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Calcium balances of premenopausal women consuming cheese- compared to spinach- and tofu containing diets

Landis, William Hathaway, Ph.D.

The University of North Carolina at Greensboro, 1987

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CALCIUM BALANCES OF PREMENOPAUSAL WOMEN CONSUMING CHEESE- COMPARED TO SPINACH- AND TOFU CONTAINING DIETS

by

William H. Landis

A Dissertation Submitted to
the Faculty of the Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Greensboro
1987

Approved by

Michael Lubman

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APPROVAL PAGE

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March 26, 1987

Date of Final Oral Examination

ABSTRACT

LANDIS, WILLIAM HATHAWAY, Ph.D. Calcium Balances of Premenopausal Women Consuming Cheese- Compared to Spinach- and Tofu-Containing Diets. (1987). Directed by Michael Liebman, Ph.D. 102 pp.

Two individual 8-week metabolic balance studies were conducted to compare the calcium bioavailability from cheese to that from spinach and tofu (soybean curd) in adult women. Subjects consumed each of 2 controlled diets for 3-week periods in a crossover experimental design. Calcium balance, oxalate excretion, urinary hydroxyproline, and serum alkaline phosphatase were determined from urine and fecal, and 2 fasting blood samples collected during the final 2 weeks of each 3-week dietary period.

In the first study (n=7), experimental diets were identical except for the inclusion of either 453 g of spinach (providing 600 mg of calcium) or 93 g of cheese (providing 670 mg of calcium). Mean fecal wet ($p < 0.05$) and dry ($p < 0.01$) weights were significantly greater during consumption of the spinach compared to the cheese feeding periods. Mean calcium balances of -168 (spinach) and -98 mg/day (cheese) were not statistically different although mean urinary calcium levels were greater ($p < 0.01$) during cheese (212) compared to spinach (136 mg/day) periods. Mean urinary (101 vs 17 mg/day, $p < 0.01$) and fecal oxalate (322 vs 13 mg/10 g dry wt, $p < 0.05$) were significantly greater during consumption of the spinach (high oxalate) compared to cheese (low oxalate) diets. Significant correlations were observed between mean urinary and fecal oxalate ($r=0.89$, $p < 0.01$), and corrected fecal oxalate and fecal calcium ($r_s=0.71$, $p < 0.05$) during the

spinach dietary periods. Individual data suggested a possible relationship between fecal oxalate levels and calcium balance. Urinary hydroxyproline was significantly higher ($p < 0.01$) during spinach (15.8) compared to cheese (13.4 mg/day) periods; Serum alkaline phosphatase did not differ significantly between treatments.

In the second study (n=9), tofu- (280 g providing 543 mg of calcium) were compared to cheese-containing diets (80 g providing 515 mg of calcium) for calcium availability. Significantly greater mean fecal calcium levels (829 vs 767 mg/day, p 0.05), and lower mean calcium balances (-135 vs -73 mg/day, p 0.05) were observed during consumption of the tofu- (providing 1900 mg phytate) compared to the cheese-containing (providing 1130 mg phytate) diet. Mean urinary phosphorus levels were significantly greater ($p < 0.05$) during the cheese (620) versus tofu (515 mg/day) periods. Urinary hydroxyproline and serum alkaline phosphatase did not differ significantly between dietary treatments.

ACKNOWLEDGEMENTS

The author gratefully acnowledges the following persons for their contribution toward the successful completion of this dissertation:

- Dr. Michael Liebman, for his guidance, hardwork and encouragement throughout this research project, and for his editorial assistance in the preparation of this manuscript.
- Dissertation Committee members, Dr. Karen Graves, Dr. Aden Magee, and Dr. John Jezorek, for their time, assistance and expertise.
- Coinvestigators, Linda Meredith and Carolyn Dunn, for their invaluable role in the planning and execution of this research project, and for their hardwork and dedication.
- Shih-Min Wu, for her assistance and expertise in laboratory methodology.
- My wife, Susan Landis, for her patience and encouragement during the course of the study, and for her constant support throughout my Ph.D. program.
- Finally, I wish to thank the study participants for their dedication and many sacrifices.

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CHAPTER I

INTRODUCTION

While dairy products supply the majority of calcium in the U.S. diet and calcium is relatively well absorbed from these foods (Allen, 1982), growing concern over the adequacy of calcium intakes has renewed interest about other less prominent sources of dietary calcium. Vegetable sources constitute the next best quantitative source of calcium after dairy products but in the U.S. make up only a small percentage of dietary calcium. Certain populations of the world which consume virtually no dairy foods and depend upon calcium of vegetable origin appear to display no symptoms of poor calcium nutriture (Hodgkinson, 1979). Generally the availability of calcium from plant foods is low relative to that from dairy foods due to the presence of various inhibitory compounds in plants (Allen, 1982).

Spinach has been generally regarded as a good source of calcium based upon its high calcium content relative to other plant foods. A limited number of studies have assessed calcium bioavailability from spinach and other green leafy vegetables in humans with the majority conducted over 35 years ago. Early studies shared a number of methodological limitations including small sample sizes, short balance periods, low calcium intakes or no statistical analysis. Data from this early research suggested that the daily feeding of large quantities of spinach (approximately 200 g or more) can adversely affect calcium balance in humans. Amounts less than 200 g appeared to have little or no effect upon calcium balance. Oxalic acid is a major constituent of spinach which forms calcium-oxalate salts of low solubility (Oke, 1969). The inhibition of calcium absorption by oxalate has been

attributed to the precipitation of insoluble calcium-oxalate salts in the small intestinal lumen (Earnest, Williams, & Admirand, 1979). Fiber, also found in large amounts in spinach, has been demonstrated to inhibit the absorption of calcium (Allen, 1982).

Soybeans and soybean derived products are also relatively rich plant sources of calcium but are less widely consumed in the typical U.S. diet than spinach or dairy foods. Little research has been conducted regarding calcium availability from soy products. In particular, tofu, a coagulated soy protein product, has received little scientific attention. Research has suggested that the calcium availability from whole cooked soybeans is less than that from soy milk, although calcium from soy milk was well absorbed relative to that from milk (Schroeder, Cahill, & Smith, 1946). The discrepancy in calcium bioavailability between whole soybeans and soy milk could be due to the presence of fiber in the whole soybeans. Tofu contains no fiber and therefore the calcium from tofu may be more available than products derived from whole soybeans. Soy products generally contain high levels of phytic acid which has been suggested to bind calcium, via the formation of insoluble calcium-phytate complexes in the intestinal lumen, thereby inhibiting its absorption.

Recent recommendations for increased calcium intake have been directed mainly toward women because of their high rate of bone mineral loss after menopause and the fact many women have calcium intakes below the Recommended Dietary Allowance (RDA) of 800 mg per day. If calcium intakes are to be increased to meet current recommendations, calcium bioavailability from alternate calcium-rich foods needs to be assessed.

At the periphery of investigations regarding calcium bioavailability of foods is the issue of to what degree does the daily intake of calcium affect calcium balance and bone turnover, and over the long term, risk of severe bone mineral loss in older adults. Adequate absorption and utilization of calcium from the diet is essential for the maintenance of bone health. When the entry of calcium into the body is deficient during periods of low intake and/or poor absorption, calcium balance may be sufficiently negative to induce increased bone resorption to meet homeostatic needs. A lifelong history of poor calcium nutrition could in this manner seriously deplete bone mineral reserves. The assessment of short term changes in bone turnover rates could help clarify the relationship between diet and progressive bone loss in the aged.

CHAPTER II

REVIEW OF LITERATURE

Much of the data on calcium availability from plant foods, in particular spinach and soy products, are from studies conducted before 1952 with several conducted during the early part of this century. Calcium availability from tofu, a coagulated soy protein, was last assessed in humans over 50 years ago (Adolph & Chen, 1931). Important factors influencing calcium availability from some plant foods are oxalic acid and phytic acid, found in large quantities in spinach and soy products, respectively. There is evidence that both of these plant constituents have the capacity to bind and possibly interfere with its absorption by the human gastrointestinal tract.

Studies Regarding Calcium Bioavailability from Spinach

Because of its high calcium content and popularity, spinach has been the most extensively studied green leafy vegetable with regard to the bioavailability of calcium. Calcium bioavailability from spinach was assessed in several early studies conducted during the first half of this century. These studies were characterized by one or more of the following limitations: short balance periods, small sample sizes, low calcium intakes and/or lack of statistical analysis. The effect of spinach upon calcium balance observed in these early studies appeared to be dose dependent. Studies feeding 200 g or more of spinach per day reported adverse effects on calcium balance (Sherman & Hawley, 1922; Mclaughlin, 1927; Fincke & Garrison, 1938) while those studies feeding less than 200 g of spinach

did not observe this effect (Schlutz, Morse, & Oldham, 1933; Bonner, Hummel, Bates, Horton, Hunscher, & Macy, 1938; Johnston, McMillan, & Falconer, 1952).

Fincke and Garrison (1938) assessed the effects of spinach or kale on calcium balance in two adult women. The kale diet contained 134 g of kale contributing approximately 85% of 400 mg of total dietary calcium and the spinach diet contained 490 g of cooked spinach contributing 80% of 445 mg of total dietary calcium. Calcium balance on the kale diet was -26 and -49 mg of calcium per day for the 2 subjects in contrast to -149 and -164 mg of calcium per day for the same subjects on the spinach diet. Balance figures were averages of two 3-day balance periods. Given equal fiber contents on the 2 diets, the markedly lower calcium balances observed in the subjects during spinach consumption are probably the result of the higher oxalic acid content of spinach compared to kale.

McLaughlin (1927) compared the effect of 275 g of spinach and comparable levels of calcium from milk on calcium balance of 7 adult women. Each metabolic balance period was 6 days in duration. Total daily calcium intakes on the milk and spinach diets were 493 and 509 mg, respectively. Mean calcium balance was positive on both diets, however, mean daily calcium balance on the spinach diet was lower than that on the milk diet by 55 mg of calcium.

Sherman and Hawley (1922) assessed the utilization of calcium from vegetables in 3 female subjects aged 5, 6 and 13 years. Mixed diets containing 500 mg of calcium from milk were fed for 27 days. During days 10-18, an average of 266 g of spinach and 264 g of carrots were added to

the diet contributing an additional 400 mg of calcium to the overall intake. The investigators assumed that if the calcium in spinach and carrots was roughly equal in bioavailability to that from milk, the addition of 400 mg of calcium from spinach and carrots would cause calcium balances to increase markedly above levels observed during the milk-only balance periods. Mean calcium balance of the 3 subjects during the spinach supplemented period was roughly equivalent to that observed during the milk-only test period, suggesting that very little if any of the calcium from spinach and carrots was available for absorption.

Sherman and Hawley (1922) conducted another feeding experiment using 3 different children of similar age to the previous experiment (aged 6, 10, & 13 years) to compare the utilization of calcium from spinach and carrots with that from milk. Using essentially the same study design as previously described, milk supplied the majority of calcium during the first 9 and final 6 days of the metabolic balance experiment. For 15 days between the milk-only dietary periods, milk was replaced with an amount of spinach (255 g) and carrots (121 g) equal in calcium content to that supplied by half of the original milk serving. Overall calcium intakes on the spinach-carrot-milk and milk-only diets were 957 and 996 mg per day. Calcium balance was considerably lower on the vegetable (+142 mg calcium/day) compared to that on the milk-only diet (+311 mg calcium/day).

Another set of early studies where spinach was fed at levels less than 200 g per day reported no apparent adverse effects on overall calcium balance. Bonner et al. (1938) reported no deleterious effects of dietary spinach

on calcium retention in children. Ten children, aged 5 to 8 years, were fed basal diets with and without 100 g of pureed spinach for 15 days. No difference in mean calcium balance was observed between diets. Total daily calcium intakes on each diet were approximately 800 mg although calcium intakes were slightly higher on the spinach diet due to the inclusion of the spinach supplement which represented 5 to 7 percent of the total calcium intake. A third diet was fed after the spinach diet in which oxalic acid and calcium, as calcium acetate, were added to the basal diet at levels equal to that found in the spinach supplement. The addition of pure oxalic acid had no apparent effect on calcium balance.

Johnston et al. (1952) demonstrated poor calcium absorption from spinach but overall calcium balance was unaffected with spinach feeding. Calcium balance was assessed in 6 adult women, aged 20-31 years, fed 120 g spinach daily for 8 weeks. All subjects consumed a basal diet for an initial 4-weeks, after which half of the subjects received the basal diet plus 120 g of spinach (containing 160 mg of calcium) at breakfast and the other half consumed the spinach with the evening meal. During the final 4 weeks, subjects switched and consumed spinach at the alternate time of day. The basal and spinach diets supplied approximately 820 and 980 mg of calcium per day, respectively. Fecal calcium during the spinach periods was greater than that during the basal period by an amount roughly equivalent to that contained in the spinach serving, suggesting that very little of the calcium in spinach was available for absorption. However, mean calcium balance on the spinach diets (-40 mg/day) was only slightly lower than the mean calcium balance on the basal diet (-26 mg/day).

Schlutz et al. (1933) assessed the effects of different forms of spinach on calcium retention in 4 infants. The daily feeding of 6 g of dried spinach reduced calcium retention slightly; no adverse effects on calcium balance were observed with the daily feeding of 60 g of pureed or 70 g of raw spinach.

More recently, Kelsay and Prather (1983) assessed the effect of dietary spinach on calcium balance in 12 men consuming each of three controlled diets for 4-week periods. Diet 1 was a low-fiber diet containing 102 g of spinach fed every other day. Diet 2 was high in fiber due to the inclusion of fruits and vegetables and also contained spinach every other day. Diet 3 was similar to diet 2 except that cauliflower, which is low in oxalate, replaced the spinach. A significantly lower mean calcium balance (-73 mg/day) was observed during week 4 on the high-fiber spinach diet compared with the mean calcium balance (+44 mg/day) on the low-fiber spinach diet. Calcium balance was also negative (-11 mg/day) on the high-fiber cauliflower diet but balance was not significantly different than those observed during diets 1 and 2.

Oxalic Acid Chemistry and the Effect of Oxalic Acid on Calcium Availability

An extensive review of the literarure regarding the nutritional implications of oxalic acid (oxalate) consumption on calcium metabolism has been conducted by Oke (1969) and Kelsay (1985). Most of the research on this topic has been conducted in small animal models and in livestock, and relatively few in human subjects. Studies using small laboratory animals seem to indicate an impairment of calcium utilization with oxalate feeding (Oke, 1969). Studies intended to assess the effects of oxalate consumption

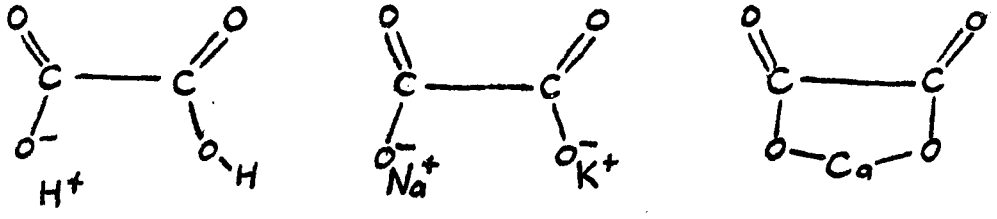
on calcium utilization often use a high-oxalate containing plant food, like spinach, as the vehicle by which oxalate is incorporated into the diet. Therefore, the solitary effect of oxalate, per se, on calcium utilization is often not directly assessed. Most plant sources of oxalate are also likely to contain high levels of fiber which has been demonstrated to interfere with the absorption of calcium (Allen, 1982).

Oxalic acid is the simplest dicarboxylic acid (Figure 1a). It is a strong acid (pK_1 = 1.46, pK_2 = 4.40) and commonly exists as a dihydrate (Fasset, 1973). In neutral and basic solutions oxalic acid readily ionizes or losses the hydrogens associated with the 2 carboxyl groups, and forms salts with various mono- and divalent cations. Neutral or soluble salts of oxalate, or those composed of monovalent cations, are readily soluble in water while oxalate salts composed of alkaline earth or divalent metal cations are less soluble.

