Zinc Transport in Caco-2 Cells and Zinc Balance in Rats: Influence of the Heat Treatment of a Casein−Glucose−Fructose Mixture

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The effects of the heat treatment of casein in the presence of glucose−fructose on Zn bioavailability were studied. Changes in Zn speciation were compared after in vitro digestion of heated (HC) and unheated mixture (C) alone and as part of the diet. The uptake and transport of digested soluble Zn was investigated in Caco-2 cells grown in bicameral chambers; balance studies were done in rats fed diets containing the different samples. After in vitro digestion, the precipitated Zn was significantly higher in HC than in C. In assays with Caco-2 cells, the amount of Zn transferred from the apical to the basolateral chamber was significantly greater when the culture medium contained raw or heated casein. However, because a larger proportion of Zn was precipitated by in vitro digestion, Zn utilization was less efficient in the presence of casein. In biological experiments, food efficiency of the heated casein−glucose−fructose diet was lower, and feeding this diet increased the urinary Zn excretion and lowered Zn absorption and retention. The effects of browning products generated during food processing should be taken into account, especially in diets containing marginally adequate levels of Zn, to prevent possible deficiency.

Keywords: *Casein; in vitro digestion; Maillard reaction; Zn bioavailability; Caco-2 cells; rats*

INTRODUCTION

A variety of heat treatments are used to make foods edible, give them a suitable texture, flavor, and aroma, destroy toxic microorganisms and antinutritive factors, and increase their shelf life. During thermal processing of foods rich in proteins and reducing sugars, the Maillard reaction (MR), also known as the nonenzymatic browning reaction, may take place. This reaction is common during industrial treatments or storage of foods, such as milk and dairy foods, because milk contains high levels of lactose and protein (Pischetrieder et al., 1998; Pizzoferrato et al., 1998). The MR produces brown polymeric pigments (melanoidins) and several compounds that contribute to the aroma and flavor of cooked foods. Controlled browning is pursued in roasting, baking, frying, and some other processes of food technology. Thus, Maillard reaction products (MRP) are consumed as a part of the habitual human diet (O'Brien and Morrissey, 1989). However, the MR may reduce the nutritional value of food and have other antiphysiological effects (Friedman, 1996).

Much effort has been devoted to investigating the influence of the MR on protein quality. Nonenzymatic browning reactions decrease the nutritional quality of protein foods during processing and storage (Hurrell, 1984; Mauron, 1981). This effect is due to the destruction of essential amino acids or a reduction in their availability (Moughan et al., 1996; Sherr et al., 1989), decreases in protein digestibility as a result of structural changes (Erbersdobler, 1977; Kato et al., 1986), and inhibition of digestive enzyme activity (Schumecher and Kroh, 1996). Moreover, the biological value of food can be reduced by browning reactions (Sgarbieri et al., 1973a,b).

There is evidence that MRP may also adversely affect mineral metabolism (Andrieux and Sacquet, 1984; Hurrell, 1990). However, the interactions between metal ions and MRP are not completely understood. Studies by Freeman et al. (1975) and Stegink et al. (1981) reported increased urinary zinc excretion in humans fed intravenously on amino acid solutions autoclaved in the presence of glucose. These authors indicated that hyperzincuria was not observed if the solutions were administered nasogastrically. Other studies have reported that orally administered MRP did not increase trace metal urinary excretion in humans; however, these subjects showed a reduction in long-term ^{65}Zn retention (Johnson et al., 1983). Tests in animals produced different results; in some studies feeding rats with MRP obtained by mild heating of glucose and glycine did not lead to changes in urinary or fecal Zn (Andrieux et al., 1980), but others found that casein−glucose MRP increased urinary Zn, with no effect on fecal Zn (Furniss et al., 1989). However, the reduction in Zn status in animals fed 0.5% glucose−glutamate MRP in a balance study was thought to result from both increased urinary Zn and increased fecal Zn (O'Brien et al., 1994).

Chemical assays indicated the ability of synthetic lysinoalanine (LAL) to form metal chelates (Hayashi, 1982); the order of stability of the LAL−metal ion complexes was described some years later (Pierce and Friedman, 1988). Rendleman (1987) reported convincing evidence that melanoidins behave like anionic polymers that form moderately stable complexes with metals. These complexes can be soluble or insoluble, and their reactivity depends on their structure. Various metal chelating compounds have been reported in different

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foods (Homma and Murata, 1994). Later studies confirmed the ability of MRPs to sequester metals (Wijewickreme and Kitts, 1997) and showed that the high molecular weight fractions possess greater metal-chelating power than the low molecular weight fractions (Yoshimura et al., 1997). Such variability in metal-chelating power was reflected as differences in calcium speciation and utilization in rats fed a diet containing untreated or heated casein in combination with glucose and fructose (Aspe et al., 1993).

