot 7H_2O$, 87.08 g; $CuSO_4 \cdot 5H_2O$, 9.82 g; $ZnSO_4 \cdot 7H_2O$, 26.38 g; and cerelose to 5,000 g.

removal of traces of muscles and tendons that could not be scraped off with a scalpel. Air-dried bones were defatted with petroleum ether in a Soxhlet apparatus for approximately eight hours. Fat-free femurs were dry ashed at 800°, dissolved in 5 ml concentrated HCl, and diluted to 50 ml. Similarly, one-gram samples of air-dried feces and samples of each diet were dry ashed, dissolved in 2 ml HCl and diluted to 50 ml. After measuring the total 7-day urine output, each sample was diluted to 250 ml. A 10 ml aliquot was transferred to a crucible, the liquid was evaporated, and the residue was dry ashed and prepared for analysis in the manner described for the fecal samples.

The serum was deproteinated with trichloroacetic acid (TCA) -- final concentration, 5% -- and mineral analyses were done on the supernatants. Failure to treat the standards in a similar manner probably resulted in the unusually high values obtained for serum calcium concentrations in this study. However, the results have been included in this report for comparison between groups. Liver, kidney and muscle were prepared for analysis by wet ashing with 30 ml concentrated HNO_3 and $HClO_3$ (1:1). The samples were boiled in a Kjeldahl apparatus until approximately 3 ml remained in the flask. The remaining liquid was quantitatively transferred to a 100 ml volumetric flask.

All samples were diluted and analyzed for calcium and magnesium by atomic absorption[6] and phosphorus by the method of Gomori (95) (see appendix). Data were analyzed for statistical significance at

---

[6]Model 453, Instrumentation Laboratories, Inc., Lexington, Mass. 02173

$p<0.05$ using analysis of variance and Scheffe's test for comparison between groups (96) (see appendix).

EXPERIMENT 2

A second experiment was designed to eliminate some of the variables which were present in experiment 1. A single source of protein (casein) was used in all diets. In experiment 1, dietary magnesium levels greatly exceeded the minimum requirements for growing rats, since the soy was unexpectedly rich in this mineral. Therefore, the effect of magnesium intake on mineral metabolism was evaluated in experiment 2 in conjunction with changes in protein level.

Before preparing the diets, samples of sodium caseinate were wet ashed and analyzed for calcium, phosphorus and magnesium. Four groups of 200 g male, Sprague-Dawley rats (10 per group) were fed diets containing 20 or 40% protein and 0.05 or 0.30% magnesium. Calcium and phosphorus levels were equivalent in all diets (1.0 and 0.05%, respectively). The dietary design is summarized in table 4, and the diet compositions are given in table 5.

Two balance studies were done with each rat; therefore, each animal was transferred across campus on two separate occassions to the building where the metabolic cages were located. Several days of adjustment were permitted before collections were begun. The first collection was made after either 28 (a_1) or 36 (a_2) days on the diets (five animals per group were used for each period), and the final collection (b) was made after 54 days for all animals. Two consecutive 48-hour collections were made for each period. An

Table 4. Design of experimental diets and actual mineral analyses of the prepared diets, experiment 2.

Design of experimental diets

Diet	Protein	Calcium	Phosphorus	Magnesium	Sodium
			%		
1	20	1.0	0.5	0.05	0.09
2	40	1.0	0.5	0.05	0.09
3	20	1.0	0.5	0.30	0.09
4	40	1.0	0.5	0.30	0.09

Mineral analyses of the prepared diets

Diet	Calcium	Phosphorus	Magnesium	Ca:P	Ca:Mg	P:Mg
		%				
1	1.13	0.36	0.05	3.14	21.65	6.90
2	1.15	0.45	0.05	2.58	21.33	8.28
3	1.16	0.40	0.26	2.86	4.40	1.54
4	1.09	0.44	0.27	2.45	4.04	1.65

Table 5. Ingredients added to each diet (%), experiment 2.

Ingredient	Diet 1	Diet 2	Diet 3	Diet 4
Sodium caseinate[1]	23.00	46.00	23.00	46.00
Cornstarch	61.93	39.93	61.54	39.54
Cellulose	4.00	4.00	4.00	4.00
Corn oil	4.00	4.00	4.00	4.00
Vitamin mix[2]	1.00	1.00	1.00	1.00
Trace mineral mix[3]	1.50	1.50	1.50	1.50
Choline Cl	0.30	0.30	0.30	0.30
$MnSO_4 \cdot H_2O$	0.02	0.02	0.02	0.02
MgO	0.10	0.10	0.49	0.49
$CaCO_3$	2.42	2.42	2.42	2.42
KH_2PO_4	0.67	0.23	0.67	0.23
K_2HPO_4	0.85	0.30	0.85	0.30
NaCl	0.21	0.20	0.21	0.20

[1]Western Dairy Products, San Francisco, Calif. 94111

[2]See footnote 2, table 3.

[3]See footnote 3, table 3.

average for each was computed. During the balance periods, deionized, distilled water was given ad libitum in place of tap water which was given previously. To avoid excessive spillage and contamination of the urine and feces, the diets were mixed with water in a Waring blender to form a thick slurry, and food cups were placed outside of the metabolic cages. The slurry was given only during the balance periods. At other times, the diets were given as a dry powder. For the 20% protein diets, the water to feed ratio was 1.5; for the 40% protein diets, it was 2.0. Before using the metabolic cages, all parts were thoroughly rinsed with deionized, distilled water. Urinary collection vessels and all glassware used for mineral analysis and sample storage were acid-washed in dilute HNO_3. Urine was collected under 3 ml toluene and acidified to prevent bacterial growth. The acid (0.5 ml 4N HCl) was added prior to collections for period a. However, sine the urinary pH[7] was determined during period b, the acid was added before storage.

After the final balance study, all animals were sacrificed with chloroform following an overnight fast and carcasses were frozen. Later, the carcasses were autoclaved[8] for one hour at 120°, followed by homogenation in a Waring blender with an equivalent weight of water. Samples (about 1 g) of the homogenate were air dried overnight and placed in a vacuum oven[9] for 24 hours. They were then wrapped in

---

[7]Corning Digital pH meter, model 109, Fisher Scientific Company, Pittsburgh, Pa. 15219

[8]American Sterilizer Company, Erie, Pa.

[9]National Appliance Company, Portland, Ore.

filter paper (Whatman #42) and defatted in a Soxhlet apparatus with petroleum ether for approximately $7\frac{1}{2}$ hours.

Samples of feed, feces and defatted carcass were wet ashed and prepared for mineral analysis. Dilutions were made with a repipeter[10] using a 1% strontium solution[11] (30.5 g $SrCl_2 \cdot 6H_2O$ plus 5 g NaCl per liter of deionized, distilled water) for calcium and magnesium analysis. Urine collections were diluted to 150 ml, filtered through Whatman #41, ashless filter paper, and refrigerated in glass bottles. Urine samples were analyzed without ashing.

Aliquots of a standard solution containing 400 ppm calcium, 200 ppm phosphorus and 100 ppm magnesium were wet ashed to determine mineral losses due to the ashing procedure. Data from samples which were wet ashed were adjusted accordingly. Standard curves were calculated with a programmable desk calculator[12] using a special program for curvilinear regression analysis. Statistical significance was evaluated at $p < 0.05$ using a two-by-two factorial design analysis of variance (96) (see appendix). Data from balance periods a_1 and a_2, although collected one week apart, were combined and reported as a single mean since no statistical differences due to time were observed.

EXPERIMENT 3

This study was designed to determine whether the calciuria produced by a high protein diet is related to the production of a

---

[10]Labindustries, 1802 Second Street, Berkeley, Calif. 94710

[11]Reagents obtained from J. T. Baker Chemical Co., Phillipsburg, N. J.

[12]Hewlett-Packard model 9100 A, Hewlett-Packard Co., Loveland, Colo.

solute diuresis. Ten male rats weighing approximately 350 g were housed individually in metabolic cages. Diets were prepared as a slurry. Five rats were given a 20% protein diet (table 6) plus a 5% sucrose solution in place of drinking water. The remaining animals were given a 40% protein diet (table 6) and deionized, distilled water to drink. After two days maintenance on the respective experimental diets, three 24-hour urine collections were made. All animals were then transferred to a control, 20% protein diet. Two 24-hour urine collections were made again after two days adjustment to the new dietary regimen. Urine volumes were measured for both treatments and controls, and the total urinary calcium excretion was determined. The data were analyzed for statistical significance ($p<0.05$) using a one-way analysis of variance and Scheffe's test (96).

EXPERIMENT 4

The purpose of this experiment was to determine whether the ratio of protein-bound to unbound calcium in the serum is altered by changing the level of protein in the diet. Fourteen rats (164-187 g) were divided into two groups (seven in each) and given either 20 or 40% protein diets similar to diets 1 and 2 in experiment 2 (table 5) and deionized, distilled water ad libitum. The animals were maintained on the diets for 38 days before blood was taken by cardiac puncture. Each animal was anesthetized with at least 0.3 ml sodium pentobarbitol (50 mg/ml). Blood was collected under mineral oil and transferred from the syringe directly to a vacutainer[13].

Table 6. Percentage dietary composition, experiment 3.

Ingredient	20% protein diet	40% protein diet
Sodium caseinate	23.00	46.00
Cellulose	4.00	4.00
Corn oil	4.00	4.00
Vitamin mix[1]	1.00	1.00
Mineral mix[2]	4.00	4.00
Choline Cl	0.30	0.30
Cornstarch	63.70	40.70

[1]See footnote 2, table 3.

[2]Salt mix No. 4164, General Biochemical Company, Laboratory Park, Chagrin Falls, O. (98).

After clotting, the blood was centrifuged (15 minutes at 1500 g) and an aliquot of serum was removed from the vacutainer anaerobically and transferred to a centriflo[14] ultrafiltration cone. The centriflo filter was soaked in deionized, distilled water for several hours and about 0.5 ml of mineral oil was placed in it before the serum was added. Serum ultrafiltrates were collected after centrifugation (20 minutes at 1500 g) and transferred quantitatively to 10 ml volumetric flasks. Further dilution with a 1% strontium solution was done and calcium concentrations determined. Total serum calcium was also determined for each animal using the TCA precipitation method described for experiment 1. Mean values were compared at $p < 0.05$ using Student's t test (97).

---

[13] Becton, Dickinson and Company, Rutherford, N. J. 07070

[14] Amicon Corporation, Lexington, Mass.

RESULTS

EXPERIMENT 1

Total weight gains for the animals in this study did not appear to be influenced by any of the dietary manipulations (table 7). There were no significant differences in weight gain due to changes in protein source or level or to changes in the calcium to phosphorus ratio. Analysis of variance, however, indicates that food intake was decreased when the protein level was increased and was greater for rats fed soy than for those fed meat. Thus, the soy diets appear to have lower feed efficiencies.

Urine volume and fecal dry weights were also influenced by protein source and protein level in this study (table 8). Lower urine and fecal outputs were observed when meat was used as the protein source. In both meat and soy groups, however, urine volumes and fecal dry weights were greater at higher protein intakes. In addition, fecal weights were significantly lowered when the calcium to phosphorus ratio was decreased.

The data presented in table 9 indicate that urinary calcium excretion is influenced by protein source and level and by the mineral composition of the diet. When a 40% soy protein diet was fed, calciuria was observed. A similar increase in urinary calcium with increased protein intake was not observed in meat-fed animals. Analysis of variance indicates that an overall decrease in urinary calcium accompanied the change in calcium to phosphorus ratio from

Table 7. Weight gain, food intake and feed efficiency, experiment 1.

Diet	Initial weights[1]	Final weights[1]	Total wt. gain[1] (27 days)	Daily wt. gain[1]	Food intake[1]	Feed efficiency[1]
	g			g/day		g wt. gain/g food
1	217.0 ± 5.2	374.4 ± 10.2	157.4 ± 6.2	5.8 ± 0.2	22.9 ± 0.3	0.25 ± 0.01
2	220.4 ± 10.2	366.2 ± 6.0	145.8 ± 9.6	5.4 ± 0.4	24.1 ± 0.3	0.22 ± 0.02
3	212.4 ± 4.1	363.2 ± 11.2	150.8 ± 12.4	5.6 ± 0.5	20.8 ± 0.6	0.27 ± 0.02
4	223.4 ± 5.9	348.0 ± 13.0	124.6 ± 8.9	4.6 ± 0.3	22.7 ± 0.8	0.20 ± 0.01
5	234.2 ± 5.2	376.4 ± 6.1	142.2 ± 7.7	5.3 ± 0.3	22.8 ± 0.4	0.23 ± 0.01
6	221.2 ± 7.3	356.2 ± 14.3	135.0 ± 9.0	5.0 ± 0.3	23.6 ± 0.8	0.21 ± 0.01
					S,L[2]	S[2]

[1]Means ± SEM, where each mean is an average of five rats.

[2]Significant differences are represented by the following symbols: S = overall effect due to protein source, L = overall effect due to protein level, SL = protein source - protein level interaction, C = overall effect due to calcium to phosphorus ratio, SC = protein source - calcium to phosphorus ratio interaction.

Table 8. Urine volumes and fecal dry weights, experiment 1.

