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Combined Impact of pH and Organic Acids on Iron Uptake by Caco-2 Cells

SUSAN SALOVAARA, ANN-SOFIE SANDBERG, AND THOMAS ANDLID*

Food Science, Department of Chemistry and Bioscience, Chalmers University of Technology, P.O. Box 5401, SE-402 29 Göteborg, Sweden

Previous studies have shown that organic acids have an impact on both Fe(II) and Fe(III) uptake in Caco-2 cell. However, to what extent this effect is correlated with the anion of organic acids per se, or with the resulting decrease in pH, has not yet been clarified. Therefore, we studied the effect of five organic acids (tartaric, succinic, citric, oxalic, and propionic acid) on the absorption of Fe(II) and Fe(III) in Caco-2 cells and compared this with sample solutions without organic acids but set to equivalent pH by HCl. The results showed that the mechanisms behind the enhancing effect of organic acids differed for the two forms of iron. For ferric iron the organic acids promoted uptake both by chelation and by lowering the pH, whereas for ferrous iron the promoting effect was caused only by the lowered pH.

KEYWORDS: Organic acids; tartaric acid; succinic acid; citric acid; oxalic acid; propionic acid; ferrous iron; ferric iron; iron absorption; iron solubility; pH; Caco-2

INTRODUCTION

Iron deficiency is one of the greatest nutritional issues in the world today. According to the World Health Organization (WHO) (*1*), as many as 4−5 billion people may suffer from iron deficiency. The problem is usually not low amounts of iron in the food but poor bioavailability. This mainly concerns the most common form of dietary iron, the nonheme iron, which is found in, for instance, cereals, legumes, and vegetables. The reason for the low bioavailability is that plant-based meals frequently contain high levels of inhibitors such as phytate (*2*) and polyphenols (*3*, *4*).

The state and solubility of nonheme iron is highly affected by pH and also by the redox potential of the environment. Generally, the oxidation potential in the gastrointestinal tract will to a large extent determine the valence state of iron (*5*). Acidity tends to increase ionization as well as favor the ferrous state, which has greater solubility at the pH of the intestine than does the ferric state (*6*). As a consequence, gastric acid may influence nonheme iron absorption (*7*). For example, gastric juice with pH values >2 showed a very limited capacity to solubilize the iron in bread, whereas the solubilization increased at pH values <2 (*8*). Both oxidation states of iron are soluble at the low pH of the stomach, but when the pH rises upon entry into the duodenum, precipitates may be formed unless iron complexes with compounds that form soluble iron chelates (*5*). This is especially true for ferric iron, which readily forms precipitated ferric hydroxide when the pH rises above 4 if it is not already chelated. Ferrous iron, on the other hand, shows less tendency to precipitate and may stay dissolved up to higher pH. In contrast to ferric iron, ferrous may form dissolved complexes with, for instance, organic acids also at neutral pH (*9*). The pH of the human small intestine varies from ∼6.4 in the duodenum to a value of 7.3 in the distal parts (*10*). During the digestion of a meal, especially when acidic chymus is delivered from the stomach to the duodenum, the pH of the intestinal lumen may fluctuate. However, the pH in the immediate vicinity of the epithelial cells has been proposed to remain fairly stable and virtually independent of changes in the luminal bulk phase (*11*, *12*).

We have previously observed that organic acids show a concentration-dependent influence on the uptake of ferrous and ferric iron in Caco-2 cells (*13*). Our results showed a correlation between absorption pattern and chemical structure of the acids. Accordingly, four-carbon dicarboxylic acids, such as tartaric, malic, succinic, and fumaric acid, showed a positive effect on both ferric and ferrous iron absorption in the cells, but to varying degrees. The number of hydroxyl groups was shown to be important. Citric, lactic, and oxalic acid (2-, 3-, and 5-carbon carboxylic acids) had a similar and very negative effect on ferrous iron and a positive effect on ferric iron absorption. Acetic and propionic acid, which are simple 2- or 3-carbon monocarboxylic acids, showed a positive effect on ferrous iron and no effect on ferric iron uptake.