Some MRP may interact with dietary micronutrients; it has been suggested that these products chelate Zn, increasing its elimination in the urine or feces. The aim of the present study was to test the influence of casein heated in the presence of glucose−fructose on Zn bioavailability and to shed light on how this MRP influences factors that can modify Zn utilization. Changes in Zn speciation after in vitro digestion were studied first. The influence on Zn uptake and transport by the enterocyte was studied in Caco-2 cells. This cell line was chosen as the experimental model because it has been shown to be useful for in vitro investigation of nutrient transport and metabolism by the intestinal absorptive epithelium (Pinto et al., 1983). In addition, balance studies in rats were done to quantify fecal and urinary Zn excretion and to follow the changes in Zn status.

MATERIALS AND METHODS

Sample Preparation. All chemicals were purchased from Merck (Darmstadt, Germany). Hammarsten casein (62.5%) and a 50% mixture of glucose−fructose (37.5%) were carefully mixed to prepare samples. The samples were assayed either without processing (casein, C) or after being heated on a stove (Selecta 2000210, Barcelona, Spain) at 150 °C for 1 h (heated casein, HC**)**. Porcelain capsules were used to heat the samples; an amount of 5 g was used in all experiments to ensure uniform thickness and internal temperature among tests. The contents of all capsules were pooled at the end of treatment and mixed thoroughly before use in the assays.

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

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.), 3.81% mineral mixture (Merck), and 0.16% vitamin mixture (Roche, Basel, Switzerland). Samples C and HC (16.90%) were used as sources of protein and a partial source of carbohydrates to prepare the C-D and HC-D diets, respectively. All diets were supplemented with DL-methionine (0.2%) (Merck). The nutrient composition of the diets is shown in Table 1.

In Vitro Digestion. The in vitro digestion technique involved two stages: gastric digestion and intestinal digestion. Shortly before use, 0.1 g of pepsin (Sigma Chemical Co., St. Louis, MO) was dissolved in 2.5 mL 0.1 M HCl. For intestinal digestion, 0.1 g of pancreatin and 0.625 g of bile salts (Sigma Chemical Co.) 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 of 0.1 M Zn). For gastric digestion, pH was adjusted to 2 with 5 M HCl, and the pepsin solution was added at a proportion of 0.05 g of pepsin/g of sample. Samples were then incubated at 37 °C in a shaking water bath at 110 oscillations/min for 2 h. For intestinal digestion, the pH of the digest was raised to pH 6 by adding 1 M $NaHCO_3$, and the pancreatin suspension was added in the proportion of 2.5 mL/g of sample. The pH was then adjusted to pH 7.5 with 1 M $NaHCO_3$; samples were incubated at 37 °C at 110 oscillation/min for 2 h.

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

	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	4.83 ± 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.

After intestinal digestion, enzymes of the digest were inactivated by incubating the digest at 100 °C for 4 min in a water bath. The samples were then centrifuged at 4000 rpm for 25 min (CS-6R Centrifuge, Beckman) to separate soluble and precipitated Zn. The supernatants were carefully separated using Pasteur pipets; aliquots of 1 mL were used to determine the concentration of Zn. The percentages of soluble and precipitated Zn were calculated from the initial Zn concentration. These supernatants were used for Zn transport and uptake experiments with Caco-2 cells.

Diet Digestion. To study the bioavailability of Zn as a part of the diet and the influence of heated or unheated casein, in vitro digestion was also done. To 6 g of each diet was added 300 mL of Milli-Q water, and gastric and intestinal digestions were carried out as described above except that intestinal digestion was performed in aliquots of 40 g of the digest and enzymes were not inactivated.

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 and Petri dishes 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 IU/mL), and 1% antibiotic−antimycotic solution. The Zn content of the complete medium was 0.008 mM. The cells were maintained at 37 °C in an incubator in a 95:5 atmosphere of air/CO_2 at 90% humidity; the medium was changed every 2 days.

At 70% confluency, cells were collected by washing three times with Hank's balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+}, followed by treatment with the same buffer with trypsin (0.25%) and EDTA (2 mM) for 5 min. After the cells were detached from the flask, complete culture medium was added to stop trypsinization. Cell viability was assessed by trypan blue exclusion and was typically 85−95%. Suspended cells were 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). The medium was changed every second day, and cells were used for transport and uptake experiments after 21 days of culture. The development of functional tight junctions during differentiation of Caco-2 cells was monitored by determining transepithelial electrical resistance (TEER) of filter-grown cell monolayers on different days after seeding, using the Millicell-ERS apparatus (Millipore). A monolayer with extremely low TEER was assumed to exhibit too much

leakage through imperfectly occluding junctions or holes in the monolayer to be useful in transcellular transport studies (Hidalgo and Borchardt, 1990). The monolayers used in this study exhibited adequate TEER values ranging from 500 to 650 Ω/cm^2.

