Long-Term Intake of a High-Protein Diet with or without Potassium Citrate Modulates Acid-Base Metabolism, but Not Bone Status, in Male Rats¹

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Abstract

High dietary protein intake generates endogenous acid production, which may adversely affect bone health. Alkaline potassium citrate (Kcit) may contribute to the neutralization of the protein-induced metabolic acidosis. We investigated the impact of 2 levels of protein intake and Kcit supplementation on acid-base metabolism and bone status in rats. Two-month-old Wistar male rats were randomly assigned to 4 groups (n = 30 per group). Two groups received a normal-protein content (13%) [NP] or a high-protein (HP) content diet (26%) for 19 mo. The 2 other groups received identical diets supplemented with Kcit (3.60%) [NPKcit and HPKcit]. Rats were pair-fed based on the ad libitum intake of the HP group. At 9, 16, and 21 mo of age, 10 rats of each group were killed. The HP diet induced a metabolic acidosis characterized by hypercalcuria, hypermagnesuria, and hypocitraturia at all ages. Kcit supplementation neutralized this effect, as evidenced by decreased urinary calcium and magnesium excretion by the HPKcit rats. Femoral bone mineral density, biomechanical properties, bone metabolism biomarkers (osteocalcin and deoxypyridinoline), and plasma insulin-like growth factor 1 levels were not affected by the different diets. Nevertheless, at 21 mo of age, calcium retention was reduced in the HP group. This study suggests that lifelong excess of dietary protein results in low-grade metabolic acidosis without affecting the skeleton, which may be protected by an adequate calcium supply. J. Nutr. 138: 718–724, 2008.

Introduction

Compared with “optimum” nutritional requirements, which are genetically determined, Western diets are characterized by an excessive protein intake, frequently associated with insufficient fruit and vegetable consumption. It has been suggested that this mismatch could lead to a number of pathological conditions (1).

In particular, an imbalanced protein-potassium ratio results in low-grade metabolic acidosis (2). Protein-rich diets are considered acidogenic due to the excess of sulphuric anions produced by the catabolism of sulfur amino acids. Moreover, low fruit and vegetable intake decreases dietary minerals and organic anions, including potassium citrate (Kcit)² and malate. Thus, the availability of alkaline organic salts potentially is insufficient to neutralize the endogenous acid production associated with excessive protein intake.

To counteract the excess dietary acid load, the homeostatic systems excrete increased amounts of urinary calcium and phosphorus (3,4). Hypercalcuria can have multiple pathological consequences (5), including nephrolithiasis, and it may contribute to the pathogenesis of osteoporosis. Acidosis promotes continual release of alkaline calcium salts from the skeleton to maintain the acid-base balance (6,7) and this may result in bone loss in the long term. Indeed, metabolic acidosis is associated with lower bone mineral density (BMD) in women (8) and rats (9). In an in vitro model for metabolic acidosis, significant bone calcium efflux was observed (10) and cell function was impaired, as evidenced by stimulation of osteoclastic bone resorption and inhibition of osteoblastic formation (11,12).

Similarly, high-protein (HP) intake and the associated acidosis are considered potentially detrimental for bone health in humans (13,14) and rats (15). However, in contrast to the well-characterized effects of dietary protein on urinary calcium excretion, the effect on bone status needs further insight. Actually, the impact of dietary protein on bone remains somewhat controversial, because several reports have emphasized that HP intake benefits bone health (16–18). Indeed, protein intake may favorably influence bone metabolism by stimulating the anabolic action of insulin-like growth factor 1 (IGF-1) on bone (19).

Fruit and vegetable intake provides potassium salts, especially Kcit. Previous investigations have reported beneficial effects of dietary potassium and potassium-rich foods (fruits and vegeta-
bles) on bone health (20–23). Given that a HP diet could induce osteopenia, it has been hypothesized that Kcit intake would neutralize the diet-induced metabolic acidosis and may be associated with a higher bone mass (24).

