Effects of Heat Treatment of Casein in the Presence of Reducing Sugars on Calcium Bioavailability: in Vitro and in Vivo Assays

Isabel Seiquer,*,† Teresa Aspe,‡ Pilar Vaquero,‡ and Pilar Navarro†

Unidad de Nutrición Animal, Estación Experimental del Zaidín, CSIC, 18100 Granada, Spain, and Instituto de Nutrición y Bromatología (CSIC-UCM), Facultad de Farmacia, Ciudad Universitaria, 28040 Madrid, Spain

Casein−glucose−fructose mixtures unheated (C) or after heating (HC) were added to a solution of ionic calcium to study calcium speciation and included in diets (C-D, HC-D) for rats. Samples and diets were digested in vitro. Supernatants of digested samples were used for transport experiments with Caco-2 cells. Total soluble and ionic calcium levels were lower and precipitated calcium levels higher with HC compared to C. Dialyzed calcium from the diets was highly ionic and lower in HC-D compared to C-D. Nondialyzed soluble calcium was also lower, whereas precipitated calcium was higher, in HC-D. HC increased calcium transport in Caco-2 cells compared to C, but transport efficiency decreased due to lower calcium solubililty after digestion. Urinary calcium increased with HC-D consumption without changes in calcium absorbed and retained. Maillard reaction products in HC decrease calcium solubility, but enterocyte metabolism and calcium absorption and retention seem to be unaffected. Nevertheless, urinary calcium losses increase.

Keywords: *Caco-2; in vitro digestion; calcium bioavailability; rat*

INTRODUCTION

Calcium is known to present a close, complex relationship with dietary protein, evidenced by its absorption, urinary excretion, balance, etc. This relationship has been described not only concerning the quantity but also the type of protein and various amino acids (*1*). In this context, the high bioavailability of milk calcium is due to, among other reasons, the existence of casein phosphopeptides (CPP), which may be released by enzymatic proteolysis during the gastrointestinal digestion of casein (*2*). These peptides have a high calcium binding capacity and can enhance calcium absorption by inhibiting the luminal precipitation of calcium salts (*3*).

Processed foods are increasingly used in the human diet, and the heat treatment of food protein produces desirable, but also undesirable, changes: cross-linking, protein degradation, Maillard browning reactions, racemization of amino acids, and, occasionally, molecular interactions among nutrients with other food ingredients (*4*, *5*). Some of theses changes may affect the nutritive value of protein and the utilization of other nutrients, including minerals. Milk proteins are among the most sensitive compounds to heat, and casein undergoes chemical and structural changes during thermal processing of milk, which may affect the profile of bioactive peptides produced during protein digestion (*2*).

One of the most common reactions related to heat-induced changes in milk proteins is the Maillard reaction (MR), which at an initial stage involves interactions between the amino group of protein-bound lysine with lactose, and it may also take place with other amino acids and sugars that are present (*6*, *7*). Adverse effects on mineral metabolism have been described among the numerous physiological and pharmacological effects of Maillard reaction products (MRP) (*8*, *9*). Brown polymeric pigments produced during the MR (melanoidins) behave as chelating agents for metal cations, forming soluble and insoluble complexes (*10*) that may affect mineral bioavailability (*11*, *12*). Rendleman (*10*) described the formation of calcium complexes with different melanoidins, which presented different calcium binding abilities. The specific ability to bind calcium may be related to structural differences of the MRP.

Animal studies have demonstrated the relationship between MRP and calcium metabolism. Diets containing MRP impair calcium absorption in axenic but not in haloxenic rats (*13*) and produce increased urinary excretion with no modifications in calcium balance (*14*, *15*). Thus, balance studies in rats are shown as a useful method to examine calcium availability in vivo.

Another model that has been introduced in nutrition research is a human colon adenocarcinoma cell line, Caco-2, which is the only intestinal cell line that differentiates spontaneously in culture, exhibiting structural and functional characteristics of mature small intestinal enterocytes (*16*). Several studies have demonstrated that Caco-2 cells provide a unique in vitro model to investigate the cellular and molecular aspects of calcium transport (*17*−*19*), and good correlation with human bioavailability has been obtained for other minerals (*20*).

The aim of this study was to assess the influence of heating casein with glucose and fructose on different aspects of calcium bioavailability by changes on the physicochemical form (calcium speciation), calcium dialyzability, calcium transport in Caco-2 cells, and calcium balance assays in rats.

* Author to whom correspondence should be addressed (telephone 34-58-572757; fax 34-58-572753; e-mail iseiquer@eez.csic.es).
† Unidad de Nutrición Animal.
‡ Instituto de Nutrición y Bromatología (CSIC-UCM).

10.1021/jf001008v CCC: $20.00
Published on Web 01/26/2001

Table 1. Nutrient Content of Experimental Diets Containing a Heated and an Unheated Casein−Glucose−Fructose Mixture[a]

	diet[a]	
	C-D	HC-D
moisture (g/100 g)	3.95 ± 0.12	4.36 ± 0.05
protein (g/100 g)	10.55 ± 0.08	10.49 ± 0.10
fat (g/100 g)	8.04 ± 0.11	7.83 ± 0.20
ashes (g/100 g)	3.30 ± 0.24	3.46 ± 0.17
Ca (mg/g)	5.93 ± 0.35	5.79 ± 0.18
Mg (mg/g)	0.46 ± 0.07	0.46 ± 0.04
Fe (μg/g)	56.20 ± 5.17	55.25 ± 4.08
Cu (μg/g)	8.05 ± 0.90	7.57 ± 0.79
Zn (μg/g)	35.08 ± 1.40	32.64 ± 1.14

[a] Values are mean ± SD. C-D, diet containing raw casein−glucose−fructose; HC-D, diet containing heated casein−glucose−fructose.

