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Organic Acids Influence Iron Uptake in the Human Epithelial  
Cell Line Caco-2

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It has previously been suggested that organic acids enhance iron absorption. We have studied the effect of nine organic acids on the absorption of Fe(II) and Fe(III) in the human epithelial cell line Caco-2. The effect obtained was dose-dependent, and the greatest increase (43-fold) was observed for tartaric acid (4 mmol/L) on Fe(III) (10  $\mu$ mol/L). Tartaric, malic, succinic, and fumaric acids enhanced Fe(II) and Fe(III) uptake. Citric and oxalic acid, on the other hand, inhibited Fe(II) uptake but enhanced Fe(III) uptake. Propionic and acetic acid increased the Fe(II) uptake, but had no effect on Fe(III) uptake. Our results show a correlation between absorption pattern and chemical structure; e.g. hydroxyl groups, in addition to carboxyls, were connected with a positive influence. The results may be important for elucidating factors affecting iron bioavailability in the small intestine and for the development of foods with improved iron bioavailability.

**KEYWORDS:** organic acids; iron absorption; Caco-2

## INTRODUCTION

Iron deficiency is one of the largest identified nutritional problems in the world today. Deficiency of iron is highly prevalent in developing countries and also in industrialized countries among vulnerable population groups, such as women of childbearing age, infants, and adolescents. Iron is an important micronutrient, especially during growth and development. The main source of iron in our diet is in the form of nonheme iron, which has a strong tendency to interact with other compounds in the meal. On average, only  $\sim 7\%$  of the nonheme iron is absorbed (1). The other type of dietary iron is in the heme form, which is much less influenced by other dietary factors, and on average, 25% is absorbed from the diet (1).

Absorption of iron is a complex process involving at least three steps: (i) digestion and release from the diet, (ii) active uptake into the enterocytes, and (iii) transport from the enterocytes to the circulation. The fraction of the total iron in the diet that is available for uptake by the intestinal brush border membranes is known as bioaccessibility. Bioaccessibility in combination with the actual absorption is defined as bioavailability. It is important to make a distinction between these terms, since bioaccessibility is often determined merely as the fraction of soluble iron, completely disregarding active absorption. Heme iron, which exists in a porphyrin complex as myoglobin or hemoglobin, is absorbed as a complex and is thus less influenced by its environment. In contrast, nonheme iron is absorbed as an ion and needs to be in a soluble state to be available for absorption. Knowledge about the mechanism for the uptake of iron has increased much during the past decade, including data

on the molecular details, such as specific proteins involved (2); however, the interaction with other compounds is not yet fully understood.

Iron has several valency states, but the only stable forms in an aqueous environment are the ferrous, Fe(II), and the ferric, Fe(III), ions. In many solutions, ferric iron precipitates as hydroxides at pH values above 4 and therefore needs to be chelated in the stomach or proximal duodenum to remain in a dissolved state. Ferrous iron, on the other hand, precipitates at pH values above 7 and may thus remain in a soluble state in the proximal intestine. Compounds that can form soluble complexes with iron ions are fragments of mucin (3), organic acids (4), certain amino acids (5), and EDTA (6). Certain chelating agents may form strong complexes with iron and consequently be poor donors of iron to the enterocytes and, hence, limit the absorption. An in vitro study on iron uptake by brush border vesicles showed that both citric acid and EDTA have high affinities for ferric iron, but were poor donors, which affected the absorption negatively (7). Several compounds can also form insoluble complexes with iron and thus make it nonabsorbable, for example, inositol hexaphosphate (8) and polyphenols (9, 10).

When studying nutrition and absorption, the most accurate results are, of course, obtained from successfully designed human studies. However, these are expensive and often hard to conduct. Therefore, in vitro model systems, such as the Caco-2 cell line, are frequently used. The cells differentiate spontaneously, both structurally and functionally, into cells resembling mature enterocytes (11). Previous research has demonstrated that Caco-2 cells are suitable to use as an in vitro model for iron absorption studies, since the cells yield data consistent with human uptake studies (12–14).