Diet	Urine volumes[1]	Fecal dry weights[1]
	ml/day	g/day
1	11.8 ± 0.5	1.93 ± 0.06
2	18.2 ± 2.7	2.31 ± 0.09
3	21.5 ± 1.0	2.17 ± 0.12
4	30.9 ± 2.0	2.81 ± 0.22
5	17.6 ± 2.3	1.43 ± 0.04
6	16.7 ± 1.1	1.76 ± 0.08
	S,L[2]	S,L,C[2]

[1]Means ± SEM, where each mean is an average of five rats.

[2]Significant differences. See footnote 2, table 7, for description of symbols.

Table 9. Urinary and fecal excretion of calcium and calcium balance, experiment 1.

Diet	Urinary Ca[1]	Fecal Ca[1]	Ca balance[1]
		mg/day	
1	3.56 ± 0.48	202.43 ± 13.15	7.19 ± 11.33
2	4.61 ± 0.65	236.28 ± 8.68	-13.54 ± 7.28
3	4.11 ± 0.68	182.96 ± 8.45	-17.86 ± 5.16
4	15.40 ± 4.38	175.21 ± 13.89	8.57 ± 8.44
5	1.61 ± 0.21	75.58 ± 2.19	36.95 ± 3.05
6	1.69 ± 0.32	89.53 ± 2.73	22.07 ± 2.16
	S,L,SL,C[2]	S,L,C[2]	S,SL,C[2]

[1]Means ± SEM, where each mean is an average of five rats.

[2]Significant differences. See footnote 2, table 7, for description of symbols.

2.0 to 1.0 with the 20% protein diets, although differences between the means for each group were not significant.

Fecal calcium excretion was lower in the high protein groups than the control for both meat and soy-based diets. A decrease in the calcium level and calcium to phosphorus ratio resulted in lowered fecal calcium excretions and higher apparent calcium retentions for diets 5 and 6. Statistical analysis of calcium balances between the first four groups indicated a protein source - protein level interaction.

Phosphorus metabolism was altered by protein source and mineral composition of the diet but not by protein level (table 10). When soy diets were fed, a greater fecal loss of phosphorus and a lower urinary phosphorus excretion were observed indicating reduced intestinal absorption of this mineral. When calcium intakes were decreased, however, fecal phosphorus losses were decreased and urinary phosphorus excretion increased. The trend towards a lower phosphorus balance with soy which was observed in the first four groups was not observed in group 6, since the phosphorus balance for this group was greater than that of group 5.

The magnesium levels of all diets in experiment 1 were greater than adequate; consequently, the magnesium balances were positive for all groups (table 11). An increase in protein intake without a change in the dietary magnesium level led to reduced magnesium retentions even though the mean urinary and fecal magnesium excretions were not significantly different. Since the magnesium levels were lower in diets 5 and 6, the lower retention of magnesium for these two groups was expected.

Table 10. Urinary and fecal excretion of phosphorus and phosphorus balance, experiment 1.

Diet	Urinary P[1]	Fecal P[1]	P balance[1]
		mg/day	
1	4.43 ± 0.74	50.79 ± 3.18	40.04 ± 1.74
2	0.99 ± 0.18	76.84 ± 3.13	37.64 ± 1.65
3	2.60 ± 1.30	43.85 ± 2.77	40.32 ± 1.40
4	0.53 ± 0.08	81.57 ± 3.33	31.68 ± 2.26
5	14.84 ± 1.72	28.39 ± 0.58	42.10 ± 2.59
6	11.11 ± 1.78	47.48 ± 1.79	60.93 ± 3.19
	S,C[2]	S,C[2]	S,C,SC[2]

[1]Means ± SEM, where each mean is an average of five rats.

[2]Significant differences. See footnote 2, table 7, for description of symbols.

Table 11. Urinary and fecal excretion of magnesium and magnesium balance, experiment 1.

Diet	Urinary Mg[1]	Fecal Mg[1]	Mg balance[1]
		mg/day	
1	8.13 ± 0.87	26.10 ± 0.54	22.74 ± 1.62
2	9.71 ± 1.53	29.45 ± 1.25	25.94 ± 0.92
3	9.02 ± 1.86	27.61 ± 1.39	12.84 ± 1.81
4	9.24 ± 2.48	28.78 ± 2.31	9.15 ± 2.22
5	3.98 ± 0.79	14.60 ± 0.54	14.89 ± 1.10
6	1.86 ± 0.74	13.86 ± 0.85	14.44 ± 1.56
	C^2	C, SC^2	L, C^2

[1]Means ± SEM, where each mean is an average of five rats.

[2]Significant differences. See footnote 2, table 7, for description of symbols.

Mineral analysis of bone and soft tissues indicated that none of the dietary parameters were responsible for altering the tissue content of calcium, phosphorus or magnesium in rats over the course of one month (tables 12-16). The fat-free dry weights of bones from animals in group 4, however, were lower than those of the animals in either of the first two groups. No significant changes in kidney weights were observed as a result of high protein intakes (table 17).

EXPERIMENT 2

Figure 1 shows the mean weight gains for the four groups of animals. There were no significant differences between the initial weights; however, the total weight gains for those animals on the 40% protein diets were significantly lower than those of animals consuming 20% protein diets (table 18). Differences in weight gain were probably related to the lower average food intakes and the initial delay in weight gain observed in the high protein groups. The weight gains for animals on diets 2 and 4 were not significantly lower for the period between days 14 and 39, however, suggesting that once they had adjusted to their high protein diets, the animals were able to attain a greater feed efficiency. Since food spillage was high, the values given in table 18 for feed intake and feed efficiency may not be accurate. More reliable calculations could not be made from data collected during the balance periods since the rats gained more weight either because of water retention or a greater food consumption when the food was given as a slurry.

The urinary output (ml/day) was greater for the 40% protein groups than the 20% protein groups (table 19). Although fluid intakes were

Table 12. Calcium content of soft tissues, experiment 1.

Diet	Muscle[1]	Kidney[1]	Liver[1]	Serum[1]
	mg/g wet wt.			mg/100 ml
1	0.096 ± 0.013 (5)	0.095 ± 0.019 (5)	0.058 ± 0.006 (3)	15.53 ± 0.94 (5)
2	0.084 ± 0.007 (3)	0.094 ± 0.013 (5)	0.128 ± 0.057 (5)	15.27 ± 1.06 (5)
3	0.094 ± 0.009 (5)	0.118 ± 0.015 (4)	0.121 ± 0.034 (5)	15.99 ± 2.09 (5)
4	0.114 ± 0.009 (6)	0.159 ± 0.032 (6)	0.170 ± 0.023 (6)	16.02 ± 1.80 (6)
5	0.107 ± 0.014 (5)	0.098 ± 0.007 (5)	0.276 ± 0.066 (5)	19.03 ± 0.98 (4)
6	0.119 ± 0.026 (5)	0.084 ± 0.028 (5)	0.093 ± 0.004 (4)	18.44 ± 1.06 (4)

[1]Means ± SEM. Number in parenthesis denotes sample size.

Table 13. Phosphorus content of soft tissues, experiment 1.

Diet	Muscle[1]	Kidney[1]	Liver[1]	Serum[1]
		mg/g wet wt.		mg/100 ml
1	2.583 ± 0.051 (5)	2.807 ± 0.163 (5)	2.788 ± 0.130 (5)	7.80 ± 0.42 (5)
2	2.607 ± 0.032 (4)	2.794 ± 0.248 (4)	2.691 ± 0.052 (5)	8.48 ± 0.54 (5)
3	2.583 ± 0.028 (5)	2.930 ± 0.042 (5)	2.904 ± 0.096 (5)	8.24 ± 0.59 (5)
4	2.648 ± 0.048 (6)	3.010 ± 0.059 (6)	2.839 ± 0.120 (6)	8.65 ± 0.67 (6)
5	2.629 ± 0.039 (5)	3.146 ± 0.079 (5)	2.727 ± 0.059 (5)	8.90 ± 0.63 (5)
6	2.634 ± 0.021 (5)	3.055 ± 0.074 (5)	2.725 ± 0.057 (5)	7.88 ± 0.26 (5)

[1]Means ± SEM. Number in parenthesis denotes sample size.

Table 14. Magnesium content of soft tissues, experiment 1.

Diet	Muscle[1]	Kidney[1]	Liver[1]	Serum[1]
	mg/g wet wt.			mg/100 ml
1	0.103 ± 0.009 (5)	0.075 ± 0.002 (5)	0.092 ± 0.004 (5)	4.10 ± 0.68 (5)
2	0.096 ± 0.003 (4)	0.078 ± 0.005 (5)	0.088 ± 0.004 (4)	4.12 ± 1.40 (5)
3	0.095 ± 0.002 (5)	0.073 ± 0.002 (5)	0.088 ± 0.005 (5)	4.48 ± 0.55 (5)
4	0.107 ± 0.006 (6)	0.082 ± 0.004 (6)	0.084 ± 0.004 (6)	4.04 ± 0.34 (6)
5	0.097 ± 0.003 (5)	0.080 ± 0.002 (5)	0.091 ± 0.006 (5)	3.66 ± 0.47 (4)
6	0.099 ± 0.002 (5)	0.080 ± 0.003 (4)	0.081 ± 0.004 (5)	2.92 ± 0.17 (4)

[1]Mean ± SEM. Number in parenthesis denotes sample size.

Table 15. Femur fat-free dry weights and ash, experiment 1.

Diet	Fat-free dry wts.[1]	Ash[1]
	mg	%
1	551 ± 15	65.02 ± 0.22
2	568 ± 8	63.05 ± 0.70
3	542 ± 9	65.40 ± 0.30
4	496 ± 10*	62.96 ± 0.41
5	538 ± 5	61.95 ± 1.20
6	529 ± 11	64.42 ± 0.36

[1]Means ± SEM, where each mean is an average of ten samples.

*Significantly less than diets 1 and 2.

Table 16. Mineral content of femurs, experiment 1.

Diet	Calcium[1]		Phosphorus[1]		Magnesium[1]	
	mg/g fat-free bone	mg/g ash	mg/g fat-free bone	mg/g ash	mg/g fat-free bone	mg/g ash
1	225.0 ± 6.6	345.9 ± 9.8	137.7 ± 4.1	210.8 ± 6.8	4.95 ± 0.25	8.08 ± 0.12
2	213.3 ± 5.0	338.4 ± 7.4	171.4 ± 6.1	270.7 ± 11.6	5.35 ± 0.28	8.47 ± 0.44
3	212.2 ± 6.7	324.4 ± 9.9	153.9 ± 2.8	234.9 ± 3.9	5.38 ± 0.09	8.24 ± 0.12
4	216.0 ± 5.7	343.5 ± 9.0	155.5 ± 6.1	249.6 ± 8.9	5.67 ± 0.08	7.50 ± 0.10
5	211.8 ± 4.0	342.9 ± 8.9	155.4 ± 3.4	246.6 ± 9.6	5.23 ± 0.08	8.49 ± 0.20
6	223.9 ± 6.5	348.1 ± 11.9	154.1 ± 5.2	238.1 ± 7.2	4.93 ± 0.08	7.65 ± 0.11

[1]Means ± SEM, where each mean is an average of ten samples.

Table 17. Kidney, percent body weight, experiment 1.

Diet	Kidney, percent body weight[1]
	%
1	0.718 ± 0.023
2	0.706 ± 0.047
3	0.760 ± 0.021
4	0.756 ± 0.033
5	0.674 ± 0.019
6	0.721 ± 0.018

[1] Means ± SEM. where each mean is an average of five rats.

