Effect of Soybean Phytate Content on Calcium Bioavailability in Mature and Immature Rats†

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The effect of phytate content on calcium bioavailability from soy flour and isolated soy protein was studied in infant and adult rats. Soybeans were grown hydroponically in nutrient culture containing various levels of phosphorus to produce a range in phytate content and simultaneously labeled with ^{45}Ca. Absorption of ^{45}Ca from test meals prepared from the labeled soy flour or from formula prepared from isolated soy protein was calculated by comparing the accumulation of radionuclide by femurs and tailbones after the labeled test meal consumption to the accumulation of radioactivity by the target bones after intraperitoneal injection of label where the intestinal barrier is bypassed. Although dephytinizing resulted in the soy flour with the most available calcium, calcium absorption from the various flours was high and differences were generally small. Varying the concentration of endogenous phytate in soybeans had little effect on calcium absorption. Absorption of ^{45}Ca from isolated soy protein was very high in infant rats compared to that in mature rats.

Soy is recognized as a nutritionally significant food product, but because of its high phytate content the inclusion of soy in the diet may compromise mineral nutriture (Allen, 1982; Cheryan, 1980; Erdman, 1979). In general, phytate is considered to inhibit mineral absorption by forming phytate–mineral complexes. It is the insolubility of these complexes at intestinal pH (Graf, 1983; Zemel, 1984), as demonstrated in vitro, that potentially inhibits absorption of minerals such as calcium, zinc, and iron.

Calcium absorption and utilization from diets containing added sodium phytate, whole wheat, oatmeal, unpolished rice, and soy products (McCance and Widdowson, 1942a,b; Harrison and Mellanby, 1939; Henry and Kon, 1945; Mollgaard et al., 1946; Walker et al., 1948; Schroeder et al., 1946; Cullumbine et al., 1950), all high in phytate phosphorus, have been reported to be reduced when assessed by the traditional balance technique in both animals and humans. Balance techniques, however, cannot distinguish calcium from a specific food source from the rest of the diet. Another confounding factor is that endogenous secretions of calcium cannot be distinguished from unabsorbed calcium, resulting in an overestimation of excretion. More recently, the use of radioactive or stable calcium isotopes has provided a method that allows for a more direct measure of bioavailability (Weaver, 1985).

Another limitation of some of the early investigations, and more recently in vitro studies (Graf, 1983; Zemel, 1984), is the addition of sodium phytate to the diet or test medium. In vitro studies provide valuable information about the chemical nature of phytate, and they provide relative biological significance.

The purpose of this study was to determine the effect of various endogenous levels of phytate in soy flour test meals and formula prepared from isolated soy protein on calcium bioavailability in rats. Endogenous phytate levels in soybeans were manipulated by altering the total phosphorus present in the nutrient solution of the growing soybean plants. A second objective was to compare calcium absorption in immature and mature rats.

METHODS

Preparation of Labeled Soybean Products with Various Phytate Contents. Two-week-old soybean seedlings (*Glycine max* L. Merr. Century) were transplanted to 16-L pots containing Chaney's nutrient solution (Weaver, 1985) with 3, 6, or 31 μg/g phosphorus. The plants were grown in a noncirculating hydroponic system as described by Weaver (1985). Starting at 6 weeks of age, ^{45}Ca as $^{45}CaCl_2$ (1.85–3.7 MBq/pot) was added weekly for 6 weeks. The soybeans, harvested at maturity, were processed to a dehulled, defatted flour (Levine et al., 1982) for experiment 1 or isolated soy protein (Weaver et al., 1984). Due to low remaining levels of ^{45}Ca radioactivity, isolated soy protein was extrinsically labeled with $^{45}CaCl_2$ by dropwise addition of an aqueous solution of the label to aliquots of the isolated protein, allowing the label to dry and thoroughly mixing the isolated protein with a mortar and pestle.