MATERIALS AND METHODS

Sample Preparation. All chemicals were purchased from Merck (Darmstadt, Germany). Hammarstein casein (62.5%) was carefully mixed with 37.5% of a glucose−fructose mixture (w/w) to prepare samples, which were assayed either without processing (C) or after being heated in a stove at 150 °C for 1 h (HC).

The development of the brown color that occurs during the heat treatment of the sample was measured with a Milton Roy Spectronic 1201 (Rochester, NY) spectrophotometer. Aliquots (0.1 g) of the complete untreated or heated mixture were dissolved in Milli-Q water (Milli-Q Ultrapure Water System, Millipore Corp., Bedford, MA) (10 mL), and browning intensity was determined by measuring the absorbance at 420 nm. The determination of furosine in the samples was quantified using the method of Resmini et al. (*21*).

Diet Preparation. Semisynthetic diets, prepared according to National Research Council recommendations, had the following composition: 37.5% starch (Central Ibérica de Drogas, S.A., Madrid, Spain), 29.83% sugar (Confisa, S.A., Madrid, Spain), 8% pure olive oil (Carbonell, Córdoba, Spain), 5% cellulose (Central Ibérica de Drogas, S.A), 0.2% DL-methionine (Merck), 3.81% mineral mixture (Merck), and 0.16% vitamin mixture (Roche, Basel, Switzerland). The only component that varied in the diets was that samples C and HC (16.90%) were used as a source of protein and as a partial source of carbohydrates (diets C-D and HC-D, respectively). The nutrient composition of the diets is shown in Table 1.

Calcium Speciation Assays. To a standard solution containing 1.5 mL of ionic calcium (pH 7, Calcium-pH Qualicheck 52360, Radiometer, Copenhagen, Denmark) was added 30 mg of the samples. This was then stirred for 2 h and centrifuged at 1000*g* for 25 min (CS-6R Centrifuge, Beckman). As a result, total calcium appeared in three forms: ionic calcium, nonionic soluble calcium, and precipitated calcium. Ionic calcium was analyzed in the supernatant (ICA 1, Radiometer), and total soluble calcium (including ionic and nonionic forms) in the supernatant and precipitated calcium were quantified by atomic absorption spectrometry (AAS, see below).

In Vitro Digestion. In vitro digestion of isolated samples and diets was carried out. The in vitro digestion technique of Miller et al. (*22*) was used, modified to our requirements. It comprised two stages: gastric digestion and intestinal digestion. Shortly before use, 0.4 g of pepsin (Sigma Chemical Co., St. Louis, MO, P-7000) was dissolved in 2.5 mL of 0.1 M HCl. For intestinal digestion, 0.1 g of pancreatin and 0.625 g of bile salts (both from Sigma Chemical Co., P-3292 and B-8756, respectively) were dissolved in 25 mL of 0.1 M $NaHCO_3$.

Sample Digestion. One gram of each sample was suspended in a final volume of 10 mL (8.5 mL of Milli-Q water and 1.5 mL of a stock solution 0.1 M Ca). The in vitro digestion of the samples was carried out as described previously (*12*). Briefly, the pH was adjusted to 2, the pepsin solution was added at a proportion of 0.05 g of pepsin/g of sample, and samples were incubated at 37 °C in a shaking water bath at 110 oscillations/min for 2 h for the gastric digestion. The pH of the digest was raised to pH 6 for intestinal digestion, and the pancreatin suspension was added at the proportion of 2.50 mL of pancreatin suspension/g of sample. The pH was then adjusted to pH 7.5, and samples were incubated at 37 °C at 110 oscillations/min for 2 h.

After intestinal digestion, the samples were centrifuged at 1000*g* for 25 min (CS-6R centrifuge, Beckman) to separate soluble and precipitated Ca. The supernatants were carefully separated, and the percentages of soluble and precipitated Ca were calculated from the initial concentration by AAS. These supernatants were used for transport experiments with Caco-2 cells.

Diet Digestion. In vitro availability assays using Miller's technique (*22*) as modified by Vaquero et al. (*23*) were performed on the diets. Milli-Q water (300 mL) was added to 6 g of each diet, and gastric and intestinal digestions were carried out as described above, except that intestinal digestion was performed for 4 h in aliquots of 40 g of the digest. During pancreatic digestion, dialysis of the diets took place through a 12000 cutoff dialysis tubing (Medicell International Ltd., size 9 36/32) containing a solution of sodium bicarbonate equivalent to the titratable acidity. Dialyzed and nondialyzed samples were collected after 1, 2, 3, and 4 h. Nondialyzable fractions were centrifuged for 15 min at 1000*g* (CS-6R centrifuge, Beckman) to separate the soluble and insoluble forms of calcium. After 1, 2, 3, and 4 h of intestinal digestion, the percentages of total dialyzed, ionic dialyzed, soluble nondialyzed, and precipitated dietary calcium were analyzed.

Cells. *Cell Culture.* Caco-2 cells were purchased from the European Collection of Cell Cultures (ECACC) at passage 20 and used in experiments at passages 22−30. All cell culture media and cell culture grade chemicals were obtained from Sigma Chemical Co. Culture flasks were purchased from Corning Costar (Cambridge, MA). Cells were grown 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 maintained at 37 °C in an incubator in an atmosphere of air/CO_2 (95:5) at 90% humidity, and the medium was changed every 2 days.

