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In Vitro Iron Availability from Iron-Fortified Whole-Grain Wheat Flour

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Iron deficiency is the most common nutritional disorder worldwide. Iron fortification of foods is considered to be the most cost-effective long-term approach to reduce iron deficiency. However, for fortified foods to be effective in reducing iron deficiency, the added iron must be sufficiently bioavailable. In this study, fortification of whole-grain wheat flour with different sources of iron was evaluated in vitro by measuring the amount of dialyzable iron after simulated gastrointestinal digestion of flour baked into chapatis and subsequent intestinal absorption of the released iron using Caco-2 cell layers. The dialyzability of iron from iron-fortified wheat flour was extremely low. Additions of 50 mg/kg iron to the flour in the form of ferrous sulfate, Ferrochel amino acid chelate, ferric amino acid chelate taste free (TF), Lipofer, ferrous lactate, ferrous fumarate, ferric pyrophosphate, carbonyl iron, or electrolytic iron did not significantly increase the amount of in vitro dialyzable iron after simulated gastrointestinal digestion. In contrast, fortification of flour with SunActive Fe or NaFeEDTA resulted in a significant increase in the amount of in vitro dialyzable iron. Relative to iron from ferrous sulfate, iron from SunActive Fe and NaFeEDTA appeared to be 2 and 7 times more available in the in vitro assay, respectively. Caco-2 cell iron absorption from digested chapatis fortified with NaFeEDTA, but not from those fortified with SunActive Fe, was significantly higher than from digested chapatis fortified with ferrous sulfate. On the basis of these results it appears that fortification with NaFeEDTA may result in whole-grain wheat flour that effectively improves the iron status.

KEYWORDS: Iron deficiency; anemia; absorption; bioavailability; fortification; Caco-2; NaFeEDTA

INTRODUCTION

Iron deficiency is the most common nutritional disorder in the world. It is believed to affect 20−50% of the world's population (*1*). Iron deficiency results in the depletion of body iron stores, which ultimately causes anemia. Functional consequences associated with iron deficiency and/or anemia include decreased physical capacity and cognitive impairment (*1*). For people whose diets mainly consist of plant foods, bioavailability of dietary iron is considered the most important determinant of iron deficiency. When the diet is mainly vegetarian, iron bioavailability is estimated to be <5% (*2*). The low bioavailability of iron in plant foods is caused by the presence of substances that inhibit iron absorption such as phytate (*3*).

Strategies for combating iron deficiency include iron supplementation, food diversification, and food fortification. Food fortification is being recognized as a sustainable, relatively simple, and realistic way to reduce and prevent iron deficiency (*4*, *5*). However, fortification of foods, such as cereal flours, is difficult as the added iron either causes organoleptic changes during storage and preparation, has a low bioavailability, or is too expensive (*5*). In general, water-soluble iron compounds (such as ferrous sulfate) have an acceptable bioavailability but cause unacceptable organoleptic changes during storage and/or preparation of the flours. In contrast, water-insoluble iron generally does not cause organoleptic changes during storage and/or food preparation of cereal flours, but it may be poorly absorbed. Forms of iron that do not affect the taste and quality of the food and may have a high bioavailability (such as heme iron, NaFeEDTA, and Ferrochel amino acid chelate) are relatively expensive (*4*, *5*).

The objective of this study was to determine the relative availability of different iron-containing compounds from fortified whole-grain wheat flour baked into chapatis. The tested compounds included water-soluble iron compounds (i.e., ferrous sulfate and ferrous lactate), ferrous fumarate, which is poorly soluble in water but soluble in diluted acid, iron compounds that are poorly soluble in water and diluted acid (i.e., ferric pyrophosphate, carbonyl iron, and electrolytic iron), and protected iron compounds [i.e., Ferrochel amino acid chelate, ferric amino acid chelate taste free (TF), Lipofer, SunActive Fe, and NaFeEDTA]. Ferrochel amino acid chelate and ferric amino acid chelate taste free (TF) are complexes of one molecule of ferrous and ferric iron and two or three molecules of glycine, respectively (*6*). Lipofer is a complex of ferric pyrophosphate, starch, and lecithin. SunActive Fe is a micronized, dispersable

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ferric pyrophosphate produced from ferric chloride and sodium pyrophosphate using a dispersion technique. This technique results in the production of very small ferric pyrophosphate particles with relatively high bioavailability (*7*). Relative availability of each compound was determined by measuring in vitro dialyzable iron after simulated gastrointestinal conditions (*8*−*10*) in conjunction with in vitro intestinal absorption using the Caco-2 cells (*11*−*13*).