Preparation of Dephytinized Soy Flour. To prepare a low phytate soy flour, the flour processed from beans grown in 6 μg/g phosphorus nutrient medium was treated with phytase (*myo*-inositol-hexakisphosphate 6-phosphohydrolase, Sigma Chemical Co., St. Louis, MO). A slurry of 10% soy flour was prepared and the pH adjusted to 5.15. Phytase was added at 5% of protein and the mixture stirred for 3 h at 54 °C. The reaction was stopped by heating for 10 min at 65 °C. The mixture was freeze-dried.

Rat Feeding Studies. *Experiment 1. Calcium Bioavailability from Soy Flour in Mature Rats.* Forty-two male Sprague-Dawley rats, 6 weeks old (150–170 g) (Harlan Industries, Indianapolis, IN), were individually housed in stainless steel cages on a controlled 12-h light–dark cycle. The animals were fed a basal soy-based diet containing 20% protein from soy flour, 8% fat from corn oil, 5% nonnutritive fiber, and vitamins and minerals to conform with the AIN 76A diet prescription ad libitum for 6 days followed by 5 days of meal training by fasting for 12-h each day. On day 12 after a 12-h fast, 3-g test meals prepared using the labeled flour (Table I) were fed to four groups ($n = 8$) of rats. A fifth group ($n = 10$) received an intraperitoneal injection of 18.5 Bq of ^{45}Ca in 0.5 mL of 0.9% saline 2 h after receiving an unlabeled 3-g meal of the basal diet. The amount of radioactivity provided adequate counts without perturbing normal calcium levels in the animals.

Experiment 2. Calcium Bioavailability from Formula Prepared from Isolated Soy Protein in Immature Rats. Mid-cycle pregnant (approximately 9 days of gestation) Sprague-Dawley rats (Harlan Industries) were housed in individual Nalgene

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Table I. Composition of Test Meals (Experiment 1)[a]

component (g)	dephytinized[b] (6 μg/g)[c]	low phytate (3 μg/g)[c]	medium phytate (6 μg/g)[c]	high phytate (31 μg/g)[c]
sucrose	1.71	1.71	1.71	1.71
soy flour (^{45}Ca labeled)	0.9	0.9	0.9	0.9
fiber (Alphacel)	0.15	0.15	0.15	0.15
corn oil	0.24	0.24	0.24	0.24
by analysis				
total Ca, mg	3.7	4.1	4.1	3.1
^{45}Ca, Bq[d]	2.2	2.2	2.2	1.48
protein, mg	520	590	541	494

[a] Amount of each component in 3 g of test meal. [b] Dephytinized flour prepared as described in the text. [c] The level of phosphorus in the nutrient culture of hydroponically grown soybeans. [d] Level of activity at the time of administration of test meals.

maternity cages in a temperature- and light-controlled room (23 °C, 12-h light–dark cycle), allowed free access to deionized water, and fed a pelleted stock diet (Wayne Rodent Blox 8604-00, Wayne Animal Research Diet, Chicago, IL).

At delivery, pups were allowed mother's milk at will. Two days after birth, rat pups were distributed for suckling among the mothers to maintain a litter size of eight to nine pups until the beginning of the study. Weights were taken daily both as a primary index of health status and as a means of identification.

On day 14 postpartum, pups were separated from dams with each treatment group housed in a separate Nalgene cage and fasted for 18 h. Pups were randomly assigned to two treatment groups (n = 10) and gastrically intubated with 1 mL of ^{45}Ca-labeled test formula using a 1-mL tuberculin syringe and an 8-cm animal feeding needle with a ball diameter of 2.25 mm (American Scientific Products, McGaw Park, IL). The ^{45}Ca-labeled test formulas were prepared by combining 5.5 g of protein-free diet powder (Product 80056, Mead Johnson Nutritionals, Evansville, IN), 1 g of extrinsically labeled isolated soy protein prepared from soybeans produced with two different phytate levels, 10 mg of sodium, 30 mg of potassium, 25 mg of chloride, and water to 40 mL. The administered dose was 14.8 Bq of ^{45}Ca in 25 mg of isolated protein. Homogeneity of test diets was maintained by continuous stirring on a magnetic stir plate. A third group was given unlabeled formula and 2 h later was injected with 0.1 mL of 0.9% saline containing 12.2 Bq of $^{45}CaCl_2$.