It was clear from the previous data that the effect of organic acids on iron uptake was not a matter of pH only. The aim of the present study was to clarify the relative impact from the anion and from the lowered pH, respectively. To attain this we investigated both the solubility of iron and the absorption. Two in vitro methods were utilized, iron dialyzability and iron

* Author to whom correspondence should be addressed [telephone (46)31-3351353; fax (46)31-833782; e-mail ta@fsc.chalmers.se].

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absorption, by the epithelial cell line Caco-2, which has previously been extensively used for iron absorption studies (*14*−*16*).

MATERIALS AND METHODS

Chemicals. Dulbecco's modified Eagle's medium (DMEM) with glucose (4.5 g/L) and l-glutamine, nonessential amino acids (NEAA), penicillin/streptomycin (PEST), and trypsin-EDTA were purchased from PAA Laboratories GmbH (Linz, Austria). Fetal calf serum was obtained from Biotech Line AS (Slangerup, Denmark), and $^{55}FeCl_3$ and $^{59}FeSO_4$ were obtained from NEN Life Science Products (Perkin-Elmer Life Sciences Inc., Zaventem, Belgium). The LCA cocktail, Ultima-FLO AP, used for scintillation counting, was purchased from Packard Bioscience B.V. (Groningen, The Netherlands). All other chemicals were purchased from Sigma-Aldrich (Stockholm, Sweden).

Iron Dialyzability Assay. Samples consisting of 2 or 4 mmol/L organic acids (citric, oxalic, propionic, succinic, and tartaric acid) were mixed in an uptake buffer (130 mmol/L NaCl, 10 mmol/L KCl, 1 mmol/L $MgSO_4 \cdot 7\ H_2O$, 5 mmol/L glucose, and 50 mmol/L HEPES, pH 6.8) (*17*). The pH was measured, and control samples with corresponding pH values but without organic acids were prepared. Thereafter, 10 μmol/L $FeCl_3$ or $FeSO_4$ was added to the samples. The samples were mixed and left to equilibrate for 30 min at room temperature. Inserts, fitting six-well plates (Transwell, Costar Europe Ltd., Badhoevedorp, The Netherlands), were fitted with a dialysis membrane (Spectra/Por 1, MWCO 8−12 kDa) held in place by a Teflon ring. One milliliter of the uptake buffer was pipetted into the wells, and the inserts were placed into the wells. The sample solution (1.5 mL) was then placed into the upper chamber formed by the insert ring. The plates were incubated at 37 °C for 1 h on an orbital shaker (50 rpm). After the incubation, the insert ring was removed, and 0.8 mL samples from the lower chambers were collected to measure dialyzed iron.

Iron Analysis. Iron in the dialyzed samples was analyzed by high-performance ion chromatography (HPIC) coupled with UV−vis detection (*18*). Briefly, the samples (0.8 mL) were pretreated with 0.1 mL of HCl (0.5 mol/L) and 0.1 mL of ascorbic acid (0.11 mol/L), mixed, and injected into the HPIC.

Cell Line and Culturing Conditions. Caco-2 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and used between passages 31 and 43. Stock cultures were maintained in 75-cm^2 flasks (TPP, Trasadingen, Switzerland) in complete medium in an atmosphere of 95% air and 5% CO_2 at 37 °C. The complete medium contained basal DMEM with 10 mL/L 100× NEAA, 10 mL/L 100× PEST, and 100 mL/L fetal calf serum. For the uptake studies cells were grown in 12-well plates (TPP) with a seeding density of ∼100000 cells/cm^2. The medium was changed every other day and the day before using the cultures for experiments. Experiments were performed using differentiated cultures at 13−15 days postseeding.