MATERIALS AND METHODS

Chemicals and Solutions. All chemicals were from Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands) unless otherwise stated. Dulbecco's Modified Essential Medium (DMEM, high glucose), fetal bovine serum, penicillin/streptomycin solution [containing 10000 units/mL penicillin G sodium and 10000 μg/mL streptomycin sulfate in 0.85% (w/v) NaCl], 1% (v/v) nonessential amino acids solution (NEAA), and Hanks' Balanced Salt Solution (HBSS, 10× concentrated) were from Gibco (Rockville, MD). Ten times concentrated phosphate-buffered saline (PBS) solution was from Roche Diagnostics Nederland BV (Almere, The Netherlands). Ultima Gold scintillation solution was from Packard Bioscience (Groningen, The Netherlands). Ferrochel ferrous amino acid chelate and ferric amino acid chelate taste free (TF) were from Albion (Clearfield, UT). Food grade NaFeEDTA (Ferrazone) was from Akzo Nobel (Arnhem, The Netherlands). SunActive Fe was from Taiyo Kagaku Co (Yokkaichi, Japan). Lipofer was from Lipofoods S.L. (Barcelona, Spain). Ferrous lactate, ferrous fumarate, ferric pyrophosphate, carbonyl iron, and electrolytic iron were from Acros Organics (Geel, Belgium). ^{55}Fe in the form of ferric chloride was from Perkin-Elmer (Boston, MA). Whole-grain wheat flour was from Hindustan Lever Ltd. (Mumbai, India).

Preparation of Chapatis. Iron-fortified flour was prepared by mixing 500 g of whole-grain wheat flour with 50 mg/kg iron from different sources for 10 s in a Stephan UM 5 Universal mixer. In this study we evaluated the iron availability of ferrous sulfate, ferric amino acid chelate TF, Ferrochel amino acid chelate, ferrous lactate, NaFeEDTA, ferrous fumarate, ferric pyrophosphate, SunActive Fe, Lipofer, carbonyl iron, and electrolytic iron. Dough was made by transferring 500 g of flour and 340 mL of water to the cup of a Hobart N50 mixer and gently mixing at level 1 for 4 min. The dough was subsequently divided in portions of 40 ± 1 g. For preparing chapatis, each portion was flattened with a pizza engine to slices of ∼15 cm in diameter and ∼1.5−2 mm in thickness. The flattened dough was baked on an electric crêpe cooking plate for 2 min on both sides at a plate temperature of 240−250 °C. Chapatis were subsequently stored at −20 °C until determination of in vitro dialyzable iron.

Cell Culture. Caco-2 cells were obtained from American Type Culture Collection (ATCC) and used in experiments at passage 30−40. Caco-2 cells were cultured in 25 or 75 cm^2 culture flasks (Corning Costar, Badhoevedorp, The Netherlands). The culture medium consisted of DMEM (high glucose) supplemented with 20% (v/v) fetal bovine serum, 1% (v/v) penicillin/streptomycin solution, and 1% (v/v) NEAA. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. For uptake and transport experiments, cells were seeded on the filters (Transwell, 1 cm^2, Costar, Badhoevedorp, The Netherlands) at a density of 300000 cells/cm^2 and cultured for at least 21 days. Twenty-four hours before the uptake and transport experiments were begun, cells were cultured in DMEM with 100 μM deferoxamine to deplete the cells of iron.