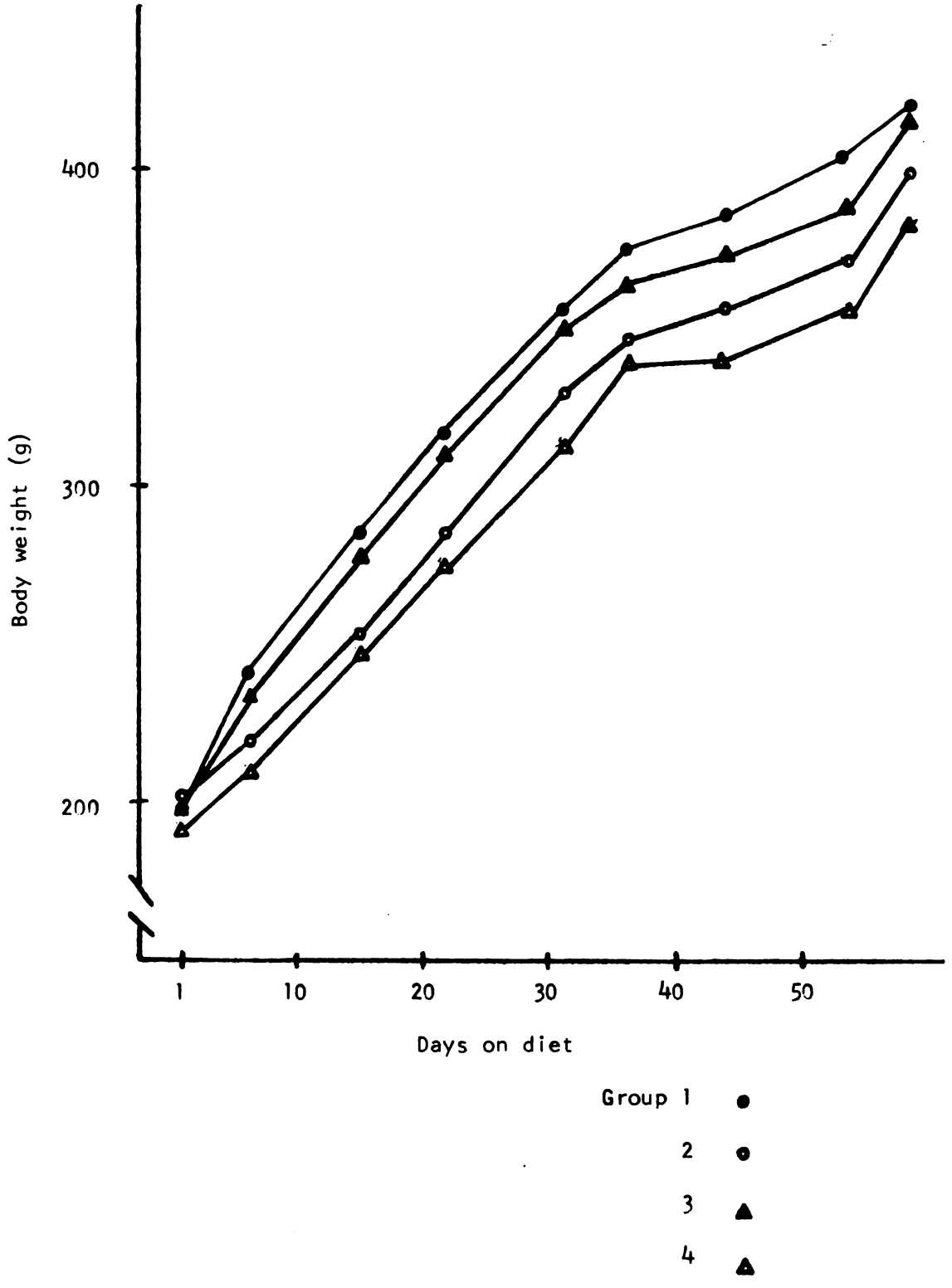


Figure 1. Average weight gains, experiment 2. (Group 1 = 20% protein and 0.05% magnesium; 2 = 40% protein and 0.05% magnesium; 3 = 20% protein and 0.26% magnesium: and 4 = 40% protein and 0.26% magnesium.)

Table 18. Weight gain, food intake and feed efficiency, experiment 2.

Diet	Initial weights[1]	Final weights[1]	Total wt. gain[1] (57 days)	Daily wt. gain[1]	Food intake[1]	Feed efficiency[1]
		g		g/day		g wt. gain/g food
1	197.7	419.9	222.2	3.90	21.2	0.18
2	203.4	399.4	196.0	3.44	18.5	0.19
3	197.2	413.3	216.1	3.79	21.4	0.18
4	193.4	383.9	190.5	3.34	18.5	0.18
SEM	5.2	7.0	7.1	0.13	0.4	0.01
		P[2]	P[2]	P[2]	P[2]	

[1]Means for ten rats.

[2]Significant differences are represented by the following symbols: P = overall effect due to protein level, M = overall effect due to magnesium level, PM = protein level - magnesium level interaction, T = overall effect due to time, PT = protein level - time interaction, MT = magnesium level - time interaction, PMT = protein level - magnesium level - time interaction.

Table 19. Food intake, fecal dry weights, urinary volumes and urinary pH during balance periods, experiment 2.

Diet	Food intake[1]	Fecal dry wt.[1]	Urinary vol.[1]	Urinary pH[1]
	g/day		ml/day	
1a[2]	23.6	1.89	27.9	
b	24.2	2.02	31.5	7.95
2a	22.7	1.85	45.0	
b	23.5	2.02	46.8	7.26
3a	23.0	1.94	28.2	
b	25.5	2.35	31.5	7.71
4a	22.0	1.98	44.3	
b	23.3	2.26	48.3	7.33
SEM	0.6	0.06	3.3	0.09
	P,T[3]	M,T[3]	P[3]	P[3]

[1]Means for ten rats.

[2]Small letters indicate balance period: a was collected after 28 or 36 days on the diet, and b was collected after 54 days.

[3]Significant differences. See footnote 2, table 18, for description of symbols.

not measured, it was observed that the rats in groups 2 and 4 drank more, also. The total fecal output, on the other hand, seemed to be related to the dietary magnesium levels, being significantly greater for the high magnesium groups.

The urinary pH values determined at the final balance period were significantly lower for the high protein groups. The values were still within the range of neutrality, thus, there was no evidence for the production of an acidosis with the high protein intakes. Whether the differences in dietary phosphorus levels (table 4) were great enough to account for differences in urinary pH is a matter of speculation.

The results from the balance collections are summarized in tables 20-22 and figures 2-5. High protein levels in the diet affected the renal excretion of calcium creating a calciuria. Despite the increase in urine volume, however, the concentration of calcium in the urine in the 40% protein groups was no different from that found in the 20% protein groups. Thus, there appears to be a direct relation between the total urinary calcium excretion and the urine volume.

At normal magnesium levels, an increase in protein intake was accompanied by a reduction in fecal calcium loss and a more positive calcium balance. However, the addition of both protein and magnesium to the diet caused a decrease in calcium retention. An increase in magnesium alone was sufficient to increase urinary calcium excretion. When a high magnesium intake was accompanied by an increase in dietary protein, the apparent % absorption of calcium and the calcium balance were significantly reduced in comparison with the control.

Table 20. Effect of dietary protein and magnesium levels on calcium balance, experiment 2.

Diet	Urinary Ca[1]		Fecal Ca[1]	Ca balance[1]	Apparent % Absorption[1]
	mg/day	mg/ml	mg/day	mg/day	
1a[2]	3.13	0.120	202.4	58.7	22.92
b	2.95	0.098	211.8	57.7	22.28
2a	6.21	0.122	175.8	79.9	32.99
b	4.99	0.107	195.6	69.5	27.66
3a	4.53	0.160	188.3	72.8	29.20
b	4.40	0.141	229.9	62.8	21.82
4a	7.18	0.162	177.8	53.6	25.49
b	6.10	0.126	209.6	37.2	17.50
SEM	0.71	0.019	12.8	6.4	1.68
	P,M[3]	M[3]	T[3]	PM[3]	PM,T[3]

[1]Means for ten rats.

[2]See footnote 2, table 19.

[3]Significant differences. See footnote 2, table 18, for description of symbols.

Table 21. Effect of dietary protein and magnesium levels on phosphorus balance, experiment 2.

Diet	Urinary P[1]		Fecal P[1]	P balance[1]	Apparent % Absorption[1]
	mg/day	mg/ml	mg/day	mg/day	
1a[2]	22.6	0.818	68.4	-7.7	19.15
b	20.8	0.668	70.1	-4.1	20.24
2a	20.2	0.457	47.9	33.3	53.19
b	27.1	0.586	54.6	23.2	48.00
3a	10.1	0.371	58.4	24.2	37.07
b	10.2	0.330	77.2	15.6	25.03
4a	11.3	0.270	54.8	31.6	44.02
b	14.7	0.297	64.9	21.7	42.78
SEM	1.5	0.061	2.5	1.9	1.63
	M[3]	P,M[3]	P,PM,T[3]	P,M,PM[3]	P,PM[3]

[1]Means for ten rats.

[2]See footnote 2, table 19.

[3]Significant differences. See footnote 2, table 18, for description of symbols.

Table 22. Effect of dietary protein and magnesium levels on magnesium balance, experiment 2.

Diet	Urinary Mg[1]		Fecal Mg[1]	Mg balance[1]	Apparent % Absorption[1]
	mg/day	mg/ml	mg/day	mg/day	
1a[2]	2.00	0.077	8.7	1.6	29.18
b	1.86	0.062	8.2	2.6	35.26
2a	2.88	0.062	6.8	2.5	44.68
b	2.69	0.058	6.4	3.2	48.56
3a	7.00	0.281	37.0	15.6	39.06
b	7.10	0.227	48.3	11.7	28.29
4a	6.90	0.161	29.4	23.1	50.48
b	6.46	0.133	38.4	17.5	37.91
SEM	0.35	0.015	1.4	1.3	2.20
	M[3]	P,M,PM[3]	P,M,PM,T,MT[3]	P,M,PM[3]	P,MT[3]

[1]Means for ten rats.

[2]See footnote 2, table 19.

[3]Significant differences. See footnote 2, table 18, for description of symbols.

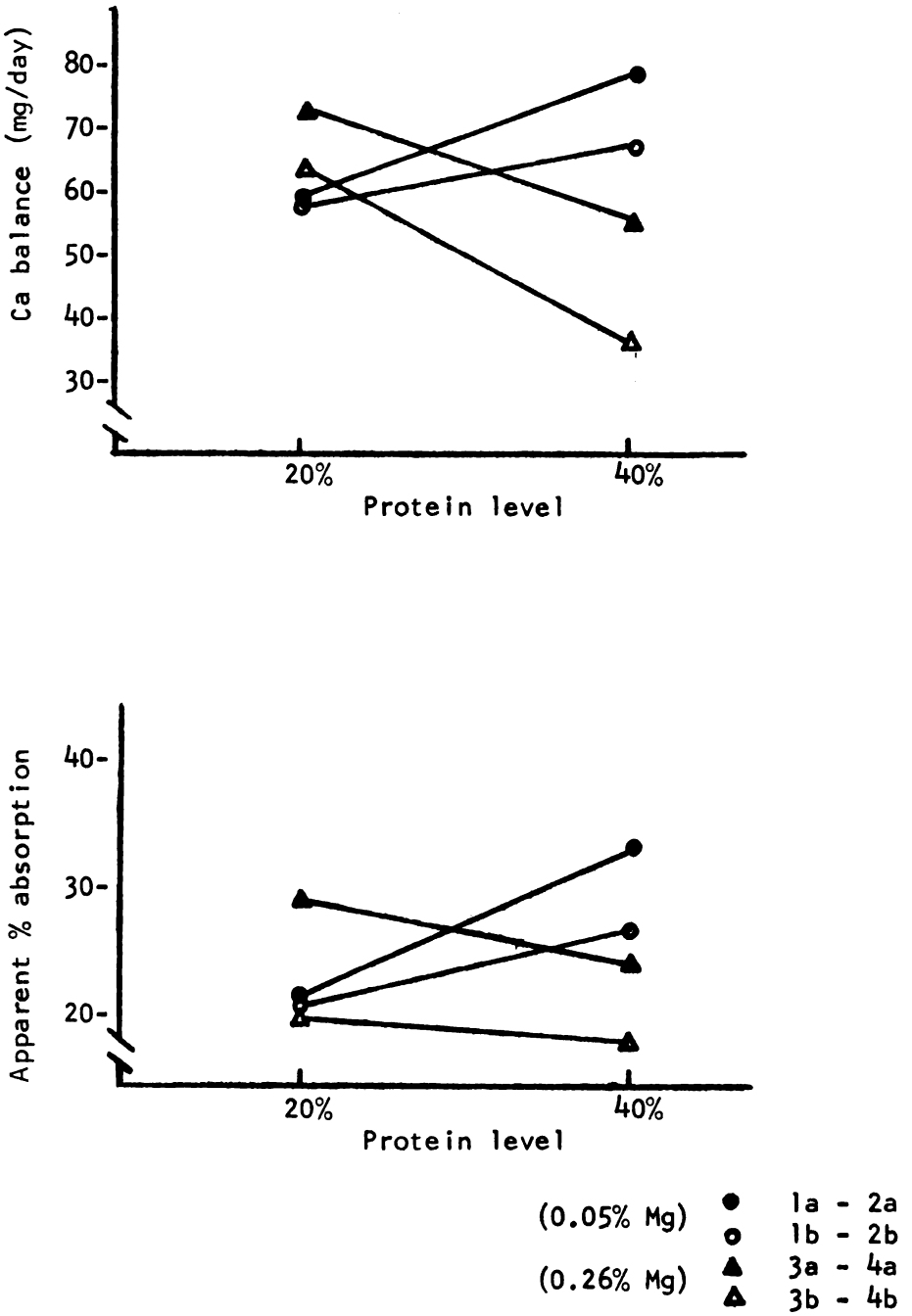
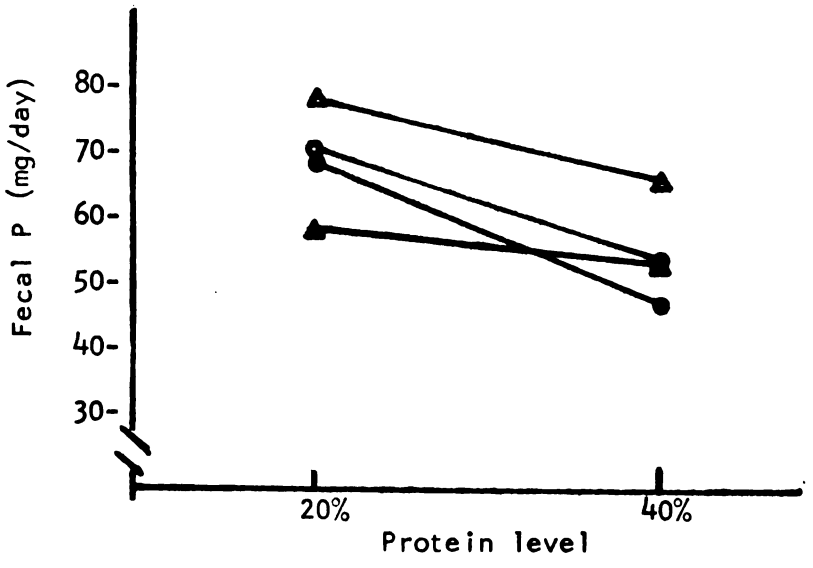
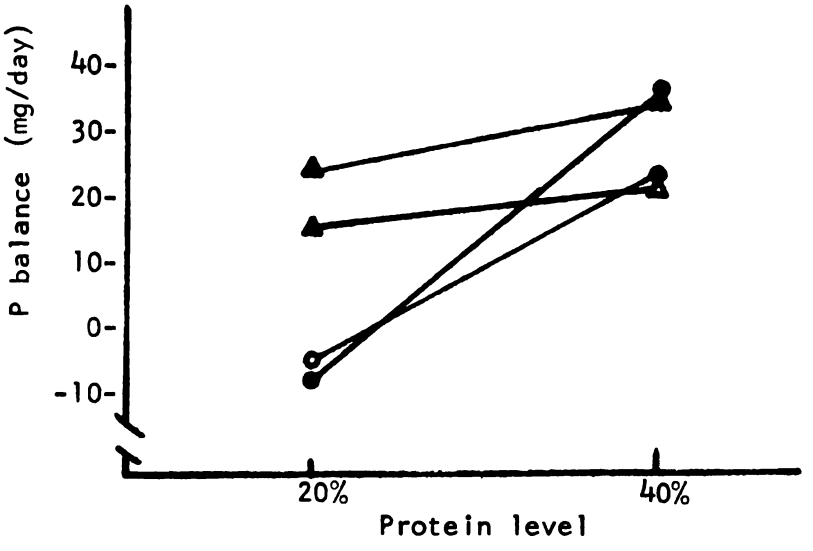