Thus, long-term investigations are needed to completely define the role of HP intake and acid-base balance in age-related bone loss. The goal of this study was to carefully identify the long-term effects of protein content in the diet on bone status and to test if alkaline-forming supplements had favorable effects on bone health. Using rats as a model, the study was designed to address 2 specific questions: 1) does a long-term HP diet (26%) have beneficial or detrimental effects on bone status when compared with a normal-protein diet (NP) (13%); and 2) does Kcit supplementation modulate the bone response to dietary protein intake.

Materials and Methods

Animals and experimental design

The study was conducted in accordance with the regional Ethics Committee for animal experiments in France. A total of 120 male Wistar rats (2 mo old, mean weight 316 g) were purchased from Institut National de la Recherche Agronomique. The rats were housed individually in plastic cages at 25°C, in 12-h-light/12-h-dark cycles and had free access to water. We measured body weight and food intake weekly. Male rats were chosen to eliminate the potential confounding effect of hormonal fluctuations.

At the beginning of the study, the rats were randomly assigned to 4 groups (n = 30). Two groups received diets containing either NP content (13% (g/100 g) or HP content (26%). The other 2 groups received identical diets supplemented with Kcit (3.60%) (NPKcit and HPKcit) (Table 1). All diets were purchased from Institut National de la Recherche Agronomique. All rats were fed a measured amount of diet, corresponding to 90–95% of the mean ad libitum intake of the HP groups, which had the lowest food intake. The pair-feeding protocol allowed us to match the energy intakes of the groups, providing healthy, nonobese control rats for the study (25). All diets contained the same amount of lipid, fiber, vitamins, and minerals, except potassium (0.36 vs. 1.66% in supplemented groups). Thus, all rats consumed similar amounts of dietary calcium (0.25%) and phosphorus (0.20%) (Ca/P ratio = 1.25).

At 9, 16, and 21 mo of age, 10 rats from each group were killed. One week before the end of the study, the rodents were transferred to individual metabolic cages specially designed for rats. After a 3-d adaptation period, food intake was controlled daily, and urine and feces were collected for 3 consecutive days. At the end of the experiment, the rats were deprived for 12 h then killed by injection of a lethal dose of anesthetic (Imagen 1000, Merial; 0.75 mL/kg body weight and Vetranquil 1%, Ceva santé animale, Libourne; 0.25 mL/kg body weight). Blood samples were collected from the abdominal aorta and frozen at –20°C until analysis. Femurs were cleaned from adjacent tissues. Left femurs were harvested in saline solution (9 g NaCl/L) and frozen (–20°C) until mechanical testing. Right femurs were kept in 80% alcohol until BMD was measured.

Biochemical analyses

Minerals. Urine samples were appropriately diluted in lanthanum oxide solution. Urinary calcium and magnesium concentrations were determined by atomic absorption spectrophotometry (AA800, Perkin Elmer). Sodium and potassium concentrations were measured by atomic emission spectrophotometry. A total of 200 mg of dried samples (food and feces) was dried-ashed (10 h at 500°C) and extracted until discoloration at 130°C in HNO3-H2O2 (2:1) (Suprapur; Merck). For calcium determination by atomic absorption spectroscopy, the final dilution was performed with lanthanum oxide. We calculated calcium retention (µmol/24 h), defined as [calcium intake – (urinary calcium excretion + fecal calcium excretion)].

Organic anions. Urine was diluted 400-fold with milli-Q water. Chloride, phosphate, sulfate, and citrate concentrations were determined by ionic chromatography (DX320, Dionex). The anions were separated on a 4 × 250-mm AS 11 column/AG 11 precolumn (flow rate 1 mL/min). An EG40 eluent generator controlled the elution using an OH− gradient (0.5–35 mmol/L in 20 min) and the conductivity detector was preceded by an automated storage and retrieval system self-regenerating suppressor.

IGF-1. Serum IGF-1 concentrations were measured using a 2-site immunoenzymometric assay (OCTEIA rat/mouse IGF-1 kit, IDS). The sensitivity of the assay was 82 µg/L. Intra- and interassay variations were 5.7 and 10.7%, respectively.