The primary species of neutral or soluble oxalate in plants are sodium ($NaHC_2O_4$) and potassium salts (KHC_2O_4) (Figure 1b): free oxalic acid ($C_2O_4H_2$) is rarely present (Oke, 1969). Insoluble salts of oxalate in plants exist chiefly as the calcium salt (CaC_2O_4), with small quantities existing as the magnesium salt (MgC_2O_4) (Figure 1c). Calcium oxalate salts are insoluble in neutral or basic solutions but become increasingly soluble in acid. The interference with calcium absorption by oxalate has been attributed to the precipitation of insoluble calcium-oxalate salts in the relatively basic environment of the intestinal lumen (Earnest et al., 1979)

Ionic bonding characterizes the attractive forces occurring between monovalent cations, like Na^+ and K^+, and oxalate molecules. Covalent bonds

characterize the attractive forces between divalent cations such as Ca^{2+} and Mg^{2+}, and oxalate. Ionic bonds between atoms are weaker than covalent bonds. On this premise, compounds characterized by ionic bonding will more readily dissociate in aqueous solutions compared to compounds characterized by covalent bonds. The nature of the attractive forces between monovalent and divalent cations and oxalate, in oxalate salts, may account for the difference in solubility between Na^{+}- and K^{+}-oxalate salts (high solubility) and Ca^{2+}- and Mg^{2+}-oxalate salts (low solubility). It has been suggested that calcium ions may interact with or adhere to oxalate in the lumen of the gut in a variety of different molecular configurations which could effect solubility and binding properties and subsequent calcium availability from these salts (Hodgkinson, 1979).



(a) (b) (c)

Figure 1. (a) The structure of oxalic acid with one of its carboxyl hydrogens dissociated from the molecule, (b) oxalate molecule in a salt form with monovalent cations Na^{+} and K^{+} demonstrating electrostatic or ionic bonding, (c) oxalate molecule as a Ca^{2+} - salt demonstrating the more stable covalent bonding scheme between the 2 oxygen molecules of oxalate and Ca^{2+}.

The results from studies in which oxalate was fed to rats suggests that oxalate consumption may have a negative effect on the utilization of calcium (Kelsay, 1985). In a study by Fincke and Sherman (1935), rats

were fed diets for 60 days in which the majority of calcium was supplied by milk or half of the milk was replaced with equal quantities of calcium in dried spinach. The calcium from the spinach diet was poorly utilized compared to that from the milk diet. A third diet in which kale replaced spinach resulted in calcium utilization comparable with that from milk, suggesting that the high oxalate content of spinach relative to kale, which is low in oxalate, may have been responsible for the lower availability of calcium from that vegetable.

Tidall and Drake (1937) reported decreases in calcium retention in rats fed a basal diet with added pure oxalic acid and calcium carbonate compared with animals fed a basal diet with only calcium carbonate added. A decrease in retention was even more pronounced after dried spinach was added to the diet at levels supplying oxalic acid in amounts similar to those fed in the supplemental diet.

Pure oxalate was fed to humans in a study by Bonner et al. (1938). Ten children were fed a basal diet, a basal diet plus 100 g of spinach, and a basal diet in which pure oxalate and calcium were fed at levels equivalent to those found in 100 g of spinach. These researchers observed no differences in calcium balance between dietary treatments.

The actual physical and chemical interactions that occur between oxalate, calcium, fiber and other dietary compounds in the lumen of the small intestine are most likely complex and essentially undetermined. Given that much of the calcium in spinach is bound to oxalic acid, as a calcium-oxalate salt, an unanswered question is whether oxalate in this form can dissociate and form new salts with other dietary minerals

during digestion. Studies by Bonner et al. (1938) and Johnston et al. (1952) demonstrated that adding approximately 100 g of spinach to human diets prevented the absorption of an amount of calcium equal to that fed in the spinach which suggested that only the spinach calcium was blocked from absorption.

In contrast to the above studies, researchers have found decreases in the availability of calcium from foods consumed in the diet other than the oxalate source fed. In a human study, Pingle and Ramasastri (1978) reported low calcium bioavailability from amaranthus, a high oxalate containing plant, compared to that from milk. These researchers also reported a decrease in the availability of milk calcium when amaranthus was fed with milk. In a rat study conducted by Spiers (1939), the utilization of calcium from milk was partially impaired when dried spinach was added to test diets.

Dietary fiber is a major constituent of spinach. Several types of fiber have been shown to inhibit calcium absorption (Allen, 1982) which may partially explain the relatively low degree of calcium availability from some plant sources. Kelsay and Prather (1983) fed 12 adult men 2 diets containing equal amounts of spinach and oxalate but differing in total fiber content. Significantly lower mean calcium balances were reported on the high-fiber compared to the relatively low-fiber spinach-containing diet. These researchers suggested that the combination of oxalate and fiber in the gut could accentuate the sequestering of minerals and inhibit their absorption more effectively than would either component alone. Tisdall and Drake (1938) found that the addition of dried spinach,

rich in fiber and oxalate, reduced total body calcium in rats more than diets in which purified oxalic acid and calcium, but no additional fiber, were supplemented at levels equal to that in the dried spinach.

The presence of significant populations of oxalate degrading bacteria (Oxalobacter formigenes) has been recently demonstrated in the gastrointestinal tract of humans (Allison, Cook, Milne, Gallagher, & Clayman, 1986). A major factor affecting the degree to which calcium is utilized from vegetables possessing high levels of oxalic acid could be the concentration and activity of gastrointestinal oxalate degrading microbes in the alimentary tract of an individual.

Studies Regarding Calcium Availability from Tofu and Other Soybean Products

Soybeans contain relatively high concentrations of calcium compared to other vegetable foods but are less widely consumed in the U.S.diet than are spinach or dairy products. Soy products could represent a significant source of calcium for vegetarians and other individuals characterized by low daily intakes of dairy foods. Coagulated soy protein products, such as tofu, when produced using calcium-salts as precipitating agents, are an even richer soybean source of calcium. Whole soybeans contain large amounts of phytate and fiber, each of which has been demonstrated to interfere with calcium absorption (Allen, 1982). Tofu contains no fiber and therefore the calcium in tofu may be more available than calcium from products derived from whole soybeans. Little research has assessed the bioavailability of calcium from tofu.

The calcium bioavailability from soybeans and soy products, as well as from other legumes, has been assessed in a small number of studies conducted during the 1930's and 1940's. Pittman (1932) reported consistently negative calcium balances ($\bar{x}$ = -113 mg/day) for 3 female subjects fed a diet in which 80-85% of the total dietary calcium was provided by Navy beans. Calcium intake on the experimental diet averaged only 310 mg per day and the diets were reported to be marginal in vitamin D. In contrast, Adolph and Chen (1932) found the availability of calcium from soybean curd and from cow's milk were approximately equal. These researchers assessed calcium balance during two 4-day periods in 3 adult men fed diets in which 77% of the dietary calcium was supplied by milk or soybean curd. Total calcium intake was 450 mg per day on each diet.

A more rigorous balance study conducted by Schroeder, Cahill, and Smith (1946) was used to compare calcium availability from evaporated cow's milk, soybean milk, whole cooked soybeans and calcium sulfate supplements. Thirteen male subjects were fed a basal diet and 3 of the 4 test diets for 20 days. The calcium provided by each supplement accounted for between 60 and 80% of the total calcium intake on each diet. Mean total calcium intakes during the evaporated cow's milk, soybean milk, whole soybean and calcium sulfate periods were 638, 695,639 and 655 mg per day, respectively. The average percent utilization of calcium from whole cooked soybeans was relatively low (10.4%), while percent utilization from soybean milk (22.6%) compared favorably with that from evaporated cow's milk (29.1%) and the calcium sulfate supplement (23.7%). The less efficient absorption of calcium from whole soybeans relative to that from

soy milk was partially attributed to an inhibitory effect of fiber in the whole soybeans.

Negative calcium balances were intentionally induced during the basal period in the same study by feeding low calcium diets. The sudden change in calcium intake from the basal to the experimental dietary periods may have influenced calcium utilization during the succeeding experimental periods. One third of the subjects consumed the soy milk diet immediately following the calcium deficient basal period which may increased the ability of the gastrointestinal tract to absorb calcium from this source. The remaining subjects consumed the calcium sulfate supplemented diet following the basal period.

Forbes, Weingarter, Parker, Bell, and Erdman (1979) found calcium from a variety of soybean products, such as full fat soyflour, freeze-dried soy beverage, and soy concentrate, to be relatively well absorbed by rats. The calcium from the soy based diets compared favorably with the availability of calcium from a casein diet supplemented with calcium carbonate.

Phytic Acid Chemistry and the Effect of Phytic Acid on Calcium Availability

Phytic acid (myo-inositol 1,2,3,4,5,6-hexakis(dihydrogen phosphate)) (Figure 2a) is a major constituent of soybeans and soy products constituting approximately 1.4% by weight (Graf & Eton, 1985). Phytic acid is a strong acid and forms a variety of salts with many dietary elements, such as sodium, potassium, zinc, copper, iron, magnesium, and calcium. A common salt species of phytate is sodium-phytate (Figure 2b). Calcium salts are poorly soluble and are readily formed under alkaline conditions

(Oberleas, 1973). Calcium can interact with phytate to form salts in several ways, including the formation of salts in combination with other minerals, the most notable being zinc (Figure 2c). Phytate is generally considered to decrease the availability of calcium in the diet of humans (Allen, 1982), possibly via the precipitation of calcium-phytate salts in the intestinal lumen. Calcium-zinc phytate complexes have been demonstrated to reach maximum precipitation levels at a pH of 6, the approximate pH of the human small intestine (Oberleas, 1973). No clear consensus has been established regarding the effect of phytate on calcium availability.

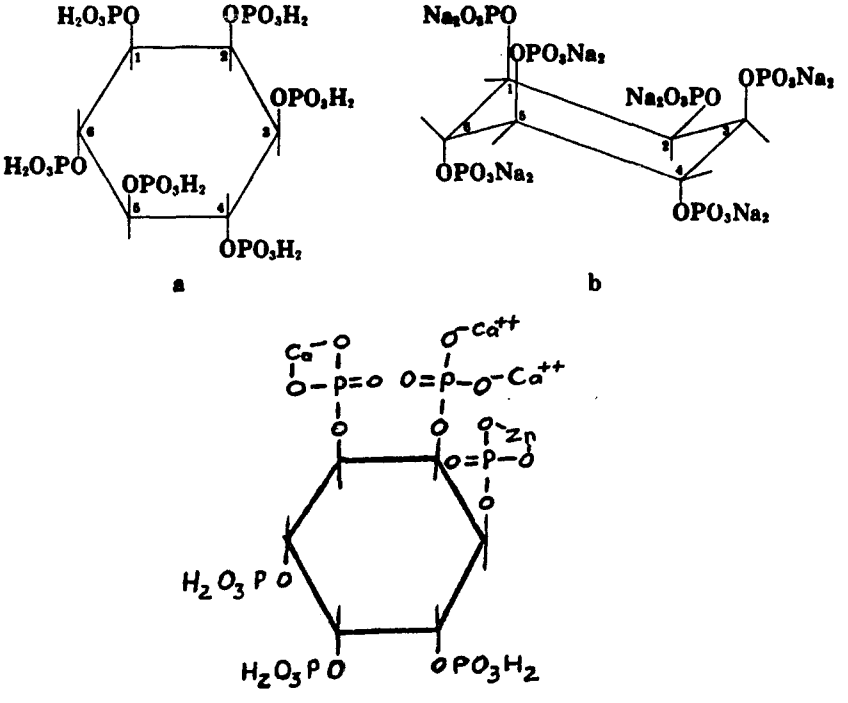


Figure 2. (a) Structure of phytic acid, myo-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate), (b) structure of sodium-phytate salt (hexasodium salt), (c) Hypothetical scheme for the binding of calcium and zinc to the phosphate groups of phytate.

Morris and Ellis (1985) conducted 2 feeding studies to determine the effects of dietary phytic acid on calcium bioavailability. In the first study, 10 men were fed identical 15-day diets differentiated by the addition of 0.2 or 2.0 g of phytic acid. Thirty-six grams of wheat bran supplying 2.0 g of phytate and an identical portion of dephytenized wheat bran were baked into muffins for daily consumption. The high and low phytate diets contained 1100 and 1110 mg of calcium per day, respectively, and were equal in fiber content. Mean calcium balance on the high phytate diet (208 mg/day) did not differ significantly from that on the low-phytate diet (184 mg/day), suggesting that the presence of large quantities of dietary phytate had no effect on the utilization of calcium.

In the second study, 12 men consumed 3 levels of phytate (0.5, 1.7 and 2.9 g/day) as sodium phytate added to muffins. The muffins also contained 8.65 g of fiber as the water soluble fraction of dephytenized bran. All 3 diets supplied an average of 740 mg of calcium per day. Mean apparent calcium absorption decreased significantly with each increasing level of dietary phytate. Six of the 12 subjects excreted more calcium in the feces than was consumed when fed the highest phytate diet, while all subjects on the low and intermediate phytate diets had positive apparent calcium absorption rates. Although molar ratios of phytic acid to calcium on the high-phytate diets from study 1 (0.11) and 2 (0.14) were comparable, individuals consuming the high-phytate diet from study 2 were more likely to exhibit negative calcium balances. The authors suggested that the 300 mg per day higher calcium intake during study 1 may have promoted the more positive calcium balances observed.

Reinhold, Lahimgarzadeh, Nasr, and Hedayati (1973) reported decreases in calcium balance in 2 out of 3 adult men fed diets supplemented with 1.85 g of phytic acid, creating a total dietary phytate intake of 2.5 g on the diet, compared to balances observed on a low phytic acid control diet. When Tanok bread, supplying approximately 3.0 g of phytate per day, replaced the phytate supplement of the first experimental period, all subjects were in markedly negative calcium balance averaging a net calcium loss of 200-300 mg of calcium per day. Calcium balance returned to positive levels during the final balance period when white bread containing no phytate replaced Tanok bread in the diet.

Research by Graf and Eaton (1985) contradicts the widely held belief that calcium-phytate complexes are generally insoluble and poorly absorbed by the human intestine. The solubility of calcium-phytate complexes was determined using in vitro studies at pH levels approximating those found in the human small intestine. At lower calcium/phytate ratios, calcium-phytate complexes were found to be very soluble. Solubility was lowest at a calcium-phytate ratio of 6:1 (Ca_6^{2+} - phytate). These researchers also determined the effect of phytate on the absorption of ^{45}Ca administered by gastric gavage in mice. The calcium from the calcium-phytate was absorbed to the same extent as unchelated calcium. These results suggested that phytate did not adversely affect calcium absorption. The authors added a note of caution concerning the extrapolation of results from in vitro solubility studies, and in vivo studies with mice fed purified controlled diets, to human dietary situations in which mixed diets generate a much more complex luminal environment.

Recent studies have demonstrated the presence of phytate splitting enzymes in the human intestine (Bitar & Reinhold, 1972). Phytase or phosphomonoesterase enzymes remove phosphorus from phytate molecules during digestion. Elevated urinary phosphorus excretion levels on a high phytate diet may indicate an increase in phosphorus absorption due to enhanced release of phosphorus from phytate. Calcium balance and phosphorus excretion data for the 3 subjects in the study by Reinhold et al. (1979) suggested that the concentration and activity of these enzymes could vary among individuals. The subject least affected by phytate feeding in terms of calcium balance exhibited significantly higher urinary phosphorus levels during the phytate feeding period compared to the non-phytate control period: fecal phosphorus excretion was constant. Decreases in calcium balance for the other 2 subjects consuming phytate were accompanied by significant increases in fecal phosphorus while urinary phosphorus was unchanged.

Urinary Hydroxyproline and Serum Alkaline Phosphatase as Indices of Bone Resorption

Calcium balance is partially dependent upon the efficiency of calcium absorption and utilization from the diet. Since 99% of total body calcium is stored in the skeleton, calcium balance often reflects bone balance (Parfitt, 1983). Chronically severe negative calcium balance suggests a net loss and positive calcium balance a net gain in bone mineral. Other indirect measures of bone turnover rate are urinary hydroxyproline and serum alkaline phosphatase. Measurement of these indices, combined with calcium balance data, could provide valuable information regarding the

effects of quality and quantity of dietary calcium upon short-term bone turnover rates among adult women, and may lend insight into the issue of longterm dietary calcium intake and age related bone mineral loss.

Urinary hydroxyproline is a widely accepted measure of bone resorption (Dull & Hennenman, 1963; Nordin, 1976; Prockop & Sjoerdsman, 1961). Hydroxyproline is a nonessential amino acid which occurs almost exclusively in collagen. About 40% of total body collagen is present in the skeleton (Smith & Nordin, 1964). Urinary hydroxyproline derived from soft tissues represents a relatively constant fraction of the total urinary hydroxyproline pool (Nordin, 1976). Therefore, on a hydroxyproline free diet, changes in hydroxyproline excretion will normally reflect changes in bone turnover.