Zinc Uptake and Transport. Cell monolayers were covered with three different solutions containing the same Zn concentration (1 mM). In the first assay the uptake solution contained no casein (DMEM); in the second, the solution contained nontreated casein (DMEM-C) and in the third, heated casein (DMEM-HC). Supernatants of digested samples were used as the source of casein and Zn. Uptake solutions were incubated at 37 °C until the experiments were started. Twenty-one days after initial seeding, 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. A test buffer (2.5 mL) was then added to the basolateral chamber. The test buffer contained 130 mmol/L NaCl, 10 mmol/L KCl, 1 mmol/L $MgSO_4$, 5 mmol/L glucose, and 50 mmol/L HEPES at pH 7. Uptake solutions were added to the apical chamber, at a volume of the sample supernatant necessary to bring the final Zn concentration to 1 mM, plus 0.3 mL of DMEM 5× and deionized water to bring the final total volume to 1.5 mL. Because the volumes of the digestion supernatants needed to reach the same final concentration of Zn were different for the C and HC samples, the volumes needed to reach 1.5 mL were also different. Therefore, a 5× concentrated culture medium was prepared; when 0.3 mL was added and the solution was then diluted to 1.5 mL with sterile deionized water, the nutrients in the final volume were always in the right proportion. To determine the initial cellular Zn content, assays of DMEM with no added Zn were done (0.008 mM Zn).

Cell cultures were then incubated at 37 °C in a humidified air/CO_2 atmosphere for 12 h. After incubation, medium from the apical compartment was aspirated, the filter insert was removed, and the cell surface was washed twice with ice-cold buffer containing 150 mM NaCl, 1 mM EDTA, and 10 mM HEPES, pH 7, to remove nonspecifically bound metal and residual medium. The membrane with the cell monolayer was cut out, tranferred to melting pots, and reserved to determine the amount of Zn internalized in cells. To calculate the Zn transported across the cell monolayer, buffer from the basolateral chamber was removed; to ensure complete collection, the wells were washed twice with deionized water.

Cell viability after 12 h of exposure to the uptake solutions was assessed by trypan blue exclusion. Viability was never <80%.

Biological Experiments. Sixteen weanling Wistar rats weighing 40 ± 0.5 g, provided by the Laboratory Animal Services 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 55−70% 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 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, moreover, feces and urine were collected to perform the Zn balance. Urine and feces were collected as a 1-week pool for each animal during 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, filtered (Whatman No. 41 filter papers, ashless, Whatman, Maidstone, U.K.), and diluted with Milli-Q water. To control for possible contamination during the collection of feces and urine, blank capsules were manipulated in the same way as those used for the animals. After a final period of 5 days, the animals were sacrificed and their liver, spleen, and a segment of dorsal skin were removed, weighed, and frozen at −20°C until Zn was analyzed.

Table 2. Zn Transport and Uptake in Caco-2 Cells after 12 h of Exposure to 1.0 mM Zn Solutions

solution[a]	transport[b] (μg/well)	% transport[b]	cell content[b] (μg/well)	% uptake[b]
DMEM	27.68 ± 3.75[b]	28.22 ± 3.82[b]	16.34 ± 1.34[b]	16.16 ± 1.37[b]
DMEM-C	33.26 ± 1.76[c]	33.91 ± 1.79[c]	4.82 ± 0.60[c]	4.41 ± 0.61[c]
DMEM-HC	37.82 ± 0.28[c]	38.57 ± 0.29[c]	3.86 ± 0.03[c]	3.42 ± 0.03[c]

[a] DMEM, culture medium; DMEM-C, culture medium containing raw casein−glucose−fructose; DMEM-HC, culture medium containing heated casein−glucose−fructose. [b] Different superscripts in the same column indicate significant differences ($P \leq 0.05$). Values are means ± SD of at least three wells from a representative experiment.

