Decreased Citrate Improves Iron Availability from Infant Formula: Application of an In Vitro Digestion/Caco-2 Cell Culture Model[1]

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ABSTRACT We have applied an in vitro digestion/Caco-2 cell culture model to the assessment of iron availability from human milk and a generic cow's milk–based infant formula. Experiments were designed to determine the availability of iron from human milk relative to infant formula and whether known promoters of iron absorption would increase Caco-2 cell iron uptake and availability from the infant formula. In addition, we sought to determine if decreasing the citrate concentration in the infant formula would increase the iron uptake. Although approximately twice as much iron was in solution from digests of the infant formula relative to that of human milk, smaller or equal amounts of iron were taken up from the infant formula relative to the human milk digest. These results are qualitatively similar to in vivo studies. Addition of known iron uptake promoters to infant formula did not enhance Caco-2 cell iron uptake from the infant formula digest, indicating that the iron in the infant formula existed predominantly in a tightly bound unavailable form(s). Enzymatic pretreatment of the infant formula with citrate lyase and oxalacetate decarboxylase decreased the citrate concentration by 67% and resulted in a 64% increase of iron in solution, which corresponded to a 46% increase in the cell iron uptake. Iron uptake from the ''low citrate'' formula plus cysteine was 102% greater relative to the nontreated formula. The results indicate that too much citrate can reduce iron uptake, particularly if it is present at concentrations greater than promoters such as ascorbic acid and cysteine. J. Nutr. 128: 257–264, 1998.

KEY WORDS: • *Caco-2* • *in vitro digestion* • *iron availability* • *infant formula* • *citrate*

In an effort to reduce the global micronutrient crisis or "hidden hunger," the international nutrition community has indicated an urgent need for a fast, inexpensive and accurate method for determining trace mineral availability from foods, particularly iron and zinc. Such need originates from the global effort to improve the density and availability of these minerals in staple plant foods. Improving the nutritional quality of staple foods is essential to developing a sustainable solution to micronutrient malnutrition (Combs et al. 1996, Graham and Welch 1994).

Traditional methods for determining food iron availability involve animal or human feeding trials. These methods are expensive, time consuming and impractical for large-scale applications such as those stated above. A rapid and inexpensive in vitro technique would significantly enhance human and animal studies of iron availability because it could be used to refine experimental objectives, resulting in more productive use of funds for in vivo feeding trials. Such a method may also eliminate the need for many animal trials.

We have developed an in vitro model in which foods undergo simulated peptic digestion followed by intestinal digestion in the presence of Caco-2 cell monolayers. Caco-2 cells are a human intestinal epithelial cell line and have been widely accepted as a model for human iron absorption. Our model includes a reasonable simulation of human gastrointestinal conditions, coupled with a human-derived component capable of giving relative estimates of available iron.

In this study, we demonstrate how use of this model can improve the iron availability of infant formula. Infant formula was chosen as the food of study because almost all of the iron present in commercial infant formula is added as $FeSO_4$. We recognize that extrinsic radiolabeling of food iron is controversial for foods in which a large percentage of the total iron is intrinsic to the food or food ingredients (Consaul and Lee 1984). Therefore, the concern of adequate radiolabeling was negated in this study by the choice of a food product in which essentially all of the iron was added extrinsically.

MATERIALS AND METHODS

Unless otherwise stated, all chemicals, enzymes and hormones were purchased from Sigma Chemical (St. Louis, MO).

Cell culture. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) at passage 17 and used in experiments at passages 25–33. Cells were seeded at a density of 50,000 cells/cm^2 in collagen-treated 6-well plates (6-well cell culture cluster dishes, Costar, Cambridge, MA). The cells were grown in Dulbecco's Modified Eagle Medium (GIBCO, Grand Island, NY) with 10% v/v

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TABLE 1

Ingredients and composition of the infant formula used in this study

Ingredient	Amount	Composition	Amount
Dried nonfat bovine milk, *g/L*	45.5	Protein, *g/L*	15.9
Soybean oil, *g/L*	24.5	Fat, *g/L*	40.6
Corn oil, *g/L*	16.3	Lactose, *g/L*	79.7
Lactose, *g/L*	57.9	Calcium, *g/L*	0.57
Potassium citrate monohydrate, ($C_6H_5O_7K_3 \cdot H_2O$), *g/L*	0.22	Phosphorous, *g/L*	0.42
Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$), *g/L*	0.017	Potassium, *g/L*	0.78
Vitamin A (retinol palmitate), *g/L*	0.0011	Zinc, *g/L*	0.01
Vitamin E (α-tocopherol acetate), *g/L*	0.0122	Vitamin A, *IU/L*	2232
Vitamin C, *g/L*	0.0892	Vitamin E, *IU/L*	18.8
Lecithin, *g/L*	0.416	Vitamin C, *IU/L*	0.07
		Energy, *kJ/L*	3116

fetal calf serum (GIBCO), 25 mmol/L HEPES and 1% antibiotic antimycotic solution (GIBCO). The cells were maintained at 37°C in an incubator with a 5% CO_2-95% air atmosphere maintained at constant humidity, and the medium was changed every 2 d. The cells were used in the iron uptake experiments at 14-d postseeding. Under these conditions, the amount of cell protein measured in each well was found to be highly consistent from well to well within each culture plate.

Infant formula and human milk. A generic infant formula without added iron was prepared according to commercial specifications. The ingredients and chemical composition of the formula are shown in **Table 1.** Human milk samples were donated from excess samples collected under approved research protocols. The human milk and infant formula were analyzed for Fe concentration by inductively coupled plasma emission spectrophotometry. The measured intrinsic concentration of iron in the human milk and infant formula was 2.66 and 1.94 μmol/L, respectively.

In Vitro digestion of human milk and infant formula. A diagram of the in vitro digestion/Caco-2 cell system is shown in **Figure 1.** Porcine pepsin (800–2500 units/mg protein), pancreatin (activity = 4 × USP specifications) and bile extract (glycine and taurine conjugates of hyodeoxycholic and other bile salts) were purchased from Sigma Chemical and used without further preparation. Shortly before use, 0.1 g pepsin was dissolved in 2.5 mL of 0.1 mol/L HCl. For the intestinal digestion, 0.1 g pancreatin and 0.6 g bile extract were dissolved in 50 mL of 0.1 mol/L $NaHCO_3$. ^{59}Fe-labeled $FeSO_4$ in 0.05 mol/L H_2SO_4 was mixed with the sample to achieve 0.296 MBq of ^{59}Fe and a total iron concentration of 10 μmol/L in the initial 10-mL sample. The ^{59}Fe-labeled $FeSO_4$ was added to the sample ~ 12–16 h before the experiment and kept at 4°C.