Urinary hydroxyproline excretion has been correlated with the rate of calcium resorption which accompanies certain metabolic bone disorders characterized by bone loss. Increased urinary hydroxyproline levels have been observed in association with Pagets disease, hyperparathyroidism, hyperthyroidism, and osteomalacia (Dull & Henneman, 1963; Nordin, 1976; Laitinen, Nikkila, & Kivirikko, 1966); below normal levels have been observed in hypothyroidism (Laitinen et al., 1966).

The higher than normal levels of urinary hydroxyproline observed among osteoporotics by some researchers (Nordin, Aaron, Speed, & Crilly, 1981; Smith & Nordin, 1964) is consistent with the supposition that osteoporosis results from patterns of net bone resorption and bone mineral loss. While dramatic bone loss characterizes a final phase in osteoporosis, studies have not consistently demonstrated elevated urinary

hydroxyproline levels in cases of post-menopausal osteoporotics relative to nonsymptomatic age-matched controls (Laitinen et al., 1966; Klein, Lafferty, Pearson, & Curtiss, 1964; Aloia, Cohn, Zanzi, Abesamis, & Ellis, 1978).

Urinary hydroxyproline and hydroxyproline/creatinine ratio, an indice correcting for body mass, have been demonstrated to be sensitive indicators of short-term changes in bone turnover in reponse to calcium intake. Horowitz, Need, Philcox, & Nordin (1982) reported a significantly decreased hydroxyproline/creatinine ratio in 14 post-menopausal osteoporotic women after only 8 days of calcium supplementation at a level of 1 g per day. Smith and Nordin (1964) reported significantly decreased mean 24-hour urinary hydroxyproline levels among 42 osteoporotic patients given 1100 mg of calcium, as calcium glycerophosphate, for 7 days. A significant positive correlation was observed between urinary hydroxyproline output and rate of bone resorption.

Osteoblast and chondroblast cells in bone contain the highest concentrations of alkaline phosphatase found in the body. Other alkaline phosphatase rich tissues include the liver, kidney, intestinal mucosa, brain and leukocytes (Chiandussi, Greene, & Sherlock, 1962). Serum levels of alkaline phosphatase are appreciably elevated only in association with diseases of the skeleton and liver (Nordin, 1976). Thus, if liver function is normal, changes in serum alkaline phosphatase probably reflect changes in bone turnover. Higher than normal serum alkaline phosphatase levels among individuals with degenerative bone diseases have been documented

(Klein et al., 1964; Nordin, 1976). Serum alkaline phosphatase has also been reported to be highly correlated with urinary hydroxyproline (Klein et al., 1964).

The disease most frequently investigated in relation to adequacy of dietary calcium is age-related osteoporosis. While many researchers suggest lifelong histories of inadequate calcium intake is a primary cause of low bone mineral mass late in life, scientific evidence is conflicting (Gordon & Vaughan, 1986). Bone mass and rate of mineral loss throughout life are influenced by other factors such as genetic predisposition, exercise, smoking, body size and other dietary nutrients. However, understanding the short-term interaction between calcium intake and bone metabolism could elucidate the role of habitual calcium intake in age-related osteoporosis.

CHAPTER III

METHODOLOGY

Two separate 8-week metabolic balance studies that utilized identical experimental designs were conducted during 1985. The first balance study, conducted in the Spring, was designed to compare the bioavailability of calcium from diets in which the majority of dietary calcium came from either spinach or cheese (Spinach study). The second balance study, conducted in the Fall, differed only in that tofu replaced spinach as the test food (Tofu study).

Subjects

Demographic characteristics for subjects in the Spinach and Tofu studies are presented in Table 1. Seven caucasian women, with ages ranging from 20-42 years, a mean age of 31 years, participated in the Spinach study. Mean body mass index (BMI) at baseline was 25.5 (kg/m^2). A BMI of 27.0 corresponds to a weight level approximately 20% over ideal body weight based on Metropolitan Life Height and Weight tables (1983). Only one subject was characterized by a BMI greater than 27.0. Eleven women (9 caucasian, 1 Black, 1 Spanish American), with ages ranging from 20-39 years, a mean age of 27 years, participated in the Tofu study. Mean BMI was 22.4; one subject was judged to be over 20% ideal body weight based on a BMI exceeding 27.0. A consent form (Appendix A) was signed by each subject before participation in each metabolic study. These studies were approved by the Human Research Review Committee of the University of North Carolina at Greensboro.

Table 1
Demographic Data for Spinach and Tofu Study Subjects at Baseline

Study	Variable			
	Age (yr)	Weight (kg)	Height (cm)	BMI (kg/m^2)
Spinach Study[a]	31 ± 9	62.7 ± 7.2	164 ± 6	23.5 ± 3.6
Tofu Study[b]	27 ± 6	60.2 ± 7.6	163 ± 6	22.4 ± 2.8

[a] n = 7
[b] n = 9

All subjects were judged to be healthy and to meet the study criteria based on responses to a preexperimental questionnaire (Appendix B). Subjects were recruited from the UNC - Greensboro campus and the local Greensboro area through the use of flyers and by word of mouth. Volunteers for the studies were screened from participation for the following reasons: users of oral contraceptives, users of prescription or over-the-counter drugs known to interfere with calcium metabolism, or having a history of menstrual irregularity, metabolic or bone disease, or gastrointestinal disorders.

Each subject completed a 7 day food record before the start of each balance study to estimate usual dietary intake and to identify those subjects having excessively high or low calcium intakes. All subjects reported either light or moderate physical activity levels and agreed to maintain their prestudy levels throughout the study. Administration of a daily questionnaire (Appendix C) elicited a continuous record of daily physical activity, general health condition, degree of satisfaction with the study, and menstrual information.

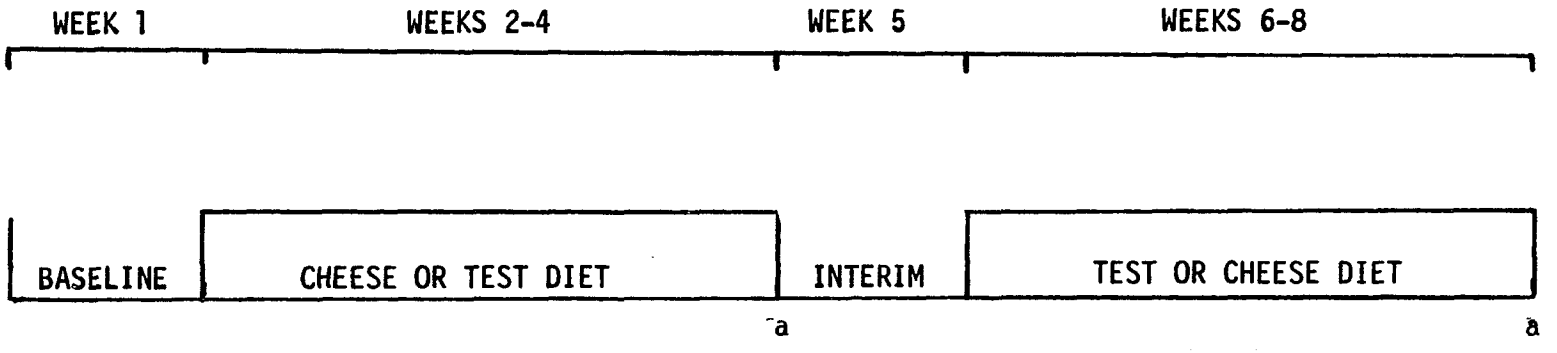
Experimental Design

The experimental design used in both the Spinach and Tofu studies is presented in Figure 1. The total duration of each metabolic study was 8 weeks: Week 1 (baseline period), Weeks 2-4 (experimental feeding period I), Week 5 (interim period), Weeks 6-8 (experimental feeding period II). During the 1 week baseline and interim periods, the majority of dietary calcium was provided in approximately equal amounts by cheese and the test food (spinach or tofu), thereby allowing subjects to become habituated to the ingestion of the test foods and to provide subjects with common calcium intakes prior to the consumption of the experimental diets.

Subjects were randomly assigned to the 2 dietary treatments at the start of each study and consumed both experimental diets in a crossover design thereby allowing each subject to act as her own control. During the Spinach study, 4 women consumed a spinach- and 3 a cheese-containing diet during the first 3-week feeding period and then switched to the alternate diet during the second 3-week feeding period. With respect to the Tofu study, 4 and 5 women were assigned to the tofu- and cheese-containing diets, respectively, during the first feeding period.

A nonabsorbable fecal marker, polyethylene glycol (PEG) ($[HO(-CH_2-CH_2-O-)_nH]$, MW 3350, Union Carbide Corp., Danbury, CT) was administered in capsule form 3 times daily (1230 mg/day) with meals during each 3-week experimental feeding period. During the last 2 weeks of each 3-week feeding period, all urine and feces were collected and 2 duplicate diet composites were made for calcium balance determinations. Given that

Experimental Design



[a]Serum alkaline phosphatase determined from 2 fasting blood samples at the end of each test period. Urinary hydroxyproline and oxalate, and fecal oxalate were determined from the last 3 samples collected at the end of each test period.

Figure 3.

daily calcium losses via skin and sweat are quite low (Gitelman & Lutwak, 1963), calcium balance was calculated using the traditional formula for whole-body balance:

Calcium Balance = Calcium Intake - (total fecal calcium + urine calcium)

Two samples were taken by venipuncture at the end of each 3-week experimental period for the determination of serum alkaline phosphatase. Urinary hydroxyproline and oxalate, and fecal oxalate were determined from the last 3 samples collected for each subject at the end of each 3-week test period.

Apparent absorption of oxalate from the spinach diet was calculated using the following formula as described by Finch, Kasidas, and Rose (1981).

$$\frac{\text{24 hr urine oxalate excretion on oxalate-rich (spinach) diet} - \text{24 hr urine oxalate excretion on low-oxalate (cheese) diet}}{\text{oxalate load per 24 hr during consumption of oxalate rich (spinach) diet}} \times 100\%$$

Experimental Diets

Experimental diets were fed using 2-day rotating menus in each metabolic study. Composition of the experimental diets fed during each study were identical except for the inclusion of either spinach or cheese (Spinach study, Table 2), or, tofu or cheese (Tofu study, Table 3). Daily intake levels of spinach and cheese, and tofu and cheese, were designed to provide similar quantities of calcium within each study.

Breakfast and dinner were prepared and consumed each day at the study center and lunch was packed for consumption outside the study center.

Table 2
Composition of the Spinach Study Experimental Diets

Meal	Food	Day 1 Weight (g)	Day 2 Weight (g)
Breakfast	Egg	55	55
	Whole wheat toast	44	44
	Margarine	10	10
	Orange juice	168	168
Lunch	Cheese	40	40
	or		
	Spinach	196	196
	Brown rice	144	-
	Egg noodles	-	122
	Tuna fish	-	93
	Pear	138	-
	Apple	-	140
	Graham crackers	28	-
Dinner	Cheese	53	53
	or		
	Spinach	257	257
	Perch	120	-
	Potato	-	154
	Lima beans	92	-
	Carrots	-	92
	Whole wheat bread	22	-
	Whole wheat roll	-	86
	Margarine	20	20
Snack	Pretzels	56	-
	Cashews	-	36
	Raisins	-	14

Table 3
Composition of the Tofu Study Experimental Diets

Meal	Food	Day 1 Weight (g)	Day 2 Weight (g)
Breakfast	1 Egg	44	44
	2 slices whole wheat bread	63	-
	1 English muffin	-	68
	Orange juice	186	186
	Banana	-	126
	Margarine	10	10
Lunch	Cheese	40	40
	or		
	Tofu	140	140
	2 slices rye bread	78	-
	2 slices whole wheat bread	-	63
	Fig bars	59	-
	Apple	-	153
Dinner	Cheese	40	40
	or		
	Tofu	140	140
	Brown rice	198	-
	Steamed carrots	78	-
	Spaghetti	-	143
	Spaghetti sauce	-	127
	Green beans	-	55
	Pineapple	-	107
	French roll	-	41
	Whole wheat roll	60	-
	Margarine	15	15
Snack	Peanuts	40	-
	Raisins	15	-
	Graham crackers	-	15
	Peanut butter	-	20

Frozen whole leaf spinach, tofu (made with calcium sulfate as a precipitant), and cheddar cheese were purchased in bulk at the beginning of the appropriate study. Spinach was prepared using a microwave oven for thawing and cooking. A standardized procedure to drain the spinach and tofu of excess water ensured that the water content per weighed serving remained constant throughout the studies. For lunch, tofu was fried using a commercial cooking spray and served in a sandwich. For dinner, tofu was cooked using a microwave oven and served either with rice or spaghetti.

Deionized water and herbal tea prepared with deionized water were permitted ad libitum. One serving of decaffeinated coffee and up to 2 sugar-free soft drinks per day were allowed. Subjects were weighed on alternate days before breakfast and those exhibiting more than a 1 kg decrease in body weight during a 3-week experimental feeding period consumed 1 to 2 sucrose containing soft drinks daily as a caloric supplement to help maintain body weight. Daily beverage consumption was recorded, and samples were obtained for mineral analysis. Subjects were instructed to consume all foods provided and to consume no other foods during the course of these studies.

Prestudy 7-day food records and experimental diets were coded and analyzed using the Nutritional Analysis System maintained by Louisiana State University (Baton Rouge, LA). This system is a computerized data bank of food composition information from sources including USDA Agricultural Handbook No.8 (1976-1980), scientific journals, and food manufacturers and processors. Nutrient composition of the diets fed during the Spinach and Tofu studies are presented in Table 4.

Table 4
Nutrient Composition of the Experimental Diets of the Spinach and Tofu Studies Based on Food Composition Data

Variable	Spinach Study Periods		Tofu Study Periods	
	Spinach	Cheese	Tofu	Cheese
Energy (kcal)	1622	1834	1723	1817
Protein (gm)	81	84	63	61
Protein (%)	19	18	15	14
Carbohydrate (gm)	216	190	253	247
Carbohydrate (%)	50	41	59	54
Fat (gm)	56	85	51	65
Fat (%)	31	41	27	32
Crude Fiber (gm)	10.0	6.0	7.8	7.5
Calcium (mg)	783	856	909	905
Phosphorus (mg)	1224	1353	1138	1195
Vitamin D (IU)	271	281	164	173
Oxalate (mg)*	2555	99	-	-
Phytate (mg)*	-	-	1900	1130

*Based on laboratory analyses conducted at the University of North Carolina at Greensboro and the University of Wyoming.

The spinach and cheese experimental diets fed during the Spinach study differed quantitatively in macronutrient composition reflecting fat and carbohydrate differences between 453 g of cooked spinach and 93 g of cheese. The cheese diet supplied more daily energy (1830 vs 1620 kcal), more total fat (85 vs 56 g), and less carbohydrate (190 vs 216 g) compared to the spinach diet. Protein levels were similar in the cheese- (84 g) and spinach-containing diets (81 g). The substitution of spinach for

cheese in the basic diets also resulted in higher crude fiber (10.0 vs 6.0 g) and oxalic acid levels (2555 vs 99 mg) in the spinach compared to the cheese diets. Daily servings of spinach and cheese provided 600 mg (77%) and 670 mg (78%) of the calcium, respectively, contained in the 2 experimental diets. Daily calcium and phosphorus intakes were slightly greater during consumption of cheese (856 and 1353 mg, respectively) than during consumption of the spinach diet (783 and 1224 mg).

Macronutrient composition of the tofu and cheese experimental diets fed during the Tofu study differed primarily in energy and fat content reflecting differences in these components between 280 g of tofu and 80 g of cheese. The cheese diet supplied more energy (1817 vs 1723 kcal) and fat (65 vs 51 g) compared to the tofu diet. Protein (61 vs 63 g), carbohydrate (247 vs 253 g) and crude fiber levels (7.5 vs 7.8 g) were similar in the cheese and tofu diets, respectively. Phytic acid intake was greater during the tofu (1900 mg) compared to the cheese diet (1130 mg) reflecting the high concentration of phytate in tofu and the absence of this dietary component in cheese. Daily servings of tofu and cheese provided 514 mg (56%) and 512 mg (57%) of the total dietary calcium, respectively. The tofu and cheese diets provided similar daily intakes of calcium (915 vs 905 mg, respectively) and phosphorus (1138 vs 1195 mg).