In Vitro Estimation of Iron Dialyzability. Iron dialyzability was estimated using an in vitro assay based on publications by Miller and co-workers (*9*) and Luten and co-workers (*8*). All glass vessels were incubated overnight in 10% (v/v) HNO_3 in water and subsequently rinsed with demineralized water. Four chapatis (corresponding to 100 g of flour) were suspended in water and homogenized for 10 s with a Braun blender (type 4142) set at maximum speed. The suspension was added to a vessel of the dissolution apparatus (VanKel VK700, Varian, Bergen op Zoom, The Netherlands). Subsequently, water, 0.45 g of pepsin, and HCl were added to the vessel, yielding a 900 mL suspension of chapatis in simulated gastric fluid of pH 2.0. A 20 g sample was taken from the homogeneous suspension for determination of total iron. After 120 min of incubation at 37 °C and continuous mixing, two samples of 20 g were taken from the suspension. One sample was mixed with 5 mL of 0.1 M $NaHCO_3$ containing 20 mg of pancreatin and 62.5 mg of bile extract and titrated to pH 7.5 with 0.5 M NaOH to yield a simulated intestinal solution. The sample was incubated in a water bath at 37 °C for 30 min, after which the pH was determined and readjusted to 7.5 if necessary. The total amount of 0.5 M NaOH added to the sample was used to determine the amount of $NaHCO_3$ needed to adjust the pH of the simulated gastric solution to pH 7.5. The other gastric digest sample was transferred to an Erlenmeyer flask, to which a dialysis bag (Spectra/Por 7 MWCO 8.000) was added, filled with water and the determined amount of $NaHCO_3$ to adjust the pH of the suspension to pH 7.5. After 30 min of incubation at 37 °C and continuous mixing in a water bath (Lauda MS/2 set at 100 rotations per min), 5 mL of 0.1 M $NaHCO_3$ containing 20 mg of pancreatin and 62.5 mg of bile extract was added to the flask. The suspension was further incubated with the dialysis bag for another 2 h at 37 °C with continuous mixing. Hereafter, the dialysis bag was removed, and a sample of the content of the bag was taken to determine the amount of dialyzable iron. Dialyzable iron is expressed as the percentage of total iron present in the digest, assuming that it had equilibrated across the dialysis membrane by the time the content of the dialysis bag was collected.

Measurement of Total Iron Content. Total iron of the wheat flour was determined by inductively coupled plasma atomic emission spectroscopy. Briefly, samples were digested in 5 mL of 65% nitric acid and 0.5 mL of 30% hydrogen peroxide in closed vessels in a microwave oven at high temperature and high pressure (110 bar). After digestion, the volume was adjusted to 50 mL using demineralized water and sprayed into the inductively coupled plasma of the plasma emission spectrometer (Perkin-Elmer 3300 DV inductively coupled plasma-optical emission spectrometer). The emission of the individual elements was measured at specific wavelengths, and concentrations were quantified from standard solutions.

Measurement of Iron in Dialysate. Dialyzable iron (sum of Fe^{2+} and Fe^{3+}) was determined using a Hitachi 912 analyzer and reagents for the analysis of iron in human serum based on FerroZine (Roche Diagnostics Nederland BV, Almere, The Netherlands). The analyses were performed according to the instructions of the supplier of the reagents, using the dialysate of the in vitro iron dialyzability assay instead of serum.

Iron Absorption by Caco-2 Cells. Iron uptake and transport from digested chapatis were measured using the dialysis samples of the in vitro iron dialyzability assays and human colon carcinoma cells (Caco-2). Twenty-four hours prior to addition to the cells, the osmolarity of dialysis samples was adjusted to ∼300 mOsmol/kg (Osmomat 030, Gonotec GmbH, Berlin, Germany) and pH 7.4 by adding 10% (v/v) of a 10× concentrated HBSS solution to the sample. As a tracer, 5 kBq of ^{55}Fe (13.5 μL of $FeCl_3$ of a 370 kBq/mL stock solution in HBSS) was added to the samples. Samples were stored at 4 °C.

