Calcium, Iron, and Zinc Uptake from Digests of Infant Formulas by Caco-2 Cells

Mónica Jovaní, Reyes Barberá, Rosaura Farré,* and Esperanza Martín de Aguilera†

Nutrition and Food Chemistry, Faculty of Pharmacy, University of Valencia, Avda Vicente Andrés Estellés, s/n. 46100, Burjassot Valencia, Spain

Our aim was to estimate the bioavailability of calcium, iron, and zinc from infant formulas using a model that includes in vitro digestion and a Caco-2 cell culture to estimate the uptake. The cell culture conditions were selected, and uptake assays were carried out first with calcium, iron, and zinc standard solutions, and then with the soluble fraction of enzymatic digests of an adapted milk-based and a soy-based infant formula. It was not possible to measure the uptake of calcium, iron, and zinc from standard solutions added to the cell cultures in amounts similar to those present in infant formula digests with our method. The fact that it was, however, possible in the case of enzymatic digests suggests the presence of components in the digests that enhance mineral uptake. When mineral uptakes were expressed as percentages of the mineral present, statistically significant differences were found in the case of calcium between the uptake from the milk- and the soy-based formulas. For iron and zinc no such differences were observed.

Keywords: *Mineral; uptake; Caco-2 cells; infant formula*

INTRODUCTION

An understanding of the bioavailability of calcium, iron, and zinc from infant formulas (IF) is important because this foodstuff is often the only, or the main, dietetic source of these minerals for infants. The ideal is to study bioavailability in humans, but human studies are time-consuming, costly to perform, and impractical for large-scale applications. In addition, the common use of radioisotopes in these studies implies a possible hazardous effect that should be taken into account, especially because the infant subjects who would be receiving radiolabeled IF are particularly vulnerable.

In vitro methods have been proposed as an alternative to in vivo methods for estimating mineral bioavailability in IF. Most of the in vitro methods consist of a simulation of gastrointestinal digestion followed by determination of how much of the element is soluble or dialyzes through a membrane of a certain pore size (*1*−*6*). The element solubility or dializability can be used to establish trends in the bioavailability or relative bioavailability values of calcium, iron, or zinc in IF. But these methods estimate only the fraction of the element available for absorption, which is the first step of the in vivo process of mineral absorption (*7*).

In vitro mineral bioavailability assays based on in vitro digestion and on measurements of the soluble or dialyzable element have been improved by incorporating a human colon carcinoma cell line, Caco-2, that shows many of the functional and morphological properties of mature human enterocytes (*8*−*10*).

The Caco-2 model offers a relatively rapid, inexpensive, and versatile system for determining the effects of various aspects of the meal itself or the digestion conditions on cell mineral uptake (*11*).

The most serious disadvantage of Caco-2 cells is the transformed nature of the cells, as they are derived from a colon carcinoma; and the question that arises is to what extent normal metabolic processes are maintained in these cells. These cells are absent a mucin layer, which may play a significant role in intestinal iron absorption, and they have a transepithelial resistance that is much higher than that in the human small intestine and resembles that of the human colon (*7*).

Because of the many similarities between differentiated Caco-2 cells and enterocytes, the cell line has been useful in studies of intestinal iron uptake and transport (*12, 13*). In addition, Caco-2 cells have proven to have distinct uptake mechanisms for calcium (*14*) and zinc (*15*).

In vitro models combining solubility or dialysability and the uptake and/ or transport by Caco-2 cells have been used mostly to estimate iron bioavailability in foods (*11, 16*−*18*). Moreover, Caco-2 cells grown on bicameral filters were used to examine the calcium transport from different mineral waters after in vitro digestion (*19*) and to assess zinc transport from a casein−glucose−fructose mixture (*20*).

It is important to emphasize that the only study on IF that used an in vitro digestion/ Caco-2 cell culture model was the one carried out by Glahn et al. (*21*) to estimate the availability of iron from an IF and to determine the effect that known promoters or enhancers of iron absorption had on it.

The aim of our study was to estimate and compare the bioavailability of calcium, iron, and zinc from milk- and soy-based IFs using a model that combines the in vitro digestion and mineral uptake by Caco-2 cells. A prior selection of the optimal conditions of the assay was carried out.

To our knowledge, no other studies evaluating calcium, iron, and zinc uptake and availability from IF and that combine simulated digestion methods with mineral

* To whom correspondence should be addressed (telephone 34-96-3864950; fax 34-96-3864954; e-mail Rosaura. Farre@UV.es).

† Department of Physiology, University of Valencia.

10.1021/jf010106t CCC: $20.00
Published on Web 06/20/2001

uptake by intestinal epithelial cell culture have been carried out. One interesting characteristic of this model is that radiolabeling of the mineral present in the food is unnecessary and represents a significant advantage in terms of time, cost, and ease of use.

MATERIAL AND METHODS

The protocol includes two steps: the in vitro digestion and the cell mineral uptake assay.

Samples. Two IFs, one adapted milk-based and the other soy-based, were provided in their commercial packages by a Spanish manufacturer.