Analytical Procedures

During each 2 week balance period, urine was collected in acidified gallon plastic containers (containing 10 ml concentrated HCL). Feces were collected in large polyethylene bags. Twenty-four hour urine volumes were recorded, duplicate aliquots were taken and further acidified by adding 3 ml concentrated HCL/100 ml urine. Daily urine aliquots were stored frozen and at room temperature. Aliquots of urine in quantities proportional

to total daily volume were taken each day for 1 week, combined, homogenized by shaking, and the resulting homogenate was used as a 7-day urine composite. Two 7-day urine composites were made corresponding with the 2 weeks of each metabolic balance period. Daily fecal samples were weighed and homogenized with deionized water in a stainless steel blender. Duplicate aliquots were taken for moisture and mineral determinations. A more detailed description of the step-by-step procedure used for the processing of daily fecal samples is given in Appendix D. Duplicate portions of daily diet composites were collected on 2 occasions during a metabolic balance period, homogenized, and aliquots stored frozen for later analyses.

Dried fecal and diet samples were wet ashed with concentrated nitric and perchloric acids and diluted to appropriate volumes prior to mineral analysis. Fecal, urinary, and dietary calcium levels were determined by atomic absorption spectrophotometry (AAS) according to standard procedures (Willis, 1961). A Perkin-Elmer model 272 Atomic Absorption Spectrophotometer was used for all calcium analyses. A 0.5% solution of lanthanum chloride was added to all samples to reduce the formation of calcium phosphates during AAS analysis.

Oxalate in food and feces was extracted by incubating 0.1 g of dry sample in 10 ml 3% EDTA solution at 70°c for 15 minutes. An optimum EDTA solution concentration for the extraction of oxalate was determined by measuring oxalate extraction rates with a range of EDTA levels from a single sample. Maximum extraction of oxalate was attained with a 3% EDTA solution. The concentration of oxalate in each sample was determined

using a kit from Boehringer Mannheim (Indianapolis, IN). According to this method, oxalate is first decarboxlyated to form formic acid and CO_2 by oxalate decarboxylase. The formic acid is oxidized to bicarbonate by nicotinamide adenine dinucleotide (NAD) in the presence of the enzyme formate dehydrogenase. The increase in reduced NAD (NADH) is proportional to the original oxalate concentration and is determined by measuring absorbance at 340 nm with a standard spectrophotometer.

Oxalate excretion data were based on analyses of the last 3 samples collected at the end of the spinach and cheese feeding periods for each subject. Fecal oxalate is expressed as the concentration in dry feces (mg oxalate/10 g dry fecal wt) and as total fecal oxalate per day. Total fecal oxalate levels were standardized using PEG data to correct for variations in daily fecal output; daily fecal calcium levels for this same time period were similarly corrected for use in assessing fecal oxalate-calcium relationships. The urinary and fecal oxalate data were assumed to be representative of oxalate excretion trends over the entire experimental feeding periods.

Urinary oxalate and creatinine were determined using kits purchased from Sigma Chemical Co. (St, Louis, MO). The urinary oxalate method is based on the extraction of oxalate from urine and its oxidation to hydrogen peroxide and CO_2 by oxalate oxidase. Hydrogen peroxide reacts with 3-methyl-2-benzothiozolinone hydrazone (MBTH) and 3-[dimethylamino] benzoic acid (DMBA) in the presence of peroxidase to yield an indamine dye. The concentration of this end product was determined by assessing absorbance at

590 nm. Urinary creatinine was determined using a colorimetric method based on the development of a yellow/orange color when the metabolite is treated with alkaline picrate reagent.

Urinary hydroxyproline was measured using the method of Kivirikko, Laitinen and Prockop (1976). Peptide bound hydroxyproline in urine is first subjected to acid hydrolysis. Hydroxyproline is then oxidized with Chloramine-T in the presence of alanine to produce Δ'-pyroline-4-hydroxy-2-carboxylic acid and pyrrole-2-carboxylic acid. Oxidation is halted by the addition of thiosulfate. Upon heating, the pyrrole is extracted into toluene and reacted with p-dimethylaminobenzaldehyde to form an amber color. The concentration of the metabolite was determined colorimetrically by measuring absorbance at 560 nm. Random samples were chosen and assayed for hydroxyproline according to the method described by Bergman and Loxley(1970) as a validity check. A more detailed description of the procedure used in this study is given in Appendix E.

Daily fecal PEG levels were determined according to the turbimetric method of Malawer and Powell (1967). The method is based on the creation of an oil-in-water emulsion of the water soluble polyethylene glycol when trichloroacetic acid is added. Interfering substances are first precipitated by adding a $Ba(OH)_2$ - $ZnSO_4$ - $BaCl_2$ solution and removed by filtration. Gum arabic acts as an emulsifying agent to stabilize the oil-in-water emulsion. The concentration of PEG is determined spectrophotometrically based on absorbance at 650 nm. A detailed description of the procedure used in the present study for the determination of PEG in feces is given in Appendix D.

Fecal polyethylene glycol levels were measured daily and the ratio of recovered PEG/dry fecal solids determined for each subject during the last 2 weeks of each 3-week experimental period. At the end of a balance period, the daily ratio of PEG/dry fecal solids per subject was plotted and a point determined at which the ratio was judged to be relatively constant. At this point, the preexperimental diet was assumed to have been eliminated from the gut. Days in which the PEG/dry fecal solids ratio deviated greatly from equilibrium for a subject occurred primarily during the first part of the balance period when the preexperimental diet was being eliminated; these days were excluded from balance calculations. A ratio of the total amount of PEG consumed to the total amount recovered in the feces for days in which equilibrium was established was used to correct for calcium provided by the experimental diet remaining in the gut at the end of a balance period. The average number of days, in each study, in which individual calcium balances were calculated were 7.5 and 8.5 days, Spinach and Tofu studies, respectively.

Serum alkaline phosphatase was measured using a kit purchased from Sigma Chemical Co. (St. Louis, MO). The method is based upon the hydrolysis of p-nitrophenyl phosphate by alkaline phosphatase, yielding p-nitrophenol and inorganic phosphate. When made alkaline with sodium hydroxide, p-nitrophenol is converted to a yellow complex. The intensity of color formed is proportional to the level of phosphatase activity and is measured spectrophotometrically at 400-420 nm.

Statistical Analysis

An analysis of variance with subjects as the blocking variable was employed to detect significant differences in dependent variables in response to consumption of the 2 (test food and cheese) experimental diets. The use of subjects as a blocking variable allowed for the removal of intersubject variation during each analysis of variance procedure. Treatment order was determined not to be an important source of variation. Pearson correlation coefficients were determined to detect associations between dependent variables. A Spearman rank correlation statistic for nonparametric data was determined to assess the degree of correlation between fecal oxalate, fecal calcium and calcium balance data during the Spinach study.

CHAPTER IV

RESULTS AND DISCUSSION

The present studies were designed to assess the calcium availability from spinach and tofu compared to cheese, with cheese representing a standard or highly available food source of dietary calcium. The results of these studies suggested that the calcium availability from cheese was superior to that from tofu and probably spinach. A comparison of calcium availability from spinach and tofu is not possible because of the provision of different amounts of calcium by the test foods; and the use of different experimental diets and subject populations in the Spinach and Tofu studies. Thus, the discussion will focus primarily on the comparisons made between each of the test foods with their respective cheese diets.

Prestudy Dietary Intakes

Prestudy dietary intakes of subjects in the Spinach study are presented in Table 5. Spinach study participants were characterized by relatively high mean daily calcium (1034 mg) and phosphorus intakes (1579 mg), and a relatively low crude fiber intake (5.6 g). Mean vitamin D intake was adequate based on the RDA established for this nutrient. Other prestudy dietary intakes appeared to be representative of typical U.S. intake levels.

Mean prestudy intakes of calcium (832 mg) and phosphorus (1158 mg) for subjects in the Tofu study exceeded the RDA for these nutrients and the mean vitamin D intake was adequate. Intakes of protein, fat and carbohydrate were within range of levels considered typical for the U.S. population. Crude fiber intake was relatively low (4.2 g) (Table 5).

Table 5
Mean (±SD) Prestudy Dietary Intakes for Spinach and Tofu Study Subjects Based on Dietary Recall Data

Variable	Spinach Study[a]	Tofu Study[b]
Energy (kcal)	2046 ± 814	1561 ± 394
Protein (g)	77 ± 26	60 ± 13
Protein (%)	15 ± 4	16 ± 3
Carbohydrate (g)	247 ± 140	180 ± 65
Carbohydrate (%)	48 ± 8	45 ± 5
Fat (g)	85 ± 30	68 ± 15
Fat (%)	37 ± 5	39 ± 5
Crude Fiber (g)	5.6 ± 4.1	4.2 ± 2.2
Calcium (mg)	1034 ± 394	832 ± 284
Phosphorus (mg)	1579 ± 800	1158 ± 315

[a] $n = 7$
[b] $n = 8$

Body Weight Alterations

Changes in mean body weight during the 3-week experimental periods were not significantly different between test periods in the Spinach and Tofu studies. In the Spinach study, weight changes for both 3-week test periods were typically negative and averaged -1.2 kg. In the Tofu study, weight changes ranged from 1.5 to -2.5 kg and overall weight changes averaged -0.4 kg for both test periods.

Fecal Weight and PEG Recovery Data

Mean fecal wet and dry weights and moisture levels during the Spinach study are presented in Table 6. Mean fecal dry ($p < 0.01$) and wet ($p < 0.05$) weights were significantly greater during the spinach compared to cheese feeding period. Fecal moisture did not differ significantly between the 2 diets. Relative differences in intestinal transit time between the 2 experimental diets could be approximated by computing the average cumulative recovery of fecal PEG. Mean PEG recovery during the last 2 weeks of the spinach feeding period was 101% compared with 91% for the cheese feeding period. Although not statistically different, the higher percent recovery rate of PEG during the spinach period may suggest a slightly decreased intestinal transit time compared with that on the cheese diet.

Table 6
Mean (±SD) Fecal Dry and Wet Weight and Moisture during Experimental Feeding Periods for the Spinach Study

	Test Period	
	Spinach	Cheese
Wet Weight (gm/day)	192 ± 64[a]	141 ± 44
Dry Weight (gm/day)	49 ± 13[b]	39 ± 49
Moisture (%)	74 ± 3	72 ± 6

[a]Significantly different between spinach and cheese dietary periods ($p < 0.05$)
[b]Significantly different between spinach and cheese dietary periods ($p < 0.001$)

Mean fecal wet and dry weights and moisture levels during the Tofu study did not differ significantly between the tofu and cheese consumption periods (Table 7). Mean cumulative recovery of fecal PEG during the final 2 weeks of the tofu and cheese periods was 77% and 82%, respectively, thus suggesting little relative difference in intestinal transit time between consumption of the tofu and cheese diets.

Table 7
Mean (±SD) Fecal Dry and Wet Weight and Moisture during Experimental Feeding Periods for the Tofu Study

	Test Period	
	Tofu	Cheese
Wet Weight (gm/day)	143 ± 35	147 ± 39
Dry Weight (gm/day)	39 ± 6	41 ± 10
Moisture (%)	72 ± 5	72 ± 5

The significantly greater mean wet and dry fecal weights during consumption of the spinach- compared to the cheese-containing diet may be attributed to the high fiber content of spinach. Fecal moisture content was nearly identical during consumption of the 2 test diets. One action of dietary fiber could be to increase stool weight by increasing total water content without affecting percent moisture (Eastwood & Robertson,1983). This could partially explain the significantly higher mean wet fecal weight for the spinach- compared to the cheese-containing diet. Bacterial content of stools typically accounts

for more than one-half of total fecal solids (Eastwood & Robertson,1980). Certain types of fiber have been suggested to influence stool weight by increasing fecal flora mass (Stephen & Cummings,1980). Thus, the higher mean dry fecal weight on the spinach compared to the cheese diet could be accounted for by a fiber-induced increase in bacterial content of the stools during the spinach feeding period. The lack of significant differences in fecal dry and wet weights between the 2 experimental feeding periods during the Tofu study is consistent with the fact that the tofu- and cheese-containing diets were almost identical in fiber content.

While relatively few balance studies have employed a quantitative fecal marker such as PEG, its validity as a fecal marker for calcium, and other minerals, has been demonstrated (Allen, Raynolds, & Margen, 1979). The use of quantitative fecal markers for metabolic balance studies offer several advantages over the more conventional and frequently used qualitative markers such as nonabsorbable dyes. One advantage is that the elimination of preexperimental intestinal contents from the body can be accurately estimated based on trends in the ratio of PEG/dry fecal solids. Second, dietary calcium remaining in the gut at the end of a balance period can be quantitatively assessed from calculations based on the ratio of total recovered PEG to total PEG consumed for a specific time period. Third, variation among individuals in day to day fecal output can be standardized by using PEG recovery data enabling more valid intersubject comparisons of fecal calcium levels. Last, relative differences in intestinal transit time between individuals or groups can be approximated by using cumulative PEG recovery data.

The mean cumulative recovery of PEG over the last 14 days of each 3-week test period during the Spinach study was 95% of the dose administered during that time period. Individual recoveries ranged from 59 to 124%. Mean cumulative recovery of PEG from the Tofu study was 80% and individual recoveries ranged from 59 to 106% during the last 2 weeks of the experimental feeding periods. Differences in overall cumulative PEG recovery rate between the Spinach and Tofu studies was probably influenced by the greater laxative effect of spinach (high fiber) compared to tofu (low fiber).

Individual recoveries greater than 100% can be attributed to the recovery of PEG administered during the first week of the 3-week experimental period in addition to that ingested during the subsequent 2-week balance period. Subjects with recovery rates approaching 95% or more by the end of the balance period can be considered "ideal" subjects for balance studies. However, balance data from subjects exhibiting "non-ideal" recovery rates, below approximately 90%, appear to be valid if PEG can be demonstrated to be fully equilibrated with intestinal contents (i.e., achievement of a constant ratio of PEG to dry fecal solids) (Allen et al.,1979). Variation in cumulative PEG recovery rates among individuals primarily reflects variation in fecal flow rather than an inability to recover PEG from the feces or losses of PEG from the gut through partial absorption or intestinal degradation.

The time required to attain a constant ratio of PEG to dry fecal solids varies among individuals. Of 6 subjects fed PEG in a study by Allen et al. (1979), one required 4-7 days before equilibrium occurred,

4 required between 8-10 days, and 1 required between 15-17 days. In the Spinach and Tofu studies, all subjects achieved PEG equilibration at some point with the majority requiring 7-11 days. One subject in the Spinach study took approximately 16 days, and 1 subject in the Tofu study took 19 days for PEG to become equilibrated with intestinal contents.

Calcium Availability from Spinach

Calcium intake, excretion and balance data during the Spinach study are presented in Table 8. Fecal calcium and overall calcium balance were not significantly different between dietary periods. Subjects varied appreciably in their ability to maintain calcium balance during spinach compared to cheese feeding periods. Three of the subjects exhibited similar calcium balance during ingestion of the 2 test diets whereas 2 subjects who maintained positive calcium balances during the cheese period had negative calcium balances of -160 and -510 mg/day during spinach consumption. In only 4 of a total of 14 individual balance periods did subjects exhibit near zero or positive calcium balances.

Table 8
Mean (±SD) Calcium Intake, Excretion and Balance Data During the Experimental Feeding periods for the Spinach Study

	Test Period	
	Spinach	Cheese
Intake (mg/day)	783 ± 4	856 ± 3
Fecal (mg/day)	817 ± 175	706 ± 115
Urinary (mg/day)	135 ± 50	205 ± 75[a]
Balance (mg/day)	-168 ± 189	-55 ± 147

[a]Significantly different between spinach and cheese dietary periods ($p < 0.01$)

The magnitude of the difference in mean calcium balances between the spinach and cheese diets suggested that the availability of calcium from the spinach diet was considerably lower than that from the cheese diet, although this was not supported statistically. Fecal excretions of calcium were 104% and 82% of the intake, during ingestion of the spinach and cheese diets, respectively, which not only suggested a greater absorption of calcium from cheese compared to spinach, but also a very low absolute absorption of calcium from spinach, per se. Estimates of true absorption of calcium from the test diets also suggested a lower utilization of calcium from the spinach- (5%) compared to the cheese-containing diet (28%). True absorption estimates were based on the assumption that endogenous fecal calcium losses averaged approximately 130 mg per day (Heaney & Skillman, 1964). The lack of statistical evidence for differences in these calcium balance and fecal calcium data could be partially explained by the particularly high intersubject variation for these variables and the relatively low subject number.

Most of the research regarding calcium availability from spinach was conducted during the first half of this century. The results of these studies can be categorized according to the dose of spinach fed. Studies feeding spinach at relatively high levels (200 g or more/day) reported adverse effects on calcium balance (Sherman & Hawley,1922; McLaughlin,1927; Fincke & Garrison,1938). Studies feeding less than 200 g per day did not report unfavorable effects on calcium balance (Schlutz et al.,1933; Bonner et al.,1938; Johnston et al.,1952). Inferences regarding rela-

tionships between daily spinach dosage and calcium balance based on these early studies are tenuous because of inherent limitations in the study designs including short balance periods, low subject numbers, low calcium intakes and lack of statistical analysis.