Peptic and intestinal digestions were carried out on a rocking platform shaker (Reliable Scientific, Hernando, MS) in an incubator at 37°C with a 5% CO_2/95% air atmosphere maintained at constant humidity. The intestinal digestion was conducted in the upper chamber of a two-chamber system in 6-well plates, with the cell monolayer attached to the bottom surface of the lower chamber. The upper chamber was formed by fitting the bottom of an appropriate-sized Transwell insert ring (gift from Costar) with a 15,000 molecular weight cut-off (MWCO)[3] dialysis membrane (Spectra/Por 2.1, Spectrum Medical Industries, Houston, TX). The membranes were soaked in deionized water before use. The dialysis membrane was held in place with a "quad" ring (#Q4121 366Y, Sealing Devices, Lancaster, NY). This method has been described in detail elsewhere (Glahn et al. 1996).

To start the peptic digestion, the pH of each sample was adjusted to pH 2.0 with 5.0 mol/L HCl. The sample was transferred to a 50-mL screw-cap culture tube; 0.5 mL of the pepsin solution was added per 10 mL of sample. The tube was capped, placed horizontally, incubated for 60 min on the rocking shaker and rocked at speed #7 (55 oscillations/min). For the intestinal digestion step, the pH of the sample (also referred to as the "digest") was raised to pH by adding 1 mol/L $NaHCO_3$ dropwise. Then 2.5 mL of the pancreatin-bile extract mixture was added per 10 mL of original sample. The pH was adjusted to pH 7.4 with NaOH, and the volume was brought to 15 mL with 120 mmol/L NaCl.

Preparation of the 6-well culture plates with cell monolayers. Immediately before the intestinal digestion period, the growth medium was removed from each culture well and the cell layer was washed twice with 37°C Hanks' Balanced Salt Solution (HBSS, GIBCO), which contained 5.6 mmol/L glucose and was buffered to pH 7.4 with 25 mmol/L HEPES. A fresh 1.0-mL aliquot of HBSS covered the cells during the experiment. The insert ring, fitted with a dialysis membrane, was then inserted into the well, thus creating the two-chamber system. Then a 1.5-mL aliquot of the intestinal digest was pipetted into the upper chamber. The plate was covered and incubated on the rocking shaker at 55 oscillations/min for 120 min.

When the intestinal digestion was terminated, the contents of the upper and lower chambers and the dialysis membranes were removed for ^{59}Fe assay. Nonspecifically bound iron was then removed from the surface of the cells by a method that has been described in detail elsewhere (Glahn et al. 1995). This method does not alter cell viability or integrity. In brief, the cells were washed with three 1-mL aliquots of 140 mmol/L NaCl, 5 mmol/L KCl with 10 mmol/L PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], referred to as "stop" solution. Then they were incubated for 10 min at room temperature in an iron removal solution consisting of 5 mmol/L bathophenanthroline disulfonate and 5 mmol/L sodium dithionite (BioChemika MicroSelect grade, Fluka Chemical, Ronkonkoma, NY), freshly dissolved in stop solution. Finally, the removal solution was aspirated and the cell layer washed twice with 1.5-mL aliquots of stop solution. Then the cell layer was solubilized in 4 mL of 0.5 mol/L NaOH and transferred to a counting vial for ^{59}Fe counting and protein assay.

Caco-2 cell iron uptake from balanced salt solutions. In one series of experiments, we determined the effects of varying nitrolotriacetic acid (NTA) or citric acid to Fe ratios on Caco-2 cell iron uptake from HBSS. These studies were conducted using 24-well plates (1.9 cm^2, Costar) by using cell culture methods described above. In these studies, various amounts of ascorbic acid, NTA or citric acid were combined with $^{59}FeCl_3$ in 0.1 mol/L HCl. HBSS buffered with 25 mmol PIPES at pH 6.7 served as the basal solution and was added to the Fe after formation of the chelate/Fe complex. The Fe concentration of these solutions was 10 μmol/L, and the chelate to iron ratios were 2:1, 5:1 and 20:1. Solubility of these solutions was determined via centrifugation immediately before use. Aliquots of these solutions were then placed on the cell monolayers and Fe uptake was determined as described previously (Glahn et al. 1995).

Experimental design. Each series of experiments presented in this manuscript represents 3–5 replications of the experimental protocol. Each experimental treatment was performed in duplication for each replication of the experiment. The duplicates were then averaged to represent the value for that replicate. The position of each experimental treatment in the multiwell plate was different for each replication

[3] Abbreviations used: HBSS, Hank's balanced salt solution; IF, infant formula; MWCO, molecular weight cut-off; NTA, nitrilotriacetic acid.

within a series of experiments. Replicates of each experiment were conducted on separate days. The exact number of replicates is noted in the table or figure legend.

The first series of experiments was designed to determine the relative availability of iron added to infant formula (IF) relative to that of human milk. In these experiments, the radiolabeled Fe was added to human milk and IF at either 10 or 200 μmol/L. These concentrations were chosen because 10 μmol/L has been reported to be an average concentration of iron in mature human milk, whereas 200 μmol/L is a typical concentration of iron in commercial IF (Fomon 1993). The second series of experiments sought to determine if addition of iron uptake promoters to the IF could improve iron uptake and availability. These promoters were added at various time points during the digestive process and, for some time points, were combined with the radiolabeled iron before addition. These time points were designated as follows: Control (no added promoter, iron added to sample 12–16 h before experiment with gentle mixing on platform shaker at 4°C); Prepepsin (promoter added to sample immediately before start of pepsin digestion, iron added to sample 12–16 h before experiment with gentle mixing on platform shaker at 4°C); Postpepsin (promoter and added iron combined at pH 2 in 0.1 mmol/L HCl, added to digest 30 min after start of pepsin digestion); Intestinal (promoter and iron combined at pH 2 in 0.1 mmol/L HCl and added to intestinal digest immediately after addition of pancreatin-bile and pH adjustment to 7.4). By adding the promoters and or iron at various points in the digestion process, we sought to determine if the digestion process altered the interaction of the promoter with the added iron. HBSS was used as a control in place of IF to illustrate the promoter effect on iron uptake and availability. Each replicate of these experiments included all of the time points; this was achieved by using multiple pepsin digestion tubes and the multiwell plates. For example, the effects of addition of ascorbic acid to digests of HBSS at all of the time points were determined in duplicate wells in each replicate. Promoters and digests of HBSS or IF were alternated randomly until sufficient replicates were obtained.