In vitro Digestion of Infant Formulas. Enzymes and bile salts were purchased from Sigma Chemical Co (St. Louis, MO): pepsin (porcine, cat. no. P-7000) - 1.6 g of pepsin was suspended in 10 mL of 0.1 N HCl; pancreatin (porcine, cat. no. P-1750); and bile extract (porcine, cat. no. B-8631) - 0.2 g of pancreatin and 1.25 g of bile extract were dissolved in 50 mL of 0.1 M $NaHCO_3$. All glassware was washed with detergent and concentrated nitric acid and rinsed with distilled deionized water (DDW) before use. DDW was used throughout the experiments.

Solubility and Dialyzability. To obtain the mineral fraction to be used in the cell uptake assays solubility and dialyzability methods were applied.

Solubility Method. Distilled deionized water (80 mL; Millipore−Milli Q DDW (Millipore Ibérica S. A.; Barcelona, Spain)) was added to 10 g of IF, and the pH was adjusted to 2.0 with 6 N HCl. After 15 min the pH value was checked, and if necessary it was readjusted to 2. Then 3 g of freshly prepared pepsin solution was added. The sample was made up to 100 g with water and incubated in a shaking water bath at 37 °C for 2 h. The gastric digest was kept in ice for 10 minutes to stop the pepsin digestion.

Prior to the intestinal digestion step, the pH of the gastric digests was raised to pH 5 by dropwise addition of 1 M $NaHCO_3$. Then 25 g of the pancreatin−bile extract mixture was added and the incubation continued for an additional 2 h. To stop the intestinal digestion the sample was maintained for 10 min in an ice bath. The pH was adjusted to 7.2 by dropwise addition of 0.5 M NaOH.

The intestinal digest was heated for 4 min at 100 °C to inhibit the sample proteases and was then immersed in an ice bath to cool. Aliquots of 20 g of the heated sample were transferred to polypropylene centrifuge tubes (50 mL, Costar) and centrifuged at 3500*g* for 1 h at 4 °C. Then the supernatants (soluble fraction) were transferred and pooled. Glucose (5 mM final concentration) and HEPES (50 mM final concentration) were added to the soluble fraction and finally, water was added to adjust the osmolarity to 310 ± 10 mOsm/kg (Freezing point osmometer, Osmomat 030).

Dialysis Method. A modification of the Miller method (*22*) was used. The validation and harmonization of this method were carried out within the framework of the EC−Flair Concerted Action no. 10 (*23*). Briefly, pepsin and hydrochloric acid were added to IF (10 g) and the pH was adjusted to 2 with 6 N HCl. The mixture was then incubated for 2 h at 37 °C in a shaking water bath. A dialysis bag (molecular mass cutoff of 10−12000 Da) containing 25 mL of water and an amount of $NaHCO_3$ equivalent to the titrable acidity, previously measured, was placed in the flasks together with 20-g aliquots of the pepsin digest. Incubation was continued for 30 min, the pancreatic−bile salt mixture (5 mL) was added, and incubation was again continued up to 2 h.

The dialysis bag (dialysate) content was removed, and glucose (5 mM final concentration) and HEPES (50 mM final concentration) were added. Sodium chloride was used to adjust the osmolarity to 310 ± 10 mOsm/kg.

Dialysates need not be heated because the digest proteases, which damage Caco-2 cells, remain outside the bags.

Cell Culture. Caco-2 cells were obtained from the European Collection of Cell Cultures (ECACC 86010202, Salisbury, UK). Stock cell cultures were maintained in 75 cm^2 plastic flasks (IWAKI brand, Japan) at pH 7.4 in minimum essential medium (MEM; Gibco BRL Life Technologies, Paisley, Scotland) supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS), 1% v/v nonessential amino acids (Gibco), 1% v/v L-glutamine (Biowhittaker Europe, Verviers Belgium), 1% v/v sodium piruvate (Biowhittaker), $NaHCO_3$ (0.22% p/v), 1% v/v HEPES (Biowhittaker), 1% v/v antibiotic solution (penicillin−streptomycin) (Biowhittaker), and 0.1% v/v fungizone (Gibco). Incubator conditions were 37 °C, 95% air, 5% CO_2; and 95% relative humidity. The medium was changed every other day. The cells were detached with trypsin−EDTA solution (2.5 g/L trypsin, 0.2 g/L EDTA) at 70−80% confluency. As a consequence of fungal growth episodes in the cell culture, probably related to the high degree of humidity in Valencia, especially in autumn and spring, it was necessary to use fungizone.

Cells were examined periodically for changes in maturation by using an Olympus inverted-phase contrast microscope (CK2 model).

Cells were used in experiments at passages 53−55. Background concentrations of Ca, Fe, and Zn in the culture medium were 1.56 mM Ca, 7.52 μM Fe, and 5.51 μM Zn.

For calcium, iron, and zinc uptake assays, cells were seeded at ≈50,000 cells/cm^2 in 75-cm^2 plastic flasks. Microscopic examination of the cultures revealed that confluence was reached after 4−5 days of growth, and the experiments were conducted on day 15−18. Cells were fully differentiated, as evidenced by maximal activities of sucrase and alkaline phosphatase. Under these conditions, the amounts of cell protein and enzymatic activities found in each flask do not vary.