More recently, Kelsay and Prather (1983) reported lower mean calcium balances in 12 adult men fed a high-fiber diet containing 102 g of spinach every other day (-73mg/day) compared to a similar high-fiber diet in which cauliflower replaced spinach (-11 mg/day). Calcium balance was positive and significantly higher during ingestion of a relatively low-fiber diet with spinach added every other day than during consumption of the high-fiber, spinach diet. The mean calcium balance of -168 mg/day observed in the present study during spinach ingestion can be explained by the considerably larger quantities of spinach fed (453 g) on a daily basis.

A study by Fincke and Garrison (1938) utilized levels of spinach (490 g/day) similar to those contained in the spinach diet of the present study. The subjects, 2 adult women, exhibited a low mean calcium balance of -149 mg/day. With the exception of spinach, the diets utilized in the study by Fincke and Garrison (1938) were relatively low in fiber and total calcium intake averaged only 450 mg per day.

The higher mean calcium intake on the cheese (856 mg) compared to the spinach diet (783) in the present study was balanced by an equally greater urinary calcium level (205 vs 135 mg/day, respectively). A highly significant negative correlation between individual mean fecal calcium and mean calcium balance ($r = -0.99$, $p < 0.001$) suggests that fecal calcium excretion was the major factor affecting calcium balance in the Spinach study.

Urinary calcium was significantly greater ($p < 0.01$) during the cheese (205 mg/day) as compared to the spinach feeding period (135 mg/day). Daily urinary calcium levels are an immediate reflection of the relation between the rates of glomerular filtration and tubular reabsorption (Lemann, Adams & Gray, 1979). However, calcium in the urine must ultimately be derived either from absorbed dietary calcium or from resorbed bone calcium and is therefore a function of dietary calcium, absorptive capacity and bone breakdown (Nordin, 1976). Thus, given equivalent calcium intakes, low urinary values suggest malabsorption of calcium, and high values, efficient absorption or high levels of bone resorption. The finding of a significantly greater urinary calcium level during the cheese compared to the spinach feeding period could partially be attributed to the slightly higher calcium intake during cheese consumption but also suggested a more efficient absorption of calcium from cheese.

Alterations in urinary calcium levels in response to apparent changes in calcium absorption have been reported by Walker, Walker and Wadvalla (1974). These researchers reported significantly higher 6-hour urinary calcium levels after ingestion of a test meal of milk compared to a test meal of swisschard, a high oxalate plant food. The test meals contained similar levels of calcium.

Marshall, Cochran and Hodgkinson (1972) reported a decrease in urinary calcium from 193 to 125 mg/day when control subjects were switched from a diet containing calcium at a level of 1000 to 250 mg/day. A decrease in urinary calcium of 68 mg reported by Marshall et al. (1972)

in response to a decrease in intake of 750 mg suggests that the relatively small daily calcium intake differences between the spinach- and cheese-containing diets in the present study is not the primary factor responsible for the significantly lower urinary calcium excretion during spinach consumption. Rather, differences in calcium bioavailability between the 2 experimental diets is the more likely explanation for the 80 mg difference observed in urinary calcium.

Oxalic acid intake, excretion and apparent absorption data are presented in Table 9. Spinach contributed 2456 mg of the total daily oxalic acid intake of 2555 mg in the spinach diet while the total oxalate content of the cheese diet was 99 mg per day. The concentration of oxalate in the feces (mg/10 g dry wt) was significantly greater ($p < 0.05$) during consumption of the spinach- compared to the cheese-containing diet. Total fecal oxalate (mg/day) and fecal oxalate as a percent of intake (%) both corrected for variation in daily fecal output, were also higher during consumption of the spinach compared to the cheese diet. The concentration of oxalate in urine (mg/day) was statistically greater ($p < 0.01$), and urinary oxalate as a percentage of intake lower, during consumption of the spinach-containing diet.

A number of studies have demonstrated low rates of dietary oxalate absorption (Hodgkinson, 1977; Kelsay & Prather, 1983). Since oxalate is catabolized to varying extents in the gut of humans (Allison, Cook, Milne, Gallagher, & Clayman, 1986), the degree of degradation rather than absorption is the more important determinant of fecal oxalate levels. A negative correlation between fecal and urinary oxalate levels might have

been expected based on the assumption that greater fecal losses would decrease the amount of oxalate available for absorption and subsequent excretion via the urine. However, the highly positive correlation between urinary and fecal oxalate observed during spinach consumption suggests that urine and fecal oxalate are both dependent upon the degree of oxalate degradation. With extensive oxalate degradation, less total oxalate is available for absorption and fecal excretion resulting in low fecal and urinary levels. Conversely, diminished oxalate degradation would be associated with high luminal oxalate concentrations allowing for greater absolute absorption resulting in increased urinary levels and greater fecal losses. Increases in urinary oxalate associated with increased oxalate absorption have been previously demonstrated (Finch, Kasidas & Rose,1981).

Table 9
Mean (±SD) Oxalate Intake, Excretion and Apparent Absorption Data during Experimental Feeding Periods for the Spinach Study

	Test Period	
	Spinach	Cheese
Intake (mg/day)	2555 ± 11	99 ± 1
Fecal Oxalate		
mg/10 g dry wt	322 ± 280[a]	13 ± 9
Corrected (mg/day)[b]	1729 ± 1776	34 ± 21
Corrected (% of intake)[b]	68 ± 69	35 ± 21
Urinary Oxalate		
mg/day	101 ± 54[c]	17 ± 3
% of intake	4 ± 2	17 ± 3
Apparent Absorption (%)	3.3 ± 2.1	—

[a]Significantly different between spinach and cheese dietary periods ($p < 0.05$).
[b]Adjusted for variation in daily fecal output using PEG recovery data.
[c]Significantly different between spinach and cheese dietary periods ($p < 0.01$).

Urinary oxalate as a percentage of intake usually decreases as intake increases (Kelsay & Prather, 1983). In the present study, urinary oxalate represented 17 and 4% of total intake during consumption of the cheese and spinach diets, respectively. Urinary oxalate levels on the low oxalate (cheese) diet were comparable with levels reported by other researchers (Kelsay & Prather, 1983), although levels on the spinach diet were higher than those reported in the literature for other high-oxalate diets. Kelsay and Prather (1983) reported urinary oxalate values ranging from 30 to 67 mg/day for a relatively low fiber, spinach-containing diet providing approximately 480 mg of oxalate/day and from 24 to 40 mg/day on similar diets providing 190 mg of oxalate/day in which cauliflower replaced spinach. In another study (Archer, Dormer, Scowen & Watts,1957), urinary oxalate levels ranging from 76 to 124 mg/day were observed in 4 healthy adults fed an average of 2250 mg of sodium oxalate/day. In the present study, urinary oxalate ranged from 55 to 200 mg/day during spinach consumption and from 13 to 21 mg/day during cheese consumption. The apparent absorption of oxalate from the spinach diet was low ($\bar{x}$ = 3.3%), but was consistent with levels reported in other studies (Finch et al.,1981; Prenen, Boer & Dorhout Mees,1984). In spite of a low apparent absorption, the extremely high oxalate intake provided by the spinach diet resulted in very high urinary levels.

Mean corrected fecal oxalate as a percentage of intake was 68% and individual values ranged from 9 to over 100% during consumption of the spinach diet. The 3 subjects who exhibited fecal oxalate levels over 100% of 3-day intake levels may have been characterized by minimal

gastrointestinal oxalate degradation. These subjects had an average calcium balance of -256 mg per day during the spinach feeding period. A mean value of 73% for fecal oxalate as a percentage of intake has been previously reported for subjects ingesting a high-fiber, high-oxalate diet (Kelsay & Prather, 1983).

Individual data related to oxalate excretion and apparent absorption, fecal calcium and calcium balance during the spinach feeding period are presented in Table 10. Subjects varied greatly in their excretion levels of oxalate during the spinach feeding period. Given identical intake levels of oxalate, intestinal degradation of oxalate must have varied widely among subjects. High correlations were observed between fecal oxalate (mg/10 g dry wt) and both urinary oxalate ($r = 0.90, p < 0.01$) and apparent oxalate absorption ($r = 0.90$, $p < 0.01$) during the spinach feeding period. Slightly lower but statistically significant correlations were observed when corrected fecal oxalate (mg/day) was correlated with urinary oxalate ($r = 0.79$, $p < 0.04$), and with apparent oxalate absorption ($r = 0.77$, $p < 0.05$).

Fecal oxalate data during the spinach period conformed to a non-normal distribution. Spearmans rank correlation statistic for non-parametric data indicated a significant correlation between corrected fecal oxalate per day and overall mean corrected fecal calcium per day ($r_s = 0.71$, $p = 0.04$). To further explore relationships between fecal oxalate, fecal calcium and calcium balance, subjects were subdivided into high and low oxalate excreters. Mean fecal and urinary oxalate excretion levels were high for subjects 1,4,5 (619 mg/10 g dry wt and 151 mg/day)

Table 10
Individual Data for Oxalate Excretion and Absorption, Fecal Calcium and Calcium Balance during the Spinach Feeding Period

Subject Number	Fecal Oxalate[a]	Corrected Fecal Oxalate (mg/day)	Fecal Calcium (mg/day)	Calcium Balance (mg/day)	Urinary Oxalate (mg/day)	Apparent Oxalate Absorption (%)
1	572	3544	807	-97	104	3.4
2	135	340	593	+67	70	2.2
4	669	2955	862	-160	200	7.2
5	616	4247	1145	-511	150	5.0
7	102	337	665	-18	55	1.5
8	105	454	853	-291	69	1.9
9	54	219	793	-169	62	1.8

[a]mg/10 g dry weight

in contrast with levels for subjects 2,7,8,9 (99 mg/10 g dry wt and 64 mg/day). Mean corrected fecal calcium per day was calculated for the final 3 fecal samples of the spinach balance period to correspond with the samples for which fecal oxalate levels were assessed. Mean daily fecal calcium for this period for the high oxalate excreters (1067 mg) was considerably greater than that for the low oxalate excreters (794 mg). Overall mean calcium balance for the high oxalate excreters (-256 mg/day) was considerably lower than that for the oxalate excreters (-103 mg/day).

These individual oxalate intake and excretion data suggested that individuals varied greatly in their ability to metabolize oxalate in the gut. Assuming that a significant proportion of calcium in spinach, or calcium present in any diet containing high levels of oxalate, is ingested in a form bound to oxalate, or can bind to oxalate within the gastrointestinal tract, then a relationship between the fecal excretion of oxalate and calcium would be expected. A significant rank correlation between total corrected fecal oxalate per day and mean fecal calcium per day helped to confirm this hypothesis.

Overall data from the present study provided only moderate support for the contention that the degree of oxalate degradation is an important determinant of calcium balance in subjects ingesting spinach-containing diets. A moderate correlation was observed between corrected fecal oxalate/day and calcium balance/day, calculated for the same 3-day period at the end of the spinach feeding period ($r = -0.58, p = 0.17$). Subjects could be clearly subdivided into high and low oxalate excreters during spinach consumption. Three subjects exhibited very high fecal and urinary oxalate

levels while 4 subjects exhibited relatively low levels (Figure 4). The high excreters, or those presumed to be less able to degrade oxalate in the gut, had a lower average calcium balance (-256 mg/day) than did the low excreters (-103 mg/day).

The presence of significant populations of oxalate degrading bacteria (Oxalobacter formigenes) has been recently demonstrated in the gastrointestinal tract of humans (Allison et al.,1986). Oxalate degradation rates increase in the gastrointestinal contents of animals after the addition of oxalate to the diet (Allison & Cook,1981). Very few studies have investigated the relationships between overall oxalate consumption, oxalate degradation and oxalate degrading bacterial concentration and activity in humans. Adaption to high oxalate feeding has been hypothesized based upon evidence that some populations of the world have typical dietary intakes high in oxalate yet appear to exhibit no clinical signs of oxalate toxicity or calcium deficiency (Hodgkinson, 1977).

Spinach has been reported to contain approximately 6.3 g of total fiber per 100 g (Paul & Southgate, 1978). An inhibitory effect of fiber upon calcium absorption is well established (Allen, 1982). Several mechanisms have been proposed based on the physical and chemical properties of dietary fiber to explain the inhibitory effect of fiber on calcium availability. The ingestion of some types of fiber have been demonstrated to decrease intestinal transit time which could reduce calcium absorption by decreasing the time allowed for digestion and absorption. Fiber may also act as a physical barrier to calcium absorption by diluting intestinal contents. Some varieties of fiber can bind calcium

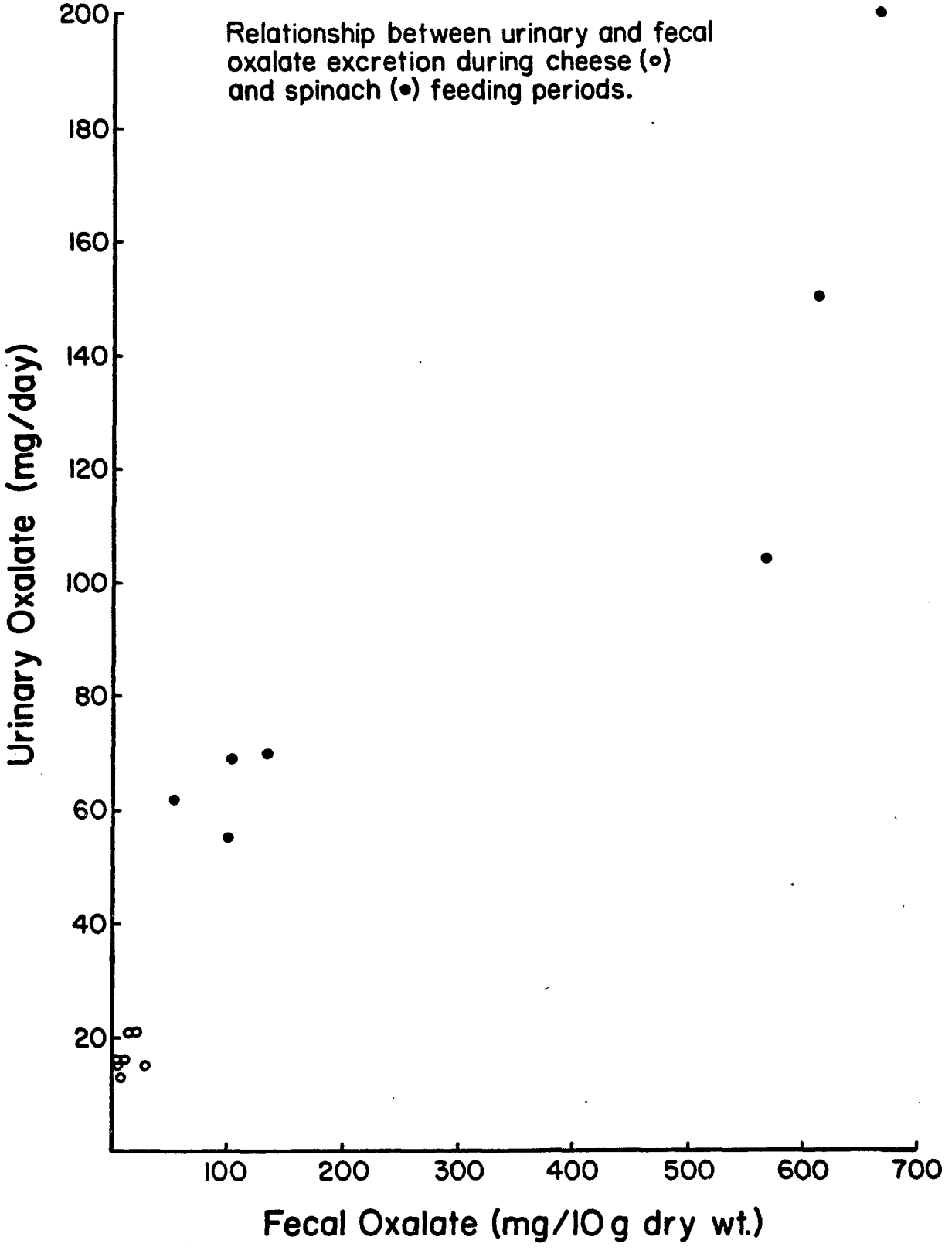


Figure 4. Individual values for urinary (mg/day) and fecal oxalate (mg/10 g dry wt) excretion during the experimental feeding periods for the Spinach study. Mean calcium balance was -256 and -103 mg/day for the high (n=3) and low (n=4) excreters of oxalate, respectively.

intraluminally and increase fecal excretion via an interaction between calcium and negatively charged side groups of fiber polymers (Allen, 1982).

