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Iron Uptake by Caco-2 Cells from NaFeEDTA and $FeSO_4$: Effects of Ascorbic Acid, pH, and a Fe(II) Chelating Agent

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Sodium iron(III) ethylenediaminetetraacetate (NaFeEDTA) has considerable promise as an iron fortificant because of its high bioavailability in foods containing iron absorption inhibitors. In this study, uptakes of iron from NaFeEDTA, $FeSO_4$, and $FeCl_3$ by Caco-2 cells were compared in the absence or presence of ascorbic acid (AA), an iron absorption enhancer; at selected pH levels; and in the absence or presence of an iron absorption inhibitor, bathophenanthroline disulfonic acid (BPDS). Ferritin formation in the cells was used as the indicator of iron uptake. Uptake from all three Fe sources was similar in the absence of AA. Adding AA at a 5:1 molar excess as compared to Fe increased uptake by 5.4-, 5.1-, and 2.8-fold for $FeSO_4$, $FeCl_3$, and NaFeEDTA, respectively. The smaller effect of AA on uptake from NaFeEDTA may be related to the higher solubility of NaFeEDTA and/or the strong binding affinity of EDTA for Fe^{3+}, which may prevent AA and duodenal cytochrome b from effectively reducing EDTA-bound Fe. Uptake was inversely related to the pH of the media over a range of 5.8−7.2. Because uptake by DMT-1 is proton-coupled, the inverse relationship between pH and Fe uptake in all three iron sources suggests that they all follow the DMT-1 pathway into the cell. Adding BPDS to the media inhibited uptake from all three iron compounds equally. Because BPDS binds Fe^{2+} but not Fe^{3+} and because only Fe^{2+} is transported by DMT-1, the finding that BPDS inhibited uptake from NaFeEDTA suggests that at least some iron dissociates from EDTA and is reduced just as simple inorganic iron at the brush border membrane of the enterocyte. Taken together, these results suggest that uptake of iron from NaFeEDTA by intestinal enterocytes is regulated similarly to uptake from iron salts.

KEYWORDS: Iron bioavailability; iron uptake; NaFeEDTA; ferritin; Caco-2 cells

INTRODUCTION

Iron deficiency affects one-third of the world's population (*1*, *2*). Poor bioavailability of dietary nonheme iron is considered a major factor contributing to the problem (*3*). Many different forms of iron ranging from iron salts to iron chelates to elemental iron powders have been approved as iron sources for food fortification (*4*, *5*). In many cases, the efficacy of these fortificants is strongly influenced by iron absorption enhancers and inhibitors in the diet (*6*). For example, ascorbic acid (AA) can reduce Fe^{3+} to the more soluble Fe^{2+} and thus is a potent enhancer of nonheme iron absorption (*7*). On the other hand, phytic acid and polyphenolic compounds, often found in plant-based diets, inhibit iron absorption by forming stable and insoluble aggregates or by chelating iron with high affinity so that iron is not available for absorption by brush border iron transport proteins even if it is soluble, thereby preventing the iron from entering intestinal epithelial cells (*8*−*10*). Recently, there has been renewed interest in using sodium iron(III) ethylenediaminetetraacetic acid (NaFeEDTA) in food fortification because of its high stability in long shelf life foods, good solubility at low to near neutral pH environments, and superior iron bioavailability in foods containing iron absorption inhibitors (*11*−*13*).

NaFeEDTA was provisionally considered to be safe by the Joint Expert Committee on Food Additives of FAO/WHO for use in food fortification and nutritional intervention programs in the past decade (*14*). In January 2006, the U.S. Food and Drug Administration approved a GRAS notice by Akzo Nobel Chemicals (Arnhem, The Netherlands), in which Akzo Nobel stated that, in their opinion, NaFeEDTA is GRAS for addition to soy, fish, hoisin, teriyaki, and sweet and sour sauces (*15*). The efficacy of NaFeEDTA in combating iron deficiency anemia has been demonstrated in human studies in China, Thailand, and Central America (*16*−*18*). One of the concerns for using NaFeEDTA as a food fortificant is that the high bioavailability of this compound may lead to iron overload. In order to understand how NaFeEDTA is accumulated in the body, we must first assess how NaFeEDTA is regulated at the site of uptake.