Figure 2. Effect of protein level on calcium balance and apparent % absorption of calcium at two levels of dietary magnesium, experiment 2. (a = data from balance collections made at 28 or 36 days; b = data from collections made at 54 days; 1 and 2 = diets with adequate magnesium intakes (0.05%); and 3 and 4 = diets with high magnesium intakes (0.26%))



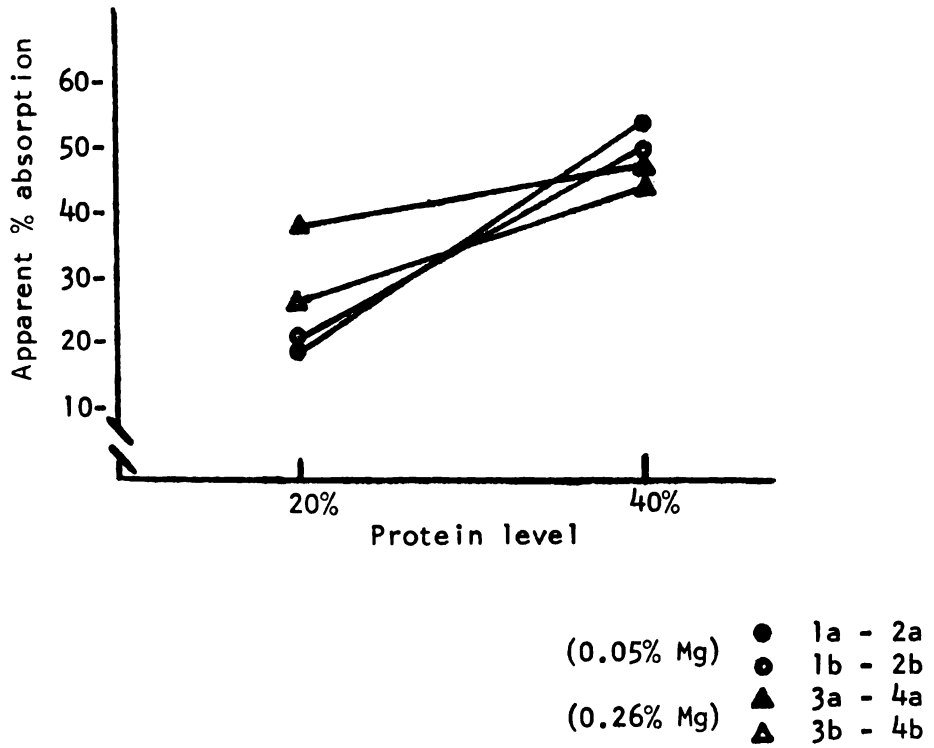


(0.05% Mg) ● 1a - 2a
○ 1b - 2b
(0.26% Mg) ▲ 3a - 4a
△ 3b - 4b

(cont'd)

Figure 3. Effect of protein level on fecal phosphorus excretion, phosphorus balance, and apparent % absorption of phosphorus at two levels of dietary magnesium, experiment 2. (See figure 2 for description of symbols.)

Figure 3. cont'd.



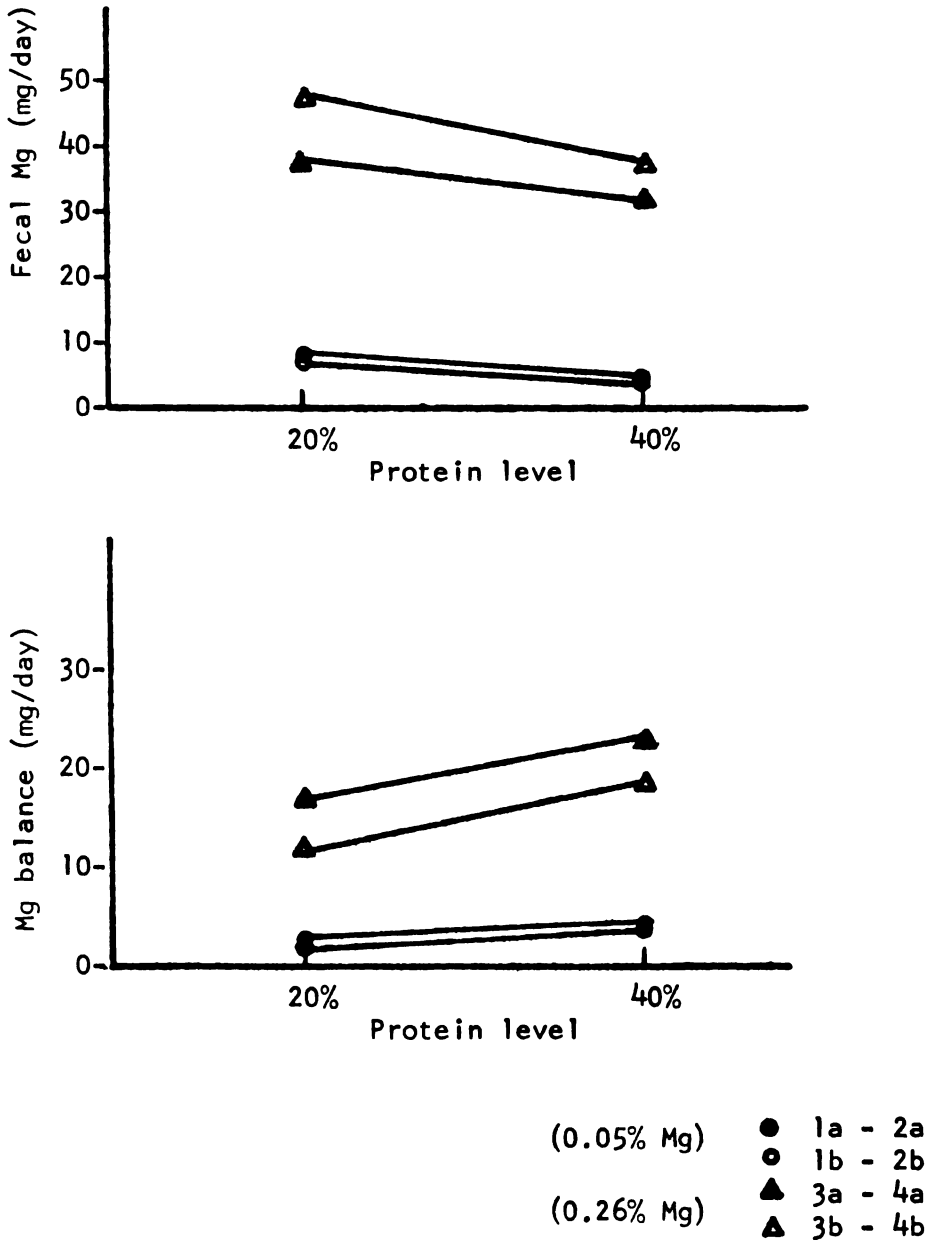


Figure 4. Effect of protein level on fecal magnesium excretion and magnesium balance at two levels of dietary magnesium, experiment 2. (See figure 2 for description of symbols.)

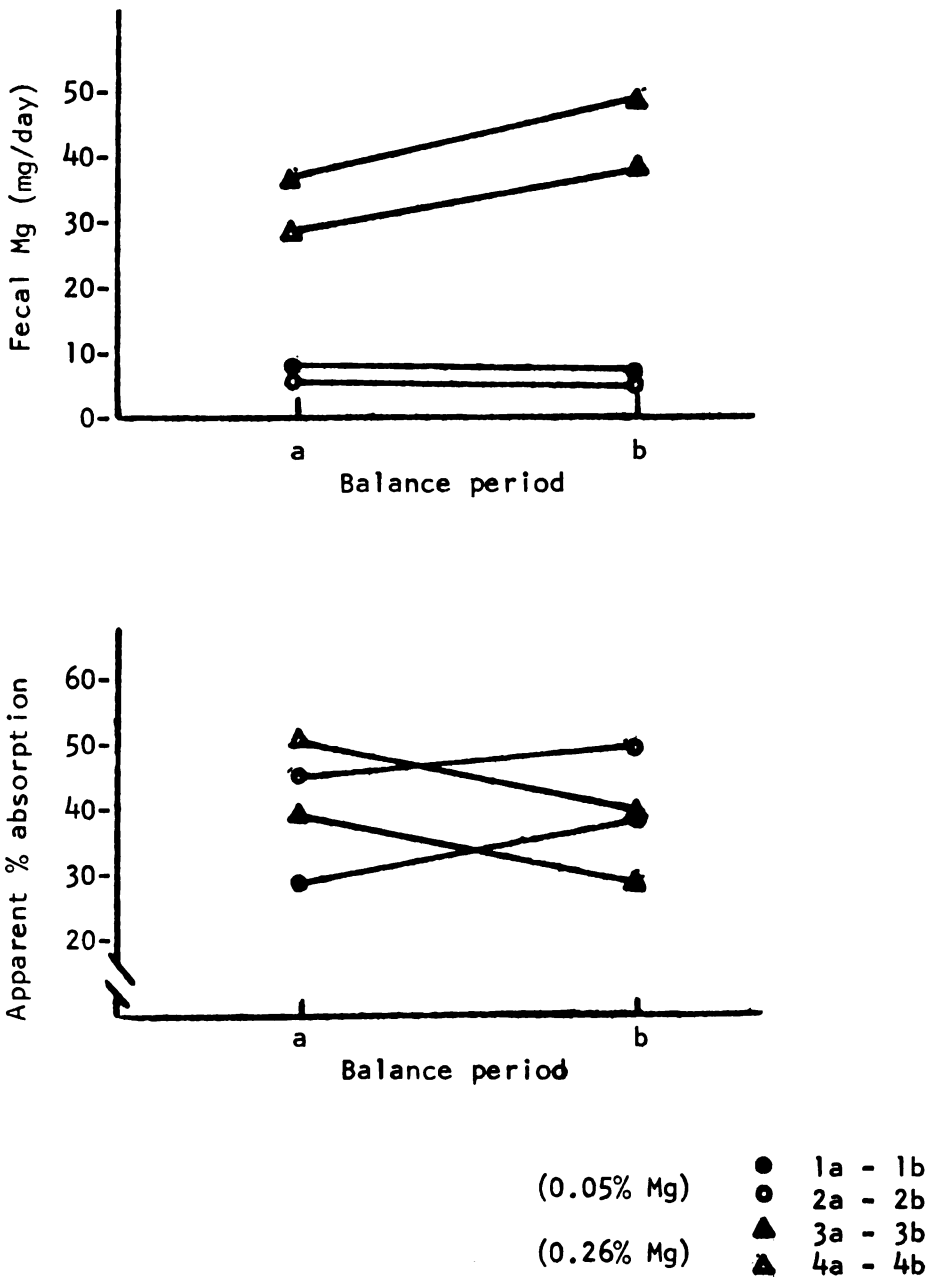


Figure 5. Effect of time on fecal magnesium excretion and apparent % absorption of magnesium at two levels of dietary magnesium, experiment 2. (a = data from balance collections made at 28 or 36 days; b = data from collections made at 54 days; 1 = diet with 20 % protein and 0.05% magnesium; 2 = diet with 40% protein and 0.05% magnesium; 3 = diet with 20% protein and 0.26% magnesium; and 4 = diet with 40% protein and 0.26% magnesium)

The excretion of calcium in the urine was not significantly affected by the length of time on the diets. However, fecal losses were enhanced and the apparent % absorption decreased with time, but not sufficiently to decrease the calcium balances significantly.