At the start of the experiment the cells were washed twice with HBSS to remove the culture medium containing deferoxamine. Subsequently, the transepithelial electrical resistance (TEER) was measured. Only those filters that had a TEER of $>200\ \Omega\cdot cm^2$ at the beginning and end of the experiment were included. Hereafter, the basolateral compartment was filled with 1.5 mL of HBSS and the apical compartment filled with 0.5 mL of sample. Transport was measured after 2 h of incubation at 37 °C (Memmert type TV 30 u, Schwabach, Germany) under continuous shaking (IKA MTS 4, Staufen, Germany). Basolateral samples of 1 mL were collected and mixed with 9 mL of scintillation solution and the amount of ^{55}Fe determined (β-counter, Packard). Subsequently, the monolayer was washed with PBS (pH 7.4). TEER was measured again to monitor for any damage to the monolayer during the experiments. Hereafter, the monolayer was washed twice with 0.1 mM EDTA in PBS. The cells were lysed and harvested by the addition of 1 mL of PBS containing 1% (w/v) sodium dodecyl sulfate. Finally, the lysed and harvested cells were diluted in scintillation solution, and the amount of ^{55}Fe was measured. Iron uptake and transport were calculated on the basis of the amount of iron in dialysate samples added

Table 1. Iron Content, Percentage of in Vitro Dialyzable Iron, and Calculated Relative Iron Dialyzability of Chapatis Made from Whole-Grain Wheat Flour Fortified with 50 mg/kg Iron from Different Sources, As Indicated[a]

fortificant	total iron content (mg/kg of flour)	in vitro dialyzable iron (% of total iron)	relative dialyzability[b]
none	30 ± 3[c]	2.1 ± 0.8[d]	2.6
ferrous sulfate	62 ± 4	0.8 ± 0.2	1.0
Ferrochel amino acid chelate	65 ± 7	0.9 ± 0.3	1.2
ferric amino acid chelate TF	63 ± 10	0.9 ± 0.2	1.1
ferrous lactate	66 ± 6	0.9 ± 0.2	1.1
NaFeEDTA	76 ± 4	5.7 ± 0.4[d]	7.0
ferrous fumarate	70 ± 10	0.8 ± 0.3	1.0
ferric pyrophosphate	73 ± 9	0.6 ± 0.2	0.7
SunActive Fe	70 ± 13	1.8 ± 0.7[d]	2.3
Lipofer	63 ± 11	0.9 ± 0.7	1.1
carbonyl iron	64 ± 12	0.8 ± 0.4	0.9
electrolytic iron	67 ± 4	0.9 ± 0.2	1.0

[a] Results are means ± SD of five experiments. [b] Relative to the iron dialyzability of chapatis made from whole-grain wheat flour fortified with 50 mg/kg in the form of ferrous sulfate. [c] Significantly different from chapatis made from iron fortified wheat flour (ANOVA, $p < 0.05$). [d] Significantly different from ferrous sulfate (ANOVA, $p < 0.05$).

to the apical compartment and the percentage of total radioactive iron taken up by the cells or transported into the basolateral compartment, respectively.

Statistics. Results are reported as means ± standard deviation (SD). Data were analyzed using GraphPad Prism software (San Diego, CA). Analysis of variance was used to compare the experimental groups. When overall effects were significantly different ($p < 0.05$), Dunnett's multiple-comparison analysis was used to compare the tested conditions to ferrous sulfate fortified controls.

RESULTS

The results from the in vitro iron dialyzability tests with chapatis made from whole-grain wheat flour fortified with different commercial iron sources are shown in **Table 1**. The iron content of the chapatis made from unfortified whole-grain wheat flour was significantly lower than that of chapatis made of fortified whole-grain wheat flour. However, the relative amount of dialyzable iron of chapatis made from unfortified whole-grain wheat flour was significantly higher than that of chapatis made from ferrous sulfate fortified whole-grain wheat flour. Total iron contents of the chapatis made from fortified whole-grain wheat flour were not significantly different. Only chapatis prepared from whole-grain wheat flour fortified with NaFeEDTA or SunActive Fe demonstrated significantly higher dialyzable iron concentrations than those fortified with ferrous sulfate.

