DOI:10.1021/jf9018646

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Influence of Diets Rich in Maillard Reaction Products on Calcium Bioavailability. Assays in Male Adolescents and in Caco-2 Cells

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The effects of the high intake of Maillard reaction products (MRP) on calcium availability in adolescents and across Caco-2 cell monolayers were examined. In a 2 week randomized two-period crossover trial, 18 male adolescents consumed two diets, named white diet (WD) and brown diet (BD), which were poor and rich in MRP, respectively. A 3 day balance was performed at the end of each period, and fasting blood samples were collected. Calcium solubility and absorption across Caco-2 cells were studied after the in vitro digestion of the diets. The in vitro assay showed similar solubility after the in vitro digestion and similar transport across Caco-2 cells. In accordance, calcium bioavailability in adolescents did not vary between the diets (%WD = 40.4 ± 5.1, %BD = 38.2 ± 3.6). Serum and urine biochemical parameters related to calcium status and bone metabolism remained unaltered. Only deoxypyridinoline values were significantly lower after consumption of the BD (13.0 ± 1.1 compared to 18.3 ± 2.1 nM/Mm Cr in the WD), possibly indicative of less efficient bone turnover during this period. As calcium acquired during adolescence is essential to maximize peak bone mass and to prevent osteoporosis, possible long-term effects of excessive MRP intake during this period warrant attention.

KEYWORDS: Maillard reaction products; adolescence; Caco-2 cells; calcium availability; calcium metabolism

INTRODUCTION

Adolescence is a period of rapid growth and development during which 25−50% of peak bone mass in the adult is accumulated (*1*). Calcium retention throughout the pubertal growth spurt is essential for optimizing this peak bone mass (*2*). Moreover, maximizing bone mineral accrual during growth is considered to be the main strategy for preventing osteoporosis in later life. Osteoporosis and low bone mass are currently perceived as major health threats for an estimated 44 million women and men over the age of 50 and are associated with low calcium intake (*3*). Moreover, other important consequences of the calcium deficiency, especially during infancy and adolescence, are rickets and fracture risk. The average calcium intake of adolescents is notably lower than that required all over the world (*4*−*7*), and diets are frequently not appropriate to satisfy calcium needs during the growth years (*8*). The calcium deficiency among adolescents is due to not only their high physiologic needs but also their dietary habits. It is well-known that the dietary habits of adolescents have changed in recent decades and that there is a tendency to a higher consumption of soft drinks, snacks, bakery products, and fast food (*9*, *10*). Soft drinks are displacing milk in the diets of children and adolescents, and this fact may affect variables of bone modeling and remodeling (*10*).

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On the other hand, culinary methods such as frying, roasting, grilling, or baking lead to the development of Maillard reaction products (MRP), which are nonenzymatic browning compounds produced when amino acids or proteins react with carbohydrates or oxidized lipids. MRP contribute to the aroma, color, and flavor that improve food palatability and, therefore, controlled browning is pursued through many food technology processes aimed at promoting consumer acceptance (*11*). Thus, MRP are widely consumed as part of the human diet, and particularly among adolescents, given their particular food patterns (*12*). In addition to their sensory properties, certain biological effects have been attributed to MRP, such as antioxidant activity (*7*), decreases in protein digestibility (*13*), or modifications in mineral availability (*14*, *15*). MRP may behave as anionic polymers and chelate metal cations, affecting mineral solubility at intestinal conditions (*16*). Calcium appears to bind weakly to soluble and insoluble melanoidins (*17*), whereas little calcium binding ability has been attributed to low molecular weight MRP in comparison with that observed for other metals such as copper or iron (*18*).

The effects of MRP on calcium absorption and metabolism have been studied using model systems of sugar−protein (*19*) and sugar−amino acid (*20*−*22*), implemented in cell culture systems (*19*) and in animal models (*21*, *22*). Most reported results indicate stability in the calcium balance after the intake of MRP-rich diets, but interesting findings have been published regarding impaired bone calcium concentration among animals fed MRP