Trypsinization and seeding of cells were performed as described elsewhere (*12*). At 70% confluency, cells were collected and seeded into bicameral chambers (Transwell, 24-mm diameter, 4.7-cm^2 area, 3-μm pore size, Costar) at a density of 100.000 cells/cm^2, with 2.5 mL of medium in the well (basolateral cell side) and 1.5 mL of medium in the insert (apical cell side). Cell viability was assessed by trypan blue exclusion and was typically 85−95%. The medium was changed every second day and the day before cultures were used for transport experiments. The development of functional tight junctions during differentiation of Caco-2 cells was monitored by determining transepithelial electrical resistance (TEER) as described (*12*). The monolayers used in this study exhibited adequate TEER values ranging from 500 to 650 Ω/cm^2.

Calcium Transport. Twenty-one days after initial seeding, calcium transport experiments were carried out. Spent culture medium was aspirated from the apical and basolateral chambers, and apical and basolateral cell surfaces of the monolayer were washed three times with Ca^{2+} and Mg^{2+}-free HBSS at 37 °C. Then the transport solution (2.5 mL) was added to the basolateral chamber, and test transport solutions were added to the apical chamber (1.5 mL). The transport solution contained 130 mmol/L NaCl, 10 mmol/L KCl, 1 mmol/L $MgSO_4$, 5 mmol/L glucose, and 50 mmol/L HEPES, pH 7. Three different test transport solutions containing the same Ca concentration (2.5 mM) were added to the apical chamber. The test transport solutions were as follows: transport solution containing Ca to reach a final concentration of 2.5 mM (TS); transport solution containing the C sample digest (TS-C), and transport solution containing the HC sample digest (TS-HC). Supernatants of digested samples were used as a source of

calcium, with the necessary volume added to the transport solution to bring the final Ca concentration to 2.5 mM in both cases. The test transport solutions were incubated at 37 °C until the experiments were started. Due to the different calcium concentrations of the digestion supernatants, the volumes needed to reach the same final concentration of Ca were different for the C and HC samples. Therefore, to avoid the influence due to the different volumes of digested samples, another experiment was performed with the same volume of HC digested sample as the C digested sample, which had a Ca concentration of 0.78 mM (TS-HC−low Ca). Moreover, by comparing the experiments performed with TS-C and TS-HC−low Ca solutions, we tried to simulate the physiological situation that would occur during the same intake of the two samples.

The cell cultures were then incubated at 37 °C in humidified air: CO_2 atmosphere for 4 h. To calculate the Ca transported across the cell monolayer, the buffer from the basolateral chamber was removed and, to ensure complete collection, the wells were washed twice with deionized water.

Cell viability after 4 h of exposure to the test transport solutions was assessed by trypan blue exclusion, and viability was never <85%.

Animal Assay. Sixteen weanling Wistar rats weighing 40 ± 0.5 g (mean ± standard error), provided by the Laboratory Animal Service of the Institute of Nutrition of Madrid, CSIC-UCM, were used for balance assays. Each animal was placed in an individual metabolic cage in an environmentally controlled room kept at 20−22 °C, with a 12-h light−dark cycle and 55−60% humidity.

The eight animals (four males and four females) of each experimental group were fed one of the two different diets (C-D or HC-D). Animals were allowed free access to food and demineralized water. The test involved a preliminary 4-day adaptation period during which solid intake and body weight changes were monitored, followed by a second period lasting 7 days in which solid intake and body weight were monitored and Ca balances were carried out. Urine and feces were collected daily separately and stored as a 1-week pool for this period. The feces were lyophilized (FTS System, Inc., TDS-3, New York), weighed, and homogenized (Moulinex grinder 980). Urine was collected in 0.5% v/v HCl solution and later filtered (Whatman No. 41 filter papers, ashless, Whatman, Maidstone, U.K.) and diluted.

Analytical Determinations. Diets, feces, culture media, and transport solutions were dry-ashed in a muffle furnace at 450 °C. Ashes were dissolved with $HCl/HNO_3/H_2O$ (1:1:2) (Suprapur, Merck). Ca analyses of diets, feces, urine, culture media, and transport solutions were performed by means of flame AAS in a Perkin-Elmer 1100 B spectrophotometer (Überlinger, Germany) using 0.5% lanthanum ($LaCl_3$, Merck) to avoid interferences. A stock standard solution of calcium (1 g/L) was prepared from Titrisol (Merck) ($CaCl_2$ in 6.5% HCl, 1.000 ± 0.002 g). A blank solution with lanthanum was also used.

Samples of skimmed bovine milk reference standard (certified reference material CRM 63; Community Bureau of Reference, Brussels, Belgium) were simultaneously used to check the Ca recovery (Ca value = 12.5 ± 0.2 mg/g, mean ± SD of eight determinations, certified value 12.6 ± 0.3 mg/g). The interassay coefficients of variation for Ca were 4.63% in the diet, 2.33% in feces, and 2.46% in urine. All glassware and the polyethylene samples bottles were washed with 10 N nitric acid, and demineralized water (Milli-Q) was used throughout.

The following indices were calculated from data on Ca intake and fecal and urinary calcium excretion: food efficiency = body mass gain/food intake in dry matter; apparent absorption = intake − fecal excretion; $\% A/I$ = (apparent absorption/intake) × 100; apparent retention = apparent absorption − urinary excretion; $\% R/A$ = (apparent retention/apparent absorption) × 100; $\% R/I$ = (apparent retention/intake) × 100.

Statistical Treatment. Data concerning Ca speciation in samples, Ca transport in Caco-2 cells, and Ca balance were analyzed by the one-way analysis of variance (ANOVA), followed by Duncan's multiple-range test to compare significant variations between means ($P < 0.05$). Ca speciation data after in vitro digestion of diets were studied by means of two-way analysis of variance to examine the effects of the type of diet (C-D or HC-D), of the duration of intestinal digestion (1, 2, 3, or 4 h), and of interactions between diet and duration time. Significance of the results was established at $P < 0.05$.