The final series of experiments was designed to determine if reducing the amount of citrate present in the IF would increase the iron availability of the formula. In these experiments, oxalacetate decarboxylase and citrate lyase were added to the IF with the intent of converting the citrate to oxalacetate and ultimately to pyruvate. Because oxalacetate inhibits citrate lyase, we added the oxalacetate decarboxylase before adding citrate lyase in order to deplete the oxalacetate concentration. It was our intent to obtain maximal conversion of oxalacetate to pyruvate, which should ultimately result in maximal depletion of citrate. We did not measure the oxalacetate or pyruvate concentration of the untreated or treated formula.

Addition of promoters to the samples and digest. Addition of promoters immediately before digestion, the Prepepsin time point, was from a 100 mmol/L stock solution dissolved in distilled water and prepared immediately before use. In these experiments, the volume of the HBSS or IF was 5.0 mL, and the promoter was added to achieve a concentration of 1 mmol/L; thus, 50 μL of promoter stock was added per sample. For the Control and Prepepsin time points, it is important to note that the iron had already been added to these samples 16–18 h before the addition of the promoter. Because the iron concentration in the sample was 10 μmol/L, the promoter to iron ratio was 100:1. The Control samples received 50 μL of distilled water.

The addition of the iron and promoter to the samples used for the Postpepsin and Intestinal time points was different than that mentioned above; however, the molar amounts were the same. For these samples, the iron and promoter were combined at pH 2, and were added together 30 min into the pepsin digestion period (i.e., the Postpepsin time point) or at the start of the intestinal digestion period (i.e., the Intestinal time point).

Analyses. ^{59}Fe was counted in an automatic gamma counter (Packard Auto-Gamma model 5530, Packard Instruments, Downers Grove, IL). ^{59}Fe solubility was determined as the fraction of sample ^{59}Fe remaining in the supernatant after the sample was centrifuged at 15,600 × *g* for 5 min (Eppendorf microcentrifuge model 5414, Brinkmann Instruments, Westbury, NY). Protein was measured on samples that had been solubilized in 0.5 mol/L NaOH by using a semimicro adaptation of the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). All glassware used in the sample preparation and analyses was acid-washed.

Enzymatic treatment of infant formula. Citrate lyase (Sigma no. C 0897) and oxalacetate decarboxylase (Sigma #O 4878) were used to decrease the concentration of citric acid in the IF. For these experiments, 0.03 g HEPES was dissolved in 5 mL infant formula at 23°C, and was titrated to pH 8.0 by the addition of 1 mol/L NaOH. Then 50 μL of oxalacetate decarboxylase (0.2 units/μL, dissolved in 10 mmol/L HEPES, pH 7.4) was added. The sample of IF was then allowed to incubate at pH 8.0, 23°C for 30 min. At the end of 30 min., the sample was titrated to pH 7.6 by the addition of 1 mol/L HCL and 200 μL of citrate lyase (0.05 units/μL, dissolved in 10 mmol/L HEPES pH 7.4). The sample was then allowed to incubate at pH 7.6, 23°C for another 30 min. The enzymatic reactions were stopped by freezing the sample at −20°C for 25 min; the sample was stored at 4°C overnight for use on the next day. As controls in these experiments, samples of IF received the same treatment as above, except that no citrate lyase or oxalacetate decarboxylase was added.

Measurement of citric acid. The concentration of citrate in the infant formula was measured by a modification of the pentabromo acetone method of Camp and Farmer (1967). Both untreated and treated samples were handled in the same way. The protein was precipitated by the addition of trichloroacetic acid to a concentration of 60 mmol/L and incubated 16 h at 0°C. After removal of protein by centrifugation at 90,000 × *g* for 30 min, citrate was converted to pentabromo acetone by the action of bromine. The pentabromo acetone was extracted into carbon tetrachloride; then a colored complex was formed by reaction with thiourea in the presence of borax. The resultant color was measured by its absorbance at 442 nm. The quantity of citrate was determined by comparison with a standard curve prepared at the same time.

Statistics. BMDP statistical software was used to perform all of the statistical analyses (BMDP 1991). Before analysis, data were log transformed to achieve equal variance. Least significant differences were calculated from the two-way ANOVA tables according to the methods of Milliken and Johnson (1984). Means were considered significantly different if *P* values were less than or equal to 0.05.

RESULTS

The comparison of Caco-2 cell iron uptake from digests of human milk vs. IF is summarized in **Table 2.** Cell iron uptake from the human milk was similar to uptake from the IF; however, the amount of iron that dialyzed into the bottom chamber from the IF digest was 90 and 210% greater than that of the human milk at iron concentrations of 6.67 and 133.3 μmol/L, respectively. Thus, the availability of the Fe present in the bottom chamber of the human milk digests was greater than that of the IF. Iron present in the bottom chamber of all digests was soluble as determined via centrifugation.

Figures 2–5 summarize the effects of added promoters on Caco-2 cell iron uptake from digests of HBSS and IF. Ascorbic acid increased iron diffusion into the bottom chamber by 150–200% from the HBSS digest (Fig. 2). In conjunction with the increased diffusion of iron, iron uptake from the HBSS digest increased 330–390% relative to the control. Addition of ascorbic acid to the HBSS digest increased iron diffusion or uptake to the same extent regardless of whether it was added at the Prepepsin, Postpepsin or Intestinal time point. In contrast, the amount of iron that diffused into the bottom chamber from the IF digest control was ~110% greater than that of the HBSS control digest. Addition of ascorbic acid to the IF digest had no effect on bottom chamber iron regardless of when it was added. Iron uptake from the IF digest control time point was only 35% of that observed from the HBSS control digest. Furthermore, iron uptake from the IF digest did not change with the addition of ascorbic acid at any of the time points.