Iron Uptake Assay. Prior to the uptake assay, the Caco-2 cells were washed three times with phosphate-buffered saline. Samples consisting of various concentrations (0.5−3.5 mmol/L) of organic acids (citric, oxalic, propionic, succinic, and tartaric acid) were mixed in an uptake buffer (130 mmol/L NaCl, 10 mmol/L KCl, 1 mmol/L $MgSO_4 \cdot 7\ H_2O$, 5 mmol/L glucose, and 50 mmol/L HEPES, pH 6.8) (*17*), and the pH was altered, to values between 5.1 and 7.0, by dropwise addition of 0.1 M NaOH or 0.1 M HCl. Also, control samples without organic acids, but with corresponding pH values, were prepared.

Thereafter, 10 μmol/L radioactively labeled $FeCl_3$ (containing ^{55}Fe) or $FeSO_4$ (containing ^{59}Fe) was added to the samples. The samples were mixed and left to equilibrate for 30 min at room temperature. The samples (1 mL) were applied in duplicates on the cells grown in the plates and incubated for 1 h at 37 °C in air/CO_2 (95:5) atmosphere. After incubation, the sample solutions were aspirated, and nonabsorbed iron was removed according to the method of Glahn et al. (*19*, *20*). In brief, the cells were washed repeatedly with stop solution (140 mmol/L NaCl, 10 mmol/L PIPES, pH 6.8, 4 °C) and for 10 min with removal solution (stop solution with 1 mmol/L bathophenanthrolinesulfonic acid and 5 mmol/L sodium dithionite, pH 6.8, 4 °C). Cells were lysed and harvested by the addition of 1 mL of 0.5 mol/L NaOH, the content of each well was homogenized by pipetting, and 0.8 mL was transferred to a scintillation vial. LCA cocktail was added (2.5 mL), and the samples were mixed and analyzed by a Tri-Carb 1900CA liquid scintillation analyzer (Packard Instrument, Meriden, CT) to assess the amount of absorbed iron. In addition, the total amount of added radioactivity was analyzed directly by scintillation counting of 1 mL of the sample solution. The amount of iron absorbed was calculated by multiplying the fraction of radioactive iron absorbed with the total amount of non-radioactive iron applied to the cells. For comparison, each plate had two control wells in which the sample solution contained only 10 μmol/L radiolabeled $FeCl_3$ or $FeSO_4$ and no organic acid. To obtain consistency among experiments, total iron uptake was normalized by dividing each value with the control value obtained from the same plate and multiplying it by an average of all control values (*16*).

Table 1. pH of the Sample Solution after Addition of either 2 or 4 mmol/L Organic Acid and the pK_a of the Organic Acids

org acid	no. of carboxyl groups	pH of sample solution: 2 mmol/L acid	pH of sample solution: 4 mmol/L acid	pK_{a1}	pK_{a2}	pK_{a3}
tartaric acid	2	6.3	5.1	2.98	4.34	
succinic acid	2	6.3	5.5	4.16	5.61	
citric acid	3	6.2	5.1	3.14	4.77	6.39
oxalic acid	2	6.3	5.1	1.23	4.19	
propionic acid	1	6.6	6.3	4.87		

RESULTS AND DISCUSSION

Organic acids were selected on the basis of previously reported differences in impact on iron uptake (*13*). The addition of an organic acid to the sample solution altered the pH from the original value of 6.8 despite the buffer present (**Table 1**). The major chemical structures responsible for the shift in pH are the carboxyl groups. Hence, the pH changes detected were similar for the organic acids with the same number of carboxyl groups. Even if the pH of the sample solution was altered by the addition of organic acids, the pH was above the pK_a for most of the acids (**Table 1**). Consequently, the majority of the carboxyl groups were deprotonated throughout the assay, enabling potential binding of the iron ions. It is assumed that for iron to become absorbed by epithelial cells it needs to be in a soluble form. To assess the impact of organic acids on iron solubility, we performed dialysis of the sample solutions.

