JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effect of Bread Baking on the Bioavailability of Hydrogen-Reduced Iron Powder Added to Unenriched Refined Wheat Flour

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Elemental iron powders are widely used to fortify flour and other cereal products. Our objective was to test the hypothesis that baking enhances the bioavailability of elemental iron powders by oxidizing Fe^0 to Fe^{2+} or Fe^{3+}. An in vitro digestion/Caco-2 cell culture model and a piglet model were used to measure bioavailability. Bread flour, either unfortified or fortified with hydrogen-reduced (HR) iron powder or $FeSO_4$ (300 mg Fe/kg flour), was baked into bread. For the in vitro studies, bread samples were treated with pepsin at pH 2, 3, 4, 5, 6, or 7 and subsequently incubated with pancreatic enzymes at pH 7 in a chamber positioned above monolayers of cultured Caco-2 cells. Ferritin formation in the cells was used as an index of iron bioavailability. Ferritin formation in cells fed HR Fe bread was similar to cells fed $FeSO_4$ bread when the peptic digestion was conducted at a pH 2 but lower when the peptic phase was conducted at pH 3, 4, 5, 6, or 7 ($P < 0.05$). Pig diets containing 35% dried bread were prepared and fed to cross-bred (Hampshire × Landrace × Yorkshire) anemic pigs in two studies. The rate of increase in hemoglobin Fe over the feeding period was used to calculate relative biological value (RBV), an index of iron bioavailability. In the first pig study, RBV of HR Fe added to flour prior to baking was 47.9% when compared to $FeSO_4$ fortified flour ($P < 0.05$). In the second pig study, a third treatment consisting of unfortified bread with HR iron added during diet mixing (after bread baking) was included. RBVs of the HR Fe diet (Fe added after baking) and HR Fe diet (Fe added before baking) were 40.1% and 53.5%, respectively, compared to the $FeSO_4$ diet. Differences in RBV between the HR Fe (before and after baking) and $FeSO_4$ (before baking) treatment groups were significant, but the difference between the before and after HR treatment groups was not significant. We conclude that bread baking does not enhance the bioavailability of elemental iron powders.

KEYWORDS: Hydrogen-reduced iron; baking; swine model; Caco-2; wheat flour; bread; iron bioavailability

INTRODUCTION

Iron fortification of staple foods (i.e. cereal flours) represents one of the most cost-effective and sustainable strategies for reducing the prevalence of iron deficiency in developing countries (*1*, *2*). Ferrous sulfate and other soluble iron compounds are highly bioavailable but may cause undesirable color and flavor changes due to the formation of iron complexes and lipid oxidation during long-term storage (*3*). Elemental iron powders are widely used as fortificants in cereal flours, in spite of their lower bioavailability, because they are relatively inexpensive and cause few sensory defects. Hydrogen-reduced iron is the most common elemental iron powder used to fortify cereal flour (*4*). It is produced by reducing iron oxide with hydrogen gas at high temperatures followed by milling to a fine powder. The powders used in Europe and North America typically have a particle size of 300 or 325 mesh (45 μm), whereas powders used in developing countries may have a larger size (*4*). Relative biological values (RBV) of reduced iron powders reported in the literature range from 13 to 54% in rats and 13−148% in humans (*5*).

Elemental iron powders added to flour may undergo oxidation during baking. Possible reactions include the following (*6*):

$$2Fe^0(s) + O_2(g) + 4H^+ \rightarrow 2Fe^{2+}(aq) + 2H_2O(l)$$

$$Fe^0(s) + 2H_2O \rightarrow Fe^{2+}(aq) + H_2(g) + 2OH^-$$

Since bread doughs are acidic (pH 4−7), reaction with protons is another possible mechanism (*7*):

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10.1021/jf0615395 CCC: $33.50 © 2006 American Chemical Society
Published on Web 09/27/2006

$$Fe^0 + 2H^+ \rightarrow Fe^{2+} + H_2$$

Fe^{2+} readily oxidizes to Fe^{3+} unless the pH is low or oxygen is absent. Ferric iron (Fe^{3+}), a strong Lewis acid, binds with water in aqueous systems, and these aquated iron complexes hydrolyze to form ferric hydroxides and iron oxides. In order for elemental iron powders to be bioavailable, they must be oxidized from the zero oxidation state (Fe^0) to a more soluble form.