The apparent % absorption of both phosphorus and magnesium in the gut was increased as the protein levels in the diet were raised, but no changes in urinary phosphorus or magnesium excretion due to a protein effect could be seen. High protein intakes, therefore, resulted in an increase in phosphorus retention. Magnesium balances were significantly increased only in those animals consuming high levels of magnesium as well as high levels of protein, however.

Urinary phosphorus and magnesium concentrations were inversely proportional to urinary volume so that the total urinary excretion of these minerals was the same at both protein levels when the magnesium intake was constant. The increase in dietary magnesium significantly decreased the urinary phosphorus excretion and led to an increase in phosphorus retention. As one might expect, both urinary and fecal magnesium levels were increased when excessive amounts of magnesium were ingested, but the apparent % absorption of this mineral was not affected by magnesium level during the first balance period. With time, the fecal losses of magnesium in the high magnesium groups were increased and the apparent % absorption decreased. On the other hand, apparent % absorption in groups 1 and 2 increased with time, indicating a magnesium - time interaction.

As a result of the increased magnesium content of diets 3 and 4, animals in these groups retained more magnesium. Total carcass analysis revealed a greater content of magnesium expressed as mg/g

fat-free dry weight and mg/g dry weight in animals from groups 3 and 4 (table 23). No significant differences were observed when the magnesium levels were expressed as mg/g wet weight or total g magnesium per animal, however, and no differences in carcass calcium and phosphorus content were observed between groups. The percentage of fat in the dried samples taken from animals in groups 2 and 4 was lower than that found in samples from groups 1 and 3 (table 24). This reflected the lower body weights of the animals consuming 40% protein diets.

EXPERIMENT 3

Data for experiment 3 are given in table 25. In contrast to the results in experiment 2, the average urine colume for the animals on the high protein diet was not significantly greater than that of the control group. However, the mean calcium excretion for the high protein group was greater than both the control and the group exhibiting a sucrose diuresis.

EXPERIMENT 4

The pattern of weight gain for the animals in this experiment is recorded in table 26 and figure 6. Results were similar to those observed in experiment 2; that is, the average weight gains and daily food intakes were lower for the 40% protein group than for the 20% protein group.

There were no significant differences in total ultrafiltrable calcium levels and percent ultrafiltrable calcium between treatments (table 27).

Table 23. Total carcass analysis, mineral composition, experiment 2.

Diet	mg/g fat-free wt.[1]	mg/g dry wt.[1]	mg/g wet wt.[1]	g total[1]
		Calcium		
1	37.52	25.92	10.34	4.15
2	39.03	27.74	10.64	4.08
3	38.01	25.86	10.23	4.06
4	35.88	26.78	10.19	3.74
SEM	1.33	1.09	0.46	0.18
		Phosphorus		
1	20.96	14.44	5.78	2.33
2	20.70	14.73	5.67	2.17
3	21.09	14.32	5.66	2.24
4	20.73	15.45	5.75	2.11
SEM	0.55	0.44	0.21	0.08
		Magnesium		
1	1.203	0.834	0.333	0.138
2	1.244	0.873	0.335	0.129
3	1.288	0.897	0.355	0.141
4	1.298	0.958	0.359	0.133
SEM	0.030	0.029	0.013	0.005
	M,PM[2]	M[2]		

[1]Means for ten rats.

[2]Significant differences. See footnote 2, table 18, for description of symbols.

Table 24. Lipid and water content of carcasses, experiment 2.

Diet	Lipid[1]	Water[1]
	% of dry weight	%
1	31.16	59.98
2	28.81	61.60
3	31.97	60.42
4	25.31	62.73
SEM	1.38	0.80
	P[2]	P[2]

[1]Means for ten rats.

[2]Significant differences. See footnote 2, table 18, for description of symbols.

Table 25. Urine volume and urinary calcium excretion, experiment 3.

Diet	Urine volume[1]	Urinary Ca[1]
	ml/day	mg/day
Control (20% protein)	35.21 ± 2.07 (5)	1.93 ± 1.17 (5)
High protein (40%)	47.28 ± 0.36 (5)	4.16 ± 1.86 (5)*
Control, 5% sucrose as drinking water	91.25 ±12.22 (10)*	2.56 ± 1.39 (10)

[1]Means ± SEM. Number in parenthesis denotes sample size.

*Significantly different from control.

Table 26. Weight gain, food intake and feed efficiency, experiment 4.

Diet	Initial weights[1]	Final weights[1]	Total wt. gain[1]	Daily wt. gain[1]	Food intake[1]	Feed efficiency[1]
	g			g/day		g wt. gain/g food
1	179.6 ± 3.1	371.4 ± 7.3	191.9 ± 7.1	4.92 ± 0.18	18.4 ± 0.5	0.27 ± 0.01
2	181.0 ± 8.0	333.8 ± 7.0*	152.9 ± 5.9*	3.92 ± 0.15*	15.7 ± 0.3*	0.25 ± 0.01

[1] Means ± SEM, where each mean is an average of seven rats.

*Significant differences.

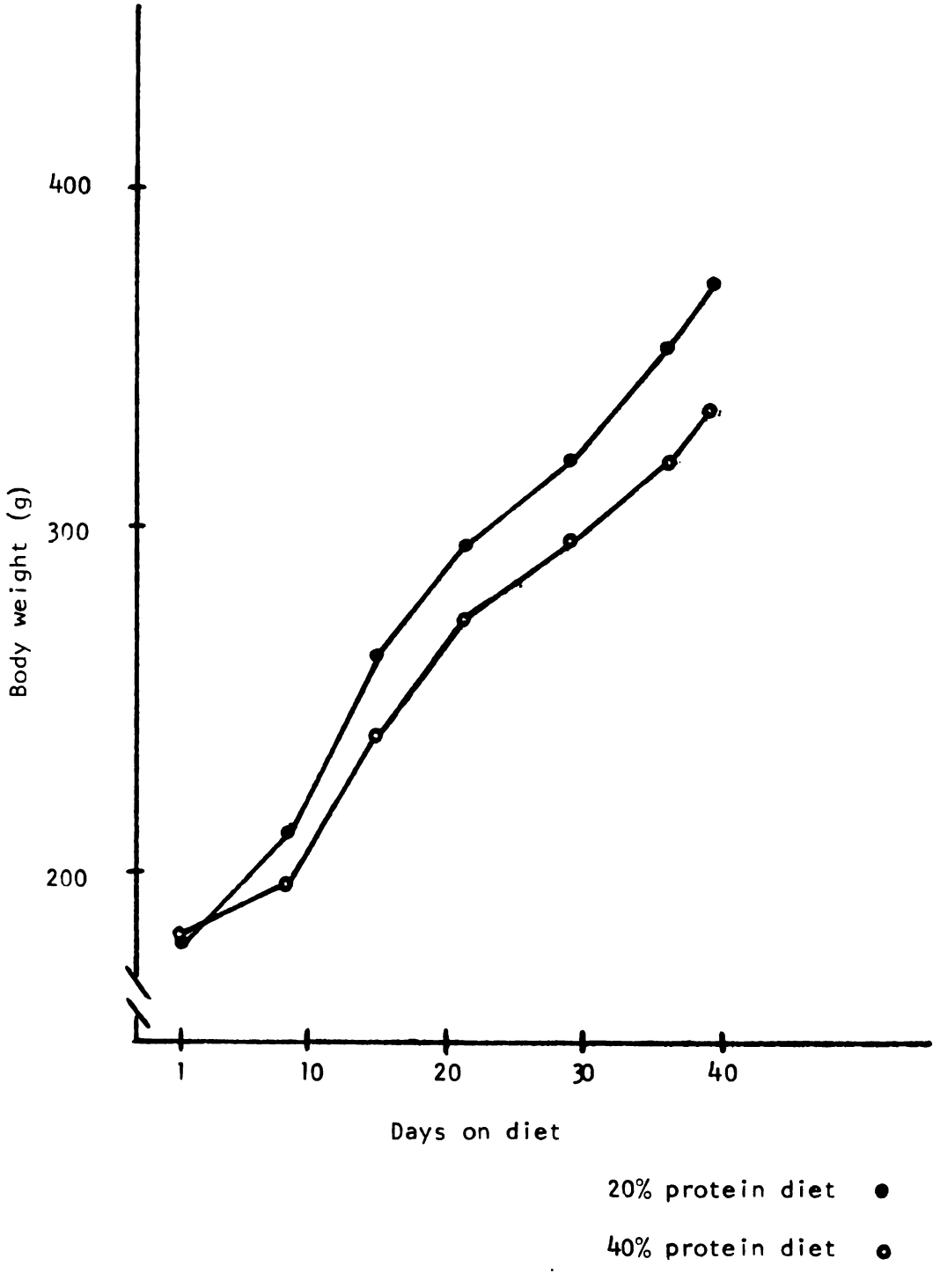


Figure 6. Average weight gains, experiment 4.

Table 27. Total serum calcium and serum ultrafiltrable calcium, experiment 4.

Diet	Total serum Ca[1]	Ultrafiltrable Ca[1]	
	mg/100 ml	mg/100 ml	%
20% protein	13.88 ± 0.19	6.04 ± 0.58	41.84 ± 5.26
40% protein	13.76 ± 0.11	6.38 ± 0.54	40.95 ± 4.50

[1]Means ± SEM, where each mean is an average of seven rats.

DISCUSSION

Because of similarities in experimental design, some aspects of experiments 1 and 2 can be compared. The only major difference between diets 1 - 4 in experiment 1 and diets 3 and 4 in experiment 2 was the protein source.

In these investigations, the protein source may have influenced the growth rate. Greater weight gains were observed when the animals were given meat or soy protein rather than casein although the initial weights were similar. Daily food intakes were greater for the rats in experiment 1, a result which may have been related to the consistency and/or palatability of the diets. The effect of environmental conditions should not be ruled out, however. Experiment 1 was conducted during the winter months while experiment 2 was conducted during the summer. An attempt to maintain constant conditions of temperature and humidity in the housing facilities was made; however, accurate records were not kept of variations.

Although the level of protein in the diet did not affect the growth rates in experiment 1, those animals which were fed casein lost weight initially in response to high protein intakes. In both experiments 2 and 4, an increase in protein level was accompanied by lower food intakes and lower weight gains. These observations are supported by the work of other investigators. For example, Colby and Frye (54) found a marked decrease in growth rates in rats

when they increased the protein levels from 24 to 50% casein. No dramatic changes in growth patterns were observed, however, when lactalbumin and gelatin were added to a casein diet in another study (51).

Protein source also appeared to affect the calcium retention in the present investigation. Those animals which were fed casein diets maintained a positive calcium balance regardless of the protein or magnesium levels. However, calcium balances for animals fed meat or soy were only slightly positive or negative. The data suggest that absorption of calcium in the gut is more efficient with casein feeding than with either meat or soy protein. Certain amino acids have been shown to promote calcium absorption (22). There is also evidence that some proteins, particularly those derived from egg whites, may have a detrimental effect on bone metabolism in the rat (20).

The production of a calciuria as a consequence of high protein intakes has been consistently observed by those who have investigated the effects of protein ingestion on calcium metabolism in both human subjects and experimental animals (12-14,16-19,21,51,56,99). This effect has been observed in most studies regardless of the protein source. In the present study, calciuria was observed in both soy and casein-fed groups. A significant increase in urinary calcium at high protein intakes was not observed when meat was given, however. Further investigation is necessary to determine whether this effect was related specifically to the protein source or to uncontrolled parameters such as individual variability.

Calciuria may result from a number of factors. Within the kidney, for example, significant increases in urinary calcium can

result from a greater rate of glomerular calcium filtration, an increase in tubular calcium secretion, or an inhibition of tubular calcium reabsorption. So far, there have been no reports than an increase in dietary protein will alter the serum ultrafiltrable calcium level, thus changing the glomerular filtration rate. But Lemann et al. (74) have shown that acidosis increases serum ultrafiltrable calcium levels in humans. In this investigation, no significant differences were observed between the ultrafiltrable calcium concentrations in rats fed either 20% or 40% protein diets (experiment 4). It is possible that the technique used was not sensitive enough to detect changes in serum ultrafiltrable calcium which would affect urinary calcium excretion in the rat. Individual variation between animals may have masked any differences due to protein intake. It is also possible that the calcium excretion is affected not by a change in the protein-bound to unbound ratio, but rather by a change in the ratio of free, ionized calcium to that which is complexed with anions such as phosphate, sulfate or citrate (72). Recent studies suggest that a secondary hyperparathyroidism is brought about by a lowering of serum ionized calcium (100). Wachman and Bernstein (31) have accredited the development of osteoporosis in humans to a secondary hyperparathyroidism resulting from high protein intakes. Using the ultrafiltration method employed in this study, changes in serum ionized calcium could not be detected. However, an ion-exchange electrode has been used successfully to determine free calcium ion concentrations in other investigations (72).