 Published on Web 10/06/2009

Table 1. Lunch and Dinner 7 Day Menus for the Diets Used for Dietary Treatments

diet	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
				White Diet			
lunch	legumes (chickpeas) salad (lettuce, tomato, etc.) strawberry yogurt bread without crust	salad of pasta boiled chicken and potatoes pears bread without crust	legumes (lentils) tuna-filled eggs rice with milk bread without crust	vegetable stew baked loin of pork and boiled potatoes syrup peach bread without crust	Russian salad with tuna legumes (beans) strawberry yogurt bread without crust	boiled potatoes, boiled eggs, and ham baked meat (veal) with vegetables custard bread without crust	salad (lettuce, tomato, etc.) stewed rice apple bread without crust
dinner	soup of pasta and chicken sausages with mashed potatoes bananas bread without crust	prawn cream baked fish custard bread without crust	tropical salad fish with cream and rice oranges bread without crust	spaghetti with tomato sauce, cheese, and ham bananas bread without crust	consommé with noodles baked fish with boiled potatoes pears bread without crust	soup of vegetables pasta with tomatoes and cheese bananas bread without crust	vegetables purée fish pudding custard bread without crust
				Brown Diet			
lunch	legumes (chickpeas) salad (lettuce, tomato, etc.) chocolate yogurt bread	salad of pasta fried chicken and fried potatoes pears bread	legumes (lentils) Spanish omelet rice with milk and cinnamon bread	sautéed vegetables griddle loin of pork and fried potatoes Torrija[a] bread	empanadillas[b] with salad legumes (beans) chocolate yogurt bread	Spanish omelet with ham meatballs (veal) with vegetables chocolate custard bread	salad (lettuce, tomato, etc.) paella apple bread
dinner	soup of pasta and chicken hamburger with fried potatoes bananas bread	purée of prawns fish croquettes caramel custard bread	tropical salad breaded fish and rice oranges bread	gratin macaroni with béchamel sauce bananas bread	consommé with noodles breaded fish with fried potatoes pears bread	soup of vegetables pizza bananas bread	vegetable cream with croutons breaded hake fish-fingers chocolate custard bread

[a] Fried bread with milk, sugar, and cinnamon. [b] Small tuna-filled breaded pastries.

diets (*21*). To the best of our knowledge, no assays have been carried out in humans on the influence of whole diets rich in MRP on calcium bioavailability and metabolism.

Given the enormous importance of calcium metabolism during adolescence, our objective in the present study was to investigate calcium bioavailability and status in male adolescents consuming MRP-rich diets, as are usually consumed by this population, and to compare these results with those corresponding to a low-MRP diet. Moreover, experiments in cell cultures using an intestinal cell line, the Caco-2 cells, were carried out to study dietary MRP effects on intestinal absorption of calcium.

MATERIALS AND METHODS

Chemicals. All chemical products and solvents, for all of the analyses performed, were of the highest grade available and acquired from Sigma (Sigma-Aldrich, St. Louis, MO) and Merck (Darmstadt, Germany).

Subjects, Diets, and Study Design. Subject selection, diet composition, and study design have been described previously (*13*, *23*). Briefly, 20 male adolescents (12.4 ± 0.34 years of age, mean ± SE) were recruited for a 2 week randomized two-period crossover trial in which they consumed two different diets with a 40 day washout period. Two 7 day menus containing the same daily servings of the different food groups and having similar contents of energy and nutrients were designed. The diets were constituted as follows: *white diet* (WD), free, as far as possible, of cooking practices in which the MR develops (i.e., frying, toasting, roasting) and foods usually containing MRP, such as bread crust or chocolate; *brown diet* (BD), rich in processed foods with an evident development of browning and, thus, rich in MRP. The BD contained breakfast cereals, baked products, chocolate, fried foods, toasted foods, breaded foods, etc. Lunch and dinner, the two main meals in the Spanish diet, were prepared by a local caterer (**Table 1**) and were distributed daily to the homes of the participants. Each 7-day menu was repeated twice during each 14-day experimental period. Breakfasts and afternoon snacks were prepared at home under the specific instructions given to the subjects and their parents. The food composition of the breakfast was as follows: whole milk with sugar, white bread without crust with margarine and fruit juice in the WD; whole milk with cocoa powder, breakfast cereals, and fruit juice in the BD. The afternoon snack was constituted as follows: whole milk with sugar, sandwich of white bread without crust with pâté or cheese and margarine in the WD; whole milk with cacao powder and pastries in the BD. The food composition of the diets was transformed into energy and nutrient values using the Spanish Food Composition Tables (*24*), under AYS44 Diet Analysis software supplied by ASDE, SA (Valencia, Spain). The overall daily contribution of energy and nutrients in the study diets was as follows: energy, 2530 kcal; fat, 107.5 g; carbohydrate, 316.9 g; protein, 90.1 g; fiber, 25.1 g; cholesterol, 311.4 mg; sodium, 1865 mg; potassium, 3826 mg; calcium, 1049 mg; phosphorus, 1595 mg; magnesium, 372 mg; iron, 17.5 mg; zinc, 8.9 mg; retinol, 1.4 mg; ascorbic acid, 117.4 mg; α-tocopherol, 11.4 mg.