In the present study, the individual effects of fiber and oxalate with respect to calcium availability could not be separated since spinach contains large quantities of both whereas cheese does not contain either of these food components. Several studies have reported lower calcium balances or overall calcium retention when subjects were fed diets rich in both fiber and oxalate versus oxalate-rich diets low in fiber (Kelsay & Prather, 1983; Tisdall & Drake, 1937).

Kelsay and Prather (1983) reported significantly lower calcium balances during consumption of a high-fiber compared with a low-fiber spinach-containing diet. These researchers suggested that the combination of oxalate and fiber in the gut might accentuate the binding of minerals and inhibit their absorption more effectively than would either dietary component alone. They also reported an apparent reduction in the intestinal degradation of oxalate (i.e., increased fecal oxalate) during ingestion of the high-fiber compared to the low-fiber diet. Another study (Tisdall & Drake,1937) reported decreased total body calcium in rats fed dried spinach, containing fiber and oxalate, compared to rats fed low-fiber diets supplemented with oxalic acid and calcium at levels equal to those in the spinach serving.

An effect of fiber in oxalate-containing diets could be to reduce the release of calcium from oxalate-calcium complexes by decreasing or interfering with the activity of oxalate-degrading bacteria. The presence of fiber in the gut could lower the degree of contact between bacteria and oxalate since high fiber intakes have been associated with a dilution of intestinal contents and decreases in intestinal transit time.

Calcium Availability from Tofu

Calcium balance was markedly negative during both the tofu and cheese consumption periods, and was significantly lower ($p < 0.05$) during the tofu compared to the cheese period (Table 11). A significantly greater level of calcium was excreted via the feces during the tofu compared to the cheese feeding period ($p < 0.05$). The majority of subjects maintained a more positive calcium balance while consuming the cheese relative to the tofu diet. One of the 9 subjects exhibited a more positive calcium balance on the tofu diet; 2 subjects exhibited similar calcium balances on the 2 diets. Overall, in only 6 of a total of 18 individual balance periods did subjects exhibit either near zero or positive calcium balance.

Table 11
Mean (±SD) Calcium Intake, Excretion and Balance Data During the Experimental Feeding periods for the Tofu Study

	Test Period	
	Tofu	Cheese
Intake (mg/day)	909 ± 17	905 ± 21
Fecal (mg/day)	824 ± 122[a]	767 ± 98
Urinary (mg/day)	219 ± 98	211 ± 98
Balance (mg/day)	-135 ± 139[a]	-73 ± 145

[a]Significantly different between tofu and cheese dietary periods ($p < 0.05$)

Results from the Tofu study suggested that calcium from tofu was not as available (i.e., absorbed to a lesser extent) as the calcium from cheese. This conclusion is based on the finding of a significantly lower mean calcium balance and higher fecal calcium during the tofu compared to the cheese feeding period. In contrast to results from the Spinach study, urinary calcium levels were similar between ingestion of the tofu and cheese diets despite a significantly higher mean calcium balance, or greater apparent absorption of calcium, during the cheese compared to tofu feeding period. The actual magnitude of difference in mean calcium balance between the test diets in the Tofu study (62 mg) was roughly one-half that observed between the test diets in the Spinach study (113 mg). The difference in apparent calcium absorption between the tofu and cheese diets, although consistent among subjects, may not have been of sufficient magnitude to elicit changes in urinary calcium output.

Other studies have demonstrated reductions in urinary calcium during consumption of high-phytate diets. Morris and Ellis (1985) reported a slight but nonsignificant decrease in mean urinary calcium from 185 to 162 mg per day when subjects were switched from a diet containing 0.2 g of phytate to a diet containing 2.0 g phytate. Reinhold et al. (1973) observed significantly decreased urinary calcium levels in 3 subjects after switching from a low-phytate control diet to a diet in which 1.85 g of phytate was added. McCance and Widdowson (1942) also reported reductions in urinary calcium when large amounts of phytate were added to the diet so as to increase the dietary phytate/calcium molar ratio above 0.3.

Very few studies have evaluated the overall calcium bioavailability from tofu. Adolph and Chen (1932) found that availability of calcium from soybean curd (tofu) closely approximated that from milk with respect to effect on overall calcium balance. However, calcium balance was determined in only 3 subjects for 7 days per experimental diet. Another study (Schroeder et al.,1946) demonstrated similar calcium availability from soybean milk and evaporated cow's milk, while the absorption of calcium from whole cooked soybeans was relatively low. The authors attributed the lower availability of calcium from whole soybeans to their high fiber content.

An inhibitory effect of dietary phytate upon zinc absorption has been well documented (Oberleas & Harland, 1981), however, the effect of phytate on the absorption of calcium is less well defined. Total phytate intakes provided by the tofu- and cheese-containing diets were approximately 1900 and 1130 mg per day, respectively, as the daily serving of tofu contained about 770 mg of phytate. A portion of the additional 770 mg of phytate provided in the tofu diet may have complexed with calcium in the gut. Thus, the formation of calcium-phytate salts during tofu ingestion may have been partially responsible for the observed differences in fecal calcium and calcium balance between experimental diets.

The ability of phytic acid to form insoluble salts with some of the mineral elements is the most likely mechanism by which phytate could interfere with calcium absorption. Recent research by Graf and Eaton (1985) demonstrated that this assumption may not be entirely valid. Using in vitro

studies, calcium-phytate salts of very high calcium/phytate ratios were found to be poorly soluble in solution whereas salts characterized by low calcium/phytate ratios were very soluble.

Several studies which involved feeding phytate at levels approximating those used in the present study have reported adverse effects on calcium metabolism. Morris, Ellis, Hill, Steele and Cottrell (1985) reported mean calcium balances of -23, -34 and -82 mg/day for 12 adult men consuming diets containing 0.5, 1.7 and 2.9 g/day of phytate, respectively. Consumption of the highest phytate diet increased the number of individuals in negative calcium balance compared to the number of negative calcium balances observed on the other diets. In an earlier study, diets containing 3.0 g of phytate from Tanok bread, when fed to 3 male adults, resulted in markedly negative calcium balances. When white bread, containing no phytate replaced Tanok in the diet, calcium balances became positive (Reinhold et al.,1973). In this same study, the ingestion of 1.85 g of pure phytic acid in leavened flat white bread, resulting in a total dietary phytate content of 2.5 g, decreased calcium balances in 2 out of 3 subjects compared to balances on a low-phytate control diet.

In contrast, one study (Morris & Ellis,1985) demonstrated no differences in calcium balance resulting from the ingestion of diets containing 36 g of wheat bran, supplying 2.0 g of phytate, and low-phytate diets containing equivalent amounts of dephytinized wheat bran. High calcium intakes (1100 mg/day) on the high-phytate diet may have partially masked the potentially adverse effect of phytate on calcium absorption.

The majority of subjects in the present study appeared to absorb calcium from the cheese diet to a greater extent than that from the tofu diet. However, 2 subjects maintained similar calcium balances on the test diets and one subject exhibited a higher mean calcium balance on the tofu compared to the cheese diet. Differences in the utilization of dietary phosphorus among subjects could help to explain the variability in the calcium utilization from the tofu-containing diets.

Phosphorus intakes were similar during the cheese (1195 mg) and tofu feeding periods (1138 mg). A significant proportion of the phosphorus in the tofu-containing diet (47%) was present in phytate. Additional phosphorus intake due to the consumption of soft drinks during the experimental feeding periods was consistent in the tofu and cheese test periods based on soft drink consumption data from daily questionnaires.

Eight out of the 9 subjects exhibited higher mean urinary phosphorus levels during the cheese- compared to the tofu-feeding period. As a group, mean urinary phosphorus was significantly higher ($p < 0.05$) on the cheese (620 mg/day) compared to the tofu (515 mg/day) diet.

There was appreciable intersubject variability in the absorption and urinary excretion of phosphorus during consumption of the tofu diet. Individual data for urinary phosphorus, calcium balance and fecal calcium during the tofu period are presented in Table 12. The 3 subjects (6,9,10) who maintained either similar calcium balances between experimental diets or a more positive balance on the tofu diet exhibited a mean urinary phosphorus level of 628 mg per day compared with 458 mg per day for subjects (1,2,3,5,7,8) who maintained appreciably lower calcium

Table 12
Individual Data for Urinary Phosphorus during the Cheese and Tofu Experimental Periods, and Calcium Balance and Fecal Calcium during the Tofu Period

Subject	Urinary Phosphorus (mg/day)			Net Change in Calcium[b] Balance From Cheese to Tofu Period (mg)	Mean Fecal Calcium during Tofu Period (mg/day)
	Cheese Period	Tofu Period	Cheese - Tofu[a]		
1	586	530	56	-162	822
2	720	385	335	-43	851
3	626	595	31	-118	978
5	556	421	135	-153	942
6	624	581	43	-28	706
8	462	379	83	-86	708
9	760	759	1	48	655
10	784	544	240	-15	977
11	465	439	26	-56	778

[a]Urinary phosphorus during cheese period minus urinary phosphorus during tofu period.
[b]Mean calcium balance during cheese period minus calcium balance during tofu period.

balances on the tofu compared to the cheese diet. Subject 9, who maintained a more positive balance on the tofu diet, exhibited the highest urinary phosphorus level (759 mg/day) observed among subjects during tofu ingestion. While the remainder of the subjects exhibited lower urinary phosphorus levels during the tofu compared to the cheese dietary periods, thereby suggesting a lower phosphorus absorption from the tofu-containing diet, subject #9 had almost identical urinary phosphorus levels during ingestion of the cheese (760 mg/day) and tofu diets (759 mg/day). These data suggest that subject #9 may have been able to effectively degrade phytate in the gut and thus utilize both the calcium and phosphorus present in calcium-phytate salts.

The presence of phytate splitting enzymes (phytases) in the human intestine has been demonstrated (Bitar & Reinhold, 1972). The concentration and activity of these enzymes could be a primary factor influencing calcium availability from tofu and other soy products which are high in phytate. Significant rates of phytate degradation would release phytate phosphorus, and perhaps phytate associated calcium, rendering these minerals available for absorption. Therefore, higher urinary levels of phosphorus might be expected among individuals who exhibit efficient gastrointestinal phytate degradation. Reinhold et al. (1973) reported that 2 out of 3 subjects exhibited lower calcium balances on a high-phytate compared to a low-phytate diet. The subject apparently unaffected by phytate feeding in terms of calcium balance also exhibited significantly increased urinary phosphorus when switching from the low- to high-phytate diet in contrast to unaltered urinary phosphorus levels in the other 2 subjects.

Based on prestudy dietary records, subjects in the Spinach and Tofu studies probably had typical diets much lower in phytate and oxalate than the levels fed in the present studies, while dairy foods appeared to be consumed on a daily basis. Although subjects consumed the dietary treatments for 3-week periods, this duration may not have been sufficient to ensure adaption to the high levels of phytate and oxalate consumed, whereas the respective cheese diets required no unusual adaptions. Relatively long periods of time may be required to develop an intestinal condition conducive to the metabolism of large loads of phytate or oxalate. In a study by Reinhold, Faraji, Abadi, and Ismail-Beigi (1981), no evidence of adaption was observed in 2 men after 3 months of consuming diets high in phytate from unleavened bread.

One factor to be considered when comparing calcium availability from test foods with that of dairy foods is the effect of the disaccharide lactose. Lactose, found in large quantities in milk and milk products, has been reported to improve calcium absorption in diets of animals and humans (Allen, 1982). The lactose present in the cheese servings used in the Tofu and Spinach studies may have enhanced calcium absorption from the cheese diets, thereby contributing to the observed differences in calcium balance and fecal calcium levels. Over a 3 week period, vitamin D intake from the experimental diets could represent another factor affecting calcium absorption. However, in both the Spinach and Tofu studies, daily vitamin D levels in the test diets were similar and thus should not have been a factor contributing to differences in calcium absorption from the test diets.

Urinary Hydroxyproline and Serum Alkaline Phosphatase Data

Mean urinary hydroxyproline and serum alkaline phosphatase data obtained during the Spinach study are presented in Table 13. All 7 subjects excreted more hydroxyproline in the urine (mg/day) during ingestion of the spinach compared to the cheese diet. As a group, mean 24-hour urinary hydroxyproline excretion ($p < 0.01$) and the hydroxyproline/creatinine ratio ($p < 0.05$) were significantly higher during the spinach feeding period compared with the cheese feeding period. Mean serum alkaline phosphatase did not differ significantly between diets.

Table 13
Mean (±SD) Urinary Hydroxyproline, Hydroxyproline/Creatinine Ratio and Serum Alkaline Phosphatase Levels during the Experimental Feeding Periods of the Spinach Study

Variable	Spinach Period	Cheese Period
Hydroxyproline (mg/day)	15.8 ± 4.9[a]	13.4 ± 3.8
Hydroxyproline/creatinine ratio	0.017 ± 0.003[b]	0.013 ± 0.004
Serum alkaline phosphatase (U/L)[c]	26.6 ± 5.3	26.4 ± 4.9

[a]Significant difference between spinach and cheese dietary periods ($p < 0.01$)
[b]Significant difference between spinach and cheese dietary periods ($p < 0.05$)
[c]U/L = International Units

In spite of a statistically significant difference in calcium balance between test diets during the Tofu study, neither 24-hour urinary hydroxyproline, hydroxyproline creatinine ratio or serum alkaline phosphatase levels were significantly different between the tofu and cheese feeding periods. Mean levels for these parameters during the experimental feeding periods of the Tofu study are presented in Table 14.

Table 14
Mean (±SD) Urinary Hydroxyproline, Hydroxyproline/Creatinine Ratio and Serum Alkaline Phosphatase Levels during the Experimental Feeding Periods of the Tofu Study

Variable	Tofu Period	Cheese Period
Hydroxyproline (mg/day)	10.4 ± 2.9	10.2 ± 4.1
Hydroxyproline/creatinine ratio	0.010 ± 0.002	0.010 ± 0.003
Serum alkaline phosphatase (U/L)[a]	26.2 ± 5.1	26.3 ± 5.4

[a]U/L = International Units

Urinary hydroxyproline is a widely accepted measure of bone resorption in humans (Nordin, 1976). Endogenous hydroxyproline is formed from the amino acid proline only after incorporation into polypeptide molecules or proteins. Hydroxyproline is present almost exclusively in collagen (Dull & Henneman,1963), and approximately 40% of the total body collagen is present in skeletal tissue (Smith & Nordin,1964). Since the input into the urinary hydroxyproline pool from soft tissues is relatively constant during consumption of a diet free of hydroxyproline, variation in urinary excretion will normally reflect variation in bone resorption (Nordin,1976). The low hydroxyproline content of the test diets used in both studies should have had little effect on urinary hydroxyproline levels. The potentially confounding effect of dietary hydroxyproline on 24-hour urine levels was further removed by the crossover component of the study design which enabled subjects to consume both dietary treatments in random order. Therefore, the

significant alterations in urinary hydroxyproline observed in the Spinach study probably reflected differences in bone resorption attributed to the high levels of spinach consumption.

Calcium influx from the gastrointestinal tract is an essential element in the maintenance of bone and plasma calcium concentrations. Low input of calcium from the gut, resulting from either low dietary intake or low absorption, could precipitate an increase in bone resorption in order to maintain plasma calcium levels, via increased parathyroid hormone levels in the blood (Heaney, Gallagher, Johnston, Neer, Parfitt, Chir & Whedon,1982). In the Spinach study of the present investigation, significantly higher mean urinary hydroxyproline levels, suggesting increased bone resorption, corresponded with greater negative mean calcium balances among subjects during consumption of the spinach compared to the cheese diet.

Changes in urinary hydroxyproline in response to changes in calcium intake and absorption have been previously reported. Horowitz et al. (1982) reported a significant decrease in mean hydroxyproline/creatinine ratio (.022 vs .017) in 14 postmenopausal women after only 8 days of calcium supplementation at a level of 1 g/day. A change in hydroxyproline ratio of similar magnitude (.017 vs .013) was observed in the present study between values obtained during the ingestion of the spinach and cheese diets. Smith and Nordin (1964) reported significantly decreased mean 24-hour urinary hydroxyproline levels among 42 osteoporotic patients given 1100 mg of supplemental calcium, as calcium glycerophosphate, for 7 days. Urinary hydroxyproline output

was correlated with calcium balance in this population. In contrast, Aloia, Cohn, Zanzi, Abesamis and Ellis (1978) reported no significant alterations in urinary hydroxyproline excretion among 32 postmenopausal osteoporotic patients given 1030 mg of supplemental calcium for periods varying from several weeks to several months.