Another possible explanation for the calciuria induced by the ingestion of high protein diets is the production of a metabolic

acidosis. Acid stress has been reported to cause significant increases in urinary calcium excretion (39). Barzel and Jowsey (38) have found that this calciuria is accompanied by an increase in bone resorption in rats. Lemann et al. (74) have indicated that acid stress inhibits renal tubular reabsorption of calcium in humans.

The acid-ash nature of high protein diets was observed during the early part of this century. Bernard (27) is frequently cited for his observation that rabbits maintained on meat diets excrete an acid urine in contrast to the alkaline urine they excrete when they consume their normal herbivorous diet. The pH of the urine reflects the production of endogenous acids in the body which result mainly from the oxidation of sulfur-containing amino acids, the formation of unmetabolizeable organic acids and the hydrolysis of protein-bound phosphate esters (29). From the present data and a recent report by Bell et al. (51), it appears that a calciuria from high protein intakes results even when the urinary pH is neutral. Another observation which suggests that an acidosis was not produced by the high protein intakes in the present investigation was the absence of an increased urinary phosphorus excretion which reportedly accompanies an acidosis (39).

With an increased protein intake, the urinary volume increases. This is probably related to the increased production of urea in the liver from amino acid degradation, and the creation of a urea diuresis. Some investigators have shown that a solute diuresis produced, for example, by saline or mannitol, may increase the urinary calcium excretion. Walser (62,63) has indicated that sodium and calcium

clearances are directly proportional during solute diuresis. Chen and Neuman (71) point out, however, that calcium excretion during mannitol diuresis is independent of water excretion. In the present study, sodium levels were kept constant in each experiment. However, when the daily calcium excretion in animals fed a 40% protein diet was compared with that of rats fed a 20% protein diet plus a 5% sucrose solution in place of drinking water (experiment 3), calcium losses significantly greater than the control were observed only in the high protein group. The average urine volume excreted by the sucrose-loaded rats was more than twice that of the high protein group. On the basis of these results, there appears to be no correlation between diuresis and calcium loss.

Recent investigators have suggested that the increase in urinary calcium excretion can be accounted for by an increase in intestinal absorption of calcium (14,16,18,51). In the present study, however, changes in fecal calcium losses which accompanied changes in urinary calcium excretion could not be detected because of their small magnitude. Since calcium balances were positive for all treatments, the higher urinary calcium losses observed with high protein intakes did not seem to be detrimental to calcium metabolism. Only about 1-4% of the total calcium excreted by rats in this investigation was excreted in the urine. Other studies in rats have likewise shown that only a small portion of the calcium excreted by the rat appears in the urine (51). In contrast, Chu et al. (19) and Linkswiler (18) have shown that a much greater proportion of the calcium excretion in humans can be found in the urine. Linkswiler et al. (18) found that approximately one half of the calcium excreted by young

men receiving 22.7 g N/day and 800 mg calcium/day was excreted via the kidneys. With lower protein intakes, the percentage of calcium in the urine was demonstrably lower. However, changes in fecal calcium excretion were much less dramatic as protein levels were altered. Negative calcium balances with high protein intakes in their studies could be related to the greatly increased urinary calcium levels. These data suggest that the rat may be a poor model for the study of protein effects on calcium metabolism in humans.

Another dietary factor besides protein level or protein source which seemed to affect the calcium excretion in the present experiments was the mineral composition. A significant increase in the calcium retention was observed in experiment 1 when the calcium and magnesium levels of the diets were decreased (diets 5 and 6). The results suggest that intestinal absorption of calcium was enhanced and urinary losses diminished with these dietary changes. Investigations by Shah et al. (101) suggest that a greater calcium retention might be expected with a dietary calcium to phosphorus ratio of 2.0 rather than 1.0, the higher ratio being more beneficial in preventing bone loss in the adult rat. On the other hand, Knapp (102) showed that increasing the calcium to phosphorus ratio in human studies led to increased urinary calcium losses. In the present investigation, there were no changes in bone mass or mineral content when the calcium to phosphorus ratio was changed from 2.0 to 1.0. Due to the experimental design, it was not possible to establish whether the changes in calcium retention were caused by the alteration of the calcium to phosphorus ratio, the reduction of magnesium levels in the

diet, or a combination of these factors. In experiment 2, however, altering the magnesium levels failed to have a consistent effect on the calcium balance.

Some investigators have shown that the metabolism of magnesium is affected by protein intakes in the same manner as calcium. At low magnesium intakes, magnesium deficiency symptoms have been made more severe and urinary magnesium losses have been enhanced in experimental animals by increasing the dietary protein levels (54, 56, 57). In the present study, the urinary magnesium excretion was not affected by increased protein intakes at either level of dietary magnesium (experiment 2).

Reports concerning the effects of dietary magnesium levels and protein intakes on the absorption and retention of magnesium have not been consistent. The data from this investigation suggest that high levels of protein reduce the fecal magnesium losses thus enhancing the retention and apparent % absorption of magnesium. An increase in magnesium retention is more pronounced when the magnesium intake is excessive. In several reports, similar effects of protein on magnesium retention have been cited (13,15). However, in these studies, urinary magnesium levels were increased. Schofield and Morrell (59), on the other hand, observed slightly reduced magnesium retention when protein levels were increased.

Similar changes in magnesium and calcium metabolism with protein intakes suggest that the divalent cations are controlled at least in part by the same mechanisms in the gut and kidney. Magnesium loading by infusion (65) or by ingestion (66) has been shown to increase urinary calcium excretion. Human studies indicate that there is a

protein-magnesium interaction such that calcium balances become more negative with an increase in both magnesium and protein than with an increase in either one by itself (55). The present studies with rats confirm these findings. Urinary magnesium excretions were not affected by the protein levels of the diets even though a calciuria was observed in the high protein groups. Nevertheless, at high magnesium intakes, the urinary calcium excretions were increased and a protein-magnesium interaction was observed.

Dietary magnesium levels appeared to affect the level of phosphorus excreted in the urine. In both experiments 1 and 2, higher dietary magnesium levels were correlated with lower urinary phosphorus levels. Possibly, the high magnesium intakes sufficiently increased the serum magnesium levels to elicit a decrease in parathyroid hormone secretion (103). Since parathyroid hormone (PTH) increases urinary phosphorus excretion (40), a decrease in PTH should result in a decreased phosphorus excretion.

Urinary phosphorus levels also appear to reflect the intestinal absorption of this element. With soy diets, greater fecal phosphorus losses were observed together with reduced urinary phosphorus outputs. Phosphates present in soy may be tied up in the phytic acid molecule making them unavailable for absorption. The data suggest, however, that phytate binding did not affect the absorption of magnesium or calcium in the first experiment.

SUMMARY AND CONCLUSIONS

Two experiments were conducted to determine primarily whether high protein intakes cause increased urinary calcium excretion in rats when the mineral composition of the diet is maintained at a constant and adequate level. In each study, 200 g, male, Sprague-Dawley rats were fed semipurified diets for a given period of time after which fecal and urine collections were taken and analyzed for calcium, phosphorus and magnesium.

In experiment 1, 32 rats were divided into six groups. Two protein sources, beef and soy concentrate, were evaluated at two levels of intake (20% and 40%) and two levels of calcium and magnesium (Ca:P:Mg = 2.0:1.0:0.6 or 1.0:1.0:0.3). Animals were fed their respective diets for a total of 34 days and one balance collection was made. Bones and soft tissues were analyzed separately for mineral content.

Increasing the protein level resulted in a calciuria only in those rats which were given soy protein. Calcium balances ranges from -17.9 to 8.6 mg/day when the calcium to phosphorus ratio was 2.0. With a calcium to phosphorus ratio of 1.0 (20% protein), however, a greater calcium retention was observed, 37.0 and 22.1 mg/day, even though the calcium content of these two diets was lower than that of the other four diets. Phosphorus balances were not affected by protein level. But, when the calcium to phosphorus ratio was reduced from 2.0 to 1.0, the phosphorus balance for soy-fed animals was significantly

increased. In all cases, fecal phosphorus losses were greater and urinary losses lower with soy than with meat. This may have been due to the presence of phytates in the soy. Magnesium balances were positive for all groups as expected since adjusting the magnesium levels to the level of the soy diets resulted in excessive intakes of this mineral. Magnesium balances were less positive at 40% than 20% protein ingestion for both protein sources and for the diets in which the calcium to phosphorus to magnesium ratio was reduced from 2.0:1.0:0.6 to 1.0:1.0:0.3. No changes in mineral composition of either soft tissues or bone was observed from any of the treatments.

To eliminate some of the variables of the first experiment and to determine the effects of high magnesium intakes as well as high protein intakes on mineral metabolism, a second experiment was conducted. Four groups of rats (10 per group) were fed diets containing 20% or 40% protein from casein. Calcium and phosphorus levels were equivalent in all diets (1.0 and 0.5%, respectively) and magnesium levels were set at either 0.05 or 0.26%. Mineral balances were determined at two separate times, after 28 or 36 days and after 54 days of feeding. For each set of data, a mean of two 48-hour collections was calculated. Total carcass analyses were also conducted.

In this experiment, weight gains were lower for those animals consuming high protein diets. Since a difference in weight gain was not observed in experiment 1, the effect may have been related to protein source. Calcium balances were positive for all four groups at both balance periods. Increasing the protein intake enhanced calcium retention when the magnesium levels were low, but decreased

retention when they were high. Both phosphorus and magnesium balances were more positive with high protein intakes due to increased apparent % absorption in the gut. A more dramatic increase in magnesium balance was observed when magnesium intakes were increased, however. Increasing the protein level of the diet led to an increase in urinary calcium. An even greater increase in urinary calcium and a decrease in urinary phosphorus was observed when dietary magnesium levels were high regardless of protein level. In both experiments 1 and 2, calcium balances reflected fecal losses. Compared with total calcium excretion, urinary calcium losses were small (about 1 to 4%). The changes in fecal calcium which accompanied the calciuria were not significant. Although changes in carcass calcium and phosphorus were not observed, there was an increase in total carcass magnesium (expressed as mg/g fat-free dry weight and mg/g dry weight) with high magnesium intakes.

Two subsequent experiments were designed to study the mechanism for calciuria induced by high protein intakes. The calciuric effect observed in experiment 2 did not appear to be caused by an acidosis since urinary pH values were near 7.0 for all four groups. In experiment 3, rats were given either a 20% protein diet with a 5% sucrose solution in place of drinking water or a 40% protein diet to determine whether the calciurea was related to a solute diuresis. Urinary calcium losses and urine volumes were compared with those observed during a control period when rats were given a 20% protein diet and water to drink. Despite greatly increased urine volumes, calcium losses during sucrose loading were not increased indicating that calciuria from high protein intakes is probably not caused by a diuresis.

In experiment 4, serum ultrafiltrable calcium concentrations were compared between rats fed either 20% or 40% protein diets similar to the first two diets used in experiment 2. No differences in serum ultrafiltrable calcium levels or in total serum calcium levels were observed, but large variations in ultrafiltrable calcium concentrations were observed within each group.

RECOMMENDATIONS

Despite the myriad of reports dealing with the effects of high protein intakes on calcium metabolism, there are yet some basic questions which remain unanswered. The fact that many factors influence the metabolism of minerals makes it difficult to compare studies done in different laboratories.

Results from the first two experiments in this investigation suggest that the retention of calcium, but not of magnesium or phosphorus, is affected by the source of protein in the diet. Further experimentation is needed, however, to confirm that the differences in calcium retention between the two experiments were due to the protein sources rather than to unaccountable differences in experimental conditions. Further study is also needed to show why the calcium retention in experiment 1 was greater with a calcium to phosphorus ratio of 1.0 than with a calcium to phosphorus ratio of 2.0. Changing the magnesium level of the diet may have had an effect on calcium retention.

Recent reports from human studies have suggested that calcium absorption in the gut is increased with high protein intakes, but there are no data to show that it is related to an increase in calcium binding protein in the intestinal mucosa. Therefore, further study is needed in this area. Due to the low percentage of calcium excreted in the urine by the rat compared to humans, differences in fecal

calcium excretion with changes in protein level were not observed in this study. It may be beneficial to use another species in which the differences in urinary and fecal calcium excretion with changes in dietary protein levels are more dramatic.

Further investigation is necessary also to support the findings in this study that suggest that the calciuria from high protein intakes is not caused by a urea diuresis. The solute diuresis produced by sucrose loading in experiment 3 may not represent the situation which results from high protein ingestion. One might, therefore, produce a urea diuresis by feeding or infusing urea into the animal. Since the rat normally excretes a large amount of protein in the urine, it may also be possible that the ratio of protein to calcium excreted in the urine is constant, and that an increase in protein excretion accompanies the increase in calcium excretion initiated by high protein ingestion.

Since there was a great deal of individual variation in the ultrafiltrable serum calcium concentrations observed in experiment 4, one might wish to take a series of blood samples from each animal over a period of time, each animal serving in the capacity of both experimental subject and control. Perhaps a larger animal would be needed since approximately 5 ml of blood per sampling is needed for the technique used in this study. One might also wish to determine the calcium ion concentration rather than the total unbound calcium concentration in the serum.