Experiment 3. Calcium Bioavailability from Formula Prepared from Isolated Soy Protein in Mature Rats. Six-week-old male Sprague-Dawley rats (Harlan Industries) were randomly assigned to three experimental groups of six animals each. Rats were housed in individual stainless steel cages, allowed free access to deionized water, and fed ProSobee powder (Mead Johnson's iron-fortified milk-free soy protein formula powder), supplemented to ensure a level of 260 mg of choline/4180 kJ of diet and an additional 0.4% DL-methionine as a basal diet ad libitum for a 3-day orientation period. Animals were then meal trained to a nocturnal feeding schedule for 7 days to ensure complete ingestion of the test meal. On the 11th day, all animals were fasted for 18 h and given an extrinsically labeled test meal. Each meal was prepared by adding 0.77 g of extrinsically labeled isolated soy protein prepared from soybeans produced with two different phytate levels to 4.23 g of protein-free diet powder (Product 80056, Mead Johnson Nutritionals). Four milligrams of sodium, 12 mg of potassium, and 10 mg of chloride were added. Each 5.0-g test meal contained 750 mg of protein and 26 mg of calcium. Two hours after the test meal, rats in the third group were injected intraperitoneally with 0.1 mL of 0.9% saline containing 12.2 Bq of $^{45}CaCl_2$. All rats were allowed basal diet ad libitum after test meals and injection.

The use of an extrinsic label in experiments 2 and 3 was necessary because processing to an isolated soy protein product reduced the total calcium and labeled calcium dramatically. Weaver et al. (1992) have shown that extrinsic labeling of wheat flour provides labeling homogeneity equivalent to that of intrinsic labeling.

Calculation of Ca Bioavailability. Thirty-six hours after receiving a test meal or injection, all animals were killed with CO_2; their left femurs and, in experiment 1, a section of tailbone (approximately the first three caudal vertebrae) were removed. Absorption of ^{45}Ca from the test meals was calculated as the ratio of ^{45}Ca accumulated in the femur or per gram tailbone from the meal to ^{45}Ca accumulated from the injection in the same target tissue expressed as a percent (Wien and Schwartz, 1983). Because it is difficult to distinguish individual caudal vertebrae in rat tails, ^{45}Ca uptake was determined on a per gram basis rather than on distinct vertebrae. The mean percent absorptions of ^{45}Ca were compared and evaluated by one-way analysis of variance and Student–Newman–Keuls multiple-range test.

Table II. Calcium, Phosphorus, Phytate, Protein, and ^{45}Ca Activity of Soy Flour with Various Levels of Phytate (Experiment 1)

treatment[a]	calcium, μg/g	^{45}Ca Bq flour[b]	protein, %	phytate, %	phosphorus, μg/g
dephytinized (6 μg/g)	4115	4.81	58	0.11	1437
low phytate (3 μg/g)	4510	4.66	57	0.34	1017
medium phytate (6 μg/g)	4540	4.70	60	0.63	1437
high phytate (31 μg/g)	3476	3.07	55	1.12	4012

[a] Level of phosphorus in the nutrient culture of hydroponically grown soybeans is given in parentheses. The moisture content of the soybeans grown on 3 and 6 μg/g phosphorus was 6%, and the moisture content was 7% for the seeds grown on 31 μg/g phosphorus. [b] Level of activity at time of processing.

Table III. Chemical Analysis of Isolated Soy Protein Prepared from Hydroponically Grown Soybeans (Experiments 2 and 3)

treatment[a]	calcium, μg/g	protein, %	phytate, %	phosphorus, μg/g
medium phytate (6 μg/g)	273.7	88	0.738	3860
high phytate (31 μg/g)	317.8	97	1.74	9335

[a] Level of phosphorus in the nutrient culture of hydroponically grown soybeans is given in parentheses.