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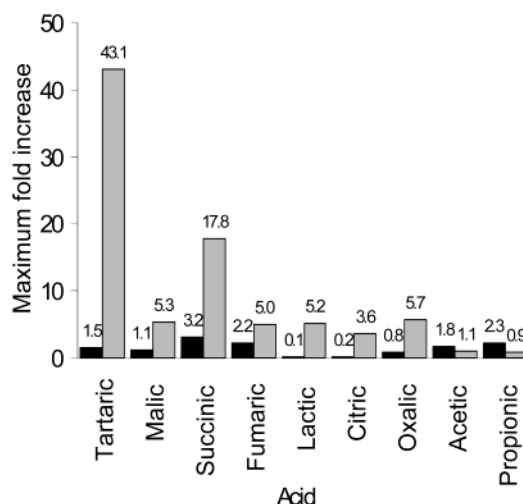
In the present communication, we report on factors affecting the absorption of iron in the human epithelial cell line Caco-2. We have studied the effect on ferric and ferrous iron uptake of nine organic acids that occur naturally in foods, such as fruits, vegetables, and lactic fermented foods. The aim was to study the dose-dependent effect of different organic acids on cellular uptake of iron and relate that to the chemical structure of the acids.

## MATERIALS AND METHODS

**Chemicals.** Dulbecco's modified Eagle medium (DMEM) with 4.5 g/L glucose and L-glutamine, nonessential amino acids (NEAA), and trypsin-EDTA were purchased from Bio Whittaker Europe (Verviers, Belgium). Fetal calf serum was obtained from Biotech Line AS (Denmark), and  $^{55}\text{FeCl}_3$  and  $^{59}\text{FeSO}_4$  were obtained from NEN Life Science Products (PerkinElmer 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).

**Cell Line and Culturing Conditions.** Two different batches of Caco-2 cells were used, one from European Collection of Cell Cultures (ECACC, Salisbury, U.K.), which was used between passages 40 and 55, and one from American Type Culture Collection (ATCC, Manassas, VA), used between passages 25 and 35. Stock cultures were maintained in 75-cm<sup>2</sup> flasks (TPP, Trasadingen, Switzerland) in complete medium in an atmosphere of 95% air and 5% CO<sub>2</sub> at 37 °C. The complete medium contained basal DMEM with 10 mL/L 100× NEAA, and 100 mL/L fetal calf serum. For the uptake studies, cells were grown in 24-well plates (TPP, Trasadingen, Switzerland) with a seeding density of 50 000 cells/cm<sup>2</sup>. 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 post seeding.

**Iron Uptake Assay.** Prior to the uptake assay, the Caco-2 cells were washed three times with phosphate buffer saline. Samples consisting of various concentrations (10–4000  $\mu\text{mol/L}$ ) of organic acids (acetic, citric, fumaric, lactic, malic, oxalic, propionic, succinic, and tartaric acid) and 10  $\mu\text{mol/L}$  radioactively labeled  $\text{FeCl}_3$  or  $\text{FeSO}_4$  were mixed in an uptake buffer (130 mmol/L NaCl, 10 mmol/L KCl, 1 mmol/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 5 mmol/L glucose, and 50 mmol/L HEPES, pH 6.8) (15). Ascorbic acid (20–1500  $\mu\text{mol/L}$ ) was also tested as a reference substance. The samples (0.5 mL) were applied in duplicates or triplicates on the cells grown in the plates and incubated for 1 h at 37 °C in air/CO<sub>2</sub> (95:5) atmosphere. After incubation, the sample solutions were aspirated, and nonabsorbed iron was removed according to Glahn et al (16, 17). In brief, the cells were washed three times with 0.5 mL of stop solution (140 mmol/L NaCl, 10 mmol/L PIPES, pH 6.8, 4 °C), followed by 0.5 mL of removal solution (stop solution with 1 mmol/L bathophenanthroline-sulfonic acid and 5 mmol/L sodium dithionite, pH 6.8, 4 °C) applied to the cells for 10 min, and finally, the cells were washed two more times with stop solution. The cells were lysed and harvested by addition of 1 mL of 0.5 mol/L NaOH; after 10 min, 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 by the total amount of nonradioactive iron applied to the cells. Since it was not possible to determine both protein content and iron uptake in the same well, only cells in six wells from each experiment were analyzed for total protein content by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). However, the protein content was fairly consistent both within and between experiments ( $202 \pm 29 \mu\text{g/mL}$ ), and therefore, an average value was used in calculating absorbed iron per microgram of cell protein. For comparison, each plate had two or three control wells in which the sample solution contained only  $\text{FeCl}_3$  or  $\text{FeSO}_4$  and