Parathyroid hormone. Plasma parathyroid hormone (PTH) concentrations were determined using an immunoradiometric assay kit (Immunotopics). The sensitivity was 1.0 ng/L. The intra- and interassay variations were 4.1 and 4.5%, respectively.

Bone biomarkers. Plasma osteocalcin (OC) concentrations were measured by an immunoradiometric assay (Immunotopics). The sensitivity was 0.01 µg/L. The intra- and interassay variations were 2.0 and 4.5%, respectively. The urinary deoxypyridinoline (DPD) concentration (nmol/L) was determined using a radioimmunoassay (Pyrilinks-D RIA kit, Metra Biosystems). The sensitivity was 2 nmol/L. The intra- and interassay variations were 4 and 6%, respectively. DPD excretion rates (nmol/24 h) were calculated using the prior 24-h urine volumes.

Physical measurements

BMD. BMD was assessed by dual-energy X-ray absorptiometry using a Hologic QDR-4500 A X-ray bone densitometer (Hologic). Total femoral BMD (T-BMD), metaphyseal BMD (M-BMD), and diaphyseal BMD (D-BMD) were determined. For M-BMD and D-BMD measurements, scans were cut and analyzed as follows: the first cut of the femur was performed at the upper quarter and the next cut at the lower quarter. D-BMD corresponded to the density of the 2nd and 3rd quarters of the femur. M-BMD was calculated as the mean of the upper and lower quarters of the femur (26).

Mechanical testing of femurs. Femoral length and mean diaphyseal diameter were measured with a precision caliper (Mitutoyo). Because of the irregular shape of the femoral diaphysis, the femoral diameter used in the calculation was the mean of the greatest and smallest femoral diaphyseal diameters. The femoral failure load was determined using a 3-point bending test (27) with a Universal Testing Machine (Instron).
Out to assess their significance. Internal correlations among variables and the Pearson test were carried differences between means. Linear regressions were performed to study Student-Newman-Keuls multiple comparison test to determine specific increased between 9 and 16 mo of age ($P$ (NP/HP), Kcit supplementation, age (9, 16, and 21 mo), and their among the groups. Thus, the main effects assessed were protein intake (NP/HP), Kcit supplementation, age (9, 16, and 21 mo), and their we used 10 rats per group. The normality of the data were verified. All rats per group, of the effect of protein intake on BMD in 8-mo-old male rats tended to decrease between 16 and 21 mo of age ($P$ = 0.07). Body weight of rats fed the diets supplemented with Kcit was weight of rats fed the NP diet, a HP diet, a NPKcit diet, and a HPKcit diet for 9, 16, and 21 mo ($P$ = 0.0001) (Table 2). Calcium intake was lower in all rats at 21 mo compared with 9 and 16 mo ($P$ < 0.0001). In parallel, fecal calcium excretion increased from 16 mo of age ($P$ < 0.0001). Fecal calcium excretion was also higher in the HP group compared with the other groups at 9, 16, and 21 mo of age ($P$ = 0.027). The urinary calcium excretion represented <1% of total calcium excretion. Calcium retention decreased during the experimental period (Table 3). This decrease was significant between 9 and 21 mo of age ($P$ < 0.0001). At 21 mo of age, the HP group showed a lower calcium retention rate than the NP, NPKcit, and HPKcit groups ($P$ = 0.044), whereas no differences were detected at the earlier stages. Calcium intake was lower in all rats at 21 mo compared with 9 and 16 mo ($P$ < 0.0001). In parallel, fecal calcium excretion increased from 16 mo of age ($P$ < 0.0001). Fecal calcium excretion was also higher in the HP group compared with the other groups at 9, 16, and 21 mo of age ($P$ = 0.027). The urinary calcium excretion represented <1% of total calcium excretion. Calcium retention decreased during the experimental period (Table 3). This decrease was significant between 9 and 21 mo of age ($P$ < 0.0001). At 21 mo of age, the HP group showed a lower calcium retention rate than the NP, NPKcit, and HPKcit groups ($P$ = 0.044), whereas no differences were detected at the earlier stages. Urinary anion excretion. As expected, rats fed Kcit excreted larger amounts of citrate in the urine (~12- to 16-fold more)