The effect of addition of cysteine to the HBSS and IF

TABLE 2

Caco-2 cell iron uptake and availability of iron from digests of human milk and infant formula[1]

Digest	[Fe] in original sample[2]	[Fe] in digest[3]	Fe in upper chamber[4]	Fe in bottom chamber[5]	Cell Fe uptake[6]	Availability of Fe in digest[7]	Availability of Fe in bottom chamber[8]
	μmol/L	*μmol/L*	*nmol*	*pmol*	*pmol/2 h*	*%*	*%*
Human milk	10	6.67	10	775 ± 35	14.1 ± 2.7	0.141 ± 0.027	1.75 ± 0.30
	200	133.3	200	7407 ± 214	141 ± 54	0.071 ± 0.027	1.79 ± 0.69
Infant formula	10	6.67	10	1476 ± 57*	10.0 ± 2.1	0.100 ± 0.021	0.66 ± 0.12*
	200	133.3	200	23,002 ± 747*	112 ± 24	0.056 ± 0.012	0.47 ± 0.08*

[1] Values are means ± SEM; $n = 5$.
[2] Concentration of Fe in the sample, added 16–18 h before the start of pepsin digestion period.
[3] Concentration of Fe in the digest at the start of the intestinal digestion period.
[4] Amount of Fe placed in the upper chamber at start of intestinal digestion period.
[5] Amount of Fe that was present in the solution of the bottom chamber at the end of the intestinal digestion period.
[6] Amount of iron taken up by the cells during the intestinal digestion period.
[7] Calculated as (Fe taken up by the cells)/(Fe placed in the upper chamber at the start of the intestinal digestion period) × 100.
[8] Calculated as (Fe taken up by the cells)/(the sum of the Fe present in the bottom chamber solution plus the cell Fe uptake) × 100.
* Indicates significant difference ($P < 0.05$) vs. human milk digest for that [Fe] in the digest.

digests on iron diffusion and Caco-2 cell iron uptake was similar to that of ascorbic acid (Fig. 3). Addition of cysteine to the HBSS digest increased iron diffusion into the bottom chamber 106–123%, and no significant difference in the amount of bottom chamber iron was observed between cysteine addition at the Prepepsin, Postpepsin or Intestinal time point. Cysteine enhanced iron uptake from the HBSS digest 420–460% regardless of when it was added. No difference in iron uptake from the HBSS digest was observed between the Prepepsin, Postpepsin and Intestinal time points. Addition of cysteine to the IF digest did not alter the amount of iron in the bottom chamber or the Caco-2 cell iron uptake at any of the time points.

Addition of cysteinyl glycine to the HBSS digest increased the amount of bottom chamber iron by 78–97%; in addition, Caco-2 cell iron uptake increased 85–122% (Fig. 4). No difference was observed between the various time points for bottom chamber iron and iron uptake. As in the previous figures, bottom chamber iron and Caco-2 cell iron uptake from the IF digest were unaffected.

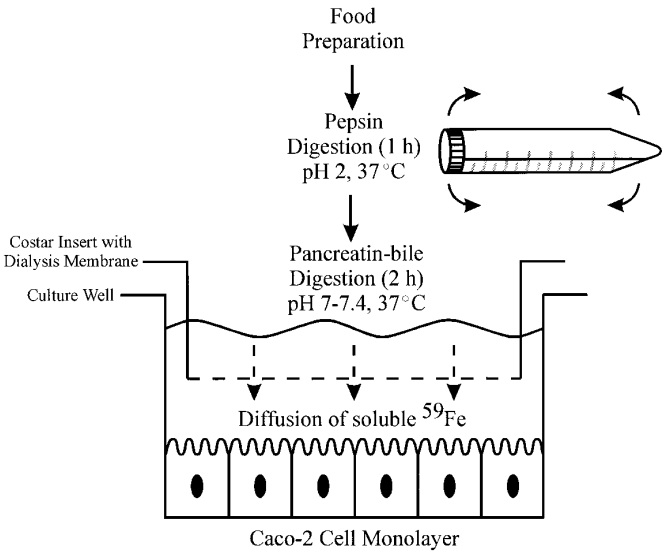

FIGURE 1 Diagram of in vitro digestion/Caco-2 cell culture model.

The addition of glutathione to the HBSS digest increased the amount of iron in the bottom chamber by 38–70% and increased the Caco-2 iron uptake by 44–85% (Fig. 5). Glutathione had no effect on the bottom chamber iron or the cell iron uptake of the IF digest. Overall, the enhancing effect of glutathione on the amount of bottom chamber iron and iron uptake was less than that of the other promoters.

The effects of varying the ratio of NTA or citric acid to Fe on iron uptake from a balanced salt solution are shown in **Figure 6.** In these experiments ($n = 4$), the iron solubility of the control solution was 2.8 ± 0.5%. Iron solubility of the ascorbic acid solution (AA/Fe of 20:1) was 99.1 ± 1.1%. The solutions containing various ratios of NTA to Fe had solubilities of 91.9 ± 1.8, 97.8 ± 0.7 and 96.1 ± 1.1% for NTA/Fe ratios of 2:1, 5:1 and 20:1, respectively. The solutions containing various ratios of citrate to Fe had solubilities of 100.0 ± 0.5, 98.7 ± 1.8 and 98.1 ± 0.6% for citrate/Fe ratios of 2:1, 5:1 and 20:1, respectively. Iron uptake from the NTA and citrate solutions decreased as the ratio of chelate to Fe increased.

The citrate concentration of the infant formula was measured before and after treatment with enzymes (citrate lyase and oxalacetate decarboxylase). Without enzyme treatment, the citrate concentration of IF was 4.64 mmol/L;after enzyme treatment, the citrate content was 1.53 mmol/L. Thus, the enzyme treatment decreased the citrate concentration of the IF by 67%. The enzyme-treated formula is subsequently referred to as the "low citrate" formula.