**Figure 1.** Maximum fold increase of Fe(II) (black bars) and Fe(III) (gray bars) uptake in the presence of specific acids within the range of our assay (0–4 mmol/L). Data are expressed as fractions of the uptake obtained with no acid added.

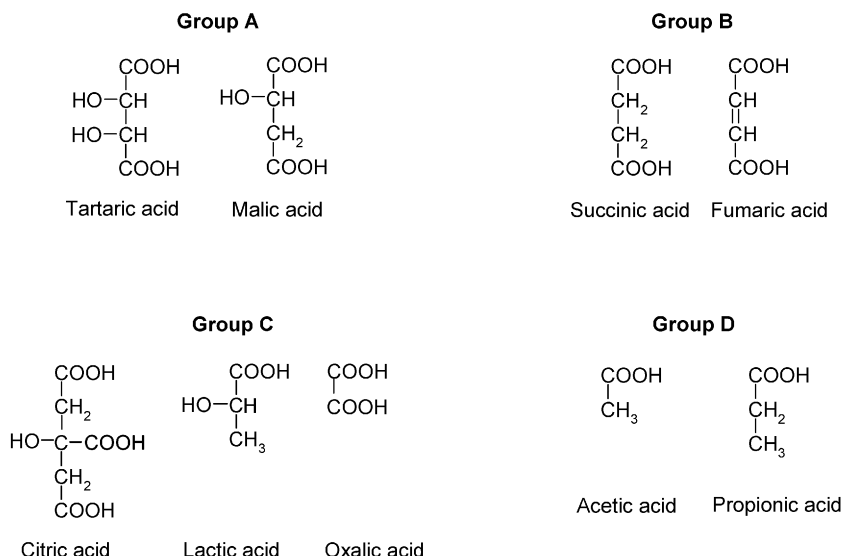
no organic acid. To obtain consistency among experiments, total iron uptake was normalized by dividing each value by the control value obtained from the same plate and multiplying it by an average of all control values (14).

## RESULTS

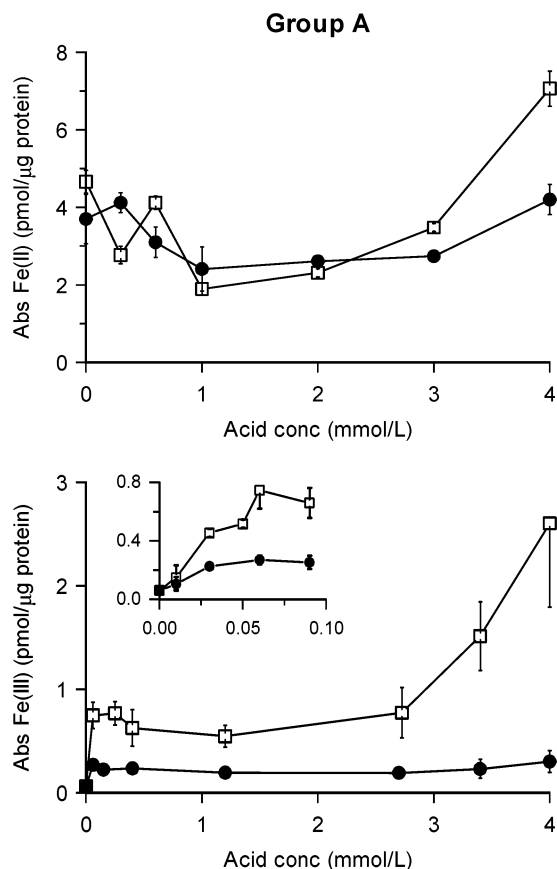
Our results show that the organic acids may be organized into four groups, designated A–D, depending on their effect on iron absorption. When Fe(II) or Fe(III) was assessed alone, without the addition of an organic acid, they showed an absorption of  $4.21 \pm 0.52$  and  $0.06 \pm 0.01 \text{ pmol}/\mu\text{g}$  cell protein, respectively. This means that the absorption of Fe(II) was  $\sim 70$ -fold higher than that of Fe(III), showing the preference for Fe(II) by the uptake system and also the negligible oxidation of ferrous to ferric iron in the samples. All of the organic acids showed a concentration-dependent influence on the iron absorption by the Caco-2 cells. **Figure 1** displays the maximum fold increase of iron uptake for the nine acids investigated.