Table 2. Percentage Distribution of Ca in the Presence of a Heated and an Unheated Casein−Glucose−Fructose Mixture

sample[a]	ionic[b]	total soluble[b]	precipitated[b]
C	42.62 ± 1.84[a]	82.20 ± 2.10[a]	17.42 ± 3.59[a]
HC	19.30 ± 0.22[b]	18.06 ± 1.17[b]	81.45 ± 0.82[b]

[a] C, unheated casein−glucose-fructose; HC, heated casein−glucose−fructose. [b] Different superscripts in each column indicate significant differences ($P \leq 0.05$). Values are means ± SD of six determinations.

RESULTS

Sample Characterization. The HC sample had an absorbance value of 0.332 ± 0.01 using the C sample as blank. The furosine content was undetected in the C sample and was 101.97 ± 0.5 mg/100 g of protein in the HC sample.

Calcium Speciation. Table 2 shows the results of the changes in soluble and insoluble calcium in the presence of the samples. When a calcium solution was added to the C sample, ~20% of the total calcium was found to be insoluble and nearly half of the total soluble calcium was in the ionic form. With the heated casein−glucose−fructose mixture, the percentage of soluble calcium was significantly lower and the percentage of precipitated calcium significantly higher, in comparison with the unheated mixture. Moreover, all of the soluble calcium was ionic calcium in the HC sample.

In Vitro Digestion Assays. After in vitro digestion of isolated samples with calcium, the percentage calcium distribution was as follows: 93.12 ± 2.33 and 0.51 ± 0.09 of soluble and insoluble calcium, respectively, with the C sample, 21.90 ± 2.29 and 73.04 ± 2.21 of soluble and insoluble calcium, respectively, with the HC sample. Differences between the samples were significant in both cases.

The results of in vitro digestion of the diets showed a greater proportion of dialyzed calcium in C-D compared with HC-D, at every hour of intestinal digestion (Table 3). For both diets the amount of dialyzed calcium tended to decrease after the second hour, especially with HC-D. Dietary dialyzed calcium was highly in ionic form, because the values of ionic calcium were very similar to those of the corresponding soluble dialyzed calcium, but in HC-D the proportion of ionic calcium with respect to total soluble dialyzed calcium was lower than in C-D, and it diminished during pancreatic digestion.

In the nondialyzed calcium, the insoluble calcium was higher than soluble calcium in any case. In HC-D, the soluble nondialyzed calcium showed a significant decrease during intestinal digestion, which, along with the decrease of ionic dialyzed calcium, contributed to a significant increase in the precipitate. In contrast, in C-D soluble nondialyzed calcium did not vary during the first and second hours; consistently, the differences between samples were significant from the second hour.

Caco-2 Cells Assays. Ca transport (Table 4) was expressed as micrograms of Ca transported to the basolateral chamber per well and as the percentage of Ca transported per well from the experimental transport solution. As calcium availability is affected by

Table 3. Percentage Distribution of Ca after in Vitro Digestion of Diets Containing a Heated and an Unheated Casein−Glucose−Fructose Mixture, at Each Hour of Intestinal Digestion

		dialyzed[a]		nondialyzed[a]	
diet[b]	hour	total	ionic	soluble	precipitated
C-D	1	22.60 ± 2.6^{ab}	21.84 ± 1.5^{a}	35.23 ± 0.7^{a}	42.17 ± 1.2^{a}
	2	23.73 ± 1.2^{a}	23.55 ± 0.3^{b}	36.07 ± 0.8^{ab}	40.19 ± 1.0^{b}
	3	22.67 ± 0.8^{ab}	23.50 ± 0.3^{b}	37.11 ± 0.6^{c}	40.21 ± 0.4^{b}
	4	21.60 ± 0.8^{b}	20.84 ± 0.7^{bb}	36.89 ± 0.5^{bc}	41.50 ± 0.3^{a}
HC-D	1	*18.99 ± 0.7^{ab}	*17.35±0.4^{a}	36.07 ± 0.1^{a}	*44.93 ± 0.7^{a}
	2	*19.63 ± 0.2^{b}	*16.22 ± 1.1^{a}	*35.07 ± 0.3^{b}	*45.30 ± 0.1^{a}
	3	*18.06 ± 0.3^{ab}	*14.74 ± 0.1^{b}	*34.13 ± 0.2^{c}	*47.80 ± 0.2^{b}
	4	*17.46 ± 0.2^{a}	*13.13 ± 0.4^{c}	*32.93 ± 0.1^{d}	*49.60 ± 0.1^{c}
ANOVA					
diet		0.0000	0.0000	0.0000	0.0000
time		0.0086	0.0000	NS	0.0000
diet × time		NS	0.0011	0.0000	0.0000

[a] Different superscripts in each column indicate significant differences for each diet between the different hours of intestinal digestion. Asterisks indicate significant differences between diets for each hour of intestinal digestion ($P \leq 0.05$). Values are means ± SD of six determinations. [b] C-D, diet containing unheated casein-glucose-fructose; HC-D, diet containing heated casein-glucose-fructose.