Cell viability was assessed by trypan blue exclusion and was typically 80−95%, and cells were recounted using a Neubauer chamber.

Brush Border Enzyme Activities. Cell differentiation in an intestinal-like epithelial cell was assessed and confirmed by measuring sucrase−isomaltase (E. C. 3.2.1.48) and alkaline phosphatase (EC 3.1.3.1) activities after partial purification of the brush border membranes, according to the following slightly modified Pinto et al. method (*8*). The cell monolayer was washed with 10 mL of phosphate buffered solution (PBS; 8 g/L NaCl, 0.2 g/L KCl, 0.91 g/L Na_2HPO_4, 0.20 g/L H_2KPO_4), and harvested with trypsin−EDTA solution at 37 °C. The cell suspension was centrifuged three times (300 g/10 min/4 °C), and the supernatants were discarded. The cell pellet was homogenized at 4 °C in 3 mL of Tris-mannitol buffer (2 mM Tris; 50 mM mannitol; pH 7.2) with Polytron (PT-2000 model) at 17000 rpm/3 min and after ultrasound bath for 5 min. An aliquot (100 μL) of cell homogenate was used for the total protein content determination. $CaCl_2$ (30 μL 1 M) was added with stirring (10 min, 4 °C) to a 10 mM final concentration. Centrifugation of the Ca^{2+}-treated homogenate (10 min, 950 g, 4 °C) yielded a clear supernatant. The supernatant was centrifuged (34758*g*, 30 min, 4 °C) to yield a small pellet (P) containing the brush border membranes. The pellet, P, was resuspended in 1 mL of distilled deionized water by ultrasound bath (30 min, 4 °C).

Alkaline phosphatase activity was determined according to Bergmeyer (*24*) with *p*-nitrophenyl phosphate as substrate.

Sucrase−isomaltase activity was determined by measuring the 3,5-diaminosalycilate formed by the action of the reducing sugars released by the enzyme on the 3,5-dinitrosalycilate reagent (*25*).

Proteins were determined according to Lowry et al. (*26*). The enzyme activities were expressed as milliunits per mg of brush border protein. One unit is defined as the activity that hydrolyses 1 μmol of substrate per min under the experimental conditions.

Protease Activity of the Digest. The protease activity of the digest was determined by an endopeptidase assay using azocasein as substrate (*27*).

Calcium, Iron, and Zinc Cell Uptake. *Standard Solutions*. Metal chelate solutions were freshly prepared before each assay. Briefly, stock solutions contained aqueous 10 mM $CaCl_2$ or 10 mM $FeCl_3 \cdot 6H_2O$ and 20 mM nitrilotriacetic acid

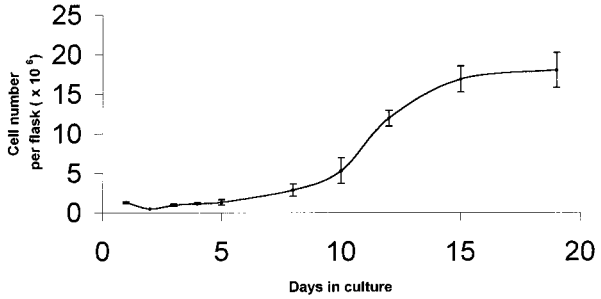

Figure 1. Cell growth curve.

(NTA) or 10 mM $ZnSO_4 \cdot 7H_2O$ and 20 mM sodium citrate in 1 mM HCl. Appropriate volumes of these stock solutions were added to the uptake buffer, the mixture was mixed well, and the mixture was completed to a volume with uptake buffer. The whole was incubated at 37 °C until the beginning of the assays.

The uptake buffer (130 mM NaCl, 10 mM KCl, 1 mM $MgSO_4$, 5 mM glucose, and 50 mM HEPES, pH 7) was prepared and incubated at 37 °C until beginning the assay.

After removing the medium from the flasks, cell monolayers were washed three times with 10 mL of phosphate buffered solution (PBS), and then appropriate volumes of soluble or dialysate digests (18−22 mL) or uptake buffer with calcium, iron, or zinc (standard solution), or uptake buffer (blank) were added to the cells. The flasks were then incubated for 1 h at 37 °C, 5% CO_2, and 95% relative humidity. Then the medium was removed and the cell monolayer was washed three times with ice-cold uptake buffer. The cells were lysed by adding 1 mL of 2% SDS (sodium dodecyl sulfate), and then a dry digestion (450 °C) was carried out.

Calcium, Iron, and Zinc Determination. Calcium, iron, and zinc soluble or dialysate contents and mineral cell content were measured by atomic absorption spectrophotometry (AAS; Perkin-Elmer, model 2380). An amount of lanthanum chloride sufficient to obtain a final content of 0.1% was added to eliminate phosphate interferences in the calcium determination. Total contents of calcium, iron, and zinc of the studied infant formulas were also measured by AAS with a prior dry destruction of the organic matter (*4*).

The differences between the calcium, iron, and zinc contents of cell cultures incubated with samples or uptake buffer (blank) give an estimation of the cellular uptake of these mineral elements. In our assays the values for calcium, iron, and zinc correspond to the amount of element taken up by cells. Mineral availability was defined as the percentage of the calcium, iron, and zinc applied to the Caco-2 cell monolayer which was taken up by the cells.