To enable analysis of the calcium content and of the MR markers in the experimental diets and to perform the Caco-2 cell experiments, the catering firm also provided the meals to the researchers; the breakfast and the afternoon snack were prepared in the laboratory following the instructions given to the participants, the ingredients being purchased at a local market. Every day, and for each diet, the edible portion of the foods was removed from all meals, weighed, and homogenized with a hand blender (Taurus, vital CM, Spain). Aliquots of each meal were mixed to obtain the 1-day sample, and aliquots of each day were mixed to obtain the diet samples (WD and BD). Aliquots of meals and diets were stored at −20 °C until the moment of analysis.

The greater development of the MR in the BD was confirmed from the analysis of MR markers in the diets (*13*). Values of hydroxymethylfurfural (HMF) and carboxymethyllysine (CML) and percentage of relative fluorescence intensity were significantly higher in the BD than in the WD (HMF, 0.94 ± 0.01 and 3.87 ± 0.03 mg/kg; CML, 6.62 ± 0.25 and 15.72 ± 0.43 mg/100 g of protein; fluorescence intensity, 7.31 ± 0.35 and

21.04 ± 0.42%, in the WD and BD, respectively). The early MRP did not differ between diets, as indicated by analysis of furosine (ε-*N*-(furoylmethyl)-L-lysine (6.99 ± 0.45 and 6.37 ± 0.15 mg/100 g in the WD and BD, respectively).

The above-mentioned software was used to calculate the intake of energy and nutrients (except calcium) from food intake data, assessed throughout the entire dietary treatments by daily record sheets, in which participants noted the details of their food consumption. If the prepared meals were not entirely consumed, the subjects were asked to weigh and record all food that was left. They also weighed and recorded the food consumed at breakfast and afternoon snack.

To determine dietary calcium utilization, we analyzed the calcium content in the diets and in the urinary and fecal samples. The 14 day period was longer than the minimum recommended of 6−7 days for calcium balances (*25*) and may be considered an adequate equilibration period, allowing gastrointestinal clearance of unabsorbed minerals from the previous diet (*26*, *7*). The balance took place over the last 3 days of each 14 day dietary treatment, in which urine and feces were collected in 24 h separated samples (*27*). Each 24 h urine sample was collected on acidified recipients, beginning with the second voiding of the day and finishing with the first voiding of the following day, and the volume from each daily sample was measured. The subjects were asked to report any problem with the collections, such as spillages or missed specimens. Individual fecal samples were weighed, diluted with 6 N HCl, and homogenized with a hand blender (Taurus, vital CM, Spain). Aliquots were frozen at −20 °C until analysis. The participants were permitted to drink tap water ad libitum, recording the volume; they were requested to provide a sample of tap water, and its calcium content was analyzed and taken into account to calculate total calcium intake. Deoxypyridinoline (DPD), creatinine, sodium, and phosphorus in urine were analyzed as described below.

At the end of each dietary treatment and after a 12 h overnight fast period, blood samples were obtained from each subject by venipuncture to measure the biomarkers of calcium metabolism for screening test. Blood collected in vacutainers with no added anticoagulant was left to clot for 30 min and then centrifuged at 1700*g* for 15 min (4 °C) to obtain serum, which was frozen at −20 °C for later analysis. Parathyroid hormone (PTH), alkaline phosphatase (AP), and calcium were determined in serum.

Body weight and height were recorded at the beginning of the study and at the end of each diet period, and body mass index (BMI = kg/m^2) was calculated.

This study was approved by the Ethics Committee of the San Cecilio University Hospital of Granada and was performed in accordance with the Helsinki Declaration of 2002, as revised in 2004. Moreover, informed consent was obtained from the parents of all of the adolescents participating in the study. Of the 20 subjects recruited, 18 completed the study, one having dropped out because of a surgical intervention, whereas a second one was found to be noncompliant.