Table 4. Ca Transport in Caco-2 Cells after 4 h of Exposure to Solutions Containing the Digest from a Heated and an Unheated Casein−Glucose−Fructose Mixture

		Ca transport[a]		
solution[b]	Ca (mM)	μg/well	%	% transport efficiency[c]
TS	2.5	37.12 ± 3.15^{a}	24.75 ± 2.09^{a}	
TS-C	2.5	58.99 ± 4.16^{b}	39.33 ± 2.77^{b}	36.64 ± 2.58^{a}
TS-HC	2.5	68.40 ± 1.30^{c}	45.59 ± 0.87^{c}	9.96 ± 0.19^{b}
TS-HC−low Ca	0.78	20.68 ± 2.60^{d}	44.13 ± 5.55^{bc}	9.64 ± 1.21^{b}

[a] Different superscripts in the same column indicate significant differences ($P \leq 0.05$). [b] TS, transport solution; TS-C, TS containing the necessary volume of the digest from the C sample to bring a final Ca concentration of 2.5 mM; TS-HC, TS containing the necessary volume of the digest from the HC sample to bring a final Ca concentration of 2.5 mM; TS-HC−low Ca, TS containing the same volume of the digest from the HC sample than the TS-C.[b] % transport efficiency = % soluble calcium × % transported calcium/100. Values are means ± SD of at least three wells from a representative experiment.

changes in Ca speciation after digestion, and due to the different soluble calcium percentages after in vitro digestion of the samples, the calcium transport efficiency was expressed by taking into account differences in calcium solubility, as follows:

$$\text{transport efficiency (\%)} = (\text{\% soluble calcium} \times \text{\% transported calcium})/100$$

The quantity of soluble Ca transferred from the apical to the basolateral compartment increased significantly with the addition of soluble casein−glucose−fructose digestion products to the transport solution. Adding the HC sample increased the calcium transport with respect to the C sample. However, the transport efficiency decreased significantly both in the TS-HC and in the TS-HC−low Ca with respect to the TS-C, due to differences in calcium solubility after in vitro digestion of the samples.

Animal Assays. The effects of feeding the HC-D diet on the body weight of animals are described in a previous paper (*12*). Data of Ca balance are shown in Table 5. Animals fed the different diets showed no significant variations in Ca intake or fecal excretion. However, there was an increase in urinary Ca in animals given the HC-D diet. Values of absorbed and retained calcium were not significantly different between groups. Biological indices are shown in Figure 1. Only the % *R*/*A* was found to be significantly lower in animals fed the HC-D diet respect to the other group.

DISCUSSION

Calcium Speciation. After addition of sample C to the calcium solution, the precipitation of calcium may be accounted for by the calcium sensitivity of α_s- and β-casein fractions at room temperature, thereby coprecipitating the element (*24*). Moreover, as casein binds calcium both by producing colloidal calcium phosphate salts inside the micelle and directly to its protein fraction (*25*), the incorporation of the samples within the calcium solution might supply ligands that bind the element and keep it soluble, decreasing the ionic fraction. Thus, due to the presence of the C sample, a small fraction of calcium precipitated and the rest remained soluble, although only half of the latter was still ionic calcium.

It is well-known that heat alters the casein micelle structure in milk and produces the dissociation of κ, β, and α_s fractions (*26*). Law (*27*) showed that in heated milk there is an increase in the level of dissociation of casein fractions, and the concentration of κ-casein in the serum increases markedly. The κ fraction is soluble at any temperature and, in addition, it plays an essential role in maintaining the micelle structure by stabilizing and preventing α_s and β fractions from precipitating in the presence of Ca. Thus, there would be a marked reduction of total soluble calcium when heated casein is added to the calcium solution, because heating could have affected the ligands that maintain the calcium in solution. In support of this, the soluble calcium content in heated goat's milk has been found to be lower than in unheated milk (*28*). Besides the influence of the denatured protein itself, the negative effect of the MRP should be considered; the marked browning and the furosine content of the HC sample indicated that the Maillard reaction has taken place. The nonsoluble pigments, melanoidins, may act as cation chelating agents that decrease the solubility of the elements by binding them and producing stable complexes. Calcium appeared to bind weakly to insoluble MRP (*10*, *29*); thus, precipitated calcium increased in the presence of the HC sample. However, in this undigested sample, binding

Table 5. Calcium Balance in Rats Fed Diets Containing a Heated and an Unheated Casein−Glucose−Fructose Mixture

	Ca (mg/day)[a]				
diet[b]	intake	fecal	urinary	absorbed	retained
C-D	56.11 ± 3.18[a]	19.24 ± 2.98[a]	1.37 ± 0.45[a]	36.95 ± 1.57[a]	35.58 ± 1.63[a]
HC-D	51.91 ± 7.72[a]	17.07 ± 3.83[a]	2.48 ± 1.18[b]	34.84 ± 4.62[a]	32.36 ± 4.39[a]

[a] Different superscripts in the same column indicate significant differences ($P \leq 0.05$). Values are means ± SD of eight animals. [b] C-D, diet containing unheated casein−glucose−fructose mixture; HC-D, diet containing heated casein−glucose−fructose mixture.

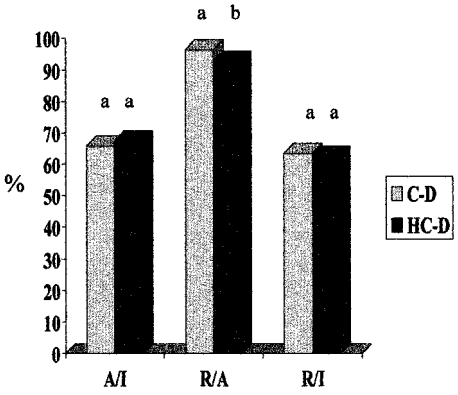

Figure 1. Biological indices in rats fed different diets. C-D, diet containing unheated casein−glucose−fructose; HC-D, diet containing heated casein−glucose−fructose. *A*, absorbed Ca; *I*, ingested Ca; *R*, retained Ca. Different letters indicate significant differences between groups ($P \leq 0.05$). Values are means ± SD for eight animals.

of Ca to MRP seems to be responsible only to a slight extent for calcium precipitation, because in other assays when the same heating conditions were applied, it was demonstrated that casein alone was able to precipitate as much as 63.82% of the calcium present (*15*).