Statistical Analysis. To assess the significant differences between mineral uptake from both milk- and soy-based IFs, the Student's test was applied (*28*).

Experimental Design. Each assay presented in this manuscript includes 5 to 10 replications (flasks) of the experimental protocol.

RESULTS AND DISCUSSION

Caco-2 Growth and Differentiation. To estimate cell proliferation and differentiation, the growth curve and the enzymatic activities of alkaline phosphatase and sucrase−isomaltase in the brush border cell membrane were used.

In the cell growth curve (Figure 1) three phases can be differentiated: a lag period from days 0 to 5, an exponential phase of quick growth from days 5 to 14, and a stationary third phase after day 14. The integrity of the cell monolayer was lost between days 20 and 25 of the culture.

Alkaline phosphatase and sucrase−isomaltase activities were measured during the growth of Caco-2 cells (Figure 2). High values of alkaline phosphatase and sucrase−isomaltase activity are related to a high degree of differentiation.

During the lag period (days 0−5) the enzyme activities were not detectable. From the day 5 onward, the enzyme activities and brush border content increased regularly. By day 15, the activities of alkaline phosphatase and sucrase−isomaltase were respectively 22.5 and 3.5 times higher than they were on day 5 (Figure 2).

Sample Preparation. First, we attempted to add the unmodified soluble digest to Caco-2 monolayers, but the digestive enzymes caused detachment of the cell monolayer from the flask, which made it impossible to separate the absorbed and unabsorbed mineral. Gangloff et al. (*17*) reported that the digest itself was toxic to cells, and used a dialysis membrane to prevent direct contact of the proteolytic active digest with the cells and cell monolayer damage. We also tried to carry out the mineral uptake assay by adding the dialysate from two adapted IFs to the cell monolayer, but dialysis dramatically reduced the amount of calcium, iron, and zinc added to cells because only the soluble forms of the elements with a molecular weight lower than the molecular cutoff of the dialysis tube dialyze. The result was a very low element content and a high variability when measuring it.

To increase the amounts of calcium, iron, and zinc added to the cell monolayer we decided to use the mineral-soluble fraction, the proteolytic activity of which had been heat-inactivated. After several assays, the minimum time (4 min) of heating at 100 °C required to prevent alteration of the integrity of the cell monolayer was selected. A proteolytic enzymatic inactivation by FBS addition to the digest was postulated by Ekmekcioglu et al. (*19*). But these authors carried out the assay with mineral water, and because of the type of samples the amount of enzymes used in the assays and their proteolytic activity were lower than in our digests. To inhibit the proteolytic activity of our digests the amount of FBS needed would be higher than 20% v/v, which is too high and unfeasible from a physiological and economic point of view.

Cell Mineral Uptake. The results obtained in the mineral-uptake assay in which the heated soluble fraction from IF (adapted milk and soy-based) was added to the cell monolayer are summarized in Table 1. In the case of calcium it was observed that in both types of formulas the uptake increased with the amount of element present (Table 1). This is consistent with the results obtained in in vitro dialysability assays (*5*) which indicated that the calcium content is the main factor affecting the dialysability of calcium from infant formulas, and there is a positive correlation between calcium content and its dialysability (*29*). The uptake of calcium from a standard solution shows a different behavior, and the calcium uptake percentage from a 560-μg calcium addition is lower than 1 (Table 2).

Statistically significant differences ($p < 0.05$) between milk- and soy-based formulas are detected when the results are expressed as percentages (Figure 3); and the values are higher for milk-based formula than for soy-based formula. Similar findings were reported by Shen et al. (*2*) in in vitro assays; with calcium dialysis percentages of 11.4 and 6.7% for soy- and milk-based IF, respectively. In the same way, Roig et al. (*5*) obtained

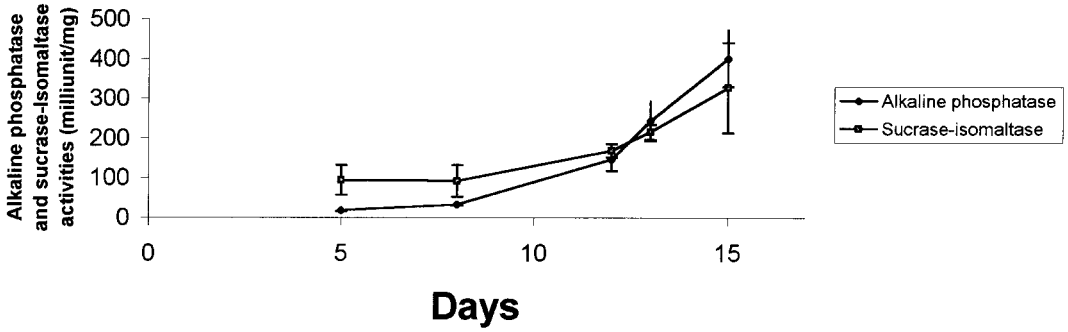

Figure 2. Brush border membrane associated enzyme activities during the growth of Caco-2 cells.