**Table 2** Body weight, urine pH, and urinary potassium, sodium, and magnesium excretion in rats fed a NP diet, a HP diet, a NPKcit diet, and a HPKcit diet for 9, 16, and 21 mo

<table>
<thead>
<tr>
<th>Age</th>
<th>NP</th>
<th>HP</th>
<th>NPKcit</th>
<th>HPKcit</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>mo</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td>$640.8 \pm 20.0$</td>
<td>$651.2 \pm 14.9$</td>
<td>$642.8 \pm 16.2$</td>
<td>$653.9 \pm 15.8$</td>
<td>$Age: P = 0.01$</td>
</tr>
<tr>
<td>9</td>
<td>$619.1 \pm 29.3$</td>
<td>$722.0 \pm 33.4$</td>
<td>$724.2 \pm 32.3$</td>
<td>$734.1 \pm 27.4$</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>$692.4 \pm 17.3$</td>
<td>$696.2 \pm 23.8$</td>
<td>$688.1 \pm 15.3$</td>
<td>$704.3 \pm 18.8$</td>
<td></td>
</tr>
<tr>
<td>Urinary variables</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>$6.66^{c} \pm 0.19$</td>
<td>$6.12^{c} \pm 0.21$</td>
<td>$8.76^{c} \pm 0.07$</td>
<td>$8.82^{c} \pm 0.12$</td>
<td>$Age: P &lt; 0.0001$</td>
</tr>
<tr>
<td>16</td>
<td>$6.18^{c} \pm 0.14$</td>
<td>$5.72^{c} \pm 0.05$</td>
<td>$8.37^{c} \pm 0.06$</td>
<td>$7.26^{c} \pm 0.47$</td>
<td>$Protein: P &lt; 0.0001$</td>
</tr>
<tr>
<td>9</td>
<td>$5.95^{c} \pm 0.03$</td>
<td>$5.55^{c} \pm 0.04$</td>
<td>$7.89^{c} \pm 0.23$</td>
<td>$8.88^{c} \pm 0.16$</td>
<td>$Age \times Kcit: P &lt; 0.0001$</td>
</tr>
<tr>
<td>Potassium, mmol/24 h</td>
<td>$2.7^{c} \pm 0.13$</td>
<td>$2.32^{c} \pm 0.07$</td>
<td>$8.57^{c} \pm 0.38$</td>
<td>$8.16^{c} \pm 0.53$</td>
<td>$Age \times Kcit: P = 0.0001$</td>
</tr>
<tr>
<td>16</td>
<td>$1.99^{c} \pm 0.15$</td>
<td>$2.01^{c} \pm 0.06$</td>
<td>$7.89^{c} \pm 0.47$</td>
<td>$7.34^{c} \pm 0.32$</td>
<td>$Age: P &lt; 0.0001$</td>
</tr>
<tr>
<td>21</td>
<td>$1.38^{c} \pm 0.16$</td>
<td>$1.33^{c} \pm 0.16$</td>
<td>$7.75^{c} \pm 0.44$</td>
<td>$7.19^{c} \pm 0.90$</td>
<td>$Protein \times Kcit: P &lt; 0.0001$</td>
</tr>
<tr>
<td>Sodium, mmol/24 h</td>
<td>$1.13^{c} \pm 0.07$</td>
<td>$1.22^{c} \pm 0.05$</td>
<td>$1.18^{c} \pm 0.07$</td>
<td>$1.27^{c} \pm 0.06$</td>
<td>$Age \times age: P = 0.039$</td>
</tr>
<tr>
<td>9</td>
<td>$1.12^{c} \pm 0.09$</td>
<td>$1.25^{c} \pm 0.05$</td>
<td>$1.13^{c} \pm 0.07$</td>
<td>$1.16^{c} \pm 0.08$</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>$1.12^{c} \pm 0.08$</td>
<td>$1.18^{c} \pm 0.10$</td>
<td>$1.15^{c} \pm 0.09$</td>
<td>$1.09^{c} \pm 0.10$</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>$1.12^{c} \pm 0.08$</td>
<td>$1.18^{c} \pm 0.10$</td>
<td>$1.15^{c} \pm 0.09$</td>
<td>$1.09^{c} \pm 0.10$</td>
<td></td>
</tr>
<tr>
<td>Magnesium, mmol/24 h</td>
<td>$44.03 \pm 7.00$</td>
<td>$57.37 \pm 6.58$</td>
<td>$47.74 \pm 8.64$</td>
<td>$49.38 \pm 6.58$</td>
<td>$Age: P = 0.001$</td>
</tr>
<tr>
<td>9</td>
<td>$68.72^{c} \pm 5.68$</td>
<td>$78.89^{c} \pm 2.06$</td>
<td>$62.55^{c} \pm 5.35$</td>
<td>$70.37^{c} \pm 0.90$</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>$72.25^{c} \pm 7.82$</td>
<td>$78.24^{c} \pm 8.64$</td>
<td>$60.48^{c} \pm 4.53$</td>
<td>$71.19^{c} \pm 4.53$</td>
<td></td>
</tr>
</tbody>
</table>