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The mechanisms involved in iron absorption have been studied intensively. It is well-established that the first step in the absorption of dietary iron is the transport of iron from the intestinal lumen into the enterocytes and that this transport involves a membrane protein called divalent metal transporter (DMT-1) (*19*−*22*). DMT-1 appears to be specific for divalent cations, so Fe^{3+} must be reduced to Fe^{2+} before being transported into the cell. An enzyme with ferric reductase activity named duodenal cytochrome b (Dcytb) has been identified near the brush border membrane of the intestinal enterocyte, and presumably functions to reduce Fe^{3+}, thereby allowing its uptake (*23*). However, it is still unclear whether EDTA-chelated Fe is transported across the intestinal mucosal barrier via the DMT-1 mechanism and whether the chelated iron is released from EDTA during or prior to absorption. Because EDTA forms an extremely stable chelate with Fe^{3+} (stability constant, Log *K*, of 25.1 for Fe^{3+} at 20 °C in 0.1 mol/L KNO_3) (*24*), a property that may explain its resistance to inhibitors of iron absorption but also raises questions about whether it will release its iron to DMT-1 prior to uptake, some have suggested that Fe does not dissociate from EDTA, and the ferric EDTA complex is thus absorbed as an intact chelate through a paracellular route (*25*); others have speculated that iron dissociates from the EDTA ligand within the lumen of the gastrointestinal tract prior to mucosal uptake due to pH changes and therefore is transported via the normal, highly regulated transcellular DMT-1 pathways for ionic iron and, presumably, is absorbed similarly to ionic iron from iron salts (*4*, *26*).

In the present study, Caco-2 cells were used to evaluate iron uptake under various experimental conditions. The Caco-2 cell line is a human adenocarcinoma cell line that has proven to be a useful model for studying iron absorption from foods and iron fortificants (*27*, *28*). The cells differentiate into polarized monolayers with characteristics such as a brush border membrane containing the enzymes present in normal absorptive epithelial enterocytes. Ferritin formation by the Caco-2 cells following exposure to an iron source has been used as a marker for iron uptake (*29*, *30*).

The aims of this study were to address the following questions: (i) Does iron disassociate from the EDTA ligand before or after uptake by the enterocytes? (ii) Is the ferric iron in NaFeEDTA reduced to Fe^{2+} prior to uptake? (iii) Does pH have the same effect on uptake from NaFeEDTA as from simple salts such as $FeSO_4$ and $FeCl_3$? We reasoned that by comparing iron uptake from NaFeEDTA with uptake from $FeSO_4$ and $FeCl_3$ in the presence and absence of AA or bathophenanthrolinedisulfonic acid (BPDS), we could make inferences about whether iron is dissociated from EDTA before uptake. In addition, we compared uptakes at different pH values since it is known that DMT1 uptake is a proton-mediated process (*22*).

MATERIALS AND METHODS

Chemicals. All chemicals were obtained from Sigma Chemicals (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ) unless stated otherwise. All reagents were prepared with ultrapure water (resistivity of 18.2 MΩ). Glassware and utensils were soaked in 10% HCl for no less than 4 h and rinsed with deionized water prior to use.

Cell Culture. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) at passage 17 and used in experiments at passages 29−35. Cells were seeded at a density of 50000 cells/cm^2 in collagen-treated 24 well plates (Costar Corp., Cambridge, MA). The integrity of the monolayer was verified by optical microscopy. The cells were cultured at 37 °C in an incubator with a 5% CO_2 and 95% air atmosphere at constant humidity, and the medium was changed every 2 days. The cells were maintained in Dulbecco's modified Eagle medium plus 1% antibiotic/antimycotic solution, 25 mmol/L HEPES, and 10% fetal bovine serum. Two days before the experiment, the growth medium was removed from each culture well, and the cell layer was washed and maintained with minimum essential media (MEM) at pH 7.0. The MEM was supplemented with 10 mmol/L PIPES, 1% antibiotic/antimycotic solution, 4 mg/L hydrocortisone, 5 mg/L insulin, 5 μg/L selenium, 34 μg/L triiodothyronine, and 20 μg/L epidermal growth factor. This enriched MEM contained less than 80 μg iron/L. All ingredients and supplements for cell culture media were obtained from Gibco (Rockville, MD). Iron uptake experiments were conducted 13 days postseeding.