Analytical Determinations. Zn concentration was determined by flame atomic absorption spectrophotometry using a Perkin-Elmer 1100 B spectrophotometer (Überlinger, Germany). Concentration was measured directly in DMEM, enriched DMEM, buffer from the basolateral chamber, and urine samples. Aliquots of supernatants of the digests, Caco-2 cells, feces, diets, livers, and spleens were dry-ashed in a muffle furnace (Selecta 366, Barcelona, Spain) at 450 °C; ash was dissolved with $HCl/HNO_3/H_2O$ (1:1:2) (Suprapur, Merck). Samples of bovine liver reference standard (BCR No. 185) were simultaneously used to check the Zn recovery. The interassay coefficient of variation for Zn was 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 the data obtained for Zn intake and fecal and urinary excretion: absorbed Zn (ingested Zn − fecal Zn); retained Zn (absorbed Zn − urinary Zn); apparent digestibility (absorbed Zn/ingested Zn × 100, %*A*/*I*); proportion of ingested Zn retained in the body (retained Zn/ingested Zn × 100, %*R*/*I*); proportion of absorbed Zn retained in the body (retained Zn/absorbed Zn × 100, %*R*/*A*).

Statistical Treatment. The results of Zn speciation after in vitro digestion and the differences between Zn balance were tested statistically using Student's *t* test. Zn uptake and transport data from Caco-2 cells were analyzed using one-way analysis of variance (ANOVA), followed by Duncan's multiple-range test to compare means with a significant variation ($P \leq 0.05$).

RESULTS

Chemical Analysis. After intestinal digestion, the percentage of soluble Zn was significantly higher with the unheated casein−glucose−fructose mixture, both in the diet (63.90 ± 1.01%) and in the isolated samples (68.10 ± 3.67%), in comparison with the heated mixture (49.29 ± 0.76 and 18.56 ± 1.12%, respectively). However, the percentage of precipitated Zn was significantly higher with heated casein in both cases (36.16 ± 1.01 versus 31.90 ± 3.67% in C-D and C; 50.74 ± 0.76 versus 81.43 ± 1.12% in HC-D and HC).

The degree of browning in the heated diluted samples was measured spectrophotometrically against the C sample. The HC sample had an absorbance of 0.332 ± 0.01.

Caco-2 Cell Assays. Zn transport and uptake were measured after 12 h of exposure to 1.0 mM Zn (Table 2). Zn transport was expressed as micrograms of Zn transported to the basolateral chamber per well and as the percentage of Zn transported per well from the experimental solution. Taking into account that Zn availability is affected by changes in Zn speciation after digestion, and due to the different percentages of soluble Zn after in vitro digestion of the isolated samples (68.10 ± 3.67 and 18.56 ± 1.12% for C and HC, respectively), the global transport efficiency in Caco-2 cells was also related to Zn content. For each sample, the initial Zn

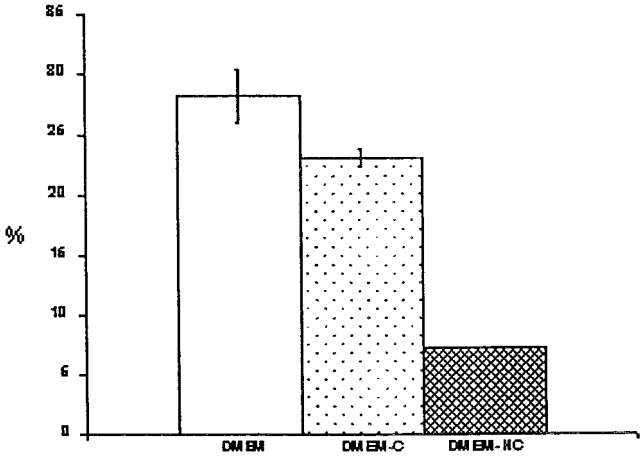

Figure 1. Percent relative Zn transport in Caco-2 cells after 12 h of exposure to 1.0 mM Zn solutions. DMEM, culture medium; DMEM-C, culture medium containing raw casein−glucose−fructose; DMEM-HC, culture medium containing heated casein−glucose−fructose. All differences between media were significant at $P \leq 0.05$. Values are means ± SD of at least three wells from a representative experiment.

Table 3. Body Weight Increase and Food Efficiency in Rats Fed Diets Containing a Heated and an Unheated Casein−Glucose−Fructose Mixture

	g/day[a]			
diet[b]	initial wt	final wt	wt increase	FE[c]
C-D	51.85 ± 1.36[b]	82.07 ± 3.99[b]	4.32 ± 0.41[b]	0.45 ± 0.03[b]
HC-D	48.77 ± 2.02[c]	71.71 ± 5.83[c]	3.28 ± 0.87[c]	0.37 ± 0.05[c]

[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 raw casein-glucose-fructose; HC-D, diet containing heated casein−glucose−fructose. [c] FE, food efficiency (ratio of body mass gain to food intake in dry matter).

necessary to obtain the same quantity of soluble Zn was calculated; the percentage of transported Zn was related with this initial quantity of Zn (Figure 1). The quantity of Zn transferred from the apical to the basolateral compartment increased significantly with the addition of casein samples to the culture medium. The addition of HC samples increased transported Zn with respect to the C samples, although the differences were not significant. However, the rate of Zn transport into the basolateral chamber, taking into account differences in Zn solubility (Figure 1), decreased significantly with DMEM-C and DMEM-HC.