In Vitro Digestion Assays. Milk proteins are precursors of many biologically active peptides that can be released by enzymatic proteolysis. Among these active peptides, the CPP can form soluble organophosphate salts and may function as carriers for different minerals, especially calcium. CPP, formed when casein is degraded by proteolytic enzymes in the digestive tract, can also be formed in vitro by trypsin digestion and to some extent by pepsin digestion of casein (*30*). According to Hansen et al. (*31*) and Meisel (*2*), soluble organophosphate salts derived from CPP keep calcium in the soluble form at neutral pH, which is considered to be important for absorption. Thus, most of the calcium was found to be soluble after in vitro digestion of the C sample, probably due to the formation of CPP.

During the thermal processing of food protein, structural and chemical changes occur and, consequently, the profile of bioactive peptides produced during protein digestion may be altered (*2*). Thus, in the HC sample, the casein structure was altered by the heat treatment. Heat induces dephosphorylation of casein, and this may produce both structural changes and a lower level of phosphopeptides. As a result, their potential effect as mineral carriers is impaired (*32*). This fact could contribute to the significant increase in the insoluble calcium fraction after in vitro digestion of the HC sample.

In the assays of in vitro digestion with dialysis of the diets, the lower proportion of ionic calcium with respect to the total dialyzed calcium found in HC-D could be due to the fact that part of the soluble dialyzed calcium may remain bound to the soluble products formed or released during digestion. Among these products, small peptides that could act as carriers should be considered. Moreover, premelanoidins and soluble melanoidins may bind the element and keep it soluble, allowing its absorption but not its metabolic utilization and, consequently, increasing the urinary excretion (*33*).

In HC-D, the soluble nondialyzed calcium showed a significant decrease during the intestinal digestion time. Therefore, C-D and HC-D diets were affected in a different way by the in vitro digestion process. It seems that during digestion of the HC sample small soluble ligands may dialyze and keep the calcium in solution, thus decreasing ionic soluble calcium, whereas others contribute to calcium insolubility. In vivo studies have shown a slowing in protein digestion due to the heat treatment of the protein (*33*), which may explain the influence of the digestion time in HC-D. Moreover, thermal treatment induces the dephosphorylation of casein, reducing calcium binding to CPP (*30*) and, consequently, decreasing calcium solubility (*34*). MRP of HC-D may also have formed insoluble metal ion complexes after in vitro digestion, as has been reported previously for glucose−glutamate MRP (*29*).

Caco-2 Cell Assays. According to the results of the present assay, calcium from the soluble fraction of the digested casein was transported across the Caco-2 monolayer more efficiently than Ca from the transport solution without digestion products. Although there is no information about the influence of casein digestion products on Ca transport in Caco-2 cells, this finding may be related to the formation of CPP during the digestion of casein. Bioactive peptides can interact with target sites of the luminal side of the intestinal tract and, furthermore, they can be absorbed (*35*). CPP released by the digestion of casein have been shown to enhance paracellular absorption of Ca in rats (*36*, *37*). However, taking into account differences in Ca solubility after in vitro digestion and considering the transport efficiency, the proportion of calcium transported into the basolateral chamber decreased significantly with TS-HC compared to TS-C. According to the results obtained by the in vitro digestion, heating casein in the presence of reducing sugars appears to decrease Ca availability as a result of increased Ca precipitation during in vitro digestion. However, the remaining soluble calcium of the digested HC sample seems to be transported across the cell monolayer more efficiently than in the C sample. This was confirmed by the experiments in which the transport solution contained the same volume of supernatant digest as TS-C (TS-HC−low Ca). In these assays, the total quantity of Ca transferred across the cell monolayer was significantly lower, due to the lower content of the sample digest, but the total digestion−absorption process presented the same efficiency for calcium transport as TS-HC.

Animal Assays. Balance studies showed that the digestive utilization of calcium did not vary after consumption of the diet containing the heated casein−glucose−fructose mixture. However, increased values of urinary calcium excretion found in animals fed the HC-D diet are in agreement with other studies in rats

fed diets containing MRP of xylose/lysine or glucose/glycine (*38*, *39*) and of glucose/glutamate (*40*). This effect, as some of these investigators have pointed out, may be ascribed to premelanoidins and soluble melanoidins, absorbable compounds that act as metal ligands and enhance elimination through the urinary pathway. This last mechanism could be related with the high proportion of calcium transported across the Caco-2 cells with the heated sample and the decrease in ionic dialyzed calcium found during in vitro digestion of the HC-D diet. Increased urinary excretion of trace elements, especially zinc, has been found following the parenteral administration of sterilized serum containing glucose and amino acids (*41*) and after the intake of diets containing different MRPs (*12*, *42*). Despite higher values of urinary calcium excretion, the retained Ca did not reach significant variations. Values of absorbed calcium did not vary either. Similarly, Andrieux and Saquet (*39*) and O'Brien et al. (*14*) showed no changes in calcium absorption or retention in conventional rats fed diets containing different MRPs. In contrast, in germ-free rats lower calcium intestinal absorption has been reported (*39*), which led to lower Ca retention. O'Brien et al. (*43*), using everted duodenal and ileal sacs from rats, showed that MRPs inhibit the duodenal and ileal transport of calcium. O'Brien and Morrisey (*8*) wonder if the inhibitory effect of MRPs on small intestinal calcium transport might constitute the action of a chelator or an inhibitor of enterocyte metabolism. The present assay with Caco-2 cells suggests that enterocyte metabolism is not inhibited in the presence of the digested MRP assayed, as the transport of soluble calcium increased when the transport solution contained the HC digested sample.

It is evident that consumption of HC-D did not modify calcium balance, and only a significant decrease in the ratio % *R*/*A* in animals fed the HC-D diet was observed (Figure 1).