Table 2 shows the results of the iron absorption from the dialysate of in vitro digested chapatis by Caco-2 cells. As shown, iron absorption is mainly determined by iron uptake into the Caco-2 cells, as transport of iron across the Caco-2 cells is <1% total iron absorption. The Caco-2 absorption of iron from the dialysate of digested chapatis prepared from whole-grain wheat flour fortified with NaFeEDTA was increased 7-fold relative to the iron absorption from dialysate of digested chapatis prepared from whole-grain wheat flour fortified with ferrous sulfate. Caco-2 cell absorption of iron from the dialysate of digested chapatis prepared from whole-grain wheat flour fortified with all other sources of iron was not higher than that of digested chapatis prepared from whole-grain wheat flour fortified with ferrous sulfate.

Table 2. Relative Iron Absorption by Caco-2 Cells from Dialysate of in Vitro Digestion Experiments with Chapatis Made from Whole-Grain Wheat Flour That Had Been Fortified with the Indicated Commercial Iron Sources[a]

fortificant	iron content[b] (ng/insert)	iron absorption[c] (% of total iron)	relative absorption[d]
ferrous sulfate	0.4 ± 0.1	2.31 ± 2.60 (2.31, 0.01)	1.0
Ferrochel amino acid chelate	0.5 ± 0.2	2.33 ± 2.29 (2.32, 0.00)	1.3 ± 0.6
ferric amino acid chelate TF	0.5 ± 0.1	2.47 ± 1.98 (2.46, 0.01)	1.5 ± 0.3
ferrous lactate	0.4 ± 0.1	2.22 ± 2.19 (2.21, 0.01)	1.1 ± 0.4
NaFeEDTA	3.4 ± 0.3[e]	1.72 ± 1.68 (1.71, 0.01)	6.9 ± 2.4*
ferrous fumarate	0.4 ± 0.1	2.35 ± 2.25 (2.35, 0.00)	1.1 ± 0.6
ferric pyrophosphate	0.3 ± 0.1	1.50 ± 1.40 (1.49, 0.00)	0.7 ± 0.4
SunActive Fe	1.0 ± 0.3[e]	2.25 ± 1.57 (2.24, 0.01)	3.6 ± 3.3
Lipofer	0.4 ± 0.3	2.92 ± 2.48 (2.91, 0.01)	1.6 ± 1.0
carbonyl iron	0.4 ± 0.2	2.00 ± 1.5 (2.00, 0.00)	1.1 ± 0.8
electrolytic iron	0.4 ± 0.1	2.40 ± 2.25 (2.40, 0.01)	0.8 ± 0.6

[a] Values are means ± SD, $n = 5$. [b] Iron content in 0.5 mL of absorption solution. [c] Percentage of iron absorption = percentage of iron uptake in the cells + percentage of iron transported across the cells after incubation. Numbers in parentheses represent percentage of uptake and transport, respectively. [d] Average iron absorption relative to ferrous sulfate. [e] Significantly different from chapati digest from wheat flour fortified with 50 mg/kg iron in the form of ferrous sulfate (ANOVA, $p < 0.05$).

DISCUSSION

The aim of the present study was to evaluate the relative availability of different sources of iron from iron-fortified whole-grain wheat flour. To this end, we determined the in vitro dialyzability and Caco-2 cell absorption of iron from wheat flour baked into chapatis.

The iron dialyzability in wheat flour is extremely low. This is in line with the general finding that iron bioavailability of cereals is low (*4*, *14*). The high phytate level of cereals is thought to be responsible for the low iron bioavailability. In fact, from an efficacy point of view, there is considerable doubt that iron fortification of phytate-rich foods, such as wheat and corn flour, is a useful strategy to combat iron deficiency (*5*). This study shows that it is feasible to produce iron-fortified flour with improved iron bioavailability that could be useful in combating iron deficiency through fortification with NaFeEDTA.