Cellular content of Zn was expressed as micrograms of Zn per well, and Zn uptake was expressed as the percentage from the initial Zn solution. The basal Zn content in Caco-2 cells was 0.50 ± 0.14 μg/well ($n = 3$). Cellular Zn content and uptake of Zn from the medium decreased significantly when raw or heated casein was added to the culture medium.

Biological Analysis. Initial and final weight, daily increase in body weight, and food efficiency of the diets were significantly higher in rats fed the C-D diet than in rats fed the HC-D diet (Table 3).

The Zn intake was significantly higher in rats fed the C-D diet than in the other group. Rats fed the HC-D diet excreted significantly more urinary Zn than rats fed the C-D diet, whereas differences in fecal Zn were not significant. Zn utilization, expressed as absorbed and retained Zn, was significantly higher when rats were fed the C-D diet (Table 4). Apparent digestibility, the proportion of ingested Zn retained in the body, and the proportion of absorbed Zn retained in the body are shown in Figure 2. All three indices were significantly reduced in rats fed the HC-D diet.

Liver and spleen weights did not differ significantly between groups. The Zn contents in the liver, expressed per liver and per gram of liver, were similar in both groups of rats. However, the Zn content in the spleen tended to decrease in animals fed the HC-D diet; the difference between groups was significant when the Zn content was expressed as micrograms per gram of spleen. In the skin there were no significant differences in the Zn content in either group of rats (Table 5).

DISCUSSION

Changes in Zn Speciation after In Vitro Digestion. *Influence of Casein.* In vitro digestion of the C-D diet led to a redistribution of the existing Zn, such that 63.90% of this element stayed soluble and 36.16% was precipitated. This precipitated Zn, consequently unusable for later absorption, seems high compared with the distribution of other elements digested from similar diets (Aspe, 1992) or after the in vitro digestion of milk (Aguirre, 1995). Earlier research showed that Zn in cows' milk is less available than that in human milk (Moynahan, 1974), probably because in cows' milk Zn is predominantly found bound to the high molecular weight protein (casein) (Blakeborough et al., 1986). Some studies have shown that Zn is bound to casein micelles in milk (Flynn and Power, 1985; Abraham et al., 1990); some also have indicated that Zn is complexed to the negatively charged phosphate groups of casein and that enzymatic dephosphorylation of the α_{s1} fraction considerably reduces zinc's binding capacity (Harzar and Kauer, 1982). However, it is known that some low molecular weight phosphopeptides derived from casein can form soluble organophosphate salts with different minerals, favoring their intestinal transport (Meisel, 1997). Human milk contains a lower concentration of casein, which is also less phosphorylated than bovine casein, which explains the poorer use of Zn from cows' milk. Dietary casein is, therefore, probably responsible for the high rate of Zn precipitation after in vitro digestion, which may limit Zn availability. This hypothesis was supported by in vitro digestion assays with isolated samples containing only Zn and the casein−glucose−fructose mixture; in these assays, the percentage of precipitated Zn was similar to that found after digestion of the diet.

Effects of Heating Casein. Results showed that in a diet containing a casein−glucose−fructose mixture heated at 150 °C for 1 h, there was a significant increase in precipitated Zn after in vitro digestion in comparison with the C-D diet. This effect was quantitatively greater when Zn was digested as an isolated sample, that is, when it was not part of a diet. Several researchers have demonstrated the effects of MRP on mineral metabolism (Johnson et al., 1981; Andrieux and Saquet, 1984), suggesting that melanoidins might behave as chelating agents for metal cations and thus influence their availability. In particular, studies in humans showed that MRP are excreted in the urine and are capable of binding urinary Zn (Johnson et al., 1981; Lykken et al., 1986). In this study, the brown color obtained after the sample had been heated, as well as its spectrophotometric absorbance, seems to indicate the formation of colored compounds and the occurrence of the Maillard reaction.