$^{1}$ Values are means $\pm$ SEM; $n = 10$. *Different from 9 and 16 mo of age, $P \leq 0.05$. †Different from 9 mo of age, $P \leq 0.05$. Means in a row with superscript letters without a common letter differ, $P \leq 0.05$. 720 Mardon et al.
than those that where not supplemented ($P < 0.001$) (Fig. 1A). At 9, 16, and 21 mo of age, citrate excretion was modulated by the protein level in the diet ($P < 0.001$); it was lower in the HP group than in the NP group and, at 9 mo, it was lower in the HPKcit group than in the NPKcit group. Similar trends were observed at 16 and 21 mo of age ($P = 0.021$). Urinary phosphate (Fig. 1B) and sulfate (Fig. 1C) excretion increased in the groups fed the HP and HPKcit diets ($P < 0.0001$) compared with those fed the NP and NPKcit diets. At 21 mo, both phosphate and sulfate excretions were lower in the HP group than in the HPKcit group ($P = 0.001$). Chloride excretion did not vary between the groups (data not shown).

**BMD.** T-BMD did not vary between 9 and 16 mo (Fig. 2A). M-BMD and D-BMD data are not represented, because T-BMD was correlated with both M-BMD ($r = 0.930; P = 0.0001$) and D-BMD ($r = 0.929; P = 0.0001$). T-BMD ($P = 0.004$), M-BMD ($P = 0.004$), and D-BMD ($P = 0.026$) were reduced at 21 mo compared with 9 and 16 mo. The level of protein intake and Kcit supplementation had no effect.

**Biomechanical properties.** Femurs from 21-mo-old rats had lower resistance to fracture (Fig. 2B) than those from 9- and 16-mo-old rats ($P < 0.0001$). The biomechanical data were positively correlated with the BMD data ($r = 0.70; P < 0.0001$). The 4 groups did not differ at any age.

**Bone biomarkers.** Plasma OC was reduced in all groups at 16 and 21 mo ($P < 0.0001$) compared with 9 mo (Fig. 2C). The urinary DPD excretion rate was similar at 9 and 16 mo of age then decreased at 21 mo ($P < 0.0001$) (Fig. 2D). Both bone biomarkers were unaffected by diet protein content and Kcit supplementation. OC was positively correlated with calcium retention ($r = 0.660; P = 0.014$).

**Plasma IGF-1.** Plasma IGF-1 concentrations were lower after 21 mo ($P < 0.0001$) compared with 9- and 16-mo-old rats (data not shown). The groups did not differ at any age.