In summary, in vivo studies seem to attenuate the negative effects of MRP found in vitro concerning calcium availability. This finding is consistent with the report of O'Brien et al. (*43*), who did not find in vivo the inhibitory effect of MRP on calcium absorption observed by everted sacs ex vivo. The data from the present study suggest that calcium is not particularly sensitive to the effect of the MRP assayed, especially when compared with other trace elements (*11*). The presence of the heated casein−glucose−fructose mixture in the diet exerted a small influence on calcium bioavailability, because only on the enhancement in urinary calcium was a negative influence observed. This finding together with some of the in vitro results deserves long-term studies concerning the influence of MRPs on calcium metabolism.

LITERATURE CITED

(1) Wapnir, R. A. *Protein Nutrition and Mineral Absorption*; CRC Press: Boca Raton, FL, 1990.
(2) Meisel, H. Biochemical properties of regulatory peptides derived from milk proteins. *Biopolymers* **1997**, *43* (2), 119−128.
(3) Naito, H. Casein phosphopeptide: intra-intestinal formation and significance in the bioavailability of calcium. In *Nutrition: Proteins and Amino Acids*; Yoshida, A., Naito, H., Niiyama, Y., Suzuki, T., Eds.; Japan Scientific Societies Press: Tokyo, Japan, 1990; pp 207−220.
(4) Friedman, M. Dietary impact of food processing. *Annu. Rev. Nutr.* **1992**, *12*, 119−137.
(5) Goddard, S. J.; Augustin, M. A. Formation of acid-heat-induced skim milk gels in the pH range 5.0−5.7: effect of the addition of salts and calcium chelating agents. *J. Dairy Res.* **1995**, *62*, 491−500.
(6) Furniss, J.; Hurrel, R. F.; Finot, P. A. Modifications of urinary zinc excretion in the rat associated with the feeding of Maillard reactions products. *Acta Pharmacol. Toxicol.* **1986**, *59*, 188−190.
(7) Friedman, M. Food Browning and its prevention: and overview. *J. Agric. Food Chem.* **1996**, *44*, 632−653.
(8) O'Brien, J.; Morrissey, P. A. Nutritional and toxicological aspects of the Maillard browning reaction in foods. *Crit. Rev. Food Sci. Nutr.* **1989**, *28*, 211−248.
(9) Finot, P. A. Metabolism and Physiological Effects of Maillard Reaction Products (MRP). In *The Maillard Reaction in Food Processing, Human Nutrition and Physiology*; Finot, P.-A., Aeschbacher, H. U., Hurrell, R. F., Liardon, R., Eds.; Birkhauser: Basel, Switzerland, 1990; pp 259−271.
(10) Rendleman, J. A., Jr. Complexation of calcium by melanoidin and its role in determining bioavailability. *J. Food Sci.* **1987**, *52*, 1699−1705.
(11) Seiquer, I.; Delgado-Andrade, C.; Navarro, P. Effects of the heat treatment of casein on Zn transport and uptake by Caco-2 cells. *Proc. Nutr. Soc.* **2000**, *59*, 134A.
(12) Navarro, P.; Aspe, T.; Seiquer, I. Zinc transport in Caco-2 cells and zinc transport in rats: influence of the heat treatment of a casein-glucose-fructose mixture. *J. Agric. Food Chem.* **2000**, *48*, 3585−3596.
(13) Andrieux, C.; Sacquet, E.; Gueguen, L. Interactions between Maillard's reaction products, the microflora of the digestive tract and mineral metabolism. *Reprod. Nutr. Dev.* **1980**, *20* (4A), 1061−1069.
(14) O'Brien, J.; Morrissey, P. A.; Flynn, A. Alterations of mineral metabolism and secondary pathology in rats fed Maillard reaction products. In *Maillard Reactions in Chemistry, Food and Health*; Labuza, T. P., Reineccius, G. A., Monner, V. M., O'Brien, J., Baynes, J. W., Eds.; Royal Society of Chemistry: Cambridge, U.K., 1994; pp 393−401.
(15) Aspe, T. Influencia del tratamiento térmico de la proteína dietética sobre la biodisponibilidad de algunos minerales. Ph.D. Thesis, Instituto de Nutrición y Bromatología (CSIC-UCM), Madrid, Spain, 1992.
(16) Pinto, M.; Robine-Leon, S.; Tappay, M.; Kedinger, M.; Triadou, N.; Dussalulx, M.; Lacroix, B.; Simon-Assman, P.; Haffer, K.; Fogh, J.; Zwiebaum, A. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol. Cell* **1983**, *47*, 323−330.
(17) Giuliano, A. R.; Wood, R. J. Vitamin D-regulated calcium transport in Caco-2 cells: unique *in vitro* model. *Am. J. Physiol.* **1991**, *260*, G207−G212.
(18) Gama, L.; Baxendale-Cox, L. M.; Breitwieser, G. E. Ca^{2+}-sensing receptors in intestinal epithelium. *Am. J. Phisiol.* **1997**, *273*, C1168−C1175.
(19) Fleet, J. C.; Wood, R. J. Specific 1, 25(OH)2D3-mediated regulation of transcellular calcium transport in Caco-2 cells. *Am J. Physiol.* **1999**, *276*, G958−G964.
(20) Angela, P. A.; Reddy, M. B. Caco-2 cells can be used to assess human iron bioavailability from a semipurified meal. *J. Nutr.* **2000**, *130*, 1329−1334.
(21) Resmini, P.; Pellegrino, L.; Batelli, G. Accurate quantification of furosine in milk and dairy products by a direct HPLC method. *Ital. J. Food Sci.* **1990**, *2* (3), 173−183.
(22) Miller, D. D.; Schricker, B. R.; Rasmussen, R. R.; Van Camper, D. An *in vitro* method for estimation of iron availability from meals. *Am. J. Clin. Nutr.* **1981**, *34*, 2248−2256.
(23) Vaquero, M. P.; Van Dokkum, W.; Bos, K. D.; Wolters, M. G. E.; Schaafsma, G.; Luten, J. B. In vitro availability of calcium, magnesium, iron, copper and zinc from white or brown bread separately or in combination with other foods. *Rev. Esp. Cienc. Tecnol. Aliment.* **1992**, *32* (1), 47−58.