Table 1. Caco-2 Cell Mineral Uptake and Availability (Means ± Standard Deviation; $n = 5-10$) of Ca, Fe, and Zn from Heated Soluble Fraction of Infant Formulas (Adapted Milk and Soy-Based)

infant formula	infant formula content (μg/g)	soluble mineral added[a] (μg)	blank[b] (μg)	uptake assay[c] (μg)	mineral uptake by cells[d, f] (μg)	estimated availability[e] (%)
			calcium			
adapted (milk-based)	3472.34 ± 18.35	327.71 ± 4.90	5.78 ± 0.79	29.50 ± 2.5	23.72 ± 2.49*	7.24 ± 0.76*
		1659.60 ± 26.57	4.98 ± 1.47	103.54 ± 10.99	98.56 ± 10.99	5.94 ± 0.66
soy-based	6212.09 ± 337.21	905.36 ± 32.15	5.78 ± 0.79	127.61 ± 9.33	121.83 ± 9.33*	13.46 ± 1.03 *
		2250.87 ± 98.45	5.75 ± 1.42	165.91 ± 5.51	160.16 ± 5.51	7.12 ± 0.24
			iron			
adapted (milk-based)	65.04 ± 1.87	64.26 ± 5.21	1.44 ±0.43	8.48 ± 1.50	7.04 ± 1.50	10.96 ± 2.34*
		78.04 ± 2.56	1.12 ± 0.10	6.50 ± 0.53	5.38 ± 0.53	6.90 ± 0.68
soy-based	91.38 ± 6.48	50.82 ± 4.25	1.15 ± 0.30	8.14 ± 0.80	6.99 ± 0.80	13.76 ± 1.58*
		87.31 ± 2.97	1.12 ± 0.10	7.15 ± 0.87	6.03 ± 0.87	6.90 ± 1.00
			zinc			
adapted (milk-based)	32.67 ± 3.63	45.55 ± 0.90	1.62 ± 0.14	5.73 ± 0.44	4.11 ± 0.44*	9.02 ± 0.97
		48.95 ± 2.68	1.83 ± 0.56	8.01 ± 1.20	6.18 ± 1.20	12.62 ± 2.46
soy-based	52.46 ± 7.04	22.63 ± 1.81	1.88 ± 0.42	5.37 ± 0.95	3.49 ± 0.95	15.43 ± 4.19*
		48.85 ± 1.94	1.62 ± 0.14	5.07 ± 0.62	3.45 ± 0.62	7.06 ± 1.26

[a] Amount of soluble mineral added to the cell monolayer. [b] Blank, cell monolayer incubated with uptake buffer. [c] Uptake assay, cell monolayer incubated with soluble mineral fraction of infant formulas. [d] Calculated by difference (c − b). [e] Calculated as [d/a] × 100.[f] An asterisk (*) indicates significant differences ($p < 0.05$) between two assays carried out in the same formula for each one of the elements.

Table 2. Caco-2 Cell Mineral Uptake and Availability (Means ± Standard Deviation; $n = 5$) of Ca, Fe, and Zn from Mineral Stock Solutions

stock solutions	mineral added[a] (μg)	mineral concn (μM)	mineral volume added (mL)	blank[b] (μg)	uptake assay[c] (μg)	mineral uptake by cells[d] (μg)	estimated availability[e] (%)
Ca	560	823.53	17	4.55 ± 0.97	7.79 ± 1.20	3.27 ± 1.20	0.58 ± 0.22
Fe	83.25	88.24	17	1.67 ± 0.38	1.67 ± 0.14		
Zn	45.9	41.22	17	1.78 ± 0.74	1.26 ± 0.32		

[a] Amount of soluble mineral added to the cell monolayer. [b] Blank, cell monolayer incubated with uptake buffer. [c] Uptake assay, cell monolayer incubated with mineral stock solutions. [d] Calculated by difference (c − b). [e] Calculated as [d/a] × 100.

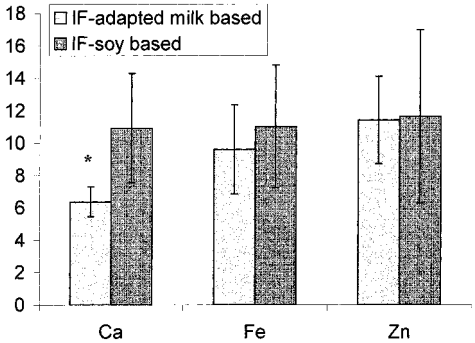

Figure 3. Caco-2 cell mineral uptake mean percentages from infant formulas (IFs). Asterisks (*) indicate significant differences ($p < 0.05$) between mean percentages of mineral uptake from milk- and soy-based infant formulas. Bars correspond to mean standard deviation.

dialysis percentages of 7.1 and 16.5 for milk- and soy-based IF, respectively.