Harvesting of Caco-2 Cells for Ferritin Analysis. The procedures used in the determination of ferritin and total protein contents of Caco-2 cells were similar to those described by Etcheverry et al. (*29*, *31*). Briefly, growth medium was first removed from the culture well by aspiration and the cells were washed twice with a solution containing 140 mmol/L NaCl, 5 mmol/L KCl, and 10 mmol/L PIPES at pH 7.0. The cells were harvested by adding an aliquot of deionized water and placing them in a sonicator (Lab-Line Instruments, Melrose Park, IL) at 4 °C for 15 min. Cells were frozen at −20 °C immediately after harvest until later analysis. The ferritin concentration and total protein concentration were determined on an aliquot of the harvested cell suspension with a one-stage sandwich immunoradiometric assay (FERIRON II Ferritin Assay, Ramco Laboratories, Houston, TX) and a colorimetric assay (Bio-Rad DC Protein Assay, Bio-Rad, Hercules, CA), respectively. Caco-2 cells synthesize ferritin in response to increases in intracellular iron concentration. Therefore, the ratio of ferritin/total protein expressed as ng ferritin/mg protein was used as an index of the cellular iron uptake.

AA Treatments. Caco-2 cells were incubated in MEM mixed with aqueous solutions of NaFeEDTA, $FeSO_4$, or $FeCl_3$, in the absence or presence of AA. The final concentration of all three iron compounds was 10 μmol/L. When added, the AA concentration was 50 μmol/L. All treatment solutions were removed from the cells after 2 h of incubation and replaced with fresh MEM without added iron or AA. Cells were incubated for an additional 22 h and then harvested. Cellular ferritin and total protein were then analyzed and compared among all treatments.

pH Treatments. Three pH levels, pH 5.8, 6.5, and 7.2, were chosen to represent the gradual increase of pH in the intestinal lumen during digestion. Regular MEM was carefully titrated with either 1 mol/L HCl, 0.1 mol/L HCl, or 0.1 mol/L NaOH to obtain pH levels of 5.8, 6.5, and 7.2. Media with the three pH levels were then supplemented with either NaFeEDTA, $FeSO_4$, or $FeCl_3$, all of which had a final concentration of 10 μmol/L. After a 2 h incubation period, treatment solutions were aspirated off the cells. Fresh medium with the corresponding pH level was added to the cell wells, and plates were returned to the incubator for overnight incubation (total incubation time was about 24 h).

BPDS Treatments. BPDS has been used in Caco-2 cell studies as a chelating agent in the media to sequester excess iron molecules (*32*). It is a strong chelator specific to ferrous (Fe^{2+}) iron. It binds Fe^{2+} and forms $Fe(BPDS)_3$, a pink-red complex that can be measured spectrophotometrically at 534 nm (*33*). Regular MEM, containing either NaFeEDTA, $FeSO_4$, or $FeCl_3$, was mixed with BPDS to achieve a final concentration of 50 μmol/L BPDS (BPDS:Fe = 5:1) and placed on the cells. All treatments were removed from the cells after 2 h of incubation, replaced with fresh media, and incubated for another 22 h before harvesting.

Statistical Analysis. Two experiments with all three treatments (AA, pH, and BPDS) were conducted on two separated days. Each treatment was performed in triplicate on both days. Results were pooled, and the average of six replications was used as the mean value for each treatment. Data were tested for normality and analyzed by analysis of variance (ANOVA) with Tukey's paired comparison using Minitab Release 14 (Minitab Inc., State College, PA). The significance was defined at $p < 0.05$.

RESULTS AND DISCUSSION

Effect of AA on Ferritin Formation. Ferritin concentrations in cells treated with the three iron sources either with or without AA are shown in **Figure 1**. Iron uptake was not significantly different among the three iron compounds in the absence of

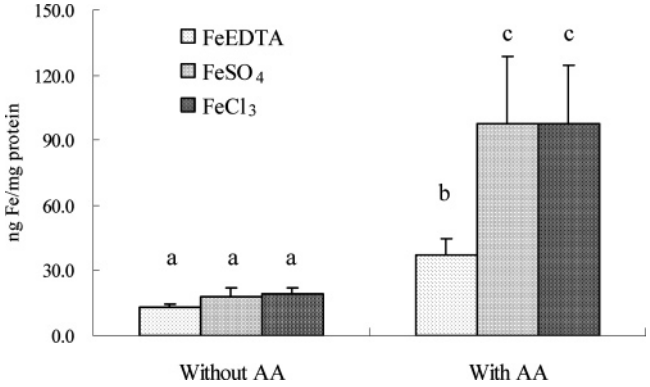

Figure 1. Effect of AA on ferritin formation in response to different iron compounds, in the absence or presence of AA. The final concentration of the three iron compounds was 10 μmol/L. The final molar ratio of AA:Fe was 5:1. Values with no letters in common are significantly different ($p < 0.05$). Values represent the mean + SD; $n = 6$.