Analysis. Total calcium was determined on soy samples and test meals that had been dried in a vacuum oven at 70 °C, ashed at 600 °C, dissolved in 1–3 mol/L HCl, diluted with 0.5% lanthanam as $LaCl_3$ in 0.5 mol/L HCl, and analyzed on a Perkin-Elmer (Norwalk, CT) 373 atomic absorption spectrophotometer. An NBS wheat flour standard (190 ± 10 μg/g) was run with all test samples and measured 194 ± 9 μg/g. Soy flour, test meal samples, rat femurs, and tailbones were dried overnight in a vacuum oven at 70 °C, ashed at 600 °C, and dissolved in 1 mol/L HCl before dilution with 14 mL of PCS liquid scintillation cocktail (Amersham, Arlington Heights, IL) for determination of ^{45}Ca with a Beckman (Fullerton, CA) LS 1800 scintillation counter.

Protein estimation was made using the Kjeldahl nitrogen method according to Section 7.015 of the AOAC *Methods of Analysis* (AOAC, 1980), and phytate was determined colorimetrically (Latta and Eskin, 1980).

RESULTS

Level of Endogenous Phytate in Soy Products. Soybean plants grown in 3, 6, and 31 μg/g phosphorus nutrient solution produced soy flour containing over a 3.5-fold difference in levels of phosphorus and phytate (Table II). Dephytinization further reduced the phytate content so that feeding studies represented a 10-fold difference in phytate content. Isolated soy protein prepared from seeds produced on two levels of phosphorus varied almost 2.5-fold in endogenous phytate and phosphorus (Table III). There was insufficient yield of low phosphorus seeds to produce isolated soy protein. Dephytinizing almost completely removed the phytate. Preparation of the isolate reduced total calcium levels 10-fold compared to the starting flour, making it necessary to extrinsically label isolated soy protein with $^{45}CaCl_2$ used to make testmeals and formulas.

Rat-Feeding Studies. *Experiment 1.* Calculated ^{45}Ca absorption from soy flour by adult rats as a percentage

Table IV. Absorption to Mature Rats of ^{45}Ca from Soy Flour Containing Various Amounts of Phytic Acid (Experiment 1)[a]

soy flour test meal[b]	^{45}Ca absorption, % of injected dose	
	femur[c]	tailbone[c]
dephytinized	70.2 ± 0.68[a]	81.4 ± 0.76[a]
low phytate	63.0 ± 0.33[b]	76.6 ± 1.03[b]
medium phytate	68.5 ± 0.80[a,b]	76.8 ± 1.04[b]
high phytate	66.0 ■ 0.46[a,b]	67.7 ± 0.74[c]

[a] Mean ± SE, n = 8. Different letter superscripts indicate significant differences among means ($p \leq 0.05$), Student–Neuman–Keuls multiple-range test. [b] Analysis of soy flour used in test meal appears in Table II. [c] Percent dose accumulated by whole femurs or per gram tailbones was compared to accumulation following an intraperitoneal injection as described in the text.

Table V. Percent ^{45}Ca Absorption from Soy Isolate Formulas in Infant Rats (Experiment 2) and from Test Meals in Adult Rats (Experiment 3)[a]

^{45}Ca source[b]	infant rat ^{45}Ca absorption,[a] % of injected dose	adult rat ^{45}Ca absorption,[c] % of injected dose
medium phytate	106.1 ± 12.0[a]	29.5 ± 4.1[a]
high phytate	101.9 ± 7.5[a]	28.1 ± 2.5[a]

[a] Percent dose accumulated by whole femurs or per gram tailbones was compared to accumulation following an intraperitoneal injection as described in the text. [b] Analysis of isolated soy protein used to prepare formulas appears in Table III. [c] Mean ± SE, n = 10. Different letter superscripts indicate significant differences among means ($p < 0.05$), Student–Newman–Keuls multiple-range test.

of injected dose using femurs and tailbones as target tissues is given in Table IV. When tailbones were used as a target tissue, increasing phytate content decreased calcium bioavailability. The difference was significant at 1.12% phytate, the highest phytate test meal. However, this trend was not paralleled when the femur was the target tissue. The poorest calcium bioavailability was observed in rats fed flour containing 0.34% phytate, the low phytate test meal. The range in calcium absorption from all test meals was only 7%. The highest calcium bioavailability was from the dephytinized soy flour test meal in both target tissues.