The effects of decreasing the citrate concentration of the IF are summarized in **Figure 7.** Similar to the results of Figure 3, addition of cysteine to the HBSS digest immediately before the start of pepsin digestion increased the amount of bottom chamber iron by 117%; also, iron uptake from the HBSS increased by 417% when cysteine was added. Once again, addition of cysteine to the IF had no effect on the amount of bottom chamber iron. However, unlike the results of Figure 3, addition of cysteine increased iron uptake from the IF by 28%. Bottom chamber iron concentration for the low citrate IF increased by 64% and uptake from the low citrate formula increased by 46% relative to the untreated formula. Addition of cysteine to the low citrate formula resulted in a smaller increase in bottom

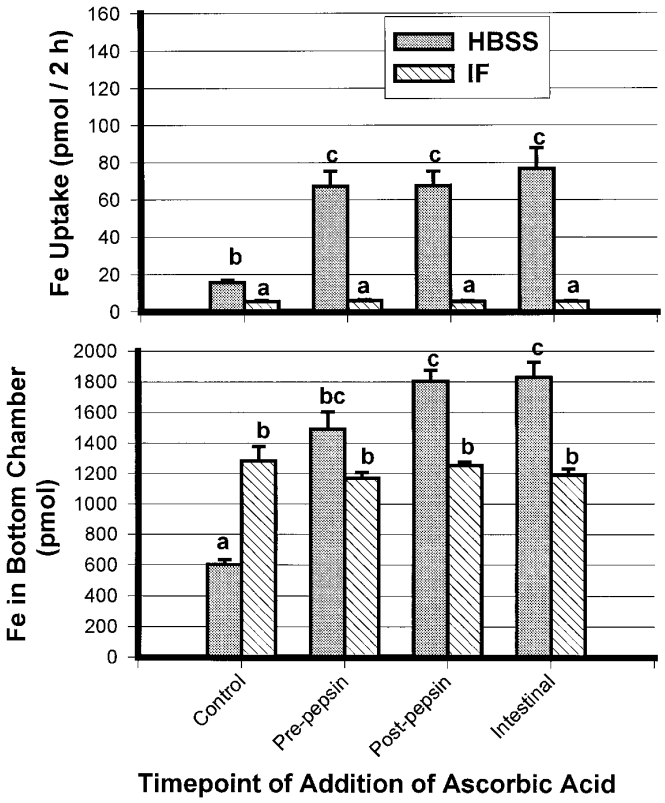

FIGURE 2 Amount of Fe present in the bottom chamber and Caco-2 cell Fe uptake at the end of the intestinal digestion period from digests of infant formula (IF) or Hank's balanced salt solution (HBSS). For the Control and Prepepsin time points, the iron had been added to these samples 16–18 h before the start of the experiment, whereas no iron or ascorbic acid was added before the experiment for the samples used for the Postpepsin or Intestinal time points. The Control time point did not receive ascorbic acid. Ascorbic acid was added at the Prepepsin time point to achieve a concentration of 1 mmol/L. For the Postpepsin and Intestinal time points, molar amounts of iron and ascorbic acid identical to those used for the Control and Prepepsin samples were combined separately at pH 2 and added 30 min into the pepsin digestion period (i.e., the Postpepsin time point) or at the start of the intestinal digestion period (i.e., the Intestinal time point). Bar values with no letters in common are significantly different ($P < 0.05$). Values are means ± SEM; $n = 3$ for HBSS; $n = 4$ for IF.

chamber iron of 40%; however, iron uptake from this digest was increased by 102% relative to the untreated formula without added cysteine.

DISCUSSION

Studies of iron availability have often used extrinsic radiolabeling to monitor the iron uptake from foods (Van Campen 1983). Use of extrinsic radiolabels involves the controversial assumption that the extrinsic radiolabel equilibrates fully with the nonlabeled intrinsic Fe of the food. In this study, the extrinsic $FeSO_4$ radiolabeled with $^{59}FeSO_4$ represented >80% of the total iron present at the lowest Fe concentration (10 μmol/L) used in our study. Our formula should be very similar to most commercial formulas in that very little Fe is intrinsic to the formula ingredients and most if not all of the iron is added as $FeSO_4$ (Fomon 1993). Therefore, the concern of adequate radiolabeling of the food iron should be minimal in this study.

In Figures 2–5, Fe and or promoter was added to HBSS or IF digests at three different points in the digestion process. The purpose was to determine if time point of addition of promoter and or Fe to the sample or digest altered the ability of the promoter to enhance iron uptake. The promoters, ascorbic acid, cysteine, cysteinyl glycine and glutathione, have been shown to enhance Caco-2 cell iron uptake from balanced salt solutions (Glahn and Van Campen 1997). In this study, addition of the promoters to the HBSS digest significantly increased the amount of bottom chamber iron and iron uptake regardless of the time point at which the iron and or promoter was added (Figures 2–5). These results indicate that iron combines well with the promoters throughout the digestion process and under these experimental conditions. It is important to note that the magnitude of the enhancement was less when glutathione was added, relative to the other promoters. This may be due to a more rapid oxidation of the glutathione, or perhaps digestion of glutathione to cysteinyl glycine and cys-