Except for NaFeEDTA and SunActive Fe, iron dialyzability from whole-grain wheat flour baked into chapatis was similar for all added iron sources and not significantly different from ferrous sulfate. Ferrous sulfate was used as a standard in comparison to other sources of iron. Ferrous sulfate dissolves completely in water and can thus enter the common pool of non-heme iron. Consequently, ferrous sulfate is thought to be absorbed to the same degree as intrinsic non-heme iron in food. We added 50 mg/kg iron to wheat flour, thereby increasing the iron content by a factor of >2. We anticipated that if ferrous sulfate entered the common pool of non-heme iron completely, the addition of 50 mg/kg iron would result in at least a doubling of the amount of dialyzable iron. However, unfortified wheat flour, which contained 30 ± 3 mg of iron/kg of flour, yielded 0.6 ± 0.3 mg of dialyzable iron/kg of flour, which is 2.1 ±

0.8% of total iron (**Table 1**). This amount of dialyzable iron from unfortified wheat flour was not significantly different from the amount of dialyzable iron from wheat flour fortified with ferrous sulfate in the same series of experiments (0.5 ± 0.1 mg/kg dialyzable iron, 0.8 ± 0.2% of total iron; **Table 1**). This result indicates that, up to the level of 50 mg/kg, the added iron does not completely enter the common pool of non-heme iron, but precipitates or complexes with components of the flour during the preparation of chapatis and/or gastrointestinal digestion.

Ferrous lactate and ferrous fumarate were included in this study as the bioavailability of iron from these iron compounds from cereals is similar to that of ferrous sulfate (*15*, *16*). Ferrous lactate is soluble in water and, like ferrous sulfate, is expected to enter the common pool of non-heme iron completely upon digestion. Ferrous fumarate is poorly soluble in water but soluble in diluted acid. It is considered a better source of iron than ferrous sulfate for fortification of wheat flour as, due to its poor solubility in water, it causes fewer organoleptic changes during storage and preparation of the flour. Depending on the pH of the stomach, it may enter the common pool of non-heme iron completely. Iron dialyzability of chapatis made from wheat flour fortified with either ferrous lactate or ferrous fumarate was indeed similar to that of chapatis prepared from wheat flour fortified with ferrous sulfate in this study.

Ferric pyrophosphate, carbonyl iron, and electrolytic iron are poorly soluble in water and diluted acid; therefore, iron from these sources cannot completely enter the common pool of non-heme iron. As a result, the dialyzability of ferric pyrophosphate, carbonyl iron, and electrolytic iron from flour has been reported to be less than that of ferrous sulfate (*17*, *18*). In this study, the dialyzability of iron from chapatis made from wheat flour fortified with either ferric pyrophosphate, carbonyl iron, or electrolytic iron was, however, similar to that of iron from chapatis made from wheat flour fortified with ferrous sulfate. This is the result of a much lower iron dialyzability of chapatis made from whole-grain wheat flour fortified with ferrous sulfate than expected, due to the apparent inability of iron form ferrous sulfate to completely enter the common pool of non-heme iron as mentioned above.

Ferrochel amino acid chelate, iron amino acid chelate TF, and NaFeEDTA are chelated forms of iron. Chelating iron is an attractive approach to increase the bioavailability of iron as it may protect iron from forming nonabsorbable complexes with food constituents. It has been reported that Ferrochel amino acid chelate and NaFeEDTA can counteract the complexation of iron with phytate (*15*, *19*, *20*). We therefore expected these compounds to have higher iron dialyzability from wheat flour than ferrous sulfate. Our study shows, however, that Ferrochel amino acid chelate and ferric amino acid chelate TF do not improve the relative iron dialyzability from chapatis made from wheat flour when compared to ferrous sulfate. This indicates that the ferrous bisglycinate and ferric trisglycinate chelates added to wheat flour and baked into chapatis cannot prevent iron complexation during the baking process and/or simulated gastrointestinal digestion. In contrast, NaFeEDTA significantly improved the dialyzability of iron from chapatis made from whole-grain wheat flour. This result demonstrates that EDTA is a useful compound in preventing iron binding to components of wheat flour and confirms previous results with cereals (*15*).