Group A consists of malic and tartaric acid, four-carbon dicarboxylic acids with one and two hydroxyl groups, respectively (**Figure 2**). The results on ferrous iron yielded a slightly hyperbolic curve (**Figure 3**). At 1 mmol/L, both tartaric and malic acid displayed a minimum Fe(II) absorption of  $1.90 \pm 0.16$  and  $2.41 \pm 0.57 \text{ pmol}/\mu\text{g}$  protein, respectively, which is significantly lower than the control. The maximum absorption,  $7.06 \pm 0.45$  and  $4.20 \pm 0.39 \text{ pmol}/\mu\text{g}$  protein, for these acids was obtained at the upper limit in our assay (4.0 mmol/L). This means a 1.5-fold increase with respect to tartaric acid, and roughly unchanged uptake for malic acid (**Figure 1**). The ferric iron absorption increased rapidly at very low concentrations up to  $\sim 0.06 \text{ mmol/L}$  of acid, with a value of  $0.75 \pm 0.13 \text{ pmol}/\mu\text{g}$  protein for tartaric acid (12.5-fold increase) and  $0.27 \pm 0.03 \text{ pmol}/\mu\text{g}$  protein for malic acid (4.5-fold increase). Above 0.4 mmol/L of malic acid, the ferric absorption leveled out and remained unaffected by additional acid in the assay. However, for tartaric acid, the absorption continued to rise fairly dramatically from 3 mmol/L, reaching  $2.60 \pm 0.81 \text{ pmol}/\mu\text{g}$  protein (43-fold increase) at the highest acid concentration in our assay (4 mmol/L).

Group B consists of succinic and fumaric acid, four-carbon dicarboxylic acids with no hydroxyl groups (**Figure 2**). The ferrous iron absorption increased linearly as a function of acid

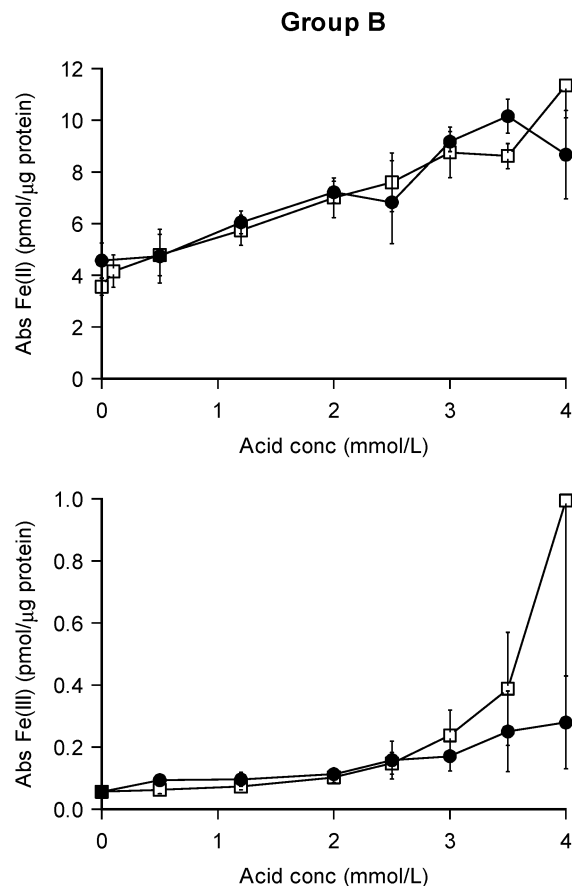


**Figure 2.** Chemical structure of the organic acids used in our assay. The acids are grouped according to their effect on Fe(II) and Fe(III) absorption.