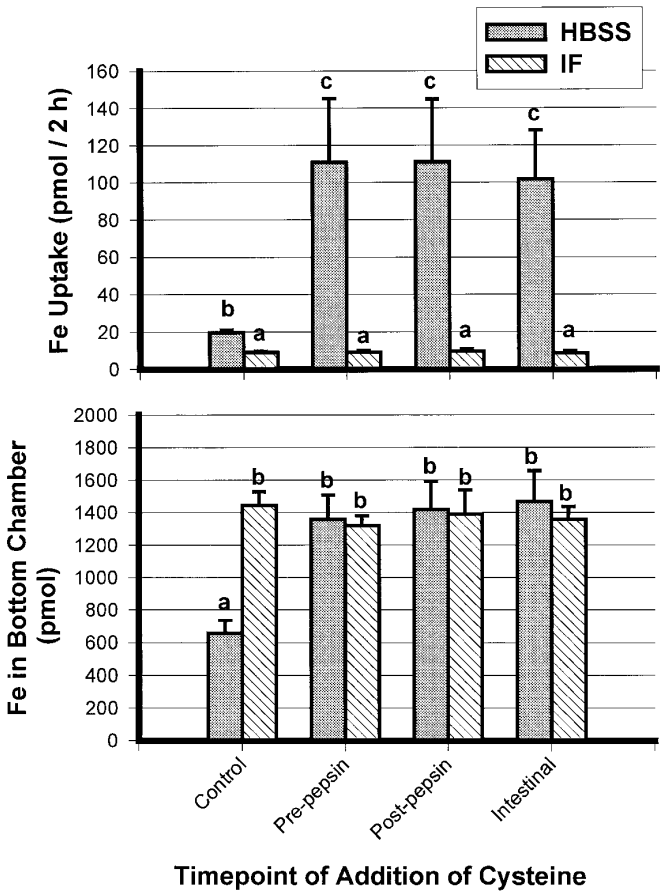

FIGURE 3 Amount of Fe present in the bottom chamber and Caco-2 cell Fe uptake at the end of the intestinal digestion period from digests of infant formula (IF) or Hank's balanced salt solution (HBSS). For the Control and Prepepsin time points, the iron had been added to these samples 16–18 h before the start of the experiment, whereas no iron or cysteine was added before the experiment for the samples used for the Postpepsin or Intestinal time points. The Control time point did not receive cysteine. Cysteine was added at the Prepepsin time point to achieve a concentration of 1 mmol/L. For the Postpepsin and Intestinal time points, molar amounts of iron and cysteine identical to those used for the Control and Prepepsin samples were combined separately at pH 2 and added 30 min into the pepsin digestion period (i.e., the Postpepsin time point) or at the start of the intestinal digestion period (i.e., the Intestinal time point). Bar values with no letters in common are significantly different ($P < 0.05$). Values are means ± SEM; $n = 3$.

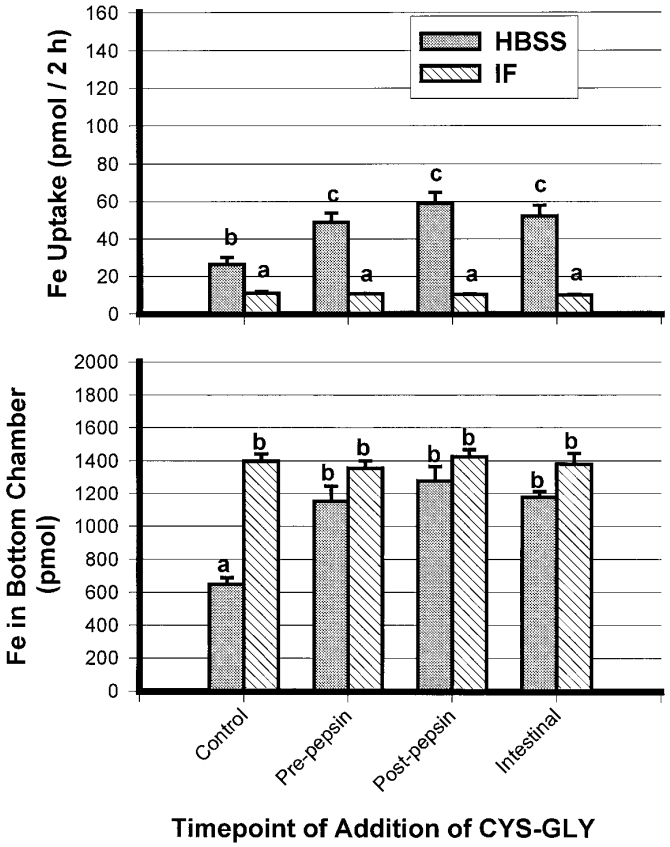

FIGURE 4 Amount of Fe present in the bottom chamber and Caco-2 cell Fe uptake at the end of the intestinal digestion period from digests of infant formula (IF) or Hank's balanced salt solution (HBSS). For the Control and Prepepsin time points, the iron had been added to these samples 16–18 h before the start of the experiment, whereas no iron or cysteinyl-glycine (CYS-GLY) was added before the experiment for the samples used for the Postpepsin or Intestinal time points. The Control time point did not receive cysteinyl-glycine. Cysteinyl-glycine was added at the Prepepsin time point to achieve a concentration of 1 mmol/L. For the Postpepsin and Intestinal time points, molar amounts of iron and cysteinyl-glycine identical to those used for the Control and Prepepsin samples were combined separately at pH 2 and added 30 min into the pepsin digestion period (i.e., the Postpepsin time point) or at the start of the intestinal digestion period (i.e., the Intestinal time point). Bar values with no letters in common are significantly different ($P < 0.05$). Values are means ± SEM; $n = 3$ for HBSS; $n = 4$ for IF.

teine via the pancreatic enzymes was necessary to enhance iron uptake. The latter possibility is supported by the results of a previous study in which the addition of glutathione to a balanced salt solution, in the absence of pancreatic enzymes, did not enhance Caco-2 cell iron uptake, yet cysteine and reduced cysteinyl glycine did enhance uptake (Glahn and Van Campen 1997). Taken together, these observations indicate that cysteine, reduced cysteinyl glycine, and possibly other cysteinyl peptides promote iron uptake. Proteins high in cysteine should promote iron uptake if digested adequately.

The results of this study show a consistent pattern of high iron solubility from the IF digest, yet low iron availability relative to the human milk and HBSS digests. Moreover, addition of promoters to the HBSS digest significantly enhanced iron solubility and uptake regardless of when the iron or promoter was added. Taken together, these observations indicate that the iron of the infant formula was in a highly soluble and relatively unavailable form. A similar trend was also observed between the human milk and the IF. These observations led us to search for components of this bovine milk-based infant formula that would bind iron and keep it relatively unavailable.

On the basis of the above observations, we suspected that citric acid might be contributing to the low iron availability of the IF. Citric acid is a good chelator of Fe, and as shown in Figure 6, increasing concentrations inhibit Caco-2 cell iron uptake from balanced salt solutions. In addition, citrate is relatively high in bovine milk, ranging from 11.2–15.1 mmol/L vs. 1.8–6.5 mmol/L in mature human milk (15 d to 15 mo postpartum, Lentner 1981). In the formula used in this study, dried nonfat bovine milk served as the main contributor of citrate (Table 1) because the amount of added potassium citrate contributed only 14.6% of the total citrate measured. From this information, it seemed reasonable that citrate may be inhibiting the iron availability of the IF.