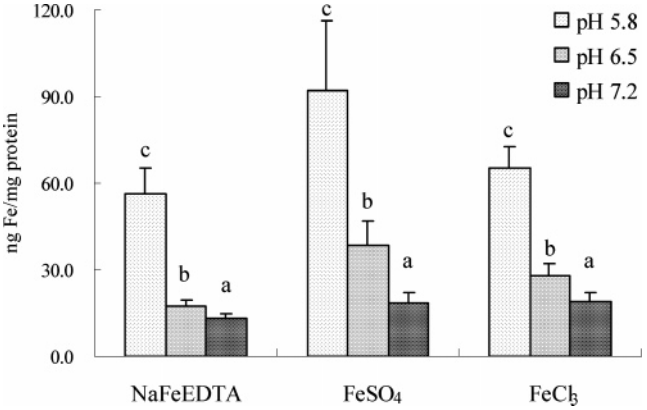

Figure 2. Ferritin formation at three pH levels: pH 5.8, pH 6.5, and pH 7.2. The final concentration was 10 μmol/L for all three iron compounds. Values represent the mean + SD; $n = 6$. Bars within an iron compound with no letters in common are significantly different (two-way ANOVA for randomized block design, $p < 0.05$).

AA. This finding indicates that at least some of the iron from FeEDTA has dissociated from the EDTA complex before being reduced by Dcytb for uptake. In the presence of AA, ferritin formation from $FeSO_4$ and $FeCl_3$ was similar and was significantly higher than that without AA (about 5.4 times higher in $FeSO_4$-treated cells and 5.1 times in $FeCl_3$-treated cells). This finding concurs with previous studies in which significant enhancing effects of AA on ferritin formation were observed (*28*, *34*, *35*). On the other hand, the addition of AA to NaFeEDTA group increased ferritin formation by 2.8-fold. This increase was significant, but the magnitude was not as dramatic as in the case of $FeSO_4$ and $FeCl_3$. In fact, in the presence of AA, ferritin formation from NaFeEDTA was about 61.8% lower than from $FeSO_4$ and from $FeCl_3$. This difference in iron uptake may be explained by the interaction of AA and NaFeEDTA. It has been proposed that the enhancing effect of AA on nonheme iron absorption comes from its reducing ability. Unlike Fe^{2+}, which can be taken up by DMT-1 directly, Fe^{3+} is first reduced by ferric reductase Dcytb or other reducing agents on the brush border side of the enterocyte. The presence of AA greatly facilitates the reduction of Fe^{3+} and maintained iron from $FeSO_4$ in Fe^{2+} state, therefore enhancing the absorption from $FeSO_4$ and $FeCl_3$ (*10*, *36*). Another added benefit of AA was proposed as a weak ligand to form a complex with iron, which increases the solubility of iron by stabilizing it from oxidation and precipitation at near neutral pH (*37*, *38*). EDTA chelates iron with high affinity, which might slow down the reduction of iron by AA as compared to the reduction of iron in $FeCl_3$. Furthermore, EDTA may compete with DMT-1 to bind iron. In fact, it has been shown that AA cannot reduce FeEDTA at pH 2.6−6.0, while the reduction of ferric iron by AA occurs slowly above pH 6.0−6.8 (*42*). On the other hand, NaFeEDTA is still quite soluble in the media (pH 7.0), which is advantageous because the chance of iron uptake increases with higher solubility. It is unlikely, however, that AA could further promote the solubility of iron from FeEDTA since EDTA is a much stronger chelating ligand for iron.

Although AA did not enhance the uptake of iron from FeEDTA as much as it did from $FeSO_4$ and $FeCl_3$, the rise in ferrtin formation from cells treated with NaFeEDTA and AA was still significant. This finding supports the hypothesis that iron dissociates from the EDTA complex prior to or during the uptake process at the apical side of the enterocyte, because AA is unlikely to affect NaFeEDTA uptake if it is acquired as an intact chelate via paracellular routes.