Available evidence regarding the relative bioavailabilities of elemental iron powders and the effects of baking on the bioavailabilities of these powders are limited and conflicting. Cook et al. (*8*) prepared radiolabeled ferrous sulfate ($FeSO_4$), sodium iron pyrophosphate (NaFePP), ferric orthophosphate, and hydrogen-reduced (HR) iron. These tagged fortificants were then baked into wheat rolls and fed to human subjects. Relative to $FeSO_4$, bioavailabilities of the NaFePP, ferric orthophosphate, and HR iron were 5, 31, and 95%, respectively; indicating that the bioavailability of hydrogen-reduced iron was equal to that of iron from ferrous sulfate. Roe and Fairweather-Tait (*9*) baked HR iron powders labeled with a stable isotope of iron into bread and compared its absorption with ferrous ascorbate using a fecal isotope balance method in human subjects. Absorption of the iron from the bread and the ferrous ascorbate was very high, 64.8% and 49.7%, respectively. In results from in vitro digestion, Caco-2 cell culture studies were similar to the aforementioned human studies although not entirely consistent. In one study, Caco-2 cell uptake of iron from electrolytic iron powder baked into bread was approximately 60% of that from ferrous sulfate fortified bread ($p < 0.05$) (*10*). In another in vitro study, iron uptakes from breads made with flour fortified with ferrous sulfate or selected iron powders, including electrolytic and hydrogen-reduced iron, were not significantly different (*11*). In sharp contrast, Hallberg et al. (*12*) reported bioavailabilities of carbonyl iron baked into wheat rolls to range from 5 to 20% that from ferrous sulfate, indicating very poor bioavailabilities of this form of elemental iron. Furthermore, they found no effect of baking on the bioavailability on this form of elemental iron. The carbonyl iron was isotopically labeled with ^{55}Fe by neutron activation. Swain et al. (*13*) reported a similar study comparing electrolytic iron and ferrous sulfate in a farina cereal matrix. Again, the iron powder was labeled by neutron activation. In agreement with Hallberg's results, iron absorption from the activated electrolytic iron was only 5−15% of that from ferrous sulfate in human subjects (*13*). Swain et al. (*13*) suggest that using neutron activation may not be an appropriate method for radiolabeling iron powders, implying that the neutron activation and subsequent storage of the powders for several years to allow for the decay of other radioactive isotopes formed during the neutron bombardment may alter the bioavailability of the powders.

Recently, we conducted studies designed to determine the fate of elemental iron powders during baking (*14*). Electrolytic and hydrogen-reduced iron powders were added to unenriched flour, and the flour was used to bake bread in single-loaf bread baking machines (Sunbeam, Boca Raton, FL). Following baking, the bread was cut into cubes, dispersed in a pH 7 buffer containing pancreatin, and incubated for 2 h at 37 °C. Pancreatin was added to digest the protein and starch in the bread, thereby facilitating the release of trapped iron powders. Following digestion, the elemental iron was extracted from the mixture using a strong magnet. The extracted iron was dissolved in HCl and measured spectrophotometrically. Only about 10% of the electrolytic iron and 5% of the hydrogen-reduced iron was recovered from the bread after baking. This suggests that more than 90% of these elemental iron powders are oxidized during the baking process and leads us to our hypothesis that the bioavailability of iron in bread made with flour fortified with elemental iron powders should be nearly the same as bread made with flour fortified with ferrous sulfate or other iron salts.

Therefore, the aim of this study was to test the hypothesis that baking improves the bioavailability of hydrogen-reduced iron powders added to bread flour. An in vitro digestion/Caco-2 cell culture model and a hemoglobin repletion pig model were used to measure iron bioavailability. Physiological and anatomical similarities between the digestive tracts in pigs and humans make pigs an attractive model for studying nutrient absorption (*15*).

MATERIALS AND METHODS

Chemicals. All chemicals and digestive enzymes were obtained from Sigma Chemicals (St. Louis, MO))or Fisher Scientific (Fairlawn, NJ) unless stated otherwise. Ingredients for cell culture media were obtained from GIBCO, Life Technologies (Rockville, MD). Reagents were prepared with double-deionized water. All experimental glassware was soaked in 1 M HCl for no less than 4 h and rinsed with deionized water.

Iron Fortificants and Flours. Hydrogen-reduced (HR)/AC-325 iron powder was donated by North American Höganäs, Inc. (Niagara Falls, NY), and the ferrous sulfate, 7-hydrate granular, ($FeSO_4 \cdot 7H_2O$) was obtained from Mallinckrodt Baker (Paris, KY). Unenriched, unbromated wheat flour (72.6% extraction rate) was purchased in 50 lb paper sacks from New Hope Mills (Moravia, NY).

Preparation of Bread. The bread was prepared in a commercial kitchen using the following recipe: unenriched flour (22.7 kg), tap water (15 L) at ambient temperature, dried yeast (283 g), iodized salt (567 g), and iron fortificant (added at a rate of 300 mg Fe per 1 kg flour). Flour fortified with either HR iron powder, $FeSO_4$, or nothing was used to prepare three batches of bread. Iron fortificants were mixed well with the salt before adding it to the rest of the ingredients. The dry ingredients were placed in a stainless steel commercial mixer (Hobart Manufacturing Company, Troy, OH), the water was added, and the combined ingredients were mixed for 10 min at medium speed. The dough was taken out of the mixer and placed on a lightly floured stainless steel countertop where it was cut into smaller pieces of dough (3−4 kg per piece), kneaded for 5 min, allowed to rise for 20−25 min, and baked for 30 min in an oven at 204 °C. The bread was removed from the oven, cooled at room temperature, and placed in sturdy plastic bags until it was dried and ground 2 or 3 days later.