The length of time allotted for the present investigation was probably too short for any differences in bone composition to be observed if high protein diets did alter the rate of bone resorption.

Unless one were to use radioactive tracers to observe bone turnover, a study of 6 months to one year in rats would be preferable to a short study of this type.

APPENDIX

APPENDIX

PHOSPHORUS ANALYSIS (95)

Reagents:

M. S. - Dissolve 5 g sodium molybdate in approximately 200 ml deionized, distilled water. Add 14 ml concentrated H_2SO_4 to 200 ml water and combine with molybdate solution. Bring solution up to a volume of one liter with water and store at room temperature in a plastic bottle.

Elon - Add 1 g p-methylaminophenol sulfate to 100 ml 3% sodium bisulfite ($NaHSO_4$). Store in refrigerator in brown glass bottle.

Procedure:

1. Add 5 ml M. S. and 0.5 ml Elon to 1 ml or 0.5 ml sample or standard (range = 0 to 50 ppm phosphorus).
2. Prepare blank using deionized, distilled water.
3. Mix and incubate at room temperature for 45 minutes before reading optical density at 700 nm.[1]

SERUM DEPROTEINATION

Procedure:

1. Add 4 ml 12.5% TCA (trichloro-acetic acid) to 1 ml serum (or use any combination which will yield a final solution of 5% TCA).
2. Mix thoroughly.
3. Centrifuge at 1500 g for 15 minutes.

---

[1]Coleman Junior IIA Linear Absorbance Spectrophotometer, model 6/20 A, Coleman Instruments, 42 Madison Street, Maywood, Ill. 60153.

4. Remove supernatant and dilute with an equivalent volume of 2% lanthanum or 1% strontium solution for calcium and/or magnesium determination by atomic absorption analysis. (Final dilution is 1/10.)

5. Prepare standards in similar manner.

STATISTICAL EVALUATION

One-way Analysis of Variance (96)

In experiment 1, a one-way analysis of variance was used to determine statistical differences between mean values for tissue mineral contents since n varied from 4 to 6. All statistical analyses for experiment 3 were done using this test (table 28).

When the value for F was greater than the critical value given in the F-distribution table (104), comparison of means was done using Scheffe's Test (96). The F value used in test was calculated by the following equation:

$$F_{\alpha,k-1,(n_T-1)-(k-1)} = \frac{(X_1-X_2)^2}{(MS_E)\left(\frac{n_1+n_2}{n_1 n_2}\right)} .$$

The critical value for F was obtained by multiplying the F from the F-distribution table by a factor of (k-1). A significant difference at $p < \alpha$ was indicated when F exceeded the critical value.

Two-way Analysis of Variance (96)

A two-way factorial design analysis of variance was used to determine differences between means for all data in experiment 1 concerning weight gain and balance collections. Two analyses were conducted on each set of data -- a comparison of groups 1 through 4

Table 28. One-way analysis of variance.

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean square (MS)	F
Treatment (Tr)	$k-1$	$\frac{(\sum X_1)^2}{n_1} + \frac{(\sum X_2)^2}{n_2} + \ldots + \frac{(\sum X_k)^2}{n_k} - \text{c.f.}^*$	$SS_{Tr}/(k-1)$	MS_{Tr}/MS_E
Error (E)	$(n_T-1) - (k-1)$	$SS_T - SS_{Tr}$	$\frac{SS_E}{(n_T-1) - (k-1)}$	
Total (T)	n_T-1	$\sum\sum X^2 - \text{c.f.}^*$		

$^*\text{c.f.} = (\sum X)^2/n_T$.

and a comparison of groups 1, 2, 5 and 6. Table 29 is an outline of the analysis for groups 1 through 4 in which a total of 20 animals (5 per group) were compared.

In experiment 2, two-way analysis of variance was also used. In stage 1, data from balance collections a_1 (28 days) and a_2 (36 days) were compared to determine whether the data could be reported as a single mean (table 30). F was evaluated at $p < 0.25$, and a_1 and a_2 were combined when no statistical differences were observed. When a_1 and a_2 could not be combined on the basis of this test, data from balance period b (54 days) was split into b_1 and b_2, and the next stage of analysis was performed using only half the total number of animals (table 31). However, since no differences between a_1 and b_1 or a_2 and b_2 were observed, data was reported as combined a and b assuming that the differences observed between a_1 and a_2 were due to individual variations among animals rather than time.

Analysis of variance indicates overall statistical differences due to various treatments. When it was desirable to know whether there were differences between specific mean values in a set of data, the following t test was used:

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{2MS_E/n}},$$ where $\bar{X}$ is a mean and n is the number of animals per group.

Standard errors were determined by the following formula:

$SEM = \sqrt{MS_B/n}$, where n is the number of animals per group.

Table 29. Two-way analysis of variance for groups 1 through 4, experiment 1. (5 animals per group)

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean square (MS)	F
Protein source (S)	1	$(\sum^{2} S^2)/10 - c.f.^*$	SS_S	SS_S/MS_E
Protein level (L)	1	$(\sum^{2} L^2)/10 - c.f.^*$	SS_L	SS_L/MS_E
Protein source - protein level interaction (SL)	1	$(\sum^{4} SL^2)/5 - c.f.^* - SS_S - SS_L$	SS_{SL}	SS_{SL}/MS_E
Error (E)	16	$SS_T - SS_S - SS_L - SS_{SL}$	$SS_E/16$	
Total (T)	19	$\sum^{20} X^2 - c.f.^*$		

$^*c.f. = (\sum^{20} X)^2/20.$

Table 30. Two-way analysis of variance, experiment 2. Stage 1, comparison of balance periods a_1 and a_2.

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean square (MS)	F
Treatment (Tr)	3	$(\sum^{4} Tr^2)/10$ - c.f.*	$SS_{Tr}/3$	
Time (Ti)	1	$(\sum^{2} Ti^2)/20$ - c.f.*	SS_{Ti}	SS_{Ti}/MS_E
Treatment - Time interaction (C)	3	$(\sum^{4}\sum^{2} C^2)/5$ - c.f.* - SS_{Tr} - SS_{Ti}	$SS_C/3$	
Error (E)	32	$SS_T - SS_{Tr} - SS_{Ti} - SS_C$	$SS_E/32$	
Total (T)	39	$(\sum^{40} X^2)$ - c.f.*		

*c.f. = $(\sum^{40} X)^2/40$.

Table 31. Two-way analysis of variance, experiment 2. Stage 2.

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean square (MS)	F
Protein level (P)	1	$(\sum^{2} P^2)/40$ - c.f.*	SS_P	SS_P/MS_A
Magnesium level (M)	1	$(\sum^{2} M^2)/40$ - c.f.*	SS_M	SS_M/MS_A
Protein - magnesium interaction (PM)	1	$(\sum^{4} PM^2)/20$ - c.f.* - SS_P - SS_M	SS_{PM}	SS_{PM}/MS_A
Error a (A)	36	$(\sum^{40} A^2)/2 - (\sum^{4} PM^2)/20$	$SS_A/36$	
Time (T)	1	$(\sum^{2} T^2)/40$ - c.f.*	SS_T	SS_T/MS_B
Protein - time interaction (PT)	1	$(\sum^{4} PT^2)/20$ - c.f.* - SS_P - SS_T	SS_{PT}	SS_{PT}/MS_B

(cont'd)

Table 31. (cont'd)

Magnesium - time interaction (MT)	1	$(\sum^{4} MT^2)/20 - c.f.^{*} - SS_M - SS_T$	SS_{MT}	SS_{MT}/MS_B
Protein - magnesium - time interaction (PMT)	1	$(\sum^{8} PMT^2)/10 - c.f.^{*} - SS_P - SS_M - SS_T - SS_{PM} - SS_{PT} - SS_{MT}$	SS_{PMT}	SS_{PMT}/MS_B
Error b (B)	36	$SS_{To} - SS_P - SS_M - SS_T - SS_{PM} - SS_{PT} - SS_{MT} - SS_{PMT}$	$SS_B/36$	
Total (To)	79	$\sum^{80} X^2 - c.f.^{*}$		

$^{*}c.f. = (\sum^{80} X)^2/80.$

BIBLIOGRAPHY

1. USDA Economic Research Service. National Food Situation, NSF - 154. Washington, D. C.: U. S. Department of Agriculture, 1975.

2. Food and Nutrition Board, National Research Council. Recommended Dietary Allowances. Washington, D. C.: National Academy of Sciences, 1974. p. 129.

3. Garn, S. M. The Earlier Gain and the Later Loss of Cortical Bone. Springfield, Ill.: Charles C. Thomas, 1970.

4. Caldwell, R. A. Observations on the incidence, aetiology, and pathology of osteoporosis. J. Clin. Path. 15: 421, 1962.

5. Trueta, J. Study of the Development and Decay of the Human Frame. Philadelphia: W. B. Saunders Company, 1968. pp. 316-327.

6. Oser, B. L. Hawk's Physiological Chemistry. New York: McGraw-Hill Book Co., 1965. pp. 256-260.

7. Lehman, D., M. B. Engel, and D. M. Laskin. Changes in soluble citrate of pigeons during egglaying. Science 130: 222, 1959.

8. Jowsey, J. and V. V. Nguyen. Bone metabolism. The acute effects of hormones, vitamin D_3 and acidosis during in vivo perfusion of adult dog forlimbs. J. Bone Joint Surg. 52A: 1041, 1970.

9. El-Maraghi, N. R. H., B. S. Platt, and R. J. C. Stewart. The effect of the interaction of dietary protein and calcium on the growth and maintenance of the bones of young, adult and aged rats. Brit. J. Nutr. 19: 491, 1965.

10. Platt, B. S. and R. J. C. Stewart. Transverse trabeculae and osteoporosis in bones in experimental protein-calorie deficiency. Brit. J. Nutr. 16: 483, 1962.

11. Heroux, O. and D. Peter. Failure of balance measurements to predict actual retention of magnesium and calcium by rats as determined by direct carcass analysis. J. Nutr. 105: 1157, 1975.

12. Sherman, H. C. Calcium requirement in man. J. Biol. Chem. 44: 21, 1920.

13. McCance, R. A., E. M. Widdowson, and H. Lehmann. The effect of protein intake on the absorption of calcium and magnesium. Biochem. J. 36: 686, 1942.

14. Johnson, N. E., E. N. Alcantara, and H. Linkswiler. Effect of level of protein intake on urine and fecal calcium and calcium retention in young adult males. J. Nutr. 100: 1425, 1970.

15. Hall, T. C. and H. Lehmann. Experiments on the practicability of increasing calcium absorption with protein derivatives. Biochem. J. 38: 117, 1944.

16. Walker, R. M. and H. M. Linkswiler. Calcium retention in the adult human male as affected by protein intake. J. Nutr. 102: 1297, 1972.

17. Anand, C. R. and H. M. Linkswiler. Effect of protein intake on calcium balance of young men given 500 mg calcium daily. J. Nutr. 104: 695, 1974.

18. Linkswiler, H. M., C. L. Joyce, and C. R. Anand. Calcium retention in young adult males as affected by level of protein and of calcium intakes. Trans. N. Y. Acad. Sci. 36: 333, 1974.

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

20. Engstrom, G. W. and H. F. DeLuca. Effect of egg white diets on calcium metabolism in the rat. J. Nutr. 81: 218, 1963.

21. Margen, S. Studies of the mechanism of calciuria induced by protein. Fed. Proc. 29: 566, 1970. (abstr.)

22. Wasserman, R. H., C. L. Comar, and M. M. Nold. The influences of amino acids and other organic compounds on the gastrointestinal absorption of calcium 45 and strontium 89 in the rat. J. Nutr. 59: 371, 1956.

23. McClellan, W. S., V. R. Rupp, and V. Toscani. Prolonged meat diets with a study of the metabolism of nitrogen, calcium, and phosphorus. J. Biol. Chem, 87: 669, 1930.

24. Tal, E. and K. Guggenheim. Effect of copper and manganese on calcification of bones of rats fed on meat. Nutr, Dieta. 7: 62, 1965.

25. Havivi, E., D. Gaster, and K. Guggenheim. Differential effects of low calcium diets on the bones of mice and rats. Nutr. Dieta. 9: 200, 1967.

26. Mazess, R. B. and W. Mather. Bone mineral content of North Alaskan Eskimos. Am. J. Clin. Nutr. 27: 916, 1974.

27. Bernard, C. in J. N. Hunt. The influence of dietary sulfur on the urinary output of acid in man. Clin. Sci. 15: 119, 1956.

28. Chan, J. C. M. The influence of dietary intake on endogenous acid production. Nutr. Met. 16: 1, 1974.

29. Relman, A. S., E. J. Lennon, and J. Lemann, Endogenous production of fixed acid and the measurement of the net balance of acid in normal subjects. J. Clin. Invest. 40: 1621, 1961.

30. Neuman, W. F. and M. W. Neuman. In the beginning there was apatite. in: Biological Mineralization, I. Zipkin, ed. New York: John Wiley & Sons, 1973.