The effects on ferric and ferrous iron uptake by Caco-2 cells by adding organic acids to a sample solution with various pH values are shown in **Figures 1** and **2**. It can clearly be seen that the decrease in pH caused by the organic acids has an impact on the uptake of iron. However, the effect differed among the various organic acids and also between ferrous and ferric iron.

When 3.5 mmol/L tartaric or succinic acid was present in the assay, the ferric iron absorption was, within the pH range tested, higher than in their absence (**Figure 1a**). The difference between tartaric and succinic acid is consistent with previous data (*13*) and demonstrates that hydroxyl groups, not only carboxyl, are important in this context. The enhancing effect observed by tartaric acid can probably be accounted for by its chelating properties, suggesting the formation of soluble acid−iron complexes. This is supported by the observation that tartaric acid improved the dialyzability of ferric iron (**Table 2**). Also, the dialyzability of ferrous iron was slightly improved by the addition of tartaric acid. The effect of succinic acid on ferric and ferrous iron dialyzability was insignificant (**Table 2**). The impact of 3.5 mmol/L tartaric or succinic acid on ferrous iron absorption did not result in any additional effect as compared to lowering of the pH by HCl (**Figure 2a**), which may explain

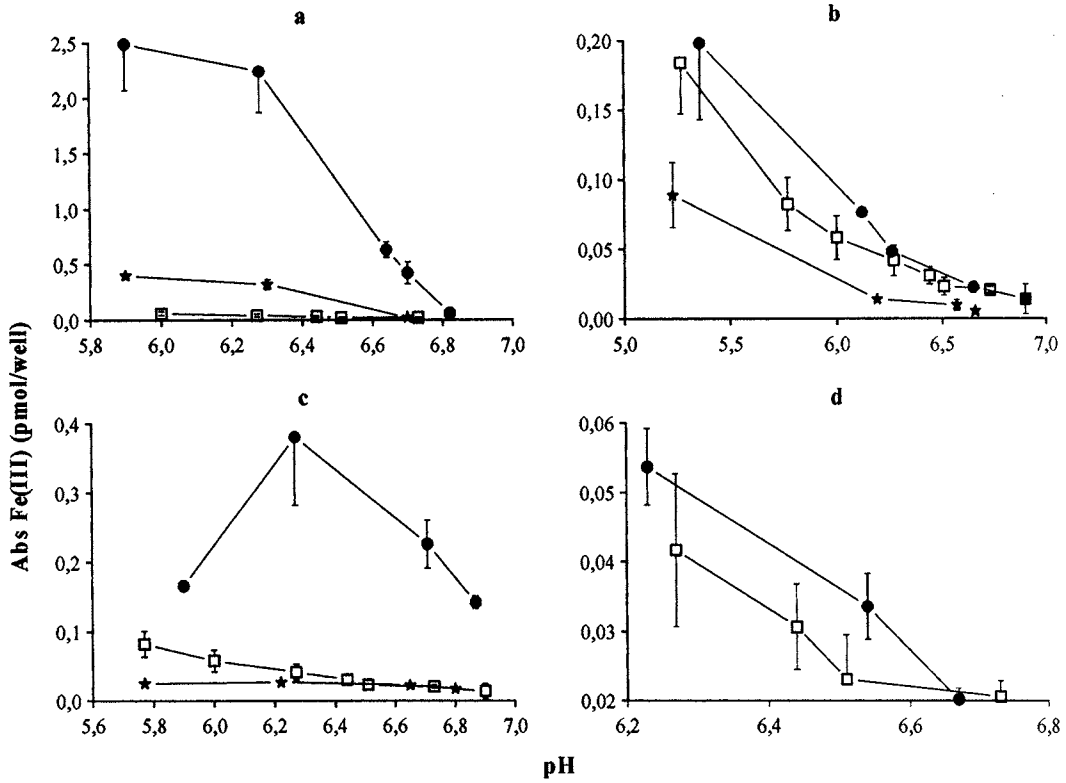

Figure 1. Absorption of Fe(III) as a function of pH. All graphs have a control curve (●) with no organic acid added and the pH altered with HCl: (**a**) tartaric acid, 3.5 mmol/L (□), and succinic acid, 3.5 mmol/L (★); (**b**) citric acid, 0.5 mmol/L (□), and 3.5 mmol/L (★); (**c**) oxalic acid, 0.5 mmolL (□), and 3 mmol/L (★); (**d**) propionic acid, 2 mM (□). Values represent mean ± SD; $n = 3$.