The bread was dried using conditions chosen to obtain a dry product without significant browning as follows. The bread was first broken up into smaller chunks and passed through a large hammer mill (Comminuting Machine, The W. J. Fitzpatrick Company, Chicago, IL) with no screen. It was then placed on stainless steel mesh trays, and loaded into a convection air dryer (Bryant Air Dryer, Bryant Manufacturing Company, Indianapolis, IN) at 54−60 °C. After drying for 30 min, the bread was put through the hammer mill for a second time with a screen in place (0.0653 cm pore size) to obtain a smaller crumb size.

Swine Diet Preparation. The diets were prepared by carefully preweighing all ingredients and thoroughly mixing them in an industrial feed mixer. A base diet (i.e. all ingredients excluding the dried bread crumbs) was first mixed to ensure consistency among the two treatment diets. The bread crumbs were then added to preweighed batches of the base diet and mixed in the feed mixer for 10 min. Since the bread crumbs without iron contributed a very small amount of iron (16 ppm Fe), most of the dietary iron came from the added iron fortificants. The treatment diets were formulated to meet or exceed current nutrient requirements for swine (Table 1) and included dried bread crumbs, ground corn, whey protein isolate (Davisco Foods International., Eden Prarie, MN), Tylan 10 antibiotics (North American Nutrition, Louisberg, OH), mineral and vitamin premixs (Dyets Inc., Bethlehem, PA), and corn oil (Dyets Inc., Bethlehem, PA). The mineral mix did not contain iron.

Table 1. Dietary Composition and Total Iron Content in Bread and Treatment Diets[a]

	g/100 g diet			
ingredients	low iron transition diet	prebaked HR Fe in diet	prebaked $FeSO_4$ in diet	HR Fe added with no baking into diet[b]
ground dent corn	70.0	35.9	35.9	35.9
bread crumbs	0	35.8	35.8	35.8
whey protein isolate	9.0	22.0	22.0	22.0
soybean meal 48%	14.0	0	0	0
corn oil	2.0	1.3	1.3	1.3
L-lysine (78% pure)	0.7	0	0	0
calcium carbonate	1.5	1.5	1.5	1.5
DL-methionine (50% eff.)	0.3	0	0	0
L-threonine (~100% pure)	0.3	0	0	0
vitamin and mineral premix[c]	1.0	1.0	1.0	1.0
sodium chloride	0.5	0.5	0.5	0.5
sodium phosphate	0.3	1.5	1.5	1.5
Tylan 20 antibiotics	0.5	0.5	0.5	0.5
total	100	100	100	100
Fe (mg/kg bread crumbs)[d]		302.4 ± 3.2	305.1 ± 1.2	15.5 ± 0.8
total dietary iron (mg/kg diet)	47.9 ± 4.7	116.5 ± 0.7	112.0 ± 2.6	115.0 ± 2.6

[a] Diets were formulated to meet or exceed the 1998 National Research Council Nutrient Requirement for Swine. [b] Additional treatment diet used in pig study #2. Iron was added after baking (during diet mixing). [c] Vitamin and mineral premix supplies (per kilogram of diet for a 10–20 kg pig): 2200 IU vitamin A, 220 IU vitamin D, 16 IU vitamin E, 0.5 mg vitamin K, 0.05 mg biotin, 0.3 g choline, 0.3 g folacin, 15 mg niacin, 10 mg pantothenic acid, 3.5 mg riboflavin, 1.0 mg thiamin, 1.5 mg vitamin B6, 17.5 μg vitamin B12, 6 mg Cu as cupric sulfate, 0.14 mg I as potassium iodate, 4 mg manganeous, 0.3 mg Se as sodium selenite, 100 mg Zn as zinc oxide. [d] By analysis. Iron concentration values are expressed as mean ± SEM, $N = 3$.

Measurement of Iron Content. Bread crumbs and swine diets were digested using a modified AOAC wet ashing method (*16*). Samples were digested in concentrated HNO_3 followed by digestion in a mixture of $HNO_3/HClO_4$. The ashed samples were analyzed for iron by inductively coupled plasma atomic emission spectroscopy (ICAP-61E Thermal Jarrell Ash Trace Analyzer, Jarrell Ash Co., Franklin, MA).

Assessment of Iron Bioavailability Using in Vitro Digestion/Caco-2 Cell Culture Model. *Measurement of Iron Bioavailability.* An in vitro digestion/Caco-2 cell culture model (*17*−*21*) was used to determine the iron bioavailabilities of the bread crumbs and pig diets. Briefly, samples were finely milled using a coffee grinder (Krups, Medford, MA) and blended into a puree in a buffer containing 140 mM NaCl and 5 mM KCl, adjusted to pH 2 with 0.1 M HCl, and incubated at 37 °C for 1 h in the presence of pepsin to simulate peptic digestion. The pepsin digests were then adjusted to pH 7 with 0.1 M $NaHCO_3$ and incubated at 37 °C for two more hours in a mixture containing bile salts and pancreatic enzymes to simulate intestinal digestion. This digestion was carried out in a small upper chamber placed above a Caco-2 cell culture monolayer in six-well plates. Caco-2 cells (at passage 30−35) were seeded at a density of 50 000 cells/cm^2. The experiment was performed 13 d post seeding since Glahn et al. (*18*) reported that the seeded cells reached confluence after 13 d. A 15 000 molecular weight cutoff dialysis membrane (Spectra/Por 2.1, Spectrum Medical, Gardena, CA) separated the contents in the upper chamber from the media immersing the Caco-2 cell layer. This membrane allowed iron released from the bread and swine diet samples to diffuse into the medium bathing the cells in the lower chamber. Presumably, the low-molecular weight, soluble iron that diffuses into the lower chamber is taken up by the cells in proportion to its bioavailability.