(24) Alais, C.; Blanc, B. Milk proteins: Biochemical and biological aspects. *World Rev. Nutr. Diet.* **1975**, *20*, 66−167.
(25) Abrahams, S. A.; Vieira, N. E.; Yergey, A. L. Unequal distribution of a stable isotopic calcium tracer between casein and whey fractions of infant formulas, human milk and cow's milk. *J. Nutr.* **1990**, *120*, 1672−1676.
(26) Singh, H.; Creamer, L. K. Aggregation and dissociation of milk protein complexes in heated reconstituted concentrated skim milks. *J. Food Sci.* **1991**, *56* (1), 238−246.
(27) Law, A. J. R. Effects of heat treatment and acidification on the dissociation of bovine casein micelles. *J. Dairy Res.* **1996**, *63* (1), 35−48.
(28) De La Fuente, M. A.; Olano, A.; Casal, V.; Juarez, M. Effects of high pressure and heat treatment on the mineral balance of goat's milk. *J. Dairy Res.* **1999**, *66*, 65−72.
(29) O'Brien, J.; Morrissey, P. A. Metal ion complexation by-products of the Maillard reaction. *Food Chem.* **1997**, *58*, 17−27.
(30) Berrocal, R.; Chanton, S.; Juillerat, M. A.; Pavillard, B.; Scherz, J.-C.; Jost, R. Tryptic phosphopeptides from whole casein: II. Physicochemical properties related to the solubilization of calcium. *J. Dairy Res.* **1989**, *56*, 335−341.
(31) Hansen, M.; Sandström, B.; Lönnerdal, B. The effect of casein phosphopeptides on zinc and calcium absorption from high phytate infant diets assessed in rat pups and Caco-2 cells. *Pediatr. Res.* **1996**, *40*, 547−552.
(32) Meisel, H.; Schlimmer, E. Casein-gebunderner phosphor und der gehalt an freien aminosäuren in unterschiedlich wärmebehandelter mich. *Kieler Milchwirtschaftl. Forschungsber.* **1995**, *47*, 289−295.
(33) Yuan, Y. V.; Kitts, D. D. Calcium absorption and bone utilization in spontaneously hipertensive rats fed on native and heat-damaged casein and soya-bean protein. *Br. J. Nutr.* **1994**, *71*, 583−603.
(34) Sato, R.; Naguchi, T.; Naito, H. The necessity for the phosphate portion of casein molecules to enhance calcium absorption from the small intestine. *Agric. Biol. Chem.* **1983**, *47*, 2415−2417.
(35) Meisel, H.; Bockelmann, W. Bioactive peptides encrypted in milk proteins: proteolytic activation and thropho-functional properties. *Antonie Van Leeuwenhoek* **1999**, *76*, 207−215.
(36) Lee, Y. S.; Noguchi, T.; Naito, H. Phosphopeptides and soluble calcium in the small intestine of rats given a casein diet. *Br. J. Nutr.* **1980**, *43*, 457−467.
(37) Kitts, D. D.; Yuan, Y. V.; Nagasawa, T.; Moriyama, Y. Effect of casein, casein phosphopeptides and calcium intake on ileal ^{45}Ca disappearance and temporal systolic blood pressure in spontaneously hypertensive rats. *Br. J. Nutr.* **1992**, *68*, 765−781.
(38) Adrian, J.; Boisselot-Lefebvres, J. Interrelations entre les produits de la reaction de Maillard et le metabolisme calcique. *Cahiers Nutr. Diet.* **1977**, *12*, 233−234.
(39) Andrieux, C.; Sacquet, E. Effect of Maillard's reaction products on apparent mineral absorption in different parts of the digestive tract. The role of microflora. *Reprod. Nutr. Dev.* **1984**, *24*, 379−386.
(40) O'Brien, J.; Morrissey, P. A.; Flynn, A. The effect of Maillard reaction products on mineral homoeostasis in the rat. In *Proceedings of EURO−FOOD TOX II*; Institute of Toxicology, Swiss Federal Institute of Technology: Zurich, Switzerland, 1986; p 214.
(41) Hurrell, R. F. Influence of the Maillard reaction on the nutritional value of foods. In *The Maillard Reaction in Food Processing, Human Nutrition and Physiology*; Finot, P. A., Aeschbecher, H. U., Hurrell, R. F., Liardon, R., Eds.; Birkháuser: Basel, Switzerland, 1990; pp 245−258.
(42) Furniss, D. E.; Vuichoud, J.; Finot, P. A.; Hurrel, R. F. The effect of Maillard reaction products on zinc metabolism in the rat. *Br. J. Nutr.* **1989**, *62*, 739−749.
(43) O'Brien, J.; Morrissey, P. A.; Flynn, A. Nephrocalcinosis and disturbances of mineral balance in rats fed Maillard reaction products. In *Nutritional and Toxicological Aspects of Food Processing*; Walker, R., Quattrucci, E., Eds.; Taylor and Francis: London, U.K., 1988; pp 177−185.

Received for review August 14, 2000. Revised manuscript received November 29, 2000. Accepted November 29, 2000.

JF001008V