In Vitro Digestion of Diets. The technique of Miller et al. (*28*), with some modifications, was followed. It comprised gastric and intestinal digestion, as described previously (*15*). Briefly, 4 g of each fresh diet sample was suspended in 10 mL of Milli-Q water. Then, pH was adjusted to 2 with 6 N HCl, a pepsin solution was added at a proportion of 0.05 g of pepsin/g of sample, and the samples were incubated at 37 °C in a shaking water bath at 110 oscillations/min for 2 h for the gastric digestion. For the intestinal digestion, the pH of the digest was raised to pH 6 with 1 M $NaHCO_3$ dropwise, and 2.50 mL of pancreatin + bile salts mixture was added. The pH was then adjusted to pH 7.5 with 1 M $NaHCO_3$, and the samples were incubated at 37 °C at 110 oscillations/min for 2 h.

After gastrointestinal digestion, the digestive enzymes were inactivated by heat treatment for 4 min at 100 °C in a polyethylene glycol bath. The samples were then cooled by immersion in an ice bath and centrifuged at 3200*g* for 60 min at 4 °C (CS-6R centrifuge, Beckman) to separate the soluble and nonsoluble fractions. The supernatants were carefully separated, and the percentages of soluble and insoluble calcium were calculated from the initial calcium content in the diets. The supernatants were reserved to be used in the Caco-2 cell experiments.

Cell Cultures. Caco-2 cells were purchased from the European Collection of Cell Cultures (ECACC) through the Cell Bank of Granada University at passage 20 and were used in the experiments at passages 30−35. In culture, this cell line exhibits many properties of normal intestinal epithelium, and it has been used as a suitable model to study calcium metabolism (*29*, *21*). Culture flasks and bicameral chambers were purchased from Corning Costar (Cambridge, MA). The cells were maintained by serial passage in 75 cm^2 plastic flasks containing high-glucose Dulbecco's modified minimal essential medium (DMEM), with heat-inactivated fetal bovine serum (15%), $NaHCO_3$ (3.7 g/L), nonessential amino acids (1%), HEPES (15 mM), bovine insulin (0.1 UI/mL), and 1% antibiotic−antimycotic solution. The cells were grown under an atmosphere of air/CO_2 (95:5) at 90% humidity and 37 °C and given fresh medium every 2 days.

Trypsinization and seeding of cells into permeable polycarbonate filter supports (Transwell, 24 mm diameter, 4.7 cm^2 area, 3 μm pore size, Costar) were performed as described elsewhere (*15*, *30*). Cell monolayer integrity during differentiation of the Caco-2 cells was monitored by determining transepithelial electrical resistance (TEER) of filter-grown cell monolayers on different days after seeding, using a Millicell electrical resistance system (Millipore, Bedford, MA). The monolayers used in this study exhibited adequate TEER values, ranging from 500 to 650 Ω/cm^2. In addition to the TEER measurements, the absorption of the phenol red marker was used to confirm the integrity of the Caco-2 cell monolayers following the procedure described by Ruiz-Roca et al. (*31*). Cell monolayers were used when the leakage rate of phenol red was lower than 2.5% per hour.

Prior to the calcium transport experiments, the final concentrations of glucose, HEPES, and osmolarity (cryoscopic osmometer Osmomat 030-D, Berlin, Germany) of the diet digests were adjusted to 5 mM, 50 mM, and 310 mOsm/kg, respectively. The pH of digests was measured, and it was always around 7. Cell viability after 2 h of exposure to the supernatant digests was assessed by trypan blue exclusion, and it was never < 85%.

Absorption experiments were carried out 21 days after initial cell seeding. Spent culture medium was aspirated from the apical and basolateral chambers, and both cell surfaces were washed three times with Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS) at 37 °C. Then 2.5 mL of the transport solution (130 mmol/L NaCl, 10 mmol/L KCl, 1 mmol/L $MgSO_4$, 5 mmol/L glucose, and 50 mmol/L HEPES, pH 7) was added to the basolateral chamber, and the supernatant digest of each diet (BD and WD) was added to the apical chamber (1.5 mL). After an incubation period of 2 h at 37 °C in a humidified air/CO_2 atmosphere, the buffer from the basolateral chamber was removed to calculate the calcium transported across the cell monolayer. To ensure complete collection, the wells were washed twice with deionized water.

Calcium transport in cell cultures is expressed as micrograms of Ca transported to the basolateral chamber per well, and as the percentage of Ca transported per well from the experimental solution. Calcium availability was expressed by taking into account differences in calcium solubility after in vitro digestion, as follows: % soluble calcium × % transported calcium/100.