Upon completion of the pancreatin-bile digestion, the upper chamber was removed and the cells were incubated for 24 h at 37 °C to allow for ferritin formation. The growth medium was then removed, and the cell monolayer was harvested from the bottom chamber by adding 18.2 MΩ deionized water and placing in a bath sonicator (VWR Intl., Bristol, CT) to promote cell disruption. Caco-2 cells synthesize ferritin in response to an increase in intracellular iron concentration (*19*). An immuno-radiometric assay (RAMCO, Houston, TX) and a spectrophotometric assay (Bio-rad, Herculies, CA) were used to determine ferritin concentration and total protein concentration of the cell culture, respectively. Cellular ferritin formation, expressed as ng ferritin/mg protein, was used as an index of iron bioavailability.

Experimental Design. Three in vitro/Caco-2-cell culture model experiments were performed to assess iron bioavailability. In the first cell culture experiment, we compared bread crumbs and dietary treatments containing either HR iron powder or $FeSO_4$ added to flour before baking. These samples were measured against a negative control sample (minimum essential culture medium, MEM, with no added iron) and a positive control sample of $FeCl_3$/ascorbic acid. In the second experiment, iron bioavailabilities of breads baked with HR Fe and $FeSO_4$ were compared using a protocol similar to the first experiment except that the pH in peptic digestion/incubation step was varied. During this step, the samples were brought to pH 2, 3, 4, or 6 before adding pepsin and incubating at 37 °C for 1 h. In a third experiment, we re-assessed the ferritin formation in cells fed bread baked with HR-fortified flour following pepsin digestion at pH 2, 3, 4, 5, 6, or 7.

Assessment of Iron Bioavailability Using a Pig Model. *Animals.* Experimental protocols were approved by the Institutional Animal Care and Use Committee at Cornell University. Crossbred piglets (Hampshire × Landrace × Yorkshire) were selected from the Cornell University Swine Farm. At birth, the piglets were injected with 50 mg Fe in the form of iron dextran (Anemi-x 100, Agripharm, Grapvine, TX). This is half the amount normally administered at birth in commercial swine operations to prevent iron deficiency (*22*). The experimental barns were maintained at 23−27 °C on a 12 h light−dark cycle.

In the first swine trial, piglets were given the low iron transition diet at age 21 days. They were weaned at age 28 days and housed in a group pen until age 42 days, when they were moved into individual pens with concrete floors. The pigs were given 3 days to acclimate themselves to their pens prior to the start of the study. At age 45 days, baseline hemoglobin (Hb) was measured, and the pigs were assigned to two groups of nine on the basis of having similar Hb concentration and body weight. Each group included five barrows and four guilts. The pigs were placed on the treatment diets once they were grouped to start the repletion period (day 0). In the second swine trial, piglets were weaned at 28 days of age and remained on the transition feed until the start of the treatment diet. Baseline Hb was measured at age 39 days, and the piglets were assigned to three groups of six on the basis of having similar Hb concentration and body weight. At age 39 days, the iron repletion period was started (day 0).

Blood Sample Collection. Body weights, measured to the nearest 0.01 kg, and blood samples were taken at day 0 (baseline), day 7, and day 16 for the first swine trial and at day 0 (baseline), day 7, and day 14 for the second swine trial following an overnight fast. Blood was collected using 6 mL sodium heparin vacutainer tubes (Bocton Diskinson Vacutainer Systems, Franklin Lakes, NJ) with 20 guage, 1 in. vacuneedles. Blood was drawn from either the subclavian vein or the vena cava.

Table 2. Effects of Iron Form on Weight and Hemoglobin Iron Gains in Piglets Fed Diets Containing 36% Dried Bread Fortified with Either Hydrogen-Reduced Iron or $FeSO_4$ (Piglet Hemoglobin Repletion Trial #1)[a]

	dietary treatments		
	HR Fe	$FeSO_4$	*p*-value[b]
body weight (kg)			
day 0	11.76 ± 0.37	11.58 ± 0.48	0.772
day 7	15.59 ± 0.44	15.84 ± 0.49	0.708
day 16	20.02 ± 0.57	21.68 ± 0.57	0.055
hemoglobin (g/L)			
day 0	110.7 ± 2.5	109.3 ± 3.3	0.727
day 7	103.3 ± 5.2	131.0 ± 8.0	0.010
day 16	120.0 ± 5.0	183.1 ± 5.1	0.000
feed intake (kg)			
overall	11.34 ± 0.49	13.81 ± 0.61	0.006
total iron intake (mg)	1494 ± 64	1771 ± 78	0.015
hemoglobin iron gain (mg)			
overall	223.7 ± 24.3	546.5 ± 27.9	0.000
HRE[c]			
overall	16.9 ± 1.7	35.3 ± 0.9	0.000
RBV[d]	47.9	100	