**Figure 3.** Absorption of Fe(II) (upper panel) and Fe(III) (lower panel) as a function of acid concentration: tartaric (□) and malic acid (●). A detailed view is inserted in the lower panel to highlight the Fe(III) absorption at low acid concentrations. Values represent mean  $\pm$  SD;  $n = 6$ .

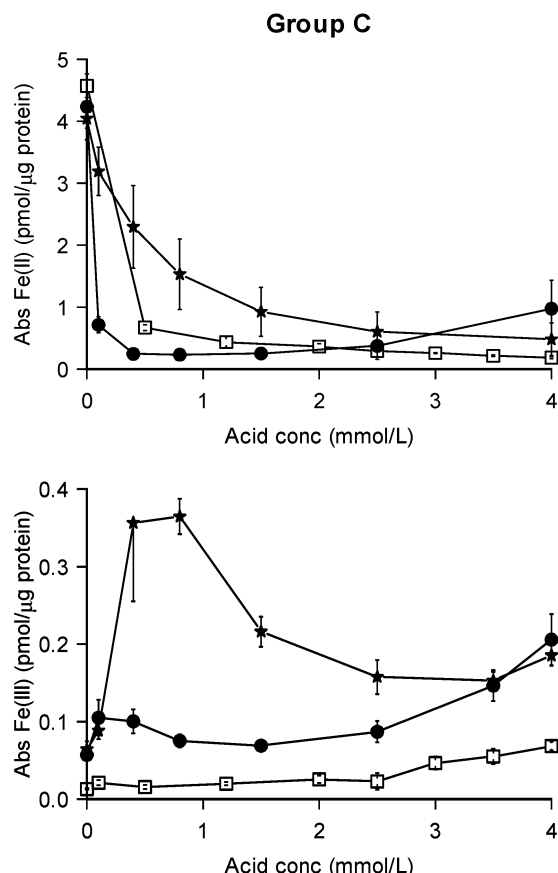
concentration, with maximal absorption of  $11.35 \pm 1.26$  pmol/ $\mu$ g protein at 4 mmol/L succinic acid (3-fold increase) and  $10.15 \pm 0.66$  pmol/ $\mu$ g protein at 3.5 mmol/L fumaric acid (2-fold increase) (**Figure 4**). To mediate an increase in ferric iron absorption, succinic and fumaric acid concentrations needed to exceed 2 mmol/L. In particular, succinic acid affected the Fe(III) uptake strongly at concentrations above 2 mmol/L, which corresponded to a nearly 18-fold increase (**Figure 1**). Unfortunately we were unable to obtain consistent results from the



**Figure 4.** Absorption of Fe(II) (upper panel) and Fe(III) (lower panel) as a function of acid concentration: succinic (□) and fumaric acid (●). Values represent mean  $\pm$  SD;  $n = 6$ .

three repetitions (each with triplicates) of 4 mmol/L succinic acid, reflected by the large standard deviation. However, all individual experiments showed a pronounced increase in Fe(III) uptake with increased acid concentration.

Group C is made up of citric, lactic, and oxalic acid, which is a structurally heterogeneous group with three, one, and two carboxyl groups, respectively (**Figure 2**). All of these showed a negative influence on Fe(II) absorption, even at very low concentrations (**Figure 5**). At the lowest acid concentrations tested, the Fe(II) absorption was  $0.67 \pm 0.05$  pmol/ $\mu$ g protein



**Figure 5.** Absorption of Fe(II) (upper panel) and Fe(III) (lower panel) as a function of acid concentration: lactic (□), citric (●), and oxalic acid (★). Values represent mean  $\pm$  SD;  $n = 6$ .