The results of Figure 7 clearly indicate that reducing the amount of citrate present in the IF increased iron availability. In addition, the results indicate that decreasing the citrate

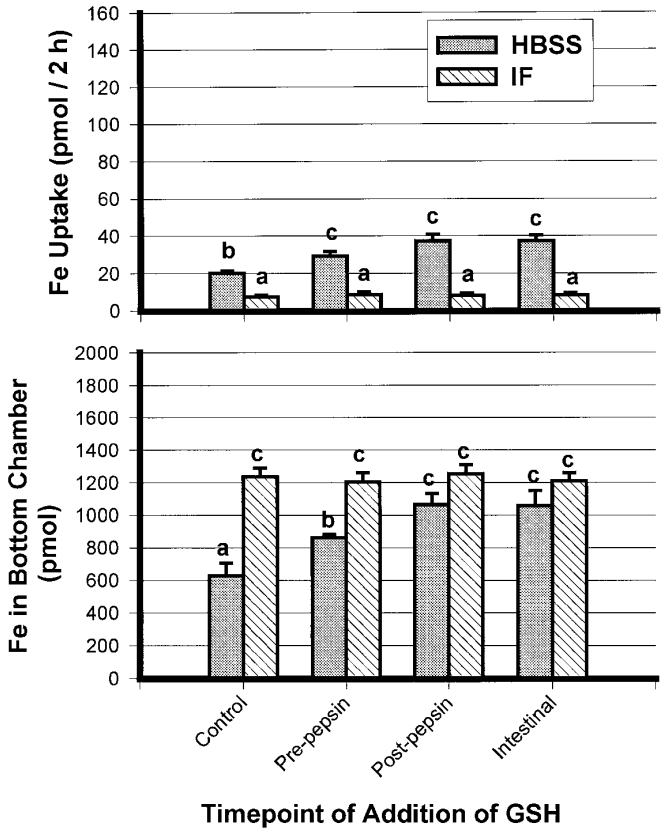

FIGURE 5 Amount of Fe present in the bottom chamber and Caco-2 cell Fe uptake at the end of the intestinal digestion period from digests of infant formula (IF) or Hank's balanced salt solution (HBSS). For the Control and Prepepsin time points, the iron had been added to these samples 16–18 h before the start of the experiment, whereas no iron or glutathione (GSH) was added before the experiment for the samples used for the Postpepsin or Intestinal time points. The Control time point did not receive glutathione. Glutathione was added at the Prepepsin time point to achieve a concentration of 1 mmol/L. For the Postpepsin and Intestinal time points, molar amounts of iron and glutathione identical to those used for the Control and Prepepsin samples were combined separately at pH 2 and added 30 min into the pepsin digestion period (i.e., the Postpepsin time point) or at the start of the intestinal digestion period (i.e., the Intestinal time point). Bar values with no letters in common are significantly different ($P < 0.05$). Values are means ± SEM; $n = 3$.

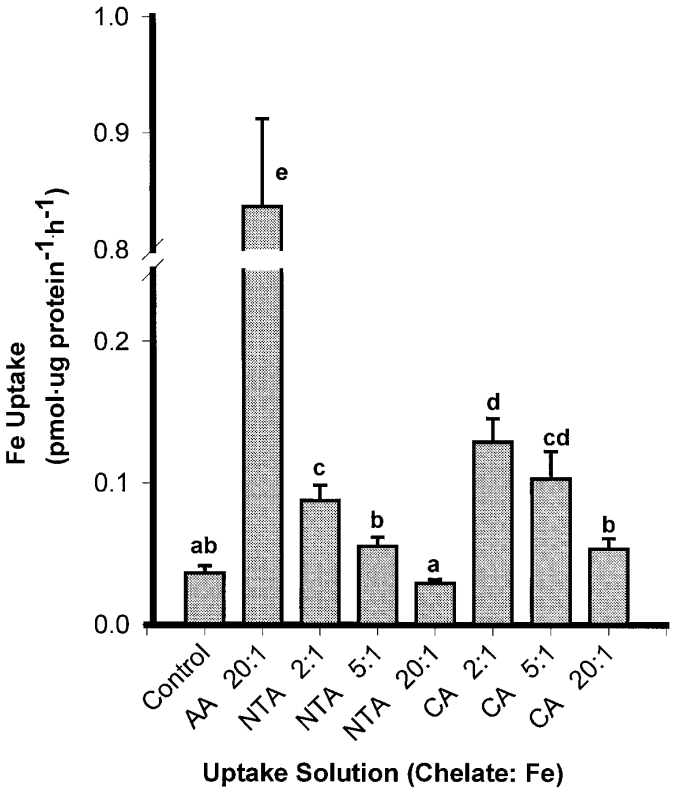

FIGURE 6 Iron uptake by Caco-2 cell monolayers from Hank's balanced salt solutions containing 10 μmol/L Fe with varying amounts (20, 50 or 200 μmol/L) of nitrilotriacetic acid (NTA) or citrate (CA) defined as a ratio relative to the Fe. The Control solution received only 10 μmol/L Fe. The ascorbic acid (AA) solution served as a positive control and contained 200 μmol/L AA and 10 μmol/L Fe. Bar values with no letters in common are significantly different ($P < 0.05$). Values are means ± SEM; $n = 4$.

concentration allows added promoters such as cysteine to enhance iron uptake. This is evident from the significant increase in Fe uptake from the low citrate IF with added cysteine relative to the low citrate IF without added cysteine. This increase in uptake occurred with even less iron present in the bottom chamber, suggesting that the iron present was a more highly available form. Overall, the results of Figures 6 and 7 indicate that citrate may decrease iron availability from milk-based IF if present at too high levels.