[a] Diets contained 35% bread fortified with either HR Fe or $FeSO_4$ added before baking. Diets were fed ad libitum. Values are means ± SEM, $N = 9$ pigs. [b] *p*-values for independent *t*-test comparing means across rows with significance at $P < 0.05$. [c] Hemoglobin repletion efficiency (HRE) values expressed as percentages. [d] Relative biological values (RBV) normalized to the $FeSO_4$ treatment group expressed as percentages.

Hemoglobin Analysis. Following blood collection, a quantitative, colorimetric determination of hemoglobin concentration was measured for each pig. Briefly, 10 μL of blood was mixed with 2.5 mL Drabkin's reagent (one vial of Drabkin's Reagent and 0.5 mL of the 30% Brij 35 solution reconstituted in 1000 mL deionized water) in a test tube. The solution was vortexed and left to stand for 15 min at ambient temperature. Mixed solutions were transferred into cuvettes and absorbance was measured in a DU Series 500 spectrophotometer (Beckman, Fullerton, CA) at 540 nm. A porcine hemoglobin standard solution was prepared from crystalline hemoglobin obtained from Sigma.

Hemoglobin Repletion Assay. Total body hemoglobin iron was calculated for each pig at day 0 and at day 16 (pig study #1) or day 14 (pig study #2) using body weight and hemoglobin concentration according to the following formula (*23*):

$$\text{Hb Fe (mg)} = \text{body weight (kg)} \times \frac{0.06\text{ L (blood)}}{1\text{ kg body weight}} \times \text{Hb (g/L)} \times \frac{3.35\text{ mg (Fe)}}{\text{Hb (g)}}$$

It was assumed that blood volume (L) was equal to 6% body weight (kg) (*24*) and that hemoglobin contains 0.335% iron (*25*). Iron intakes were calculated from the feed intake and the compositional data. Iron bioavailability was expressed as hemoglobin repletion efficiency (HRE) (*26*):

$$\text{HRE} = \frac{\text{Hb Fe, mg (final)} - \text{Hb Fe, mg (initial)}}{\text{total Fe intake, mg}} \times 100\%$$

where Hb Fe (final) and Hb Fe (initial) are total body hemoglobin iron contents at the end and beginning of the treatment period, respectively. HRE provides a reasonable estimate of iron absorption.

Experimental Design. There were two swine experiments to measure iron bioavailability. In the first swine trial, pigs were fed the treatment diets for 16 days (Table 2). On day 0, each pig received 1000 g of feed, and subsequent increases in the amount of feed were closely monitored. Spilled feed was carefully collected, weighed, and recorded once a day. The second swine trial was identical to the previous trial except that a third treatment consisting of unfortified bread with HR iron added during diet mixing (after baking) was included. The piglets were fed their individual diets for 14 days. Feed was offered at 90% of the estimated ad libidum intake to reduce variability in feed intakes.

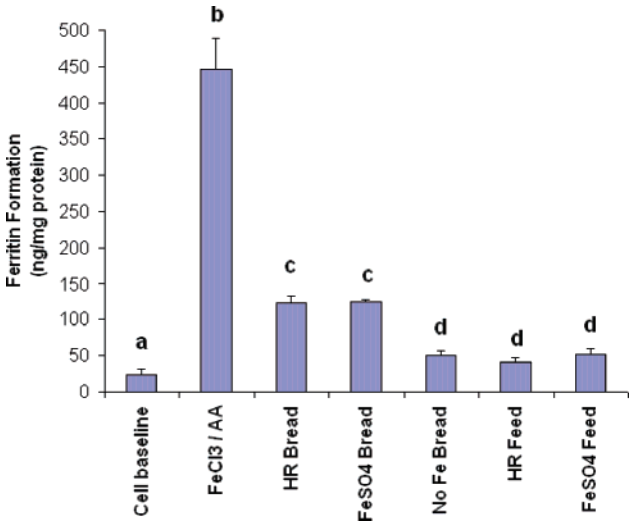

Figure 1. Relative iron bioavailabilities in unfortified and iron-fortified bread and swine feed (containing 35% bread) assessed using an in vitro digeation/Caco-2 cell model. Ferritin formation in the cells is an index of iron bioavailability. Cell baseline: growth media only; $FeCl_3$/AA: 50 μmol Fe/L, 1 mmol ascorbic acid/L; HR bread: bread baked with flour fortified with (HR)/AC-325 iron powder; $FeSO_4$ bread: bread baked with $FeSO_4$-fortified flour; no Fe bread: bread prepared with no fortified iron; HR feed: feed containing 35% HR fortified bread; $FeSO_4$ feed: feed containing 35% $FeSO_4$-fortified bread. Bar values (mean ± SEM, $n = 6$) with no letters in common are significantly different using Tukey's multiple comparisons ($P < 0.05$).