Experiment 2. Calculated ^{45}Ca absorption from isolated soy protein formulas by infant rats as a percentage of injected dose using femurs as the target tissue is given in Table V. There was no significant difference in the absorption of ^{45}Ca due to the level of endogenous phytate. The fact that ^{45}Ca absorption appeared to exceed 100% relates to the method of absorption calculation, which compares accumulation of ^{45}Ca by a target tissue to accumulation following an ip injection. In adults the ip injection bypasses the gut barrier, eliminating the effect of the gastrointestinal tract on mineral accumulation. However, in infant rats the immature gut is not yet discriminatory, and all dosed Ca could be taken up through the intestine. Slightly more ^{45}Ca reached the femur from an oral dose than from the abdominal cavity.

Experiment 3. Calculated ^{45}Ca absorption from isolated soy protein by adult rats as a percentage of injected dose using femurs as the target tissue is also given in Table V. Again, as seen with infant rats, there was no significant difference in ^{45}Ca absorption due to the level of endogenous phytate.

DISCUSSION

The natural range of phosphorus in soybeans grown in the United States is 2900–7900 μg/g, dry basis (Wolnik et al., 1983). The range in phosphorus content of the seeds grown hydroponically in this study was 1082–4314 μg/g, dry basis. Since the correlation between soybean seed phosphorus and phytate content is 0.94 (Raboy et al., 1984), the range in phytate concentration represented in this study is likely as broad as can occur in field-grown seeds but did not reach the upper levels possible. Dephytinization broadened the range of phytate used experimentally beyond that of field-grown seeds.

Despite a 10-fold difference in phytate content of soybean seeds, there was not a predictable impact on calcium absorption. This contrasts with the striking inhibitory effect of phytate on zinc absorption (Oberleas and Harland, 1981). When the tailbone was used as the target tissue for ^{45}Ca uptake from soy flour rather than the femur, small differences were observed.

No differences were seen in calcium absorption from isolated soy protein prepared from seeds containing two levels of phytate either in infant rats fed soy isolate in formula or in mature rats fed the isolate as a component of a test meal. Churella and Vivian (1989) also found no differences in weight gain or in femur and carcass calcium in weanling rats fed either infant formulas made from isolated soy protein containing phytic acid or isolate in which 86% of the phytate had been removed by ultrafiltration.

Calcium absorption from isolated soy protein was greater in infant rats than mature rats. The undeveloped infant intestine is less discriminatory than mature intestines, and calcium absorption occurs primarily by passive diffusion. Low birth weight human infants also absorb most of a calcium load; absorption of calcium from human milk is reported as 80 ± 9% (Lui et al., 1989).

In the rat model, phytic acid endogenous to soybeans is not an important inhibitor of calcium bioavailability. However, unlike humans, rats have intestinal phytase activity (Williams and Taylor, 1985). Therefore, although many foods can be inexpensively screened under closely controlled conditions using animal models, the evaluation of phytic acid as an inhibitor of mineral absorption must be ultimately evaluated in humans.

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LITERATURE CITED

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

AOAC. *Official Methods of Analysis*, 13th ed.; Association of Official Analytical Chemists: Washington, DC, 1980.

Cheryan, M. Phytic acid interactions in food systems. *CRC Crit. Rev. Food Sci. Nutr.* **1980**, *13*, 297–335.

Churella, H. R.; Vivian, V. M. Effect of phytic acid level in soy protein based infant formulas on mineral availability in the rat. *J. Agric. Food Chem.* **1989**, *37*, 1352–1357.

Cullumbine, H.; Basnayake, V.; Comottee, J.; Wichramanayake, T. W. Mineral metabolism on rice diets. *Br. J. Nutr.* **1950**, *4*, 101–111.

Erdman, J. W., Jr. Oilseed phytates: Nutritional implications. *J. Am. Oil Chem. Soc.* **1979**, *56*, 736–741.

Graf, E. Calcium binding to phytic acid. *J. Agric. Food Chem.* **1983**, *31*, 851–855.

Harrison, D. C.; Mellanby, E. Phytic acid and the rickets-producing action of cereals. *J. Biochem.* **1939**, *33*, 1660–1679.