Because of its efficacy in delivering bioavailable iron in the presence of iron absorption inhibitors, NaFeEDTA has been considered a very attractive iron fortificant. Concerns have been raised as to whether dietary AA may further enhance the bioavailability of NaFeEDTA and thus cause or exacerbate iron overload (*5*, *39*, *40*). The finding that in Caco-2 cells AA does not enhance iron absorption in NaFeEDTA compound as much as it does with simple iron salts is therefore reassuring because it suggests that NaFeEDTA is no more likely to exacerbate iron overload than $FeSO_4$ or $FeCl_3$, even in the presence of AA.

Ferritin Formation at Selected pH Levels. The ferritin formation response was tested at three pH levels: pH 5.8, pH 6.5, and pH 7.2. The results are summarized in **Figure 2**. Regardless of the iron source, the patterns of ferritin formation were qualitatively comparable, with decreased iron uptake as the pH of the media increased.

Because the active divalent metal transport process mediated by DMT-1 has been shown to be proton-coupled, it is no surprise that DMT-1-mediated metal transport is more effective as the pH decreases, and iron uptake at pH 5.8 was the highest among all three pH levels tested (*22*). As the pH increases, the efficiency of DMT-1 decreases, so does the solubility of $FeSO_4$ and $FeCl_3$, hence the decrease in iron uptake. Because iron uptake from NaFeEDTA increased at lower pH in a similar fashion as seen in $FeSO_4$ and $FeCl_3$, our finding suggests that iron from NaFeEDTA was also taken up by DMT-1 or by a proton-coupled transport mechanism similar to DMT-1. It is arguable that the decrease in iron uptake as the pH increases is due to decreased solubility of $FeSO_4$ and $FeCl_3$ alone. However, ferritin formation from NaFeEDTA, which is soluble at all three pH levels, also decreased, indicating that the pH of the media does affect the efficiency of DMT-1.

Different iron compounds responded to the change of pH in different ways. This observation was best revealed in the interaction plot of two-way ANOVA **(Figure 3)**. The change in iron uptake from $FeSO_4$ was different from those of $FeCl_3$ and NaFeEDTA and was the main force resulting in a significant interaction. On the other hand, NaFeEDTA was soluble in the cell media at all three pH levels; therefore, the observation that iron uptake from NaFeEDTA decreased in a similar fashion as $FeCl_3$ when pH increased is puzzling. One hypothesis may be that the structural complexity and binding affinity of NaFeEDTA decreased the rate of reduction by Dcytb despite the good solubility of NaFeEDTA, while decreased solubility limited the

Table 1. Ferritin Formation in the Absence and Presence of BPDS[a]

	blank	FeEDTA	$FeSO_4$	$FeCl_3$
WITHOUT BPDS (ng ferritin/mg protein)	6.8 (2.33)	13.13 (1.56)[b]	18.18 (4.13)[b]	19.12 (2.83)[b]
WITH BPDS (ng ferritin/mg protein)	2.32 (0.65)	2.36 (0.25)	2.56 (0.98)	3.10 (0.85)
significance	$P = 0.222$	$P < 0.001$	$P < 0.001$	$P < 0.001$

[a] The final concentration of BPDS was 50 μmol/L, with a ratio of BPDS:Fe = 5:1. The pH of the media was 7.0. Cells appeared to remain intact and healthy throughout the treatment. Values represent means (SD); $n = 6$. The significant difference (before vs after the addition of BPDS) was detected at $p < 0.05$. [b] The ferritin formation from all three iron compounds was significantly higher than that from the blank in the absence of BPDS but was not significantly different among them (one-way ANOVA, $p < 0.05$).

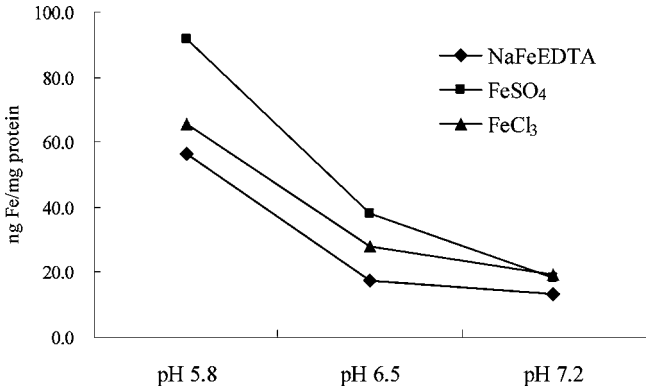

Figure 3. Interaction plot of two-way ANOVA, analyzing the response (ferritin formation, ng Fe/mg protein) for each iron compound at different pH levels. The two lines for NaFeEDTA and $FeCl_3$ have similar slope patterns, indicating that cells take up Fe from these two iron compounds in a similar fashion. On the other hand, the line representing ferritin formation from $FeSO_4$ at a different pH value had a steeper slope, suggesting that Fe^{2+} responded to Fe^{3+} differently as the pH in the cell media changed.