31. Wachman, A. and D. S. Bernstein. Diet and osteoporosis. Lancet 1: 959, 1968.

32. Reidenberg, M. M., B. L. Haag, B. J. Channick, C. R. Shuman, and T. G. G. Wilson. The response of bone to metabolic acidosis in man. Metabolism 15: 236, 1966.

33. Nichols, N. and G. Nichols, Jr. The effect of alloxan diabetes and acidosis on the mineral and water content of bone. J. Clin. Invest. 37: 1676, 1958.

34. Irving, L. and A. L. Chute. The participation of the carbonates of bone in the neutralization of ingested acid. J. Cell. Comp. Physiol. 2: 157, 1932.

35. Logan, M. A. Calcium and ammonium excretion in the urine of rabbits. J. Biol. Chem. 109: 481, 1935.

36. Fiske, C. H. and S. S. Sokhey. Ammonia and fixed base excretion after the administration of acid by various paths. J. Biol. Chem. 63: 309, 1925.

37. Farquharson, R. F., W. T. Salter, D. M. Tibbetts, and J. C. Aub. Studies of calcium and phosphorus metabolism. XII. The effects of the ingestion of acid producing substances. J. Clin. Invest. 10: 221, 1931.

38. Barzel, U. S. and J. Jowsey. The effects of chronic acid and alkali administration on bone turnover in adult rats. Clin. Sci. 36: 517, 1969.

39. Newell, G. K. and R. E. Beauchene. Effects of dietary calcium level, acid stress, and age on renal, serum, and bone responses of rats. J. Nutr. 105: 1039, 1975.

40. Chase, L. R. and G. D. Aurbach. Parathyroid function and the renal excretion of 3'5'-adenylic acid. Proc. Natl. Acad. Sci. USA 58: 518, 1967.

41. Aurbach, G. D. and L. R. Chase. Cyclic 3',5'-adenlylic acid in bone and the mechanism of action of parathyroid hormone. Fed. Proc. 29: 1179, 1970.

42. Peacock, M., W. G. Robertson, and B. E. C. Nordin. Relation between serum and urinary calcium with particular reference to parathyroid activity. Lancet 1: 384, 1969.

43. Raisz, L. G. Stimulation of bone resorption by parathyroid hormone in tissue culture. Nature 197: 1015, 1963.

44. Borle, A. B. Calcium metabolism in hela cells and the effect of parathyroid hormone. J. Cell Biol. 36: 567, 1968.

45. Shimamura, T. and A. B. Morrison, Secondary hyperparathyroidism in rats with an experimental chronic renal disease. Exp. Mol. Pathol. 15: 345, 1971.

46. Wills, M. R. Fundamental physiological role of parathyroid hormone in acid-base homeostasis. Lancet 2: 802, 1970.

47. Sie, T-L., H. H. Draper, and R. R. Bell. Hypocalcemia, hyperparathyroidism and bone resorption in rats induced by dietary phosphate. J. Nutr. 104: 1195, 1974.

48. Anderson, G. H. and H. H. Draper. Effect of dietary phosphorus on calcium metabolism in intact and parathyroidectomized adult rats. J. Nutr. 102: 1123, 1972.

49. Kreshnarao, G. V. G. and H. H. Draper. Influence of dietary phosphate on bone resorption in senescent mice. J. Nutr. 102: 1143, 1972.

50. Draper, H. H., T. Sie, and J. G. Bergan. Osteoporosis in aging rats induced by high phosphorus diets. J. Nutr. 102: 1133, 1972.

51. Bell, R. R., D. T. Engelmann, T. Sie, and H. H. Draper. Effect of a high protein intake on calcium metabolism in the rat. J. Nutr. 105: 475, 1975.

52. Guggenheim, K., A. Reshef, Y. Ben Menachem, and D. S. Bernstein. An epidemiological study of osteoporosis in Israel. Arch. Environ. Health 22: 259, 1971.

53. Haag, J. R. and L. S. Palmer. The effects of variations in the proportions of calcium, magnesium, and phosphorus contained in the diet. J. Biol. Chem. 76: 367, 1928.

54. Colby, R. W. and C. M. Frye. Effect of feeding high levels of protein and calcium in rat rations on magnesium deficiency syndrome. Am. J. Physiol. 166: 408, 1951.

55. Johnson, N. E. and H. Linkswiler. Effect of level of protein and magnesium intakes on calcium retention of older adolescent males. Fed. Proc. 28: 562, 1969. (abstr.)

56. Schwartz, R., G. Walker, M. D. Linz, and I. MacKellar. Metabolic responses of adolescent boys to two levels of dietary magnesium and protein. I. Magnesium and nitrogen retention. Am. J. Clin. Nutr. 26: 510, 1973.

57. Bunce, G. E., P. G. Reeves, T. S. Oba, and H. E. Sauberlich. Influence of the dietary protein level on the magnesium requirement. J. Nutr. 79: 200, 1963.

58. Alcantara, E. N. and H. Linkswiler. Effect of protein and of magnesium intake on magnesium balbnce of older adolescent males. Fed. Proc. 28: 562, 1969. (abstr.)

59. Schofield, A. and E. Morrell. Calcium, phosphorus and magnesium. Fed. Proc. 19: 1014, 1960.

60. Schwartz, R., F. L. Wang, and N. A. Woodcock. Effect of varying dietary protein-magnesium ratios on nitrogen utilization and magnesium retention in growing rats. J. Nutr. 97: 185,,1969.

61. Agus, Z. S., L. B. Gardner, L. H. Beck, and M. Goldberg. Effects of parathyroid hormone on renal tubular reabsorption of calcium, sodium and phosphate.. Am. J. Physiol. 224: 1143, 1973.

62. Walser, M. Calcium clearance as a function of sodium clearance in the dog. AM. J. Physiol. 200: 1099, 1961.

63. Walser, M. Ion Association. VII. Dependence of calciuresis on natiuresis during sulfate infusion. AM. J. Physiol. 201: 769, 1961.

64. Kleeman, C. R., J. Bohannan, D. Bernstein, S. Ling, and M. H. Maxwell. Effects of variations of sodium intake on calcium excretion in normal humans. Proc. Soc. Exp. Biol. Med. 115: 29, 1964.

65. Chesley, L. C. and I. Tepper. Some effects of magnesium loading upon renal excretion of magnesium and certain other electrolytes. J. Clin. Invest. 37: 1362, 1958.

66. Womersley, R. A. Studies on the renal excretion of magnesium and other electrolytes. Clin. Sci. 15: 465, 1956.

67. Heaton, F. W. and F. M. Parsons. The metabolic effect of high magnesium intake. Clin. Sci. 21: 273, 1961.

68. Samiy, A. H. E., J. L. Brown, D. L. Globus, R. H. Kessler, and D. D. Thompson. Interrelation between renal transport systems of magnesium and calcium. AM. J. Physiol. 198: 599, 1960.

69. Heaton, F. W., A. Hodgkinson, and G. A. Rose. Observations on the relation between calcium and magnesium metabolism in man. Clin. Sci. 27: 31, 1964.

70. Schachter, D., E. B. Dowdle, and H. Schenker. Active transport of calcium by the small intestine of the rat. AM. J. Physiol. 198: 263, 1960.

71. Chen, P. S., Jr. and W. F. Neuman. Renal excretion of calcium by the dog. Am. J. Physiol. 180: 623, 1955.

72. Moore, E. W. Ionized calcium in normal serum, ultrafiltrates, and whole blood determined by ion-exchance electrodes. J. Clin. Invest. 49: 318, 1970.

73. LeGrimellic, C., N. Roinel, and F. Morel. Simultaneous Mg, Ca, P, K, and Cl analysis in rat tubular fluid. IV. During acute phosphate plasma loading. Pflug. Arch. Eur. J. Physiol. 346: 189, 1974.

74. Lemann, J., J. R. Litzow, and E. J. Lennon. Studies of the mechanism by which chronic metabolic acidosis augments urinary calcium excretion in man. J. Clin. Invest. 46: 1318, 1967.

75. Prasad, A. S. and E. B. Flink. Effect of carbon dioxide on concentration of calcium in an ultrafiltrate of serum obtained by contrifugation. J. Appl. Physiol. 10: 103, 1957.

76. Osmun, J. R. Total and ultrafiltrable calcium and magnesium in human plasma: improved method and normal values. Am. J. Med. Tech. 33: 448, 1967.

77. Loken, H. F., R. J. Havel, G. S. Gordan, and S. L. Whittington. Ultracentrifugal analysis of protein-bound and free calcium in human serum. J. Biol. Chem. 235: 3654, 1960.

78. Rodan, G. A. and E. P. Katz. Calcium binding to nondialyzable components of rat blood under in vivo conditions. Am J. Physiol. 225: 1082, 1973.

79. Rose, G. A. A simple and rapid method for the measurement of plasma ultrafiltrable and ionized calcium. Clin. Chim. Acta. 37: 343, 1972.

80. Farese, G. and M. Mager. Protein-free filtrates obtained by membrane ultrafiltration application to the determination of serum constituents. Clin. Chem. 16: 281, 1970.

81. Munday, K. A. and B. W. J. Mahy. Determination of ultrafiltrable calcium and magnesium on small quantities of plasma. Clin. Chim. Acta. 10: 144, 1964.

82. Farese, G., M. Mager, and W. F. Blatt. A membrane ultrafiltration procedure for determining diffusible calcium in serum. Clin. Chem. 16: 226, 1970.

83. Toribara, T. Y., A. R. Terepka, and P. A. Dewey. The ultrafiltrable calcium of human serum. I. Ultrafiltration methods and normal values. J. Clin. Invest. 36: 738, 1957.

84. Robertson, W. G. and M. Peacock. New techniques for the separation and measurement of the calcium fractions of normal human serum. Clin. Chim. Acta. 20: 315, 1968.

85. Perry, S. W. Proteinuria in the Wistar rat. J. Path. Bact. 89: 729, 1965.

86. Linkswiler, H., M. S. Reynolds, and C. A. Baumann. Factors affecting proteinuria in the rat. Am. J. Physiol. 168: 504, 1952.

87. Hardy, R. W. F. and C. A. Baumann. Suppression of rat proteinuria by dietary glycine. J. Nutr. 69: 429, 1959.

88. Addis, T., E. M. MacKay, and L. L. MacKay. The effect on the kidney of the long continued administration of diets containing an excess of certain food elements. I. Excess of protein and cystine. J. Biol. Chem. 71: 139, 1929.

89. Jackson, H. and M. D. Riggs. The effect of high protein diet on the kidneys of rats. J. Biol. Chem. 67: 101, 1926.

90. Krebs, H. A., D. A. H. Bennett, P. deGasquet, T. Gascoyne, and T. Yoshida. Renal gluconeogenesis. The effect of diet on the gluconeogenic capacity of rat kidney-cortex slices. Biochem. J. 86: 22, 1963.

91. Kotake, T., S. Yachiku, and F. Wada. Effect of parathyroid hormone on rat kidney microsomes in vivo. J. Biochem. 66: 855, 1969.

92. Nagata, N. and H. Rasmussen. Parathyroid hormone, 3'5' AMP, Ca^{++}, and renal gluconeogenesis. Proc. Natl. Acad. Sci. 65: 367, 1970.

93. Rutman, J. Z., L. E. Meltzer, J. R. Kilchell, R. J. Rutman, and P. George. Effect of metal ions on in vitro gluconeogenesis in rat kidney cortex slices. Am. J. Physiol. 208: 841, 1965.

94. Suzuki, H. and H. Fuwa. Comparison of effects of dietary calcium and magnesium depletion on renal glucose-6-phosphatase activity in rats fed high protein and high carbohydrate diets. J. Nutr. 102: 1615, 1972.

95. Gomori, G. A. A modification of the colorometric phosphorus determination for use with the photoelectric colorimeter. J. Lab. Clin. Med. 27: 955, 1942.

96. Klugh, H. E. Statistics: the Essentials for Research. (2nd edition) New York: John Wiley & Sons, Inc., 1974.

97. Dixon, W. J. and F. J. Massey, Jr. Introduction to Statistical Analysis. (3rd edition) New York: McGraw-Hill Book Co., 1969. p. 116.

98. Draper, H. H., J. G. Bergen, M. Chiu, A. S. Csallany, and A. V. Boara. A further study of the specificity of the vitamin E requirement for reproduction. J. Nutr. 84: 395, 1964.

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

100. Coburn, J. W., M. M. Poportzer, S. G. Massry, and C. R. Kleeman. The physiochemical state and renal handling of divalent ions in chronic renal failure. Arch. Intern. Med. 124: 302, 1969.

101. Shah, B. G., G. V. G. Krishnarao, and H. H. Draper. The relationship of Ca and P nutrition during adult life and osteoporosis in aged mice. J. Nutr. 92: 30, 1967.

102. Knapp, E. L. Factors influencing the urinary excretion of calcium. I. In normal persons. J. Clin. Invest. 26: 182, 1946.

103. Aurbach, G. D. and J. M. Phang. Vitamin D, parathyroid hormone, and calcitonin. in : Medical Physiology, V. B. Mountcastle, ed. St. Louis: The C. V. Mosby Company, 1974. p. 1676.

104. Rolf, F. J. and R. R. Sokal. Statistical Tables. San Francisco: W. H. Freeman Company, 1969.