**Plasma PTH.** Plasma PTH concentrations increased at 16 and 21 mo ($P = 0.001$) compared with 9 mo of age. The 4 groups (data not shown) did not differ at any age.

### Discussion

A relatively high dietary protein intake can generate a substantial fixed acidity, which inconsistently has been reported to adversely affect bone health (13–15). Kcit could possibly be used to neutralize this acid load. We investigated the effect of 2 levels of dietary protein (13 or 26%) in the presence or absence of Kcit supplementation on acid-base metabolism and bone status. We used male Wistar rats as an experimental model for age-related bone loss (29) and monitored them until 21 mo of age.

**Characterization of HP diet-induced low-grade metabolic acidosis.** The HP diet elicited an acid load, which was characterized by decreased urine pH compared with the NP diet group at all ages (Table 2). The HP diet was associated with significant calciuria (Table 3). Hypercalcemia is correlated with net acid excretion (30) and is a well-known consequence of HP diet ingestion (31,32). In the kidney, it has been attributed to an increased glomerular filtration rate and a decreased tubular calcium reabsorption, which neutralizes $SO_4^{2-}$ generated by catabolism of sulfur amino acids (4). The changes in magnesium excretion were similar (30% increase between the NP and HP groups). This trend is consistent with previous short-term studies and suggests that magnesium, in addition to calcium, compensates for metabolic acidosis in the long term (33). Increased urinary sulfate and phosphate excretion was observed with the HP diet (Fig. 1). The increased sulfate elimination resulted from a greater sulfur amino acid intake (34). The higher phosphate excretion rate could be due to the presence of more organic phosphorus in the HP diet (35), but impaired tubular reabsorption of phosphate (15) is another possibility. Hypocitraturia was a relative excess of organic anions (Kcit: 35.95 g/kg) compared with 9 mo of age (Fig. 1A) (36). This is consistent with reports of renal acidosis resulting in decreased proximal tubular reabsorption of citrate and impaired citrate transport (37,38).

**Kcit supplementation affected acid-base metabolism.** In contrast to western diets, the NPKcit and HPKcit diets provided a relative excess of organic anions (Kcit: 35.95 g/kg) compared with sulfur amino acids (~6.9 g/kg in NP diets and 13.8 g/kg in HP diets). The protein:potassium ratio is a reliable predictor of diet net acid load and these 2 diets were previously shown to prevent metabolic acidosis (2).