The majority of studies attempting to relate hydroxyproline metabolism, bone resorption and calcium intake have been conducted in postmenopausal women. The mean age of subjects in the Spinach study (31 yr) was considerably lower than the typical ages of postmenopausal women. Generally, bone density continues to increase until approximately the third decade of life (Heaney et al.,1982). Thus, compared to postmenopausal women, subjects in the Spinach study may have been characterized by a more active state of bone turnover and therefore may have been more sensitive to alterations in dietary calcium with regard to hydroxyproline metabolism. The supposition that younger individuals have more active bone turnover is supported by data which suggest decreasing urinary hydroxyproline levels with age (Laitinen, Nikkila, & Kivirikko,1966; Aloia et al.,1978).

Essentially identical mean urinary hydroxyproline levels were observed for the tofu and cheese dietary periods during the Tofu study, despite significant differences in apparent calcium absorption and calcium balance. This outcome could be attributed to 2 factors. First, as stated earlier, the actual magnitude of difference in mean calcium balance between test diets was much smaller than that observed between the spinach and cheese diets of the Spinach study. If calcium

balance is an approximation of bone balance (Parfitt, 1983) then the relatively small difference in balance figures between diets in the Tofu study may have reflected only a slight difference in bone balance and therefore bone resorption. Secondly, the possibility that individuals characterized by more rapid bone turnover rates were over-represented in the Spinach study or underrepresented in the Tofu study cannot be discounted in light of the small sample sizes and nonrandom selection of subjects.

Serum alkaline phosphatase levels were not affected by experimental treatments within either the Spinach or Tofu study and similar levels were observed in the 2 studies. Alkaline phosphatase has been demonstrated to be a valid marker of bone turnover among patients diagnosed with bone disorders characterized by severe bone matrix or bone mineral degeneration (Klein et al.,1964; Nordin,1976). This indice has also been found to be correlated with changes in urinary hydroxyproline (Klein et al.,1964). Since alkaline phosphatase is also present in several other tissues of the body in large concentrations (Klein et al., 1964),marked changes in bone turnover rates, as in cases of metabolic bone diseases, may be necessary to elicit significant changes in serum alkaline phosphatase concentration.

Several studies have reported no differences in serum alkaline phosphatase levels between individuals with osteoporotic-related bone loss and normal controls. Nilas, Christiansen and Rødbro (1984) demonstrated no differences in serum alkaline phosphatase in 103 post-

menopausal women stratified into groups based on rate of bone loss. Bone loss was determined using photon absorptiometry over a 2-year period. In another study (Klein et al.,1964), no differences were found in mean serum alkaline phosphatase levels between 3 normal ($\bar{x}$ age = 62 yr) and 5 osteoporotic ($\bar{x}$ age = 64 yr) individuals.

CHAPTER V

SUMMARY, CONCLUSIONS AND SUGGESTIONS FOR FUTURE RESEARCH

Summary and Conclusions

Calcium balance data from the present study suggested that the calcium availability from cheese was superior to that from both spinach and tofu. Although the difference in mean calcium balance between the spinach (-168 mg/day) and cheese (-55 mg/day) dietary periods was not statistically significant, subjects consuming the spinach diet had an average net calcium loss from the body of 113 mg per day more than that during the cheese diet. Urinary calcium excretion was significantly higher during ingestion of the cheese- compared to the spinach-containing diet, further supporting the possibility of increased intestinal calcium absorption from cheese. The adverse effect of spinach ingestion upon calcium balance observed in the present study is consistent with findings from other studies in which over 200 g of spinach was fed daily. Other studies have suggested that spinach consumed in more typical quantities (i.e., less than approximately 200 g/day, less than 7 days/week) has little effect on overall calcium balance. However, there is still the possibility that the calcium furnished by smaller doses of spinach is poorly absorbed.

The relatively poor availability of calcium from spinach can most likely be attributed to the high levels of both oxalate and fiber present in spinach. The high oxalate content of the spinach diet (2555 mg/day) relative to the cheese diet (99 mg/day) resulted in significantly greater

mean fecal oxalate (322 vs 13 mg/10 g dry wt) and urinary oxalate levels (101 vs 17 mg/day) during the spinach compared to the cheese consumption periods.

Ingestion of the spinach-containing diet resulted in a large intersubject variation in fecal and urinary oxalate levels reflecting varying degrees of intestinal oxalate degradation. Corrected fecal oxalate per day was significantly correlated ($r_s = 0.71, p < 0.05$, Spearman rank correlation statistic) with mean fecal calcium per day for subjects during the spinach dietary period. Subjects could be categorized into high and low relative excreters of oxalate during spinach consumption. Subjects exhibiting high excretion levels of oxalate (n=3) had a much lower mean calcium balance (-256 mg/day) compared with subjects (n=4) characterized by relatively low oxalate excretion levels (-103 mg/day). These data suggested that during high oxalate feeding, fecal calcium and calcium balance could be related to rates of oxalate degradation in the gut.

The experimental design of the present study did not allow a differentiation between the individual effects of fiber and oxalate of spinach upon calcium absorption. However, given the demonstrated inhibitory effect of fiber on calcium absorption and the dramatic contrast in fiber content between the spinach and cheese servings, it appears likely that fiber played a significant role in decreasing the availability of calcium from the spinach diet.

Mean calcium balance and fecal calcium during ingestion of the tofu diet (-135 & 824 mg/day) were significantly different from levels observed on the cheese diet (-73 & 767 mg/day), suggesting that the calcium availability from cheese was superior to that from isocalcic quantities of tofu. In addition to fiber, which is absent from tofu, the primary dietary factor with the potential for inhibiting calcium absorption from soy products is phytate. Phytate intake during ingestion of the tofu diet was higher than that provided by the cheese diet (1900 vs 1130 mg/day) which may have contributed to the lower calcium balances observed during consumption of the tofu compared to the cheese diet.

Three subjects did equally well or better in terms of calcium balance on the tofu compared to the cheese diet. Mean urinary phosphorus for this group was higher (628 mg/day) than the level for the remaining subjects (458 mg/day), who apparently utilized the calcium from tofu to a lesser extent. Higher urinary phosphorus levels may reflect a greater catabolism of phytate in the gut, such that phosphorus and calcium associated with phytate may eventually be released and become available for absorption. A greater absorption of phosphorus from the cheese- compared to the tofu-containing diet was suggested by a significantly higher mean urinary phosphorus level during the cheese- (620 mg/day) compared to the tofu-feeding period (515 mg/day).

Twenty-four hour urinary hydroxyproline and hydroxyproline/ creatinine ratio, indirect measures of bone resorption, increased

significantly in conjunction with consumption of the experimental spinach diet relative to levels observed during consumption of the cheese diet. These data suggested that an increase in bone resorption may have occurred in response to the feeding of large amounts of spinach and are consistent with the contention that spinach derived oxalate and/or fiber can decrease overall calcium availability. No significant differences in 24-hour urinary hydroxyproline or hydroxyproline/creatinine ratio were observed between ingestion of the tofu and cheese diets of the Tofu study. Serum alkaline phosphatase did not differ significantly between test diets in either the Spinach or Tofu study.

Suggestions for Future Research

The recent discovery of oxalate degrading bacteria (Oxalobacter formigenes)in the gastrointestinal tract of humans has presented researchers with a known mode by which humans can degrade dietary oxalate and possibly utilize calcium from oxalate-rich plant foods. Subjects in the present study clearly differed in oxalate degrading capacity during consumption of the spinach diet which suggested differences in the concentration and activity of oxalate degrading bacteria in the gut. The activity of these bacteria may be a primary factor determining the ability of an individual to utilize calcium, and perhaps other minerals, from oxalate-mineral salts present in plant foods. Changes in oxalate degrading bacterial growth and activity in humans in response to high-oxalate feeding needs to be studied by quantifying oxalate degrading bacteria. There is also a need for research assessing the possibility of long term adaption to

high-oxalate feeding. Likewise, little research has been conducted regarding the effects of dietary factors that may influence luminal conditions which may affect Oxalobacter formigenes growth and activity such as other dietary minerals besides calcium, fiber (quantity and type), antibiotics (which destroy intestinal bacteria), and foods that may have an effect on luminal pH.

Additional research is needed regarding calcium availability from soybean products. Products like tofu and soymilk contain no fiber and therefore would be expected to provide a more available source of calcium than whole soybeans which contain fiber. Enzymes capable of catabolizing phytate have been demonstrated in the gut of humans, yet minerals bound in phytate complexes appear to be poorly available. Dietary as well as nondietary factors affecting phytate degrading enzyme activity and the capacity for long term adaption to high phytate feeding in human populations need to be further explored.

The enigmatic relationship between calcium intake, calcium balance and bone mineral content requires additional clarification. Data suggest that calcium intake after menopause may have less of an effect on bone density and rates of bone mineral loss than was originally suspected. Some researchers have suggested that calcium nutrition before menopause, during the bone forming decades, may be more predictive of risk of bone mineral depletion in old age. The recent recommendation by health professionals to increase calcium intake from the current RDA of 800 mg to 1000, or even 1200 mg per day for some individuals, is consistent...

with data from the present studies. Only 5 out of 16 women maintained either positive or near zero mean calcium balances during the 2 cheese dietary periods of the Spinach and Tofu studies, even though calcium intakes were above 800 mg per day and came primarily from cheese which is considered a highly available calcium source.

Lastly, relationships between adequacy of dietary calcium, calcium availability and calcium metabolism are intimately related to important endocrine systems in the body. It is assumed that the effect of dietary calcium intake on bone resorption is mediated through changes in blood parathyroid hormone and calcitonin levels. Research assessing the effect of dietary calcium, as well as other dietary components, meal composition, and meal and snacking patterns on daily or even hourly fluctuations in plasma PTH concentration would be extremely useful.

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APPENDIX A

CONSENT FORM

Consent for Participation in
Study of Calcium Bioavailability

Department of Food, Nutrition, and Food Service Management
School of Home Economics
University of North Carolina at Greensboro

I have received an explanation of the nutrition study to be conducted at the University of North Carolina-Greensboro entitled "A Comparative Study of Calcium Bioavailability from Cheese, Spinach, and Soybean Curd (Tofu) as Assessed by Calcium Balance in Adult Women." The project will be directed by Dr. Michael Liebman, faculty member in the Department of Food, Nutrition, and Food Service Management. Other investigators include William Landis, Carolyn Dunn, and Linda Meredith.

The primary study objectives are: (1) to determine the relative bioavailability of calcium provided by tofu and by spinach in comparison to calcium provided by cheese and (2) to determine whether adult women can maintain calcium balance on semi-vegetarian diets containing approximately 800 mg of calcium, the majority of which is provided by cheese, tofu, or spinach. Secondary objectives include determinations of whether plasma lipid alterations occur in response to feeding high levels of spinach or high levels of tofu and a relative assessment of zinc and iron bioavailability from semi-vegetarian diets.

As a participant in this metabolic feeding study, I understand that I will consume a rigidly controlled diet for a period of 9 weeks and will not be allowed to consume any foods or beverages other than those dictated by the study design. Although the diets which will be fed are concentrated in nutrient dense foods, I am aware that there exists the possibility that dietary intakes may not meet the Recommended Dietary Allowances for all nutrients. With the exception of an iron supplement given during the cheese consumption period, no dietary supplements will be administered. I also understand that I will collect all urine and fecal samples during weeks 3-4 and 7-9 of the 9-week study; nonabsorbable fecal markers will be administered on certain days to mark the beginning and end of the fecal collection periods. In addition, I am aware that I will be asked to donate 14-ml blood samples after an overnight fast on 6 occasions for the analysis of specific biochemical indices. I am also aware that I will be given the opportunity to participate in two iron tolerance tests (to assess iron availability from spinach) which will involve two additional blood donations per test.

The potential risks associated with participation in this study include fainting, bruising or infection from the blood drawing and some degree of psychological stress which could result from the required adherance to the study design (i.e., strictly controlled feeding and periodic collection of urine and fecal samples). There is also a very slight risk that the use of fecal markers could cause a certain degree of gastrointestinal distress in some individuals. I realize that all possible precautions will be taken to minimize these risks (e.g., use of only highly trained personnel and established protocols for the blood drawing), and that I can voluntarily withdraw from the study at any time of my choosing without incurring prejudice from the investigators.

The benefits I may gain from the study include the evaluation of numerous nutrition status parameters. This project will increase nutritionist's knowledge of: (1) the relative bioavailability of calcium provided by tofu and by spinach in comparison to calcium provided by cheese, and (2) the plasma lipid alterations which occur in response to feeding high levels of spinach or high levels of tofu.

I understand that all information will be considered private, will be treated confidentially, and that my identity will not be revealed. Dr. Liebman or one of the other members of the research staff will be available to answer questions that I may have regarding the study. They may be reached at the UNC-G Nutrition Department on weekdays at 379-5313. All of my immediate questions have been answered.

Understanding the above, I agree to participate.

Date	Signature, subject	Signature, witness

Social security number
(subject)

APPENDIX B

PREEXPERIMENTAL QUESTIONNAIRE

Pre Experimental Questionnaire

NAME:_____AGE_____PHONE_____BIRTH DATE_____

ADDRESS_____ OCCUPATION_____

_____

MEDICAL HISTORY

1.Do you have any of the following disorders?

Diabetes yes no
CHD yes no
Hypertension yes no
Hyperlipidemia yes no
Metabolic Disorders yes no
Gastrointestinal Disorders yes no
Renal Disease yes no
OTHER (please specify)_____

2.Are your bowel habits normal?(i.e., frequent constipation or diarrhea) Explain:

_____

3.Are you on any medications?_____

4.Do you take any vitamin, mineral, or protein supplements?

BRAND	AMOUNT TAKEN	FREQUENCY
_____	_____	_____
_____	_____	_____

5.Do you consume alcohol? _____never_____beers/week_____wine/week_____liquor/week

6.Do you smoke tobacco?____no____yes____packs/week

7.Do you smoke marijuana?____no____1 or more times/week___1-3 times/month___1 time/month

8. How often do you get your period? every____days

9.Do you ever skip periods on a regular basis? yes no explain:_____

_____

10.Usual # of flow days_____

11.Are your periods usually____heavy____moderate or ____light?

12.What is the usual course of your periods?____painless____cramps____varies(explain)

_____

13.Number of past pregnancies____Dates of past pregnancies___to___, ___to___

Pre Experimental Questionnaire (cont.)

14. Are you a former oral contraceptive user?_____no _____yes
From ____to____, ____to____

15. Check the corresponding column indicating the frequency with which you participate in any of the following (if never leave blank).

	4-7 days/wk	1-3 days/wk	occasionally	
Running	_____	_____	_____	miles/week____
Swimming	_____	_____	_____	miles/week____
Biking	_____	_____	_____	miles/week____
Aerobic Dance	_____	_____	_____	
Weight Training	_____	_____	_____	
Team Sports	_____	_____	_____	
Racket Sports	_____	_____	_____	
Other(specify)_____	_____	_____	_____	

16. Do you have any strong food dislikes?_____

17. Is there any family history of osteoporosis or frequent bone fractures?
_____

18. What time would be most convenient for you to have breakfast?_____
dinner ?_____

19. Estimate your consumption of the following foods:*

		Col. II
Soft Cheese	_____	oz/wk
Hard Cheese	_____	oz/wk
Cottage Cheese	_____	cups/wk
Yogurt	_____	cups/wk
Milk	_____	cups/wk
Ice Cream	_____	cups/wk
Eggs	_____	/wk
Tofu	_____	oz/wk
Spinach	_____	cups/wk
Other Green Leafy Veg. (i.e., collards, mustard greens, etc.)	_____	cups/wk

* If food isn't consumed on a weekly basis use col II to estimate monthly consumption

APPENDIX C

DAILY QUESTIONNAIRE

NAME_____

SUBJECT ID#_____

DATE_____

DAILY QUESTIONNAIRE

1. How many hours of sleep did you get yesterday?_____

2. How did you feel physically yesterday?

_____much better than usual
_____better than usual
_____as usual
_____poor
_____very poor

If poor or very poor, please explain:_____

_____

3. Did you engage in any planned physical activity yesterday (e.g. running, swimming, etc.)? If yes,describe activity, duration, and intensity._____

4. Was your day_____more active than usual_____as usual_____less active than usual?

5. Were yesterday's meals satisfying to you?_____

6. Did you experience any nausea or gastrointestinal problems?

_____

7. Are your urine and fecal samples from yesterday complete?

_____

8. Approximate yesterday's consumption of the following:

	NUMBER OF CUPS	AMOUNT AND KIND OF SWEETNER
Coffee	_____	_____
Tea	_____	_____
Herbal Tea	_____	_____
Soft Drinks	_____	_____

9. Are you becoming discouraged with the study or are there any problems? If yes, please indicate how we can correct this problem._____

10. Additional Comments: (menstrual function and medications)

APPENDIX D

FECAL PROCESSING AND PEG DETERMINATION PROCEDURE

I. Processing of Fecal Sample

1. Cut seams of polyethylene sample bag using scissors and empty contents into pre-weighed stainless steel blender. Weigh (minus blender wt.) and record.
2. Add to blender 2 to 4 times the fecal sample's weight in distilled water. Calculate beforehand the approximate weight of blender, feces and water together. With the blender and feces on a balance, add water to determined mark, transfer to blender base, homogenize, and add additional water to slurry if necessary. Slurry should be completely homogenized and assume a milkshake-like consistency. Record final slurry weight (minus blender weight).
3. Pour homogenized sample into 2 "etched" 150 ml beakers:
   A. Record beaker weight empty.
   B. "zero" beaker weight on balance
   C. Pour slurry into beakers - Wet sample weight should fall in the range of 4.50-6.00 g. This will vary according to the consistency of the slurry (ie, thick slurry-low sample weight, thin,watery slurry-higher sample wt). This will result in a dried slurry sample in the range of .35 - .60 g, suitable for direct ashing and dilution for AAS.
4. Place samples in oven at 60° - 70° C for 2 days (this was usually sufficient for complete drying). Pour approx. 50 ml of slurry into container for frozen storage.