Analytical Techniques. Analyses were performed in triplicate, when possible. Before the mineral analysis, aliquots of feeds, urine, feces, serum, and solutions from the in vitro diet digestion and cell culture experiments were completely digested by the addition of concentrated HNO_3 and $HClO_4$ and by heating at high temperatures (180−220 °C) in a sand beaker (Block Digestor Selecta S-509; J.P. Selecta, Barcelona, Spain) (*15*). Calcium concentration in drinking water was determined directly. Sodium analysis in urine and calcium analysis in all samples were performed by atomic absorption spectrophotometry in a Perkin-Elmer Analyst 700 spectrophotometer (Norwalk, CT). Standard solutions were prepared from stock Tritisol solutions (calcium, $CaCl_2$, in 6.5 g/L HCl, 1000 mg of Ca; sodium, NaCl in H_2O, 1000 mg of Na; Merck). To avoid interferences in the calcium and sodium analysis, lanthanum chloride or lithium chloride (Merck), respectively, was added to samples and standards in a final concentration of 0.3 g/L

Urine phosphorus analysis were performed with a spectrophotometric method, by which absorbance was measured at 820 nm (*32*). Standard solutions were prepared from a 1000 mg/L phosphorus solution, made using PO_4H_2K (Suprapur Merck, Darmstadt, Germany).

Pools of diet, feces, and urine were used as an internal control to assess precision. The interassay coefficients of variation for calcium were 5.02% in the diet, 3.24% in feces, and 4.09% in urine; for phosphorus and sodium in urine they were 0.73 and 2.87%, respectively. Milk powder standard (certified reference material CRM 063, Community Bureau of Reference, Brussels, Belgium) was simultaneously used to quantify

calcium, phosphorus, and sodium accuracy, yielding a value of 13.47 ± 0.04 mg/g for calcium (mean ± SD of 10 determinations; certified value = 13.49 ± 0.10 mg/g), 11.04 ± 0.03 mg/g for phosphorus (mean ± SD; certified value = 11.10 ± 0.13 mg/g), and 4.35 ± 0.02 mg/g for sodium (mean ± SD; certified value 4.37 ± 0.03 mg/g). All glassware and polyethylene sample bottles were washed with 10 N nitric acid, and demineralized water (Milli-Q Ultrapure Water System, Millipore Corp., Bedford, MA) was used throughout.

Using the data obtained for the calcium intake (I), fecal excretion (F), and urinary excretion (U), the following indices were calculated: apparent absorption ($A = I - F$), fractional absorption or digestibility ($\%A/I = A/I \times 100$), apparent retention ($R = A - U$), and utilization efficiency or bioavailability ($\%R/I = R/I \times 100$).

DPD in urine was analyzed by using an immunoassay kit, Metra DPD EIA (Quidel Corp., San Diego, CA). Urine creatinine was measured using a chemical analyzer (Bechman Systems, Synchron LX, Fullerton, CA).

PTH was determined in serum by ratio immunoassay (Incstar PTH-MN, Stillwater, MN), and serum AP was measured enzymatically by standard techniques, using paranitrophenol as the substrate.

Statistical Data Analysis. SPSS for Windows, version 13.0 (SPSS Inc., 1999−2004, Chicago, IL), was used for data entry and statistical analysis. The experimental data obtained after the crossover dietary treatments were analyzed by using the repeated-measures analysis of variance (ANOVA) to ascertain the consequences of the dietary treatment and to determine whether the order of presentation of the diets had an effect. There were no order effects and no treatment × order interaction for any of the dependent variables. When a significant effect between dietary treatments was found, post hoc comparison of means was made using Bonferroni's test. Differences were considered to be significant at $P < 0.05$. Data from calcium content in diets and from calcium absorption in Caco-2 cells were statistically tested by one-way ANOVA, followed by Duncan's test to compare means that showed significant variation ($P < 0.05$).

RESULTS AND DISCUSSION

Basal characteristics of the subjects were weight of 55.9 ± 2.9 kg and height of 1.60 ± 0.03 m (mean ± SE). Weight and height increased by 2.09 ± 0.32 kg and 2.13 ± 0.36 cm during the whole experimental period, respectively, with no significant differences between groups. The BMI (21.8 ± 1.0 kg/m^2) did not change during the study.