### Table 3

<table>
<thead>
<tr>
<th>Age</th>
<th>NP</th>
<th>HP</th>
<th>NPKcit</th>
<th>HPKcit</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ca intake, μmol/24 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1402 ± 24</td>
<td>1407 ± 21</td>
<td>1409 ± 17</td>
<td>1396 ± 16</td>
<td>Age: $P &lt; 0.0001$</td>
</tr>
<tr>
<td>16</td>
<td>1412 ± 27</td>
<td>1417 ± 16</td>
<td>1401 ± 24</td>
<td>1404 ± 25</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>1293* ± 9</td>
<td>1306 ± 16</td>
<td>1291* ± 10</td>
<td>1299* ± 18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fecal calcium excretion, μmol/24 h</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>9</td>
<td>1158 ± 38</td>
<td>1267 ± 41</td>
<td>1206 ± 37</td>
<td>1173* ± 26</td>
<td>Age: $P &lt; 0.0001$</td>
</tr>
<tr>
<td>16</td>
<td>1398± ± 24</td>
<td>1444± ± 51</td>
<td>1326± ± 36</td>
<td>1329± ± 53</td>
<td>Protein × Kcit: $P = 0.027$</td>
</tr>
<tr>
<td>21</td>
<td>1370± ± 31</td>
<td>1503± ± 56</td>
<td>1350± ± 48</td>
<td>1386± ± 51</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Urinary Ca excretion, μmol/24 h</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>9</td>
<td>8.00± ± 1.50</td>
<td>15.25± ± 2.25</td>
<td>8.50± ± 1.25</td>
<td>9.75± ± 1.75</td>
<td>Protein: $P = 0.003$</td>
</tr>
<tr>
<td>16</td>
<td>9.25± ± 2.50</td>
<td>15.50± ± 3.25</td>
<td>11.25± ± 2.25</td>
<td>11.00± ± 1.25</td>
<td>Kcit: $P = 0.041$</td>
</tr>
<tr>
<td>21</td>
<td>12.25± ± 1.25</td>
<td>17.00± ± 2.75</td>
<td>10.75± ± 2.50</td>
<td>12.50± ± 2.50</td>
<td>Protein × Kcit: $P = 0.0009$</td>
</tr>
<tr>
<td></td>
<td>Ca retention, μmol/24 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>236 ± 52</td>
<td>105 ± 49</td>
<td>198 ± 52</td>
<td>214 ± 31</td>
<td>Age: $P &lt; 0.0001$</td>
</tr>
<tr>
<td>16</td>
<td>63 ± 41</td>
<td>−32.5 ± 56</td>
<td>64 ± 53</td>
<td>64 ± 80</td>
<td>$Age × protein × Kcit: P = 0.044$</td>
</tr>
<tr>
<td>21</td>
<td>−89± ± 51</td>
<td>−214± ± 42</td>
<td>−70± ± 55</td>
<td>−78± ± 79</td>
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</tr>
</tbody>
</table>

Values are means ± SEM, $n = 10$. *Different from 9 and 16 mo of age, $P ≤ 0.05$. †Different from 9 mo of age, $P ≤ 0.05$. Means in a row with superscript letters without a common letter differ, $P ≤ 0.05$. 

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**TABLE 3 Calcium intake, fecal excretion, and urinary excretion and retention in rats fed a NP diet, a HP diet, a NP Kcit diet, and a HP Kcit diet for 9, 16, and 21 mo**

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**High-protein intake and bone health 721**
Indeed, Kcit supplementation significantly increased urine pH, consistent with previous work (22,39). The administration of Kcit resulted in increased urinary potassium (Table 2) and citrate excretion (Fig. 1) in both the NPKcit and HPKcit groups. However, the HP diet limited the effect of the Kcit supplementation on citraturia. The Kcit neutralizing effect was further confirmed by lower urinary calcium and magnesium excretion (Tables 2, 3). The Kcit probably exerted its effects by neutralizing endogenous acidity by \( \text{KHCO}_3 \) generation, glutamine sparing (40,41), and modulation of hepatic urea synthesis for removal of bicarbonate (42). Furthermore, potassium influences the expression of the proximal tubular Na/sulfate-cotransporter. Although Kcit supplementation may have restored SO\(_4^{2-}\) reabsorption through a reversal of transporter downregulation by acidosis, it increased sulfate excretion (Fig. 1C). This was consistent with data from Sabboh et al. (35), who suggested that sulfate reabsorption could not match increased generation of SO\(_4^{2-}\). Moreover, it seems the capacity of the kidney to excrete sulfate and phosphate was maintained during aging with Kcit supplementation, whereas it was altered after 21 mo in the NP and HP rats.

**HP diet and Kcit supplementation did not affect bone quality.** The sole change reported on bone status was a marked age-related bone loss at the end of the experimental period (21 mo of age) (Fig. 2). Bone density changes were consistent with the observed biomarker variations and resulted in decreased bone strength (Fig. 2B). These alterations may be related to the decreased IGF-1, a bone anabolic hormone, secretion. The lower plasma IGF-1 levels probably result in age-induced perturbation of the hypothalamo-adenohypophysial-somatotrophic axis (43,44). Secondary hyperparathyroidism has been reported in old rats and was suggested to contribute to bone alteration. In parallel, these changes were associated with the impaired ability of the kidney to excrete the dietary acid load with aging and were characterized by acidification of excreted urine and increased loss of urinary magnesium and calcium.