SunActive Fe and Lipofer are emulsified forms of ferric pyrophosphate. Like chelation, the coating of iron with emulsifiers may also protect iron from forming nonabsorbable complexes and is thus also considered to be a potentially useful approach to improve the bioavailability of iron. In this study the relative iron dialyzability from chapatis made from wheat flour fortified with SunActive Fe was significantly higher than that of chapatis made from wheat flour fortified with ferrous sulfate or ferric pyrophosphate. Due to the high standard deviations, however, there is no significant difference between the intestinal absorption of iron from digested chapatis prepared from whole-grain wheat flour fortified with ferrous sulfate, ferric pyrophosphate, or SunActive Fe. The relative iron dialyzability and absorption from chapatis made with Lipofer was not significantly higher than that of chapatis made with ferrous sulfate or ferric pyrophosphate. SunActive Fe and Lipofer are both emulsified forms of ferric pyrophosphate. They differ, however, in composition and average particle size. SunActive Fe is a micronized ferric pyrophosphate with an average particle size of ∼0.3 μm. Ferric pyrophosphate in Lipofer has an average particle size of 7 μm. This difference in particle size may explain the differences in dialyzability from whole-grain wheat flour baked into chapatis.

We used Caco-2 cells as a model for the intestinal absorption of iron as iron bioavailability depends not only on the iron solubility in the intestinal lumen but also on intestinal absorption. Caco-2 cells have been characterized for the expression of proteins that are involved in intestinal iron absorption, and the results of iron transport with Caco-2 cells correlate well with in vivo iron bioavailability data (*11*−*13*, *21*). At the moment, Caco-2 cell layers are considered to be the most suitable in vitro model of human intestinal iron absorption.

However, as can be deduced from **Table 2**, the results of iron uptake and transport measurements in Caco-2 cells need to be evaluated carefully. The average in-day variance of absorption from the several digests was 50% (range = 17−102). The between-day variance is as high as 112%. This means that it is difficult to compare the uptake and transport levels of iron in Caco-2 cells from the digests measured on different days. To obtain consistency among the individual experiments, we performed the absorption studies with all samples from each of the 12 digests on one day and normalized by dividing each value by the control value of uptake and transport from the digest containing ferrous sulfate. The relative uptake and transport values were shown to be less variable (**Table 2**). **Table 2** also shows that only a small fraction of the iron taken up by the cells is actually released at the serosal side of the cells. This is explained by the fact that transport of iron across the basolateral membrane of intestinal epithelium cells requires systemic signals (*22*), which have not yet been fully identified and characterized and which were therefore not present in the basolateral compartment of Caco-2 cells during transport studies. In preliminary studies we evaluated the effect of apo-transferrin on iron transport but did not observe a difference in iron transport across Caco-2 cells in the presence or absence of apo-transferrin in the basolateral compartment (data not shown).

Iron uptake and transport measurements in this study were performed after a trace of ^{55}Fe had been added to the digest. It is assumed that the added ^{55}Fe is representative for the total soluble iron in the system and absorbed in the same manner as the non-radioactive iron in the sample. However, this is an assumption. Addition of an intrinsic label would be preferred in this type of study, meaning that all procedures would be performed under radioactive conditions. Although this is technically feasible, it is more time-consuming and more expensive and would make this kind of experiment less versatile.

In conclusion, on the basis of the results of the in vitro iron availability testing it appears that only the addition of Na-

FeEDTA to whole-grain wheat flour may result in a food product that may be expected to effectively improve the iron status of people consuming it. However, final proof for this needs to be obtained from human absorption or efficacy studies.

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LITERATURE CITED

(1) Beard, J.; Stoltzfus, R. J. Iron-deficiency anemia: Reexamining the nature and magnitude of the public health problem. *J. Nutr.* **2001**, *131*, 563S−703S.

(2) Du, S. F.; Zhai, F. Y.; Wang, Y. F.; Popkin, B. M. Current methods for estimating dietary iron bioavailability do not work in China. *J. Nutr.* **2000**, *130*, 193−198.

(3) Hurrell, R. F. Bioavailability of iron. *Eur. J. Clin. Nutr.* **1997**, *51*, S4−S8.

(4) Hurrell, R. F. Preventing iron deficiency through food fortification. *Nutr. Rev.* **1997**, *55*, 210−222.

(5) Hurrell, R. F. Fortification: Overcoming technical and practical barriers. *J. Nutr*. **2002**, *132*, 806S−812S.

(6) Allen, L. H. Advantages and limitations of iron amino acid chelates as iron fortificants. *Nutr. Rev.* **2002**, *60*, S18−S21.