Table 4. Zinc Utilization in Rats Fed Diets Containing a Heated and an Unheated Mixture of Casein−Glucose−Fructose

	μg/day[a]				
diet[b]	Zn intake	fecal Zn	urinary Zn	absorbed Zn	retained Zn
C-D	332.6 ± 20.48[b]	241.5 ± 24.62[b]	19.85 ± 3.95[b]	91.10 ± 16.82[b]	71.25 ± 16.92[b]
HC-D	287.0 ± 44.23[c]	232.5 ± 43.09[b]	30.78 ± 10.95[c]	54.49 ± 17.55[c]	23.71 ± 13.36[c]

[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 raw casein−glucose−fructose; HC-D, diet containing heated casein−glucose−fructose.

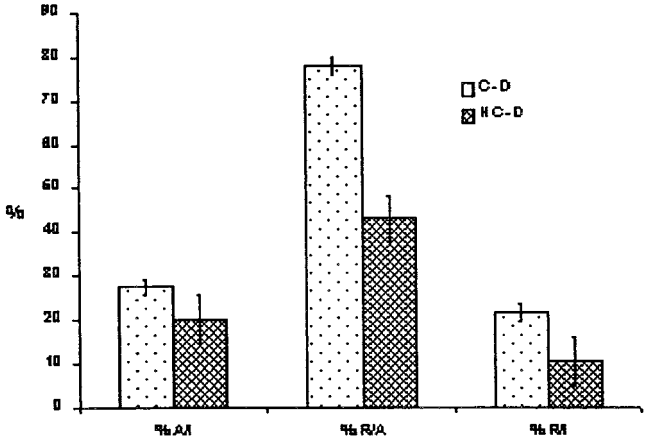

Figure 2. Biological indices in rats fed different diets. C-D, diet containing raw casein−glucose-fructose; HC-D, diet containing heated casein−glucose−fructose. *A*, absorbed Zn; *I*, ingested Zn; *R*, retained Zn. Differences between the diets were significant at $P \leq 0.05$ for all three indices. Values are means ± SD of eight animals.

Table 5. Zinc Content in the Liver, Spleen, and Skin of Rats Fed Diets Containing a Mixture of Heated or Unheated Casein−Glucose−Fructose

	diet[a]	
	C-D	HC-D
liver wt (g)	2.41 ± 0.47[b]	2.19 ± 0.29[b]
Zn (μg)	17.43 ± 6.53[b]	17.43 ± 4.19[b]
Zn (μg/g)	7.12 ± 1.77[b]	8.07 ± 2.24[b]
spleen wt (g)	0.17 ± 0.03[b]	0.19 ± 0.08[b]
Zn (μg)	4.30 ± 1.04[b]	3.75 ± 1.81[b]
Zn (μg/g)	24.42 ± 2.37[b]	20.16 ± 1.36[b]
skin Zn (μg/g)	21.87 ± 3.89[c]	24.69 ± 5.99[c]

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

The increase in precipitated Zn after in vitro digestion of isolated heated samples in comparison with that the HC-D diet may be related to the presence of other components that interfere with precipitation, thus "protecting" the element or competing with it for binding sites on chelating compounds. Other metal ions, in addition to Zn, were found in the digested diet, and the ability of MRP to complex several metal ions has been demonstrated in vitro (Homma et al., 1986; O'Brien, 1988; Rendleman, 1986) and in vivo (Gregor and Emery, 1987). O'Brien (1988) showed that a heated glucose−glutamate mixture was able to complex calcium, magnesium, zinc, and copper ions in solution, and the strength of binding followed the order $Mg^{2+} > Cu^{2+} = Ca^{2+} > Zn^{2+}$. In assays of foods, Rendleman (1986) reported that toast prepared from bread made with milk had a stronger affinity for calcium and copper compared with toast prepared from milk-free bread. Earlier findings with the same diet analyzed in the present study indicated an increase in the precipitation of calcium, magnesium, and copper when the diet contained heated casein and a decrease in ionic calcium in the soluble fraction (Aspe, 1992; Aspe et al., 1993). Results in the present study suggest that the MRP in the diet may complex metals other than Zn and that this may contribute to the lower increase in precipitated Zn compared with other isolated samples.

Caco-2 Cells Assays. A concentration of 1 mM Zn was used, because both the uptake and transport in Caco-2 cells increase linearly with Zn concentrations of 0.2−1.2 mM (Finley et al., 1995). In all assays the cells were exposed to the same amount of soluble Zn, so that the only variable that could influence transport and uptake was the presence of the raw or heated digested casein−glucose−fructose mixture. It was found that Zn from the soluble fraction of the digested casein (whether raw or heated) was transported across the Caco-2 cells more efficiently than Zn from the DMEM without casein. As noted above, this finding may be related to the formation of small peptides (casein phosphopeptides, CPP) during digestion. These peptides can form soluble organophosphate salts and may function as carriers for different minerals and trace elements (Sato et al., 1986), thus influencing Zn absorption in the intestine (Meisel, 1997). These results appear to contradict the negative effects of casein noted above; however, because a large proportion of Zn was precipitated during in vitro digestion, the global process digestion−absorption showed that Zn was used less efficiently in the presence of casein, especially when the mixture was heated previously (Figure 1). However, the quantity of soluble Zn transferred across the Caco-2 monolayer did not differ significantly between DMEM-C and DMEM-HC. In other words, heating casein in the presence of reducing sugars appears to decrease Zn availability as a result of increased Zn precipitation during in vitro digestion, but not as a result of decreased transport of the remaining free Zn.