II. Obtaining Sample Aliquots for PEG Determination

1. Into a "zeroed" unmarked 150 ml beaker pour 3.0 g of slurry (try to obtain aliqout within 2.95 - 3.05 g) and record in PEG Chart(Dilution weight) Using a 10 ml capacity auto-pipette, add exactly 6 ml of distilled water to sample, and swirl beaker till completely incorporated and homogenized. This step creates a 2:1 dilution (3X), water to slurry. At this point the original slurry is considered "the fecal sample". and therefore, this step is the first dilution of the fecal sample. A 3:1 dilution (adding 9 ml water) may sometimes be necessary if the original slurry is unsually thick. In this situation, record under Dilution weight 3:1 or 4X by the subject as a reminder for calculation purposes.
2. Label 2 Erlenmeyer flasks (50 ml) A & B, or 1 & 2, along with subject number.
3. Pour 1.0 g (.96 - 1.05 g) of slurry from step 1 into zeroed Erlenmeyers and cap.Record exact sample weight in PEG chart (Test weight).
4. Dispose of remaining sample and clean containers and equipment after each subject and use distilled water for final rinse.

III. PEG Standard Solutions and Reagents

1. Make 2% w/v (2 g/100 ml) PEG solution using distilled water and store at 4°C in dark. Shake vigorously before each use. Stock solution is good for 2 months (duration of balance study).
2. Standards:

20 mg/ml Stock Solution

0.125 ml	+	4.875 ml H_2O	=	0.5 mg/ml PEG
0.250 ml	+	4.750 ml H_2O	=	1.0 mg/ml PEG
0.375 ml	+	4.625 ml H_2O	=	1.5 mg/ml PEG
0.500 ml	+	4.500 ml H_2O	=	2.0 mg/ml PEG
0.750 ml	+	4.250 ml H_2O	=	3.0 mg/ml PEG
1.000 ml	+	4.000 ml H_2O	=	4.0 mg/ml PEG
2.000 ml	+	3.000 ml H_2O	=	8.0 mg/ml PEG
3.000 ml	+	2.000 ml H_2O	=	12.0 mg/ml PEG
3.500 ml	+	1.500 ml H_2O	=	14.0 mg/ml PEG

3. Reagents:

A. $ZnSO_4 \cdot 7H_2O$	5% w/v	8.92 g/100 ml H_2O (make large quantities)
B. $Ba(OH)_2$	0.3 N	25.70 g/ 1 L
C. $BaCl_2 \cdot 2H_2O$	10% w/v	30.30 g/ 250 ml
D. Gum Arabic		12.0 mg/1 L
E. Trichloroacetic Acid		30% w/v

IV. PEG Analysis:

1. To 1.0 ml H_2O (blank), 1.0 ml Standards amd 1.0 ml fecal homogenate in 50 ml Erlenmeyer flasks add:

   10 ml H_2O
   1.0 ml of 10% $BaCl_2$ anhydrous Mix by swirling after each addition
   2.0 ml of 0.3 N $Ba(OH)_2$

2. Add 2.0 ml of 5% $ZnSO_4 \cdot 7H_2O$

   Cap flasks and shake vigorously. Let stand for 10 minutes. While waiting, label and set up test tubes, funnels and filter paper.

3. Filter contents through single thickness Whatman no. 42 filter paper.

   Filtrate should be clear but can be discolored.

4. Transfer 1.0 ml aliquots of filtrate to 16 x 150 mm test tubes.

5. Add 3.0 ml of Gum Arabic solution and mix by inversion.

6. Add 4.0 ml of 30% TCA solution and mix by inversion 8 to 10 times.

   Differences in cloudiness among standards should be detectable after 10 - 15 minutes. Blank should display no emulsion or cloudiness properties.

7. After 60 minutes, read absorbance against blank at 650 nm.

   The emulsion was stable for at least 90 minutes. Record Absorbance in PEG Chart. Construct PEG standard curve - the line is generally curved between PEG concentrations 0.0 and 1.5, tending to straighten thereafter. Record PEG concentration of sample in PEG Chart.

V. Total PEG Determination

1. Determine exact dilution factor used for sample by dividing 9 by Dilution weight (eg, 9/2.99 = 3.01). Multiply PEG concentration ([ ]) off the standard curve by this factor. If the dilution was 3:1, divide Dilution weight into 12 (eg, 12/2.99 = 4.013). This corrects for the inaccuracies occurring during the weighing of test samples.
2. Determine Corrected Total weight for each sample by dividing (slurry) Total weight by Test weight (eg, 166/1.03 = 161.2 g).
3. For the final quantity of PEG per fecal sample, multiply Corrected Total weight by Undiluted [ ] . Average to the nearest gram.

APPENDIX E

URINARY HYDROXYPROLINE PROCEDURE

HYDROXYPROLINE METHOD

Pipet the following into screw-top tubes with 15 ml mark:
1 ml water (blank) OR
1 ml working standard (standard) OR
1 ml urine (samples)
Add 1 ml HCL (concentrated) to each tube.
Loosely screw caps on tubes. Pressure cook for 3-4 hrs at 10-15 lb pressure.

1. Add 1 ml 12N KOH to each tube.
2. Dilute with water to the 15 ml mark.
3. Mix. Centrifuge. (May want to transfer to wider-topped test tubes before centrifuging in order to facilitate next step).
4. Pipet 3 ml aliquot from each tube into Erlenmeyer flasks (in duplicate).
5. Add 1 ml water to each flask to bring volume up to 4 ml.
6. Add 1 drop phenolphthalein solution and adjust to a pale pink color with .05N KOH or .05N HCL. (May want to have other dilutions available).
7. Saturate the solution with approximately 3 g KCl.
8. Pipet 0.5 ml alanine reagent and 1.0 ml potassium borate buffer into all tubes. Mix well and allow to stand at room temperature 20-30 minutes with occasional mixing.
9. Add 1.0 ml 0.2M chloramine-T solution and mix immediately. Let stand for 25 minutes with occasional mixing.
10. Add 30 ml 3.6M sodium thiosulfate. Mix and add 5.0 ml toluene. Cap tightly.
11. Place on mechanical shaker and shake for 5 minutes.
12. Aspirate toluene layer and discard. Replace lids.
13. Place in boiling water bath for 30 minutes.
14. Cool flasks in ice water bath (or with running tap water). Add 5.0 ml toluene. Recap and shake for 5 minutes. Pour samples from flasks into large test tubes.
15. Pipet exactly 2.5 ml sample from each large test tube into spec tubes.
16. Add 1.0 ml Ehrlich's reagent while mixing rapidly. (Do not transfer any of the aqueous layer as it will cause color to fade.
17. Let stand for 30 minutes. Read absorbance against toluene blank 560 nm in a spectrophotometer.

APPENDIX F

RAW DATA

Descriptive Data for Spinach Study Subjects at Baseline

Subject	Age(yr)	Weight(kg)	Height(cm)	BMI(kg/m^2)[a]	Treatment Order
1	41	75.7	158.7	30.0	Cheese-Spinach
2	42	60.2	167.6	21.4	Cheese-Spinach
4	20	65.2	160.0	25.5	Cheese-Spinach
5	21	64.2	160.0	25.1	Cheese-Spinach
7	36	56.4	172.7	18.9	Spinach-Cheese
8	31	53.6	157.0	21.7	Spinach-Cheese
9	24	63.6	170.2	22.0	Spinach-Cheese

[a]BMI = Body Mass Index

Descriptive Data for Tofu Study Subjects at Baseline

Subject	Age(yr)	Weight(kg)	Height(cm)	BMI(kg/m^2)[a]	Treatment Order
1	31	72.3		24.7	Cheese-Tofu
2	28	72.3		27.2	Cheese-Tofu
3	24	61.6		21.1	Cheese-Tofu
5	20	60.5		22.2	Cheese-Tofu
6	20	54.8		20.6	Tofu-Cheese
8	21	51.9		19.5	Tofu-Cheese
9	31	52.5		20.7	Cheese-Tofu
10	26	57.9		19.8	Tofu-Cheese
11	39	58.7		25.7	Tofu-Cheese

[a]BMI = Body Mass Index

Individual Fecal Dry and Wet Weights, Moisture Content and Defecation Frequency Data for Subjects in the Tofu Study

Subject Number	Treatment Group[a]	No of Days with Sample[b]	Wet Weight (g/day)	Dry Weight (g/day)	Moisture (%)
1	C	14	116.3	29.9	74.9
2	C	6	173.5	52.2	69.9
3	C	14	74.4	25.1	65.8
5	C	13	171.4	45.3	72.9
6	C	12	107.6	37.1	64.8
8	C	12	161.1	40.0	74.3
9	C	8	192.4	56.5	70.1
10	C	13	152.8	44.7	70.9
11	C	14	174.0	34.7	80.4
1	T	12	161.6	37.9	76.1
2	T	7	119.7	41.1	65.0
3	T	12	90.2	29.1	67.0
5	T	11	176.4	49.2	71.5
6	T	10	102.9	32.7	67.7
8	T	12	194.7	44.3	76.9
9	T	13	152.4	41.2	72.1
10	T	13	128.2	36.5	71.5
11	T	13	163.2	35.1	78.1

[a]C = Cheese Dietary Treatment; T = Tofu Dietary Treatment

[b]Values represent the number of days during each 14-day balance period in which fecal samples were collected

Individual Fecal Dry and Wet Weights, Moisture Content and Defecation Frequency Data for Subjects in the Spinach Study

Subject Number	Treatment Group[a]	No of Days with Sample[b]	Wet Weight (g/day)	Dry Weight (g/day)	Moisture (%)
1	C	9	62.9	165.8	62.1
2	C	12	33.2	104.1	68.1
4	C	10	36.1	140.2	74.3
5	C	13	32.4	163.1	80.1
7	C	12	30.7	111.5	72.5
8	C	7	49.5	214.7	76.9
9	C	11	25.7	87.0	70.5
1	S	11	69.2	314.3	78.0
2	S	12	44.3	140.3	68.4
4	S	11	51.4	186.2	72.3
5	S	14	43.3	178.5	75.7
7	S	11	41.5	163.9	74.7
8	S	9	61.0	234.8	74.0
9	S	14	31.1	126.3	75.4

[a]C = Cheese Dietary Treatment; S = Spinach Dietary Treatment

[b]Values represent the number of days during each 14-day balance period that fecal samples were collected

Individual Urinary Hydroxyproline, Creatinine and Serum Alkaline Phosphatase Data during the Cheese (C) and Spinach (S) Test Periods for the Spinach Study

Subject Number	Treatment Group	Urinary Hydroxyproline (mg/day)[a]	Urinary Creatinine (mg/day)[a]	Serum Alkaline Phosphatase (U/L)[b]
1	C	8.6	825	28.9
2	C	11.0	1018	22.9
4	C	12.5	1150	26.1
5	C	18.2	1117	24.9
7	C	10.2	1520	22.2
8	C	15.4	1061	23.6
9	C	18.1	1037	36.4
1	S	10.1	665	31.1
2	S	12.8	998	23.2
4	S	13.3	722	29.6
5	S	20.7	1060	30.0
7	S	13.2	932	19.8
8	S	16.8	1050	20.6
9	S	23.7	1245	32.2

[a]Mean of the last 3 days of each test period

[b]U/L = International Units

Individual Urinary Hydroxyproline, Creatinine amd Serum Alkaline Phosphatase Data during the Cheese (C) and Tofu (T) Test Periods for the Tofu Study

Subject Number	Treatment Group	Urinary Hydroxyproline (mg/day)[a]	Urinary Creatinine (mg/day)[a]	Serum Alkaline Phosphatase (U/L)[b]
1	C	6.9	947	27.1
2	C	11.1	1234	30.1
3	C	19.3	1187	31.2
5	C	8.0	899	22.7
6	C	12.1	979	25.8
8	C	8.2	815	19.8
9	C	9.7	1168	18.4
10	C	11.3	1323	35.1
11	C	5.5	745	26.1
1	T	7.7	1135	29.7
2	T	6.6	703	29.2
3	T	15.2	1102	33.2
5	T	10.2	1350	28.6
6	T	13.6	994	20.2
8	T	10.2	989	19.6
9	T	12.6	1130	19.7
10	T	10.6	1229	29.8
11	T	7.3	793	25.3

[a]Mean of the last 3 samples collected at the end of each test period.

[b]U/L = International Units

Individual Total Calcium Intake, Excretion and Balance Data during the Cheese (C) and Spinach (S) Test Periods for the Spinach Study

Subject Number	Treatment Group	Balance Duration[a] (days)	Fecal Calcium (mg)	Corrected Fecal Calcium (mg)	Urinary Calcium (mg)	Dietary Calcium (mg)	Calcium Balance(mg)	
							Overall	Daily
1	C	5	5035	3701	748	4280	-169	-34
2	C	9	7853	6597	1662	7704	-555	-62
4	C	5	4768	2928	670	4280	682	136
5	C	6	4236	3063	1344	5136	729	121
7	C	7	6692	5855	1396	5992	-1259	-180
8	C	7	8326	5295	2525	5992	-1828	-261
9	C	11	9897	8571	2006	9416	-1161	-106
1	S	10	10705	8072	726	7830	-968	-97
2	S	5	5136	2963	615	3915	337	67
4	S	4	4060	3447	324	3132	-639	-160
5	S	14	12759	16025	2086	10962	-7149	-511
7	S	5	4686	3327	680	3915	-92	-18
8	S	4	4467	3413	884	3132	-1165	-291
9	S	13	10610	10313	2068	10179	-2209	-169

[a]Number of days in which calcium balance was based

Individual Total Calcium Intake, Excretion and Balance Data during the Cheese (C) and Tofu (T) Test Periods for the Tofu Study

Subject Number	Treatment Group	Balance Duration[a] (Days)	Fecal Calcium (mg)	Corrected Fecal Calcium (mg)	Urinary Calcium (mg)	Dietary Calcium (mg)	Calcium Balance(mg)	
							Overall	Daily
1	C	7	4272	4956	1372	6409	81	12
2	C	6	7861	4874	702	5576	-20	-3
3	C	10	8363	8973	1701	8910	-1764	-176
5	C	6	4406	4582	1368	5208	-742	-124
6	C	10	8103	6644	2860	8838	-666	-67
8	C	10	8773	6492	1164	9100	1444	144
9	C	2	2605	1485	802	1806	-481	-240
10	C	5	3993	4672	1405	4665	-1412	-282
11	C	10	7737	7273	1078	9168	817	82
1	T	12	8435	9869	2880	10944	-1805	-150
2	T	7	7176	5956	753	6384	-325	-46
3	T	11	8895	10763	2347	9878	-3232	-294
5	T	8	5843	7537	1719	7040	-2216	-277
6	T	10	7596	7064	2598	9274	-388	-39
8	T	6	5517	4248	863	5460	349	58
9	T	10	5420	6558	4280	8915	-1923	-192
10	T	12	11166	11724	3032	11196	-3560	-297
11	T	11	8564	8564	1215	10065	286	26

[a]Number of days in which calcium balance was based