Daily intakes of energy and nutrients were similar in both diets (**Table 2**). Only the intakes of fat and α-tocopherol were found to be significantly different between diets ($P = 0.023$ and $P = 0.005$, respectively).

Daily calcium intake did not differ significantly between the dietary treatments (**Table 3**). Values were similar to those found among Spanish adolescents of similar age (*6*), in both cases being close to the current Spanish and European recommendations for this age group of 1000 mg/day (*33*, *34*), which are clearly lower than the calcium dietary reference intake of 1300 mg/day in the United States (*35*). In both diets, milk and dairy products contributed the majority of dietary mineral (≈68%), and cereals were the second-largest contributors (≈10%), which is in agreement with the dietary calcium distribution found among European and American adolescents (*4*, *5*).

Total calcium excretion in feces (mg/day) or related to body weight (mg/kg/day) did not differ between diets. The values were similar to the mineral fecal excretion in boys and girls with similar calcium intakes (*36*, *37*). The apparent absorption and the calcium digestibility were not significantly different when the subjects consumed the MRP-rich diet with respect to the MRP-poor diet (**Table 3** and **Figure 1**). The calcium absorption values shown in the present study were higher than those observed in studies in male adolescents aged 9−14 years (*38*) and 9−17 years (*36*) and were sufficient to satisfy the high needs related to the pubertal period, which may reach 400−500 mg/day (*39*).

Table 2. Daily Intakes of Energy and Nutrients during Crossover Dietary Treatments with White and Brown Diets in Adolescent Males Aged 11−14 Years[a]

	white diet	brown diet
energy (kcal)	2176 ± 53	2271 ± 44
protein (g)	84.7 ± 2.4	80.0 ± 1.9
fat (g)	91.0 ± 2.8a	97.4 ± 2.0b
carbohydrates (g)	269.7 ± 6.8	286.6 ± 6.2
fiber (g)	20.8 ± 0.6	21.5 ± 0.6
retinol (mg)	1.04 ± 0.05	1.05 ± 0.05
ascorbic acid (mg)	105 ± 6	93 ± 5
α-tocopherol (mg)	10.6 ± 0.5a	8.8 ± 0.4b
calcium (mg)	1027 ± 36	1019 ± 21
phosphorus (mg)	1360 ± 34	1371 ± 27
magnesium (mg)	306 ± 9	305 ± 9
iron (mg)	15.8 ± 0.42	16.67 ± 0.36
zinc (mg)	8.6 ± 0.3	7.3 ± 0.2

[a] Values are means ± SE, $n = 18$. The subjects consumed the white diet (low in MRP) and the brown diet (rich in MRP) for 14 day periods with a 40 day washout period. Different letters in each file indicate significant differences (one-way ANOVA and Duncan test, $P < 0.05$).

Table 3. Dietary Calcium Utilization in Adolescent Males Aged 11−14 Years during Crossover Dietary Treatments with White and Brown Diets[a]

	white diet	brown diet
intake		
mg/day	1027 ± 36	1019 ± 21
mg/kg/day	18.4 ± 0.9	18.4 ± 0.7
fecal excretion		
mg/day	546 ± 49	583 ± 39
mg/kg/day	10.0 ± 1.1	10.7 ± 0.8
urinary excretion		
mg/day	64.2 ± 8.0	52.0 ± 7.4
mg/kg/day	1.1 ± 0.1	0.9 ± 0.1
absorption		
mg/day	481 ± 50	436 ± 37
mg/kg/day	8.4 ± 1.0	7.7 ± 0.7
retention		
mg/day	416 ± 51	384 ± 36
mg/kg/day	7.2 ± 1.0	6.8 ± 0.7

[a] Values are means ± SE, $n = 18$. The subjects consumed the white diet (low in MRP) and the brown diet (rich in MRP) for 14 day periods with a 40 day washout period. Subjects collected urine and feces on the last 3 days of each dietary period. Different letters in each file indicate significant differences (repeated measures ANOVA followed by a Bonferroni test, $P < 0.05$).

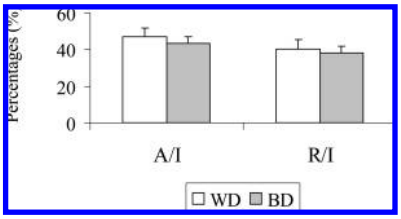

Figure 1. Calcium digestibility (%A/I) and calcium utilization efficiency (%R/I) in male adolescents aged 11−14 years after crossover dietary treatments with white diet (low in Maillard reaction products) and brown diet (rich in Maillard reaction products). A, absorbed calcium; I, ingested calcium; R, retained calcium. Values are mean ± SE, $n = 18$. The differences were not significant in any case (repeated measures ANOVA followed by a Bonferroni test, $P < 0.05$).