Statistical Analysis. Measurement of iron contents of bread crumbs and treatment diets were performed in triplicate. Iron bioavailability measurements for all in vitro Caco-2 cell treatments were independently replicated 6 times. Data were analyzed by analysis of variance (ANOVA) or Student's *t*-test. Significance was defined at $P < 0.05$. If appropriate, means were compared using Tukey's multiple comparison procedures calculated with MINITAB (State College, PA).

RESULTS AND DISCUSSION

In Vitro Caco-2 Cell Culture Model. Figure 1 shows ferritin formation in cells treated with bread (baked with flour fortified with HR iron, $FeSO_4$, or no iron) and pig feed digests. Ferritin formation in cells receiving iron-fortified bread crumbs was higher than in cells receiving the swine diets and unfortified bread. This was expected since the fortified bread contained almost three times more iron. Both bread samples produced a similar level of ferritin, which implies a similar bioavailability. These results are similar to those of Yeung et al. (*11*) who measured iron bioavailability in bread baked with flour containing added iron powders. They reported no significant differences in iron bioavailability between breads baked with HR Fe powder and $FeSO_4$.

The method employed in this study simulates peptic digestion by adjusting the pH of the food sample to 2 with HCl followed by incubation for 1 h at 37 °C in the presence of pepsin. The pH in the stomach following a meal is likely to be higher than 2 during much of the gastric phase of digestion. In young healthy subjects, the fasting gastric pH of the stomach generally ranges between 1.5 and 2 (*27*−*29*). However, following a meal, the gastric pH is elevated due to the buffering effect of the meal, in spite of increased gastric acid secretion to dilute the ingested food components (*30*). Partially digested food gradually leaves the stomach and enters the duodenum where the median pH

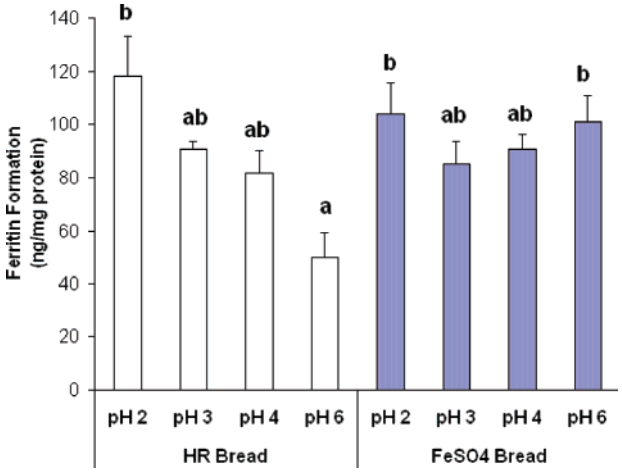

Figure 2. Ferritin formation in Caco-2 cells exposed to iron-fortified bread digests. pH during the peptic phase of the digestion varied from 2 to 6. Bar values (mean ± SEM, $n = 6$) with no letters in common are significantly different ($P < 0.05$) using Tukey's multiple comparison. Different letters indicates significant difference ($P < 0.05$) among treatments.

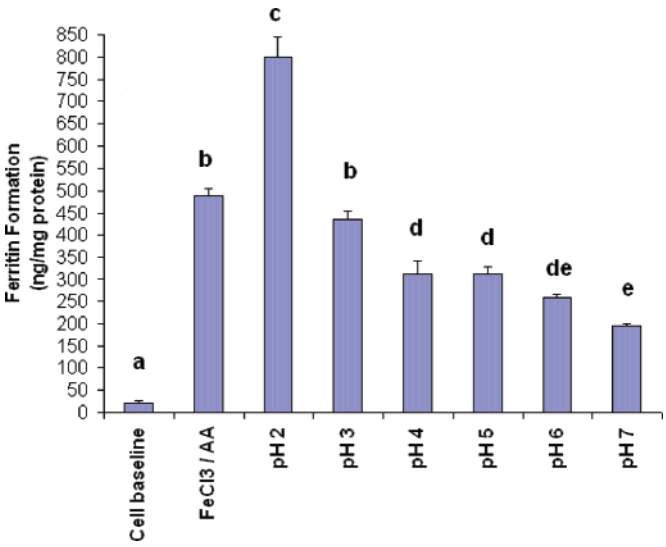

Figure 3. Iron bioavailability of HR-fortified bread samples (305 ppm Fe) with varying peptic digestion pH. Bars with no letters in common are significantly different. Error bars indicate standard error of the mean. Cell baseline: Caco-2 cells with growth medium at pH 2; $FeCl_3$/AA: 50 μmol Fe/L MEM at a ratio of AA:Fe – 20:1 at pH 2.

ranges between 5 and 7 (*31*, *32*). Therefore, additional in vitro digestion/Caco-2 cell culture experiments were conducted in which the gastric phase was carried out at higher pH conditions.