Henry, K. M.; Kon, S. K. Retention of calcium and phosphorus by the rat from whole meal bread, with and without added calcium and from white bread fortified with calcium and vitamin B-1. *J. Biochem.* **1945**, *39*, 117–122.

Latta, M.; Eskin, M. A simple and rapid colormetric method for phytate determination. *J. Agric. Food Chem.* **1980**, *28*, 1313.

Levine, S. E.; Weaver, C. M.; Kirleis, A. W. Accumulation of selected trace elements in hydroponically grown soybeans and distribution of the elements in processed soybean fractions. *J. Food Sci.* **1982**, *47*, 1283–1287.

Lui, Y.-M.; Neal, P.; Ernst, J.; Weaver, C.; Rickard, K.; Smith, D. L.; Lemons, J. Absorption of calcium and magnesium from fortified human milk by very low birth weight infants. *Pediatr. Res.* **1989**, *25*, 496–502.

McCance, R. A.; Widdowson, E. M. Mineral metabolism of healthy adults on white and brown bread dietaries. *J. Physiol.* **1942a**, *101*, 44–85.

McCance, R. A.; Widdowson, E. M. Mineral metabolism on dephytinized bread. *J. Physiol.* **1942b**, *101*, 304–313.

Mollgaard, H.; Lorensen, K.; Hansen, I. G.; Christensen, P. E. Phytic acid, its importance in metabolism and its enzyme cleavage in bread supplemented with calcium. *J. Biochem.* **1946**, *40*, 589–603.

Oberleas, D.; Harland, B. F. Phytate content of foods: Effect on dietary zinc bioavailability. *J. Am. Diet. Assoc.* **1981**, *79*, 433–436.

Raboy, V.; Dickinson, D. B.; Below, F. E. Variation in seed total phosphorus, phytic acid, zinc, calcium, magnesium, and protein among lines of *Glycine max* and *G. soja*. *Crop Sci.* **1984**, *24*, 431–434.

Schroeder, L. J.; Cahill, W. M.; Smith, A. H. The utilization of calcium in soybean products and other calcium sources. *J. Nutr.* **1946**, *32*, 413–421.

Walker, A. R. P.; Fox, F. W.; Irving, J. T. Human mineral metabolism. 1. Effect of bread rich in phytate phosphorus on the metabolism of certain mineral salts with special reference to calcium. *J. Biochem.* **1948**, *42*, 452–462.

Weaver, C. M. Intrinsic mineral labeling of edible plants: Methods and uses. *CRC Crit. Rev. Food Sci. Nutr.* **1985**, *23*, 75–101.

Weaver, C. M.; Nelson, N.; Elliott, J. G. Bioavailability of iron to rats from processed soybean fractions determined by intrinsic and extrinsic labeling techniques. *J. Nutr.* **1984**, *114*, 1042–1048.

Weaver, C. M.; Heaney, R. P.; Martin, B. R.; Fitzsimmons, M. L. Extrinsic vs intrinsic labeling of the calcium in whole-wheat flour. *Am. J. Clin. Nutr.* **1992**, *55*, 452–454.

Wien, E. M.; Schwartz, R. Comparison of in vitro and in vivo measurements of dietary Ca exchangeabilility and bioavailability. *J. Nutr.* **1983**, *113*, 388–393.

Williams, P. J.; Taylor, T. G. A comparative study of phytate hydrolysis in the gastrointestinal tract of the golden hamster (Mesocricetus auratus) and the laboratory rat. *Br. J. Nutr.* **1985**, *54*, 429–535.

Wolnik, D. A.; Fricke, F. L.; Caper, S. G.; Braude, G. L.; Meyer, M. W.; Satzger, R. D.; Keunnen, R. W. Elements in major raw agricultural crops on the United States. II. Other elements in lettuce, peanuts, potatoes, soybeans, sweeteners, and wheat. *J. Agric. Food Chem.* **1983**, *31*, 1244–1249.

Zemel, M. In vitro evaluation of the effect of ortho-, tripoly- and hexametaphosphate on zinc, iron and calcium bioavailability. *J. Food Sci.* **1984**, *49*, 1562–1565.

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