With regard to iron (Table 1), in IFs of the same type, variations in the amount of mineral present in the IF digests did not produce statistically significant differences ($p < 0.05$) in the uptake of the element. This was probably because the amounts of mineral present in the assays were similar. However, when results are expressed in percentages in relation to the soluble content, significant differences are detected ($p < 0.05$), and the percentages are lower when the amount of iron added increases. Similar results were reported by Glahn et al. (*21*), who used digests of milk-based IF and of human milk and observed that in both types of samples the uptake increased 10-fold when the iron content rose from 10 to 200 nmols. However, the increase was not proportional to the addition, as uptake percentages decreased about 50% for the highest addition. The percentages of iron uptake from a digest of milk-based IF reported by Glahn et al. (*21*) are much lower than ours (0.06−0.1% versus 6.9−10.96% in our assays), perhaps because these authors used a dialysis membrane and, therefore, the amount of mineral reaching the cell monolayer was smaller than that in our assays. Differences in experimental conditions make it difficult to compare results from different studies.

No significant differences ($p < 0.05$) were found between iron uptake mean percentages from milk- and soy-based IF (Figure 3). Several authors (*30*, *31*) reported a lower iron absorption in adult women and infants from soy-based IF than from milk-based IF. However, ascorbic acid can enhance iron bioavailability from soy-based IF, and this effect is dose-dependent. Our soy-based IF had a much higher ascorbic acid content (118.31 mg/100 g) than the milk-based IF (34.04 mg/100 g). In addition, our soy-based formula had an ascorbic acid-to-iron ratio of 4.2:1, which has been considered optimal for iron absorption from this type of formula (*30*). A dose-dependent effect of citrate on iron uptake by Caco-2 cells has also been reported (*21*). These authors reported a higher iron uptake by Caco-2 for a milk-based IF with a citrate content of 1.54 mM than when the content was 4.64 mM. Our formulas had very similar citrate contents (1.95 mM and 2.22 mM in milk- and soy-based IF, respectively). The high ascorbic acid content of our soy-based IF in comparison with the milk-based IF, together with their citric acid content could then explain the similarity in the iron uptake values of the milk- and soy-based IFs.

It has been reported that iron uptake by Caco-2 cells depends on the amount of iron available under the cell culture conditions. In model solutions with different iron chelating agents Alvarez-Hernandez et al. (*10*) showed that adding iron (500 nM) to cell monolayers incubated at three different iron levels (<0.1, 1.1, and 65 μM) results in a decrease in iron uptake when the content of iron in the medium increases.

In contrast, in cells grown in a medium with or without FBS, differences were not observed in iron uptake from model solutions with 10 μM added iron and either containing or not containing chelating agents (*32*). It has also been reported that iron uptake from a standard solution (10 μM iron and 200 μM ascorbic acid) decreases when the amount of iron in the media increases from <0.75 μM to 20 μM (*11*). However, this effect was not observed when the iron added came from digests of different foods (beef, chicken, and fish).

We used a 10% (v/v) FBS in the medium with a final iron content (in the media) of 7.52 μM, which means that 35% to the iron content of the medium is contributed by FBS. But FBS is an important component of the media and is needed to maintain the cell monolayer integrity. According to Gangloff et al. (*32*) cells grown in the absence of FBS have a lower transepithelial electrical resistance than those containing this supplement. In addition, the iron from FBS has a lower availability than added inorganic iron.

We were unable to detect (Table 2) iron uptake in the assays carried out with iron additions (83.25 μg Fe−NTA) from standard solutions similar to those coming from IF digests. The differences can be ascribed to either the chemical form of the added iron (a very low iron uptake from Fe−NTA as compared with that of Fe−ascorbate has been reported (*32*)) or the presence in the digests of iron ligands (ascorbic acid and citric acid) which are known to enhance iron bioavailability (*33*), or both.

A lower zinc uptake was observed in soy-based than in milk-based formulas (Table 1). The inhibitory effect of phytate on the bioavailability of zinc from IF has been mentioned, both in vivo (*34*) and in vitro (*6*). In standard solutions a decrease in zinc uptake by Caco-2 cells when the molar ratio between zinc and phytate was 1:10 was reported by Han et al. (*35*), who ascribed this effect to a decrease in zinc solubility caused by phytates. However, in vivo there could be dietetic and physiological factors that enhance zinc absorption, because of the competition with phytates in the intestinal lumen. Zinc is not prone to undergo redox processes, and the potential chelating effect of ascorbic acid seems insufficient to counteract the negative influence of phytic acid (*36*). On the other hand, citric acid did not seem to improve the bioavailability of zinc from soy-based IF and apparently does not counteract the negative effect of phytates (*34*). When the uptake results were expressed as a percentage of the amount of element present, no statistically significant differences ($p < 0.05$) between the milk- and soy-based IFs were found (Figure 3). As the addition of 45 μg of Zn as $ZnSO_4$ to the standard solution (Table 2), a quantity that is close to that present from soy-based IF digests, gives non- detectable uptake levels, it seems that zinc uptake by Caco-2 cells could be affected by several dietetic factors that require further study.

The results discussed above contribute to a better understanding of the bioavailability of calcium, iron, and zinc from IF and the factors affecting them. The in vitro model applied here is useful in detecting differences between formulas and to classifying them according to the mineral bioavailability.

The proposed model enzymatic digestion and Caco-2 cells allows uptake to occur simultaneously with food digestion under pH conditions similar to those found along the absorptive surface of the intestinal tract (*37*, *38*), and it is a great improvement over the use of in vitro digestion alone, which measures only mineral solubility and therefore is an incomplete measure of mineral availability (*21*, *39*).