Figure 2 shows the effect of different peptic digestion pH levels on ferritin formation from breads baked with HR iron or $FeSO_4$. These results demonstrate the complexity of accurately simulating the digestive process in humans. Unlike the bread fortified with $FeSO_4$, ferritin formation in the bread with HR Fe varied as a function of pH during the gastric phase, e.g., 118.0 ± 15.0 (ng/mg protein) at pH 2 and 49.8 ± 9.9 at pH 6 ($P < 0.05$). The higher H^+ concentration at lower pHs may increase the rate of oxidation of Fe^0 to Fe^{2+}, thereby increasing soluble Fe^{2+} concentrations and uptake of iron by the Caco-2 cells. Ferritin formation in the pH 6 gastric phase treatment was 42% of the pH 2 treatment suggesting that stomach pH can have a marked effect on the bioavailability of hydrogen-reduced iron.

Figure 3 shows the iron uptake from bread baked with flour fortified with HR Fe using the in vitro Caco-2 cell culture model under a range of peptic digestion conditions: pH 2, 3, 4, 5, 6, and 7. Ferritin formation following peptic digestion at pH 2 is significantly higher than following the other pH treatments. These results confirm the findings shown in Figure 2.

Presumably, the bioavailability of elemental iron powders is limited primarily by the rate of oxidation from the zero oxidation state to either Fe^{2+} or Fe^{3+}. This most likely occurs in the stomach where the low pH will favor the following reaction: $Fe^0 + 2HCl \rightarrow Fe^{2+} + H_2 + 2Cl^-$. In a rat hemoglobin repletion study, Swain et al. (*33*) fed purified diets fortified with six different elemental iron powders. The powders were carefully mixed into the diets to avoid any particle fracturing by the abrasive action from mixing. Also, the diets were dry powders and there was no heat treatment of the diets so it is unlikely that there were any significant chemical changes in the powders prior to feeding. The authors reported a strong correlation ($R^2 = 0.82$) between relative bioavailability measured by hemoglobin repletion and solubility in 0.1 N HCl, suggesting that the oxidation rate of Fe^0 is a major factor in the bioavailability of the powders.

These observations raise questions about the choice of pH for pepsin digestion in an in vitro model, especially when evaluating products containing elemental iron. It is well-established that gastric acid secretion affects iron absorption in humans. Skikne et al. (*34*) showed that the administration of cimetidine, a inhibitor of gastric acid secretion, decreased iron absorption in human subjects by 28 to 65% depending on the dose of cimetidine given. However, as mentioned above, the pH of stomach contents rises following ingestion of a meal and then is gradually titrated back to the pH of the empty stomach. For example, the pH of stomach contents in human subjects increased from approximately 1 during a fasting state to 4.5 following a meal of steak, potatoes, fresh vegetables, salad, and desert and then decreased back to 1 after 3 to 4 h (*30*). In our in vitro/Caco-2 cell model, we adjust the pH of the meal to 2.0 prior to adding pepsin and do not adjust it again until the end of the pepsin digestion phase. We may need to consider modifying the method to make pH adjustments more gradually.

Pig Experiments. In the first pig trial, both treatment groups showed similar increases in body weight, but final hemoglobin concentration in the baked-in $FeSO_4$ treatment group was significantly higher than in the baked-in HR treatment group (Table 2). The pigs consuming bread baked with flour fortified with $FeSO_4$ displayed a higher HRE than those in the treatment group fed bread made from HR-fortified flour. The relative biological value (RBV) of the HR Fe powder was 48% compared to $FeSO_4$.

This experiment was repeated with the addition of a third treatment group containing HR iron powders added to the diet after baking (115 mg Fe/kg diet) (Table 3). The swine were offered 90% of their estimated daily feed requirement. Unfortunately, some of the animals in all three treatment groups had diarrhea for most of the trial and this appeared to adversely affect feed intake, weight, and hemoglobin iron gain. The HRE in the baked $FeSO_4$ treatment group was significantly higher than the groups fed either of the HR Fe fortified diets. However, since the RBVs were similar to the values found in the first swine trial, the diarrhea did not appear to affect relative iron absorption. The RBV, relative to $FeSO_4$, for baked HR Fe powder and the unbaked HR Fe powder was 53.5% and 40.1%, respectively. While the RBV of the powder added before baking was higher than the powder added after baking, the difference was not significant.