(7) Fidler, M. C.; Walczyk, T.; Davidsson, L.; Zeder, C.; Sakagchi, N.; Juneja, L. R.; Hurrell, R. F. A micronised, dispersible ferric pyrophosphate with high relative bioavailability in man. *Br. J. Nutr.* **2004**, *91*, 107−112.

(8) Luten, J.; Crews, H.; Flynn, A.; van Dael, P.; Kastenmayer, P.; Hurrell, R. F.; Deelstra, H.; Shen, L.-H.; Fairweather Tait, S. J.; Hickson, K.; Farré, R.; Schlemmer, U.; Frøhlich, W. Interlaboratory trial on the determination of the *in vitro* iron dialysability from food. *J. Sci. Food Agric.* **1996**, *72*, 415−424.

(9) Miller, D. D.; Scricker, B. R.; Rasmussen, R. R.; Van Campen, D. A. An *in vitro* method for estimation of iron availability from meals. *Am. J. Clin. Nutr.* **1981**, *34*, 2248−2256.

(10) Miller, D. D.; Berner, L. A. Is solubility *in vitro* a reliable predictor of iron bioavailability? *Biol. Trace Elem. Res.* **1989**, *19*, 11−24.

(11) Au, A. P.; Reddy, M. B. Caco-2 cells can be used to assess human iron bioavailability from a semipurified meal. *J. Nutr.* **2000**, *130*, 1329−1334.

(12) Garcia, 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.

(13) Tallkvist, J.; Bowlus, C. L.; Lonnerdal, B. Functional and molecular responses of human intestinal Caco-2 cells to iron treatment. *Am. J. Clin. Nutr.* **2000**, *72*, 770−775.

(14) Uauy, R.; Hertrampf, E.; Reddy, M. Iron fortification of foods: Overcoming technical and practical barriers. *J. Nutr.* **2002**, *132*, 849S−852S.

(15) Hurrell, R. F.; Reddy, M. B.; Burri, J.; Cook, J. D. An evaluation of EDTA compounds for iron fortification of cereal-based foods. *Br. J. Nutr.* **2000**, *84*, 903−910.

(16) Hurrell, R. F.; Furniss, D. E.; Burri, J.; Whittaker, P.; Lynch, S. R.; Cook, J. D. Iron fortification of infant cereals: a proposal for the use of ferrous fumarate or ferrous succinate. *Am. J. Clin. Nutr.* **1989**, *49*, 1274−1282.

(17) Davidson, L.; Kastenmayer, P.; Szajewska, H.; Hurrell, R. F.; Barclay, D. Iron bioavailability in infants from an infant cereal fortified with ferric pyrophosphate or ferrous fumarate. *Am. J. Clin. Nutr.* **2000**, *71*, 1597−1602.

(18) Hurrell, R. F.; Bothwell, T. H.; Cook, J. D.; Dary, O.; Davidsson, L.; Fairweather Tait, S. J.; Hallberg, L.; Lynch, S. R.; Rosado, J.; Walter, T.; Whittaker, P. The usefulness of elemental iron for cereal flour fortification: A SUSTAIN task force report. *Nutr. Rev.* **2002**, *60*, 391−406.

(19) Layrisse, M.; Carcia-Casal, M. N.; Solano, L.; Baron, M. A.; Arguello, F.; Llovera, D.; Ramirez, J.; Leets, I.; Tropper, E. Iron bioavailability in humans from breakfast enriched with iron bisglycine chelate, phytates and polyphenols. *J. Nutr.* **2000**, *130*, 2195−2199.

(20) Bovell-Benjamin, A. C.; Viteri, F. E.; Allen, L. H. Iron absorption from ferrous bisglycinate and ferric trisglycinate in whole maize is regulated by iron status. *Am. J. Clin. Nutr.* **2000**, *71*, 1563−1569.

(21) Glahn, R. P.; Lee, O. A.; 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.

(22) Miret, S.; Simpson, R. J.; McKie, A. T. Physiology and molecular biology of dietary iron absorption. *Annu. Rev. Nutr.* **2003**, *23*, 283−301.

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