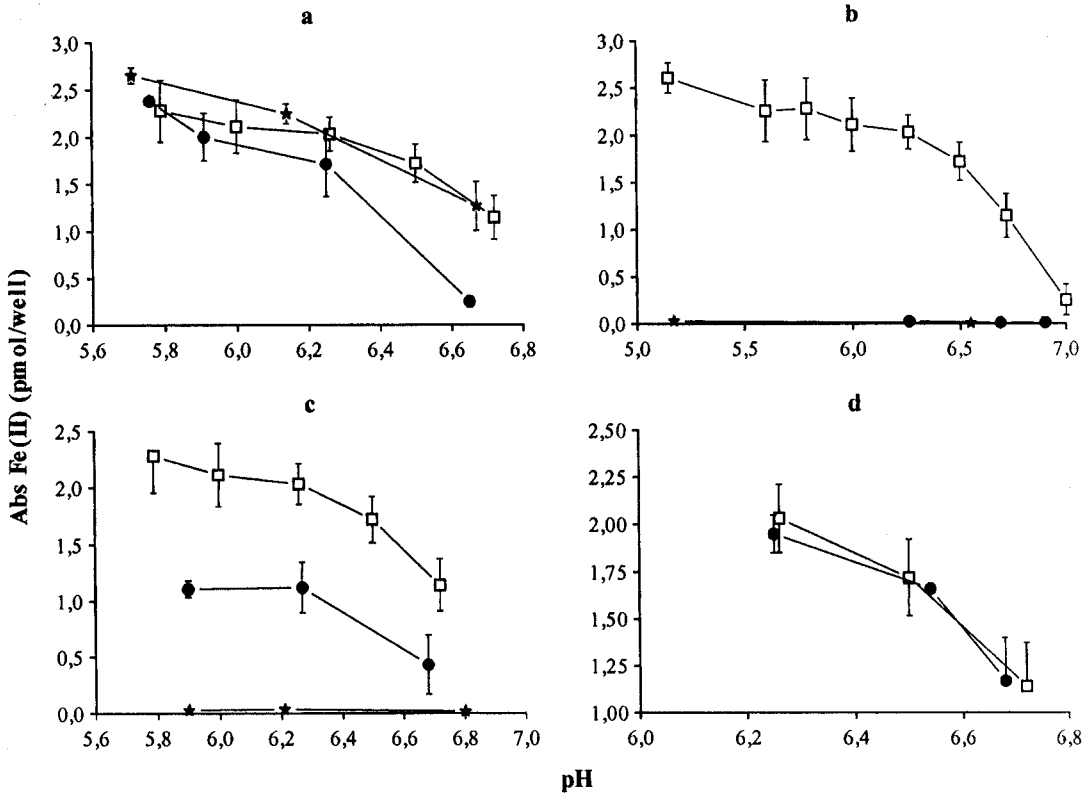

Figure 2. Absorption of Fe(II) as a function of pH. All graphs have a control curve (●) with no organic acid added and the pH altered with HCl: (**a**) tartaric acid, 3.5 mM (□), and succinic acid, 3.5 mM (★); (**b**) citric acid, 0.5 mM (□), and 3.5 mM (★); (**c**) oxalic acid, 0.5 mM (□), and 3 mM (★); (**d**) propionic acid, 2 mM (□). Values represent mean ± SD; $n = 3$.