number of Fe^{3+} ions from $FeCl_3$ in the solution. The combination of these factors resulted in a similar level of iron uptake.

Our finding shows the possibility that iron from NaFeEDTA is taken up by DMT-1. This finding is supported by studies from Yeung et al. (*41*), which showed that relative iron uptake from both FeEDTA and $FeCl_3$ decreased significantly in the presence of 100-fold molar excess of Co^{2+} or Mn^{2+}, suggesting that Fe from FeEDTA is competing with divalent metals such as Co^{2+} and Mn^{2+} for transporters on the brush border of the enterocytes. Although our data are not sufficient to conclude that DMT-1 was the sole transporter responsible for NaFeEDTA uptake, our finding, together with the research by Yeung et al., supports the hypothesis that iron dissociates from the EDTA complex before being taken up by Caco-2 cells since the uptake was regulated in a similar fashion as simple iron salts.

Effect of BPDS on the Availability of Iron. The BPDS study design and results are summarized in **Table 1**. In the absence of BPDS, ferritin formation from all three compounds was similar. In the presence of excess BPDS (BPDS:Fe = 5:1), ferritin formation decreased significantly to the level of the baseline (fresh MEM + BPDS with no iron added) and was essentially identical for all three iron sources despite differences in their valence states and binding ligands.

BPDS binds Fe^{2+} but not Fe^{3+}; therefore, it is possible that all Fe^{2+} from $FeSO_4$ was chelated by BPDS and not available for uptake. The inhibitory effect in the two treatments containing Fe^{3+} (FeEDTA and $FeCl_3$) suggested that the cells reduce Fe^{3+} to Fe^{2+} prior to uptake possibly via the ferrireductase Dcytb. The finding that iron uptake from NaFeEDTA was affected by BPDS similarly to $FeCl_3$ suggests, again, that iron dissociates from EDTA at the site of uptake and is reduced before being taken up. In this case, BPDS competes with DMT-1 for ionic Fe^{2+} and apparently has higher affinity for it because there was nearly no ferritin formation in the presence of BPDS. However, the study design was not sufficient to conclude that all iron is dissociated from EDTA prior to uptake by the cells. It is possible that some FeEDTA is acquired by the body as an intact complex via the paracellular route. It is also possible that Fe^{3+} dissociates from EDTA but precipitates out of the solution because of lowered solubility by forming $Fe(OH)_3$ and, therefore, is unavailable for reduction by Dcytb. This scenario is highly unlikely, because the $Fe(OH)_3$ formation is a slow process, and also because uptake from $FeCl_3$ was significantly higher under the same condition. It is worth mentioning that iron uptake from the blank (fresh MEM only, pH 7.0) also decreased slightly in the presence of BPDS, although not significantly. This finding suggests that trace amounts of iron from cell media were also chelated by BPDS.

Conclusion. In this study, iron absorption from NaFeEDTA was equal to that from simple iron salts such as $FeCl_3$ and $FeSO_4$ in the absence of AA. AA promoted iron uptake from all three compounds, albeit to a lesser extent with NaFeEDTA, possibly due to the good solubility of NaFeEDTA at neutral pH and the high binding affinity of iron and EDTA. Iron uptake was higher at acidic pH than at neutral pH for all three iron compounds. Because DMT-1 transports ionic Fe^{2+} via a proton-coupled mechanism, which is favored by a more acidic pH, we speculate that Fe from NaFeEDTA is released before uptake and is also transported through the apical membrane via a similar proton-coupled mechanism. During the 2 h incubation in the presence of BPDS, Fe^{3+} from NaFeEDTA and $FeCl_3$ did not induce ferritin formation, indicating that Fe^{2+} was the main source of uptake, and at least some of the iron was dissociated from EDTA and was transporter-regulated just as simple iron salts.

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