Values of fractional calcium absorption >40%, as in the present study, have been found among adolescents consuming high-calcium diets (1900 mg/day). Therefore, the diets designed for this study seemed to be adequate to achieve optimal absorption of

Table 4. Calcium Transport in Caco-2 Cells[a]

diet	transport (μg/well)	% transport	% availability
white	1.40 ± 0.07	12.58 ± 0.64	1.26 ± 0.15
brown	1.52 ± 0.20	12.65 ± 1.65	1.90 ± 0.35

[a] Values are mean ± SE of at least three wells from a representative experiment.

the dietary calcium, because they were balanced and contained the recommended servings of milk products, fruits, and vegetables (*40*). The similar calcium digestibility findings were consistent with the absence of variations observed in mineral solubility after the in vitro digestion of the diets (soluble calcium = 10.0 ± 2.61% and 15.0 ± 6.18% for WD and BD, respectively; insoluble calcium = 89.9 ± 2.61% and 85.0 ± 6.18% for WD and BD, respectively), supporting the conclusion that the in vitro gastrointestinal digestion constitutes a useful tool for predicting changes in nutrient availability, as mineral solubility in the intestinal tract is a prerequisite for it to be absorbed. The present data support that the high MRP content in the BD did not induce changes in calcium solubility and, therefore, did not affect its subsequent absorption. However, previous studies have shown a significant increase in calcium insolubility after the in vitro digestion of heated casein−sugar mixtures in a calcium solution, compared to the nonheated samples (*29*), which was attributed to the formation of Ca−MRP insoluble complexes. Because it has been described that calcium shows no particular affinity to MRP (*18*, *16*), it is possible that in a whole diet other divalent metals present (such as iron or zinc) avoid the formation of calcium−MRP chelates, thus preventing its binding and subsequent precipitation. In accordance, when glucose−lysine and glucose−methionine heated mixtures have been analyzed for their potential chelating activity in a multimineral solution at intestinal conditions, no changes were observed in calcium solubility, whereas other minerals (copper, iron, and zinc) have been significantly affected (*41*, *42*).

Results from the Caco-2 cell assay showed that the quantity of calcium from the bioaccessible fraction transferred across the cell monolayer did not differ significantly between the WD and BD and, as no differences were found in calcium solubility after in vitro digestion, calcium availability was similar in both diets (**Table 4**). On the contrary, experiments carried out in Caco-2 cells with a MRP model reveal that the presence of heated glucose−methionine or glucose−fructose−casein mixtures enhances calcium absorption, in comparison with the raw mixtures (*21*,*29*), although the glucose−lysine heated mixtures do not modify the calcium transported (*21*). Although there are only a few reports in the literature concerning the effect of MRP on calcium absorption across Caco-2 monolayers, some investigators have used this cell line to study calcium absorption from infant formulas (*43*) of from juice fruits (*44*), concluding that assays in Caco-2 cells offer a better indicator of bioavailability than solubility. The absence of differences in calcium availability between diets, as found in the present Caco-2 cell experiment, was in line with the in vivo results of calcium digestibility.

No bibliographic data were found about MRP effects on calcium digestibility in humans. Biological experiments of calcium absorption in rats fed diets containing a MRP model system from amino acid−sugar (*22*), protein−sugar (*29*), or infant formula (*45*) revealed no differences of calcium absorption due to the MRP intake. Decreases in calcium digestibility have been described in axenic but not in haloxenic rats (*20*). Therefore, it is possible that intestinal microflora in haloxenic animals hydrolyze the mineral−MRP complexes to some extent, enabling mineral absorption. The same effect may have taken place among the subjects of the present study. Moreover, the formation of MRP complexes with other minerals presenting more affinity than does calcium, and an adequate dietary calcium availability, could have contributed to maintaining mineral absorption and digestibility after consumption of MRP-rich diets.