The other major phase in the intestinal absorption of Zn is uptake by the erythrocyte. Under the current experimental conditions the uptake of soluble Zn decreased significantly with the addition of raw casein (∼73%) and heated casein (∼79%) in comparison with Zn uptake from DMEM without casein. In samples containing casein, soluble Zn may be linked to some of the digestion products, causing enterocyte uptake to decrease. Assays with Caco-2 cells have shown that the addition of certain casein phosphopeptides (CPP-Ca) at levels higher than 14 μmol/L depressed Zn binding plus uptake (Hansen et al., 1996). Because CPP can be formed in vitro by pepsin digestion of casein (Berrocal et al., 1989), the decrease in Zn uptake found in the present assays may also be due to the presence of similar compounds.

The negative influence of casein was more pronounced when it had previously been heated with glucose and fructose. Even when the cells had been exposed to the same amount of Zn, the accumulation of Zn was lower in HC cells. Consequently, casein heating may have led to a series of changes which, after digestion, produced

other factors in the supernatant that decreased the cellular uptake of Zn. Some of the MRP formed may have been responsible for this effect; these compounds, although soluble, may have chelated Zn and, thus, decreased uptake by the enterocyte. It has been suggested that Zn may form soluble complexes as a result of the presence of MRP in the intestinal lumen (O'Brien et al., 1994). The results of in vivo experiments in rats have shown that certain MRP were absorbed from the intestine and eliminated in the urine mainly as intact molecules and that their uptake by muscle cells was very low as well (Erbersdobler et al., 1981). These results suggest that some MRP may chelate Zn in the intestine, thus limiting Zn uptake by the enterocytes. Assays with Caco-2 cells by other authors (Finley et al., 1995) also showed that free ionic Zn may be able to diffuse across the cell, whereas ligand-complexed Zn may not. Under these conditions, Zn may have a stronger affinity for the ligand present in the medium than for the transporter, which is bound to the enterocyte membrane and, therefore, less available for uptake (Finley, 1995).

Biological Assays. *Body Weight and Food Efficiency*. Feeding the HC-D diet significantly slowed body weight gain, although animals weighed the same at the start of the experiment. When Zn balance was determined after the 4-day period of adaptation to the diet, mean body weights in the two groups were already significantly different, and this difference increased with time. Reduced weight gains are a common feature in animals fed MRP (Lee et al., 1981). In animals fed a diet containing casein heated with glucose at different temperatures and for different times, reduced weight gain and delayed growth were reported (Chuyen et al., 1994; Lipka and Ganowiak, 1993). This negative effect, although not always seen (Varela et al., 1997), has been attributed to reduced food intake, diarrhea (O'Brien and Walker, 1988), and antinutritional effects (Finot, 1990). Diarrhea in the test animals was not observed, although rats that consumed diet HC-D had softer feces. Food intake was not significantly reduced, but intake in the group fed diet C-D was slightly greater than in group HC-D (9.47 ± 0.19 versus 8.79 ± 0.52 g/rat/day, respectively.) In addition, food efficiency was lower in the CD-D group (Table 3), which is in agreement with an earlier study that used a similar diet (Furniss et al., 1980). The MR may reduce the nutritional value of foods by destroying essential amino acids or reducing their availability; lysine destruction is the most significant consequence of the MR in most foods, especially in milk and dairy products (Hurrell, 1984). Depending on the temperature used, significant losses of sulfur amino acids, arginine, tryptophan, and histidine may also occur in heated foods. Moreover, the development of cross-links due to the MR may further reduce the digestibility of proteins (Ersberdobler, 1977; Öste and Sjödin, 1984).

Utilization of Zn. In the HC-D group, Zn intake showed a small but significant decrease, due in part to the small variations in food intake noted above and the fact that Zn concentration was found upon analysis to be slightly lower in this diet. The lower Zn intake in these animals may have contributed to the delay in growth, although Zn deficiency seems unlikely given that hepatic reserves were not decreased, as discussed below.

Despite the decrease in Zn intake, fecal excretion in the HC-D group was similar to that in the C-D group. In relative terms, fecal excretion was greater in the HC-D group; this led to a decrease in the apparent digestibility (%A/I), the index which provides an overall quantification of the process of digestion (Figure 2). The increase in fecal Zn, together with the lower intake, led to a clear decrease in the amount of Zn absorbed. Although some authors found no increase in fecal Zn excretion after feeding of a diet rich in MRP (Furniss et al., 1989; Hurrell, 1990), the present results were similar to those reported by O'Brien et al. (1994) and support the hypothesis that melanoidins, advanced products of the MR, are not easily digestible by rats (Finot and Magnenat, 1981) or by humans (Stegink et al., 1981). These brown products can chelate with Zn, precipitate the element in the feces, and thus impede its absorption (Lykken et al., 1986). In fact, the current in vitro assays showed an increase in Zn precipitation in the presence of heated casein. This effect reduced Zn availability, as in vivo experiments confirmed.

Urinary excretion of Zn increased in animals fed the HC-D diet. This increase was of particular importance in view of the lower Zn absorption in this group. Increases in urinary Zn as a result of MRP intake have been widely reported (Furniss et al., 1989; O'Brien et al., 1994). In the current experiments, rats that consumed the HC-D diet showed considerable hyperzincuria, although it did not double the urinary Zn concentrations seen in control rats or reach the high values reported for rats fed a diet containing heated casein−glucose (Furniss et al., 1989). These findings thus support the controversial hypothesis that MRP, when consumed in food, favor the urinary excretion of Zn.

The mechanism by which MRP increase urinary Zn is unclear. Some authors have related the effect to complexation of Zn by soluble ligands that are partially absorbed and excreted in urine (O'Brien et al., 1994), whereas others have suggested that MRPs have a direct effect on the reabsorption of Zn in the kidney (Furniss et al., 1989). Rats that consumed the HC-D diet gained less weight and used protein less efficiently (∼15% decrease) than did the C-D group. It is therefore possible that Zn requirements for tissue formation were reduced in these animals and that this favored urinary excretion. The higher urinary Zn excretion reflected less efficient metabolic utilization, which was also reflected in the decrease in the %R/A (Figure 2). These results, together with the lower Zn absorption, led to a marked decrease in Zn balance both in absolute (retained Zn) and in relative terms (%R/I) (Figure 2). In rats fed casein heated at different temperatures with glucose or lactose (Furniss et al., 1989), body retention of Zn differed only in animals that consumed a casein−lactose mixture that had been heated for 3 days at 60 °C in contrast to only 1 day at 50 °C. As in the present study, these animals also gained less weight and used Zn less efficiently. Decreased Zn retention was also reported in humans who consumed a cornflake diet compared to the same diet containing corn grits porridge (Johnson et al., 1983).

Some authors have reported increases in relative spleen weight in rats fed a browned egg albumin diet (Kimiagar et al., 1980). In other studies, a significant increase in liver weight was found in rats fed 10% MRP in comparison with animals given a diet containing only 5% MRP (O'Brien and Walker, 1988; Ragot et al., 1992). According to these authors, the increase in relative liver

weight may have been due to the diet rather than to weight loss. Therefore, hepatomegaly may represent an adaptive change in response to an increased requirement for xenobiotic metabolism, as supported by the fact that some MRP are bound and metabolized in the liver (Erbersdobler et al., 1981). In the present experiments, liver and spleen weights did not change in absolute terms; however, when expressed as a percentage of body weight, the weight of both organs tended to increase (2.92 ± 0.49 versus 3.20 ± 0.64% in the liver; 0.21 ± 0.05 versus 0.26 ± 0.12% in the spleen, in C-D and HC-D groups, respectively). These differences did not reach statistical significance, possibly because the deceleration in weight gain was not very great or because the expected increase in hepatic metabolism in the HC-D group was insufficient to give rise to a significant difference. Zn content and concentration in the liver did not differ between the two groups, a finding also reported by Furniss et al. (1989) and compatible with the reported stability of Zn concentrations in tissues (Hurrell, 1990). Zn concentrations in skin were also similar in both groups. However, relative Zn concentration in the spleen was significantly lower, a finding not previously reported, the significance of which remains unknown at this time.

In summary, current findings show that heating casein in the presence of glucose and fructose reduces the bioavailability of Zn, mainly as a result of changes in solubility. Moreover, once the element has been consumed, its utilization is decreased because of impaired digestibility and reduced metabolism. It is, therefore, concluded that the effects of browning products generated during food processing should be taken into account, especially in diets containing marginally adequate levels of Zn, to prevent possible Zn deficiency.

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