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The femoral BMD was not affected by protein intake or Kcit supplementation (Fig. 2A). Indeed, the groups did not differ at any of the time points. Corresponding femoral biomechanical properties remained unchanged as well (Fig. 2B). These results are consistent with previous observations in rats by Whiting and Draper (45), Calvo et al. (46), and Goulding and Campbell (47), showing no change in femoral bone mass or macromineral composition in response to dietary protein excess (30, 35, and 60% excess, respectively) despite hypercalcemia. In contrast, using histomorphometric analysis, Amanzadeh et al. (15) observed bone loss in rats fed a 48% casein diet during 59 d and displaying hypercalciuria and hypocitraturia.

The physical bone measurements were consistent with the bone biomarker levels, which did not vary between the 4 groups (Fig. 2C,D). Yet metabolic acidosis has been demonstrated to stimulate osteoclastic resorption and inhibit osteoblastic collagen synthesis in vitro (12,48). As far as hydroxyproline can be considered as a relevant indicator of bone resorption, HP intake was associated with increased resorption levels (32,47,49). However, Creedon and Cashman (50) and Shapses et al. (51) reported no effect of HP diets on urinary excretion of collagen pyridinium crosslinks in rats and human subjects. In this study, the absence of variation in bone metabolism can be attributed to stable circulating PTH, a potent stimulator of bone resorption, levels.

Thus, the bone variables in this study lead us to conclude that long-term, low-grade metabolic acidosis, induced by a slightly hyperproteic diet (26% casein), does not affect the skeleton. However, it would be interesting to repeat the analysis with female rats that are subject to greater hormonal variations. Actually, it appears that the effects of dietary protein on bone status depend on multiple, interacting variables (52), including additional dietary factors. Protein and calcium, in particular, seem to act synergistically on human bone (53,54). An adequate calcium supply, as in this study, may reduce or offset the negative...
effects of protein on bone mass. Here, calcium retention was not significantly affected by the HP (26%) diet or citrate salt intake at 9 and 16 mo of age (Table 3), although calcium fecal excretion increased. Nevertheless, calcium retention was altered in the HP rats compared with the NP rats after 21 mo. This was a result of both higher fecal and urinary calcium excretion. Calcium retention did not vary between the NPKcit and the HPKcit groups. In summary, in the older rats, the HP diet had deleterious effects on calcium retention and Kcit supplementation could prevent this impairment. The impact of HP intake on calcium retention is controversial, because Creedon and Kashman (50) found no effect of this parameter in growing rats. Femoral BMD was unchanged in this study at 21 mo, but it is conceivable that bone density would be altered at later time points. These results remain to be confirmed in humans, given the differences suspected in intestinal calcium absorption.

The role of dietary protein in bone health is currently debated. Some authors argue that HP diets diminish bone health (13,55), whereas others speculate that it is favorable for the skeleton (17,56). Considering that dietary protein supplies the amino acid building blocks for the bone matrix and stimulates the IGF-1 system, it may have a magnitude-dependent anabolic effect on bone. The plasma IGF-1 levels in this study did not differ between the 4 groups. However, IGF-1 is an osteotrophic factor and it was previously shown to be regulated by nutritional status, especially dietary protein intake (44). Inappropriate protein intake (2.5% casein diet) impairs the IGF-1 system and has been suggested to lead to decreased bone mineral mass and fragility in rats (57). The absence of variation in IGF-1 levels in this study is consistent with the BMD values and probably reflects that our diets were less restrictive, particularly with respect to protein content.

In conclusion, the HP diet induced a low-grade metabolic acidosis, which persisted over the entire duration of the experiment. Kcit supplementation had alkalinizing effects and neutralized the increased acid load. Neither beneficial nor detrimental effects associated with HP intake or Kcit supplementation was reported on bone status or metabolism under our experimental conditions. This could be explained by an adequate calcium supply, because calcium intake may offset the effects of a protein-generated acid load on bone mass.

**Literature Cited**