Table 5. Biochemical Indices of Calcium Status and Bone Metabolism in Male Adolescents Aged 11−14 Years after the White and Brown Diets[a]

		white diet	brown diet
serum	Ca (mg/dL)	10.0 ± 0.1	9.9 ± 0.1
	PTH (pg/mL)	38.6 ± 4.4	38.6 ± 4.6
	AP (U/L)	721 ± 60	717 ± 72
urine	DPD (nM/Mm Cr)	18.3 ± 2.1a	13.0 ± 1.1b
	Cr (mM/day)	13.2 ± 1.3	14.6 ± 1.3
	Ca/Cr (mM/mM)	0.13 ± 0.02	0.11 ± 0.02
	Na/Cr (mM/mM)	11.8 ± 1.4	9.7 ± 1.1
	Ca/Na (μM/mM)	11 ± 1	10 ± 1
	P/Cr (mM/mM)	1.7 ± 0.1	1.6 ± 0.2

[a] Values are mean ± SE, n = 18. Different letters in each file indicate significant differences (repeated measures ANOVA followed by a Bonferroni test, $P < 0.05$).

It has been reported that the formation of certain mineral−MRP complexes could be responsible for increased trace element excretion in urine (*14*), especially of zinc (*30*). In humans, no significant variations have been found in urinary excretion of iron (*15*) or of major minerals such as phosphorus (unpublished data) after consumption of high-MRP diets. Few studies, performed in rats, have addressed the relationship between MRP intake and urinary calcium and have found either no significant changes in urinary calcium excretion when diets contain MRP from an amino acid−sugar model system (*20*, *22*) or increased excretion with protein−sugar MRP diets (*29*). In the present assay, urinary calcium excretion was slightly lower but not significantly different ($P = 0.15$) after consumption of the BD compared with the WD (**Table 3**), and both values were similar to those reported elsewhere in male adolescents (*38*). The low calcium affinity by advanced MRP and their preference to complex other cations present in the diet could explain the nonmodification of calcium elimination. Supporting this theory, the greater presence of browning compounds in urine found when the adolescents consumed the BD compared to the WD (*13*) was not associated with a higher elimination of calcium. Moreover, similar calcium/creatinine and calcium/sodium excretion ratios were observed in both periods (**Table 5**). In the present study, the phosphorus/creatinine ratio did not vary between the groups (**Table 5**). In view of the calcium−phosphorus relationship associated with bone metabolism, these findings suggest there were no significant metabolic changes in the subjects during the assay.

No significant changes in calcium retention and bioavailability (%R/I) (**Table 3**; **Figure 1**) were observed among the subjects after consumption of the BD, compared with the WD, in agreement with the results observed in rats fed diets containing MRP from amino acid−sugar or heated protein model- systems (*22*, *29*), but not with those for animals fed bottle-sterilized infant formula (*45*). The findings of the present assay suggest that MRP intake does not significantly affect the calcium balance in humans.

No significant differences in biochemical indices of calcium status and bone metabolism, which were within the normal range, were found after consumption of the diets (**Table 5**), except that DPD values were significantly lower after consumption of the MRP-rich diet ($P = 0.036$). An increase in DPD levels is observed during puberty as an indicator of bone turnover (*46*), and thus decreased values of this parameter after BD intake could be related to lower bone turnover during this period. It has been reported that exogenous advanced glycation end products (AGEs) proceeding from the diet (i.e., MRP) could increase

endogenous AGEs in plasma and cells (*47*, *48*). AGEs, which are formed by a nonenzymatic glycation or oxidation reaction, have detrimental effects on the mechanical and biological functions of bone and, therefore, are considered to be a class of disadvantageous cross-linking in bone (*49*). It has been suggested that AGEs formation in the bone matrix may alter bone metabolism, decrease calcium uptake, and increase calcium resorption (*50*, *51*). Thus, although no changes in calcium balance were found after consumption of the MRP-diet in the present assay, it is possible that high levels of MRP act synergistically with AGEs to produce certain negative changes in bone metabolism, manifested in a decrease in DPD values.

In summary, current findings show that high MRP intake has no apparent effects on dietary calcium bioavailability in adolescents when consumed as a part of a balanced and varied diet. However, possible metabolic changes cannot be discounted, as lower DPD urinary excretion may be related to decreased bone turnover at this age. In view of the current dietary habits of adolescents, and the well-established relationship between calcium deficiency, bone formation during the growth spurt, and, consequently, osteoporosis in the adult period, it seems of special interest to study the possible long-term effects of dietary MRP on calcium metabolism.

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Received June 1, 2009. Revised manuscript received September 10, 2009. Accepted September 22, 2009.