Iron availability assays based on in vitro digestion alone lack a living component capable of iron uptake. This living component is necessary for true determination of iron availability. With in vitro digestion alone, solubility and valence of iron are the sole estimators of iron availability, and these have been shown to be inadequate for assessing iron availability (Gangloff et al. 1996, Glahn et al. 1996, Miller and Berner 1989). Therefore, the incorporation of Caco-2 cells into an in vitro digestion model contributes a living element to the system and offers a unique approach to estimating food iron availability. In addition, the established acceptance of Caco-2 cells as a model for human intestinal iron uptake coupled with the cost saving benefits of an in vitro system makes this experimental model very attractive (Gangloff et al. 1996, Glahn et al. 1995 and 1996, Glahn and Van Campen 1997, Han et al. 1994a and 1994b).

Other investigators have coupled in vitro digestion with culture of Caco-2 cells. Garcia et al. (1996) conducted a pepsin digestion of beef, soybean protein isolates, egg albumen and bovine serum albumin. They then took the supernatants from these pepsin digests, labeled them with ^{59}Fe and incubated them for 1 h with Caco-2 cell monolayers cultured on microporous membranes. The investigators observed significantly higher ^{59}Fe uptake from beef samples vs. soybean protein, egg albumen and bovine serum albumin. These observations are in agreement with similar studies in humans, even though the samples did not undergo a pancreatin/bile digestion such as that used in this study. These results are interesting because only ~20% of protein digestion occurs in the stomach due to the action of pepsin (Guyton 1996). The majority of the protein digestion occurs in the intestine, and digestion products of beef proteins are thought to be a major factor contributing to the enhancing effect of meat on iron uptake (Martinez-Torres et al. 1981, Taylor et al. 1986). Thus it seems that beef contains factors other than protein digestion products that enhance nonheme iron uptake.

In designing our in vitro system, we sought to achieve conditions as close as possible to those of the human digestive tract. For example, our experimental system includes a pepsin digestion followed by pancreatin/bile digestion in the presence

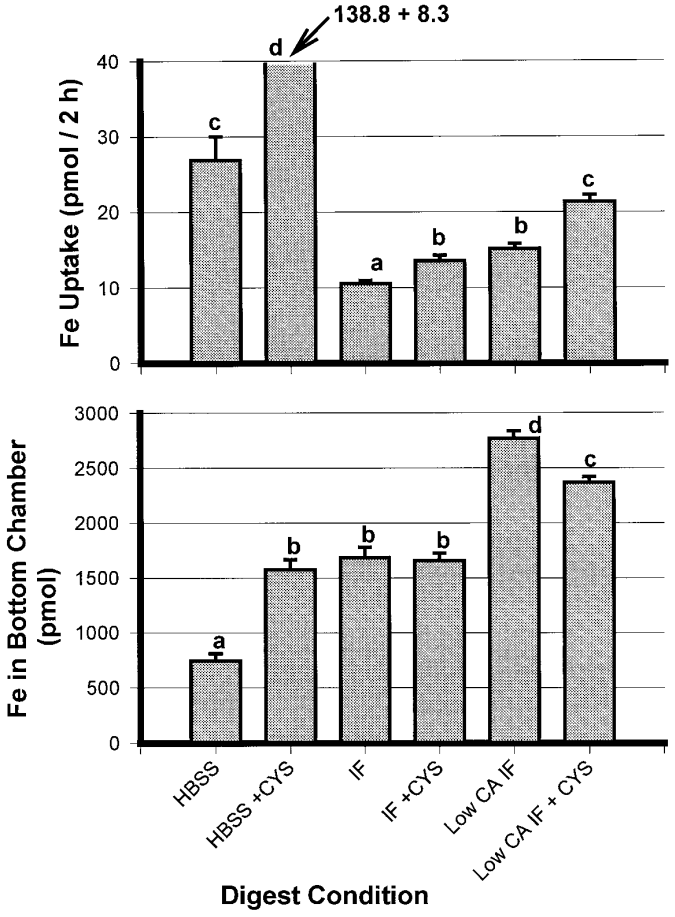

FIGURE 7 Amount of Fe present in the bottom chamber and Caco-2 cell Fe uptake at the end of the intestinal digestion period from digests of Hank's balanced salt solution (HBSS), Hank's balanced salt solution plus cysteine (HBSS + CYS), untreated infant formula (IF), untreated infant formula plus cysteine (IF + CYS), low citrate infant formula (Low CA IF) or low citrate infant formula plus cysteine (Low CA IF + CYS). The iron was added to these samples 16–18 h before the start of the experiment. Cysteine was added at a concentration of 1 mmol/L immediately before the start of the pepsin digestion. Bar values with no letters in common are significantly different ($P < 0.05$). Values are means ± SEM; $n = 4$.

of Caco-2 cell monolayers. This simultaneous digestion of foods coupled with uptake by the Caco-2 cells is certainly similar to that of the human intestinal lumen. By setting the pH to 7–7.4 in our pancreatin/bile digest, we should be achieving conditions similar to those in vivo at the surface of the intestinal epithelial cell, where an alkaline mucus layer protects the epithelium as the intestinal secretions neutralize the stomach acids (Berne and Levy 1993, Guyton 1996). In our system, the dialysis membrane serves to protect the cells from the digestive enzymes. Generation of an in vitro mucus layer similar to that of the intestinal tract would be difficult because the mucus layer of the intestinal lumen is highly regulated and maintained (Guyton 1996). Even co-culture of Caco-2 cells with a human goblet cell clone such as the HT29-MTX cell line may not provide significant protection of the cells from the digestive enzymes unless sufficient mucus production that will not rinse off can be achieved (Walter et al. 1996).

In summary, the results of this study demonstrate how our model system can be used to improve food iron availability from a food product such as IF. Caco-2 cells have repeatedly been shown to qualitatively reflect known human iron absorption mechanisms; therefore, this model should be an excellent tool for research scientists and the food industry. We would like to emphasize that this model should be considered useful to study iron availability from foods in which extrinsic radiolabeling of the food iron can be considered valid, such as infant formula in which the iron is predominantly from an extrinsic source. Similarly, this model could be useful to determine iron availability from staple foods such as wheat or corn, plants that can be intrinsically radiolabeled at adequate levels. At present, we cannot say whether this model could be useful to determine intrinsic iron availability when a significant portion of the total iron is intrinsic to the food and an extrinsic radiolabel must be used. Current efforts in our laboratory are focusing on modifying this model to eliminate the need for using radiolabeled Fe to determine iron availability.

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