the previously observed linear increase in ferrous iron absorption with increasing succinic acid concentration (*13*). In fact, 3.5 mmol/L tartaric acid lowered the ferrous iron absorption at pH values >6.3 (compared with HCl control), also consistent with our previous data. Because the phenomenon of decreased ferrous iron uptake at pH values >6.3 was observed only with tartaric

Table 2. Percentage of Dialyzed (MWCO 6–8 kDa) Ferric and Ferrous Iron from a Sample Solution with or without Organic Acids but with the Same pH Value ($n = 3$)

org acid	concn (mmol/L)	percentage dialyzed Fe(III) from a sample solution + org acid	percentage dialyzed Fe(III) from a sample solution + HCl	percentage dialyzed Fe(II) from a sample solution + org acid	percentage dialyzed Fe(II) from a sample solution + HCl
tartaric acid	2	21	5	28	18
	4	29	5	31	21
succinic acid	2	9	8	18	18
	4	2	8	20	20
citric acid	2	41	3	42	18
	4	44	7	44	242
oxalic acid	2	34	9	36	18
	4	48	7	50	22
propionic acid	2	4	6	16	13
	4	3	8	17	18

acid, and not with succinic acid, which is similar except for two hydroxyl groups, it can be assumed that the formation of a strong, inhibitory ferrous iron−acid complex is associated with the hydroxyl groups of tartaric acid. Either the resulting complex is reluctant to release Fe(II) to the uptake protein or the solubility decreases. On the basis of the minor difference in dialyzed Fe(II) between 2 and 4 mM tartaric acid, pH 4.8 and 6.3, respectively), which is not greater than the HCl control, we suggest that reluctance to release iron, rather than lowered solubility, is the dominating factor.

The action of citric acid presumably occurs through its carboxylic and hydroxyl groups, which prevent polymerization of iron hydroxides by forming soluble complexes with iron (*21*). It has previously been shown that citric acid renders a high solubility of ferrous iron but has a reluctance to donate iron to the Caco-2 epithelial cells and, hence, causes a poor absorption of iron (*22*). This was confirmed by our results that showed a high dialyzability (**Table 2**) but a nearly total inhibition of ferrous iron absorption, independent of the citric acid concentration and the pH in the solution (**Figure 2b**). The complexes formed between citric acid and ferrous iron appear to be very strong and may consist of more than one citric acid per iron ion (*23*, *24*). The dialyzability was similar for ferric and ferrous iron, but because a ferric iron solution has a very low solubility if no chelating agents are present, the pH-independent effect on dialyzability (i.e., the difference between data in the organic acid column and HCl column, **Table 2**) was larger for ferric iron than for ferrous. No difference in ferric iron uptake was observed between the sample solutions without citric acid or with low concentrations of citric acid (0.5 mM), and the increase in iron uptake was likely to be solely caused by the pH change (**Figure 1b**). Higher citric acid concentrations (3.5 mM) inhibited the ferric iron uptake by Caco-2 cells, probably for the same reasons as for ferrous iron.

The effect of oxalic acid on iron absorption (**Figures 1c** and **2c**) and on iron dialyzability (**Table 2**) was quite similar to that of citric acid. However, the decrease in ferrous iron absorption caused by 0.5 mmol/L oxalic acid was not as marked as for citric acid (**Figures 2b**,**c**). An explanation for the difference of impact between 0.5 and 3 mmol/L oxalic acid on ferrous iron absorption can be that at higher concentrations of oxalic acid the molecules may form larger complexes and render the ferrous iron even less available for absorption. The ferric iron absorption, on the other hand, was enhanced by 0.5 mmol/L oxalic acid, whereas 3 mmol/L oxalic acid had no effect at high pH values and a distinct negative effect at low pH values (**Figure 1c**). The positive influence of low levels of oxalic acid correlates well with previous data showing a peak in iron uptake at ~0.5 mmol/L (*13*).