Table 3. Effects of Baking on Iron Absorption from Diets Containing Hydrogen-Reduced Iron or $FeSO_4$ (Piglet Hemoglobin Repletion Trial #2)[a,b]

	dietary treatments			
	HR Fe added before baking	HR Fe added after baking	$FeSO_4$ added before baking	*p*-value
body weight (kg)				
day 0	9.46 ± 0.30	9.89 ± 0.41	9.27 ± 0.23	0.443
day 7	11.45 ± 0.62	11.97 ± 0.61	11.30 ± 0.44	0.734
day 14	12.49 ± 0.75	12.96 ± 0.67	12.70 ± 0.54	0.765
hemoglobin (g/L)				
day 0	109.5 ± 2.7	121.8 ± 6.7	122.8 ± 4.5	0.166
day 7	107.3 ± 5.0 a	111.8 ± 4.3 ab	127.9 ± 5.9 b	0.038
day 14	107.4 ± 7.0	111.6 ± 6.6	130.6 ± 6.5	0.076
feed intake (kg)				
overall	5.0 ± 0.6	5.7 ± 0.5	5.1 ± 0.4	0.645
total Fe intake (mg)	581 ± 74.6	655 ± 58.9	570 ± 47.1	0.618
Hb iron gain (mg)				
overall	55.7 ± 18.1 a	48.0 ± 7.5 a	104.0 ± 16.4 b	0.046
HRE[c]				
overall	8.7 ± 3.0 a	7.5 ± 1.3 a	18.7 ± 2.8 b	0.016
RBV[d]	53.5	40.1	100	

[a] Diets contained 35% bread fortified with either HR Fe or $FeSO_4$ added before baking and HR iron added during diet mixing after baking. Diets were fed according to 90% of the daily calorie intakes for young pigs. [b] Values are means ± SEM, $N = 7$ pigs. One-way ANOVA was performed to compare means across rows (F-test with df = 2, 21) Tukey's multiple comparison test was performed when F-test was significant ($P < 0.05$). Different letters across a row imply significance. [c] Hemoglobin repletion efficiency (HRE) expressed as % = (total body hemoglobin Fe gain/Fe intake) × 100. [d] Relative biological value (RBV) expressed as % = (HRE of HR Fe treatment/HRE $FeSO_4$ treatment) × 100.

As mentioned in the introduction, we conducted preliminary in vitro studies to determine the effect of bread baking on elemental iron powders. We were able to recover only about 10% of the added elemental iron with a strong magnet following baking, suggesting that the iron was oxidized during baking. Apparently either oxidizing elemental iron does not affect its bioavailability or our method of recovering iron with a magnet was not effective.

Our results from the pig studies are consistent with a recent efficacy study by Zimmerman et al. (*5*). They compared wheat-based snacks fortified with ferrous sulfate, electrolytic iron, and HR Fe in Thai women with low iron stores. Subjects ($n = 330$) were randomly assigned into four groups and received wheat flour-based snacks fortified to provide 12 mg Fe/day for 6 days/week for 35 weeks. They collected body weight information and blood samples which were used to measure hemoglobin, serum ferritin, and serum transferrin receptor. Compared to the treatment group receiving the ferrous sulfate monohydrate, the relative efficacies of the HR iron and electrolytic iron was 49% and 77%, respectively. Walter et al. (*35*) studied twelve 5- to 7-year-old children who were fed bread prepared from unfortified wheat flour with a HR iron stable isotope. Iron isotopes, ^{57}Fe and ^{58}Fe, with enrichments greater than 90% were purchased as reduced iron powder in argon gas ampoules and used without further chemical alteration. The isotope iron powder was added in a thin gelatin capsule that would melt upon bread baking to release the isotope. After 14 days, erythrocytes were analyzed for isotopic enrichment and the relative bioavailability was about 65%, compared to ferrous sulfate. Hoppe et al. (*36*) served rolls fortified with different elemental iron powders or ferrous sulfate nine weeks apart to male subjects. Blood samples were drawn every hour for 6 h after bread consumption and were measured for serum iron concentration. RBVs were obtained by comparing the increase in serum iron concentration induced by the bread fortified with elemental iron powder versus the increase induced by $FeSO_4$. They found that the RBVs for the hydrogen-reduced iron powders, i.e., AC-325 and HiSol, were 50% and 56%, respectively.

Results from numerous studies suggest that $FeSO_4$ has superior bioavailability compared to elemental iron powders when used as a fortificant in bread flour (*33*, *36*, *37*). However, ferrous sulfate can promote chemical reactions in the flour leading to undesirable changes in color and flavor during storage (*4*). Hydrogen-reduced iron powders (i.e. AC-325, North American Höganäs, Inc., Niagara Falls, NY) are known to oxidize when exposed to oxygen, moisture, and/or acid. Once oxidized, the iron must remain soluble in the digesta in order to remain bioavailable (*33*). Recent fortification guidelines published by the World Health Organization suggest that concentrations of hydrogen-reduced iron should be used at twice the concentration of ferrous sulfate in order to provide an equivalent quantity of absorbed iron (*38*).

In conclusion, baking does not enhance the bioavailability of hydrogen-reduced iron powders added to wheat flour. In general, the bioavailability of HR iron powders was about half that of $FeSO_4$ whether the iron was added before or after baking the bread (but this was not the case in the Caco-2 trial using a gastric pH of 2).

ACKNOWLEDGMENT

We thank North American Hoganas, Inc., Karl Roneker and the Cornell University Swine Farm, Delmar Crim and the Cornell University Dining Services, and Xinpei Chang, Zhiquing Chang, and Mary Bodis at USDA/ARS for technical expertise and service.

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Received for review June 1, 2006. Revised manuscript received August 4, 2006. Accepted August 16, 2006.

JF0615395