The relative low cost, ease of use, and widespread acceptance of the Caco-2 cell line make this model system an attractive alternative to in vivo studies.

LITERATURE CITED

(1) Wolters, M. G. E.; Schreuder, H. A. W.; Van den Heuvel, G.; Van Lonkhuijsen, H. J.; Hermus, R. J. J.; Voragen, A. G. J. A continuous in vitro method for estimation of the bioavailability of minerals and trace elements in foods: Aplication to breads varying in phytic acid content. *Br. J. Nutr.* **1993**, *69*, 849−861.

(2) Shen, L.; Luten, J.; Robberecht, H.; Bindels, J.; Deelstra, H. Modification of an in vitro method for estimating the bioavailability of zinc and calcium from foods. *Z. Lebensm. Unters. Forsch.* **1994**, *199*, 442−445.

(3) García, R.; Alegría, A.; Barberá, R.; Farré, R.; Lagarda, M. J. Dialyzability of iron, zinc, and copper of different types of infant formulas marketed in Spain. *Biol. Trace Elem. Res.* **1998**, *65*, 7−17.

(4) Roig, M. J.; Alegría, A.; Barberá, R.; Farré, R.; Lagarda, M. J. Calcium bioavailability in human milk, cow milk and infant formulas − comparison between dialysis and solubility methods. *Food Chem.* **1999**, *65*, 353−357.

(5) Roig, M. J.; Alegría, A.; Barberá, R.; Farré, R.; Lagarda, M. J. Calcium dialysability as an estimation of bioavailability in human milk, cow milk and infant formulas. *Food Chem.* **1999**, *64*, 403−409.

(6) Guillem, A.; Alegría, A.; Barberá, R.; Farré, R.; Lagarda, M. J.; Clemente, G. In vitro dialyzability of zinc from different salts used in the supplementation of infant formulas. *Biol. Trace Elem. Res.* **2000**, *73*, 1−8.

(7) Wienk, K. J. H.; Marx, J. J. M.; Beynen, A. C. The concept of iron bioavailability and its assessment. *Eur. J. Nutr.* **1999**, *38*, 51−57.

(8) Pinto, M.; Robine-Leon, S.; Appay, M. D.; Kedinger, M.; Triadou, N.; Dussaulx, E.; Lacroix, B.; Simon-Assmann, P.; Haffen, K.; Fogh, J.; Zweibaum, A. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol. Cell* **1983**, *47*, 323−330.
(9) Hidalgo, I. J.; Raub, T. J.; Borchardt, R. T. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology* **1989**, *96*, 736−749.
(10) Alvarez-Hernandez, X.; Nichols, G. M.; Glass, J. Caco-2 cell line: a system for studying intestinal transport across cell monolayers. *Biochim. Biophys. Acta* **1991**, *1070*, 205−208.
(11) Glahn, R. P.; Wien, E. M.; Van Campen, D. R.; Miller, D. D. Caco-2 cell iron uptake from meat and casein digests parallels in vivo studies: Use of a novel in vitro method for rapid estimation of iron bioavailability. *J. Nutr.* **1996**, *126*, 332−339.
(12) Alvarez-Hernandez, X.; Smith, M.; Glass, J. Regulation of iron uptake and transport by transferrin in Caco-2 cells, an intestinal cell line. *Biochim. Biophys. Acta* **1994**, *1192*, 215−222.
(13) Halleux, C.; Schneider, Y. J. Iron absorption by Caco2 cells cultivated in serum-free medium as in vitro model of the human intestinal epithelial barrier. *J. Cell Physiol.* **1994**, *58*, 17−28.
(14) 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.
(15) Raffaniello, R. D.; Lee, S.; Teichberg, S.; Wapnir, R. A. Distinct mechanisms of zinc uptake at the apical and basolateral membranes of Caco-2 cells. *J. Cell Physiol.* **1992**, *152*, 356−361.
(16) García, M. N.; Flowers, C.; Cook, J. D. The Caco-2 cell culture system can be used as a model to study food iron availability. *J. Nutr.* **1996**, *126*, 251−258.
(17) Gangloff, M. B.; Glahn, R. P.; Miller, D. D.; Van Campen, D. Assessment of iron availability using combined in vitro digestion and Caco-2 cells culture. *Nutr. Res.* **1996**, *16*, 479−487.
(18) Glahn, R. P.; Olivia, A. L.; Yeung, A.; Goldman, M. I.; Miller, D. D. Caco-2 cell ferritin formation predicts nonradiolabeled food iron availability in an in vitro digestion/Caco-2 cell culture model. *J. Nutr.* **1998**, *128*, 1555−1561.
(19) Ekmekcioglu, C.; Pomazal, K.; Steffan, I.; Schweiger, B.; Marktl, W. Calcium Transport from mineral waters across caco-2 cells. *J. Agric. Food Chem.* **1999**, *47*, 2594−2599.
(20) Navarro, P.; Aspe, T.; Seiquer, I. Zinc transport in Caco-2 cells and zinc balance in rats: Influence of the Heat Treatment of a casein−glucose−fructose mixture. *J. Agric. Food Chem.* **2000**, *48*, 3589−3596.
(21) Glahn, R. P.; Lai, C.; Hsu, J.; Thompson, J. T.; Guo, M.; Van Campen, D. R. Decreased citrate improves iron availability from infant formula: Application of an in vitro digestion/Caco-2 cell culture model. *J. Nutr.* **1998**, *128*, 257−264.
(22) Miller, D. D.; Schicker, B. R.; Rasmussen, R. R.; Van Campen, D. An in vitro method for estimation of iron availability from meals. *Am. J. Clin. Nutr.* **1981**, *34*, 2248−2256.
(23) Luten, J.; Crews, H.; Flynn, A.; Van Dael, P.; Kastenmayer, P.; Hurrell, R.; Deelstra, H.; Shen, L.-H.; Fairweather-Tait, S.; Hickson, K.; Farré, R.; Schlemmer, U.; Frohlich, W. Interlaboratory trial on the determination of the in vitro iron dialysability from food. *J. Sci. Food Agric.* **1996**, *72*, 415−424.
(24) Bergmeyer, H. U., Ed. Phosphatase, alkaline. In *Methods of Enzymatic Analysis, vol. 2*; Verlag Chemie Weinheim/Academic Press: New York and London, 1974; pp 496−497.
(25) Bacon, J. S. D. Methods for measuring transglycosylase activity of invertases. In *Methods in Enzymology*; Colowick, S. P., Kaplan, N. O., Eds.; Academic Press: New York, 1955; pp 258−260.
(26) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.
(27) Sarath, G.; De la Motte, R. S.; Wagner, W. Protease assay methods. In *Proteolytic Enzymes: a Practical Approach*; Beynon, R. J., Bond, J. S., Eds.; IRL Press: Oxford, England, 1989; p 28.
(28) Box, G. E. P.; Hunter, W. G.; Hunter, J. S. Significance Tests and Confidence Intervals for Means, Variances, Proportions and Frequencies. In *Statistics for Experimenters. An Introduction to Design, Data Analysis, and Model Building.* John Wiley & Sons: New York, 1978; pp 115−117.
(29) Roig, M. J.; Alegría, A.; Barberá, R.; Farré, R.; Lagarda, M. J. Effects of different infant formula components on calcium dialysability. *Eur. Food Res. Technol.* **1999**, *209*, 93−96.
(30) Gillooly, M.; Torrance, J. D.; Bothwell, T. H.; MacPhail, A. P.; Derman, D.; Mills, W.; Mayet, F. The relative effect of ascorbic acid on iron absorption from soy-based and milk-based infant formulas. *Am. J. Clin. Nutr.* **1984**, *40*, 522−527.
(31) Davidsson, L.; Galan, P.; Kastenmayer, P.; Cherouvrier, F.; Juillerat, M.-A.; Hercberg, S.; Hurrell, R. F. Iron bioavailability studied in infants: the influence of phytic acid and ascorbic acid in infant formulas based on soy isolate. *Pediatr. Res.* **1994**, *36*, 816−822.
(32) Gangloff, M. B.; Lai, C.; Van Campen, D. R.; Miller, D. D.; Norvell, W. A.; Glahn, R. P. Ferrous iron uptake but not transfer is down regulated in Caco-2 cells grown in high iron serum-free medium. *J. Nutr.* **1996**, *126*, 3118−3127.
(33) Lönnerdal, B. Effects of milk and milk components on calcium, magnesium and trace element absorption during infancy. *Physiol. Rev.* **1997**, *77*, 643−669.
(34) Lönnerdal, B.; Cederblad, A.; Davidsson, L.; Sandström, B. The effect of individual components of soy formula and cow's milk formula on zinc bioavailability. *Am. J. Clin. Nutr.* **1984**, *40*, 1064−1070.
(35) Han, O.; Failla, M. L.; Hill, A. D.; Morris, E. R.; Smith, J. C. Inositol phosphates inhibit uptake and transport of iron and zinc by a human intestinal cell line. *J. Nutr.* **1994**, *124*, 580−587.
(36) Sandström, B.; Cederblad, C. Effect of ascorbic acid on the absorption on zinc and calcium in man. *Internat. J. Vit. Nutr. Res.* **1987**, *57*, 87−90.
(37) Berne, R. M., Levy, M. N., Eds. Digestion and absorption. In *Physiology*, 3rd ed.; Mosby-Year Book Inc.: St. Louis, MO, 1993; pp 688−716.
(38) Guyton, A. C., Hall, J. E., Eds. Secretory functions of the alimentary tract. In *Textbook of Medical Physiology*, 9th ed.; W.B. Saunders Company: Philadelphia, PA, 1996; pp 815−832.
(39) Miller, D. D.; Berner, L. A. Is solubility in vitro a reliable predictor of iron bioavailability? *Biol. Trace Elem. Res.* **1989**, *19*, 11−24.

Received for review January 24, 2001. Revised manuscript received May 2, 2001. Accepted May 2, 2001. This study is part of the project ALI 97-0890 financially supported by the CICYT (Spain). Mónica Jovaní benefits from a grant by the Generalitat Valenciana.

JF010106T