Propionic acid previously showed a linear increase in ferrous iron uptake but no effect on ferric iron. Because propionic acid is a moncarboxylic acid, it has only a modest effect on the pH of the sample solution (**Table 1**). The increase of ferrous and ferric iron absorption with the addition of propionic acid seemed to be linked to the shift in pH (**Figures 1d** and **2d**). The previously observed lack of effect on ferric iron absorption by increasing concentrations of propionic acid is probably explained by the moderate influence on pH for all propionic acid concentrations without adjustment by HCl (pH 6.5−6.8). The anion itself did not seem to influence the uptake, which was confirmed by the lack of effect on iron dialyzability (**Table 2**).

The previously observed positive effects of the organic acids on iron absorption seem to mainly be caused by the pH change for the ferrous iron and by the combination of the anion of the organic acid and by the pH for the ferric iron absorption. The explanation for an increase in ferrous iron transport, but not in ferric iron transport, at lower pH is associated with the absorption mechanisms for iron. Ferrous iron is transported across the cell membrane by the divalent metal transporter 1 (DMT1; DCT1/Nramp2). This ferrous iron transport is proton-coupled (*25*), and the absorption has been shown to increase at a lower apical pH (*26*). This effect is probably connected to the increased membrane proton gradient (proton motive force; *P*) at lower pH, which would cause an increase in the driving force of protons, and coupled ions, across the cell membrane. The ferric iron, on the other hand, can also be transported by DMT1 but needs then first to be reduced or, alternatively, be transported by other proteins, such as the β_3-integrin and mobilferrin pathway (IMP), which is specific for ferric iron (*27*). Because the absorption of ferric iron to some extent was dependent on the organic acid added and its concentration and not only on the pH per se, we suggest that the uptake mechanism of ferric iron is less influenced by pH than the ferrous iron uptake is. Instead, the chelating properties of the organic acids seem to be more important.

Human trials have resulted in contradictory data concerning the effect of citric acid on iron bioavailability. When citric acid (1 g) was added to a rice meal (3 mg of $FeSO_4$), the iron absorption increased by 200% (*28*), and when the same rice meal also contained 33 mg of ascorbic acid, the addition of 0.75 g of citric acid increased the iron absorption by 49% (*21*). However, when citric acid (1 g) was added to a basal meal (4.3 mg of native iron) consisting of maize, rice, and black beans, the iron absorption decreased by 67% (*29*). Obviously, there is a difference in the meal composition among the three studies; for example, the last study probably contains more polyphenols. However, our results may propose an explanation for these diverging results, even if our experimental setup lacks the important digestion step. According to our results, citric acid is a strong inhibitor of ferrous iron absorption independent of the pH of the solution, whereas the effect by low concentrations on ferric iron absorption is slightly enhancing. The pH changes during the digestion, and the varying components of the meal matrix make it hard to predict the valency state of iron at the moment of complexation with citric acid. The resulting effect on iron uptake is so dependent on the valency state of iron that this may explain the varying results from human studies.

To conclude, the pH of a solution does influence the absorption of ferrous and ferric iron in Caco-2 cells. Because the addition of organic acid alters the pH of the sample solution to a certain extent, the pH could explain some of the effects

observed in regard to organic acids and iron absorption, especially with ferrous iron. Tartaric acid, succinic acid (both 3.5 mmol/L), and oxalic acid (0.5 mmol/L) promoted an additional effect on ferric iron absorption beyond the pH effect per se, probably due to formation of soluble iron−acid complexes. The effects of citric acid (0.5 mM) and propionic acid (2 mM) on ferric iron absorption were due to only the decrease in pH, and no effect could be linked to the anion of the acid. Furthermore, the effect of tartaric and succinic acid (both 3.5 mmol/L) and propionic acid (2 mmol/L) on ferrous iron absorption was also only a pH effect, and no additional enhancing effect by the organic acids tested could be detected, probably mainly as a result of the solubility of ferrous iron at physiological pH. Instead, both citric and oxalic acid, which formed complexes with ferrous iron, inhibited the uptake by the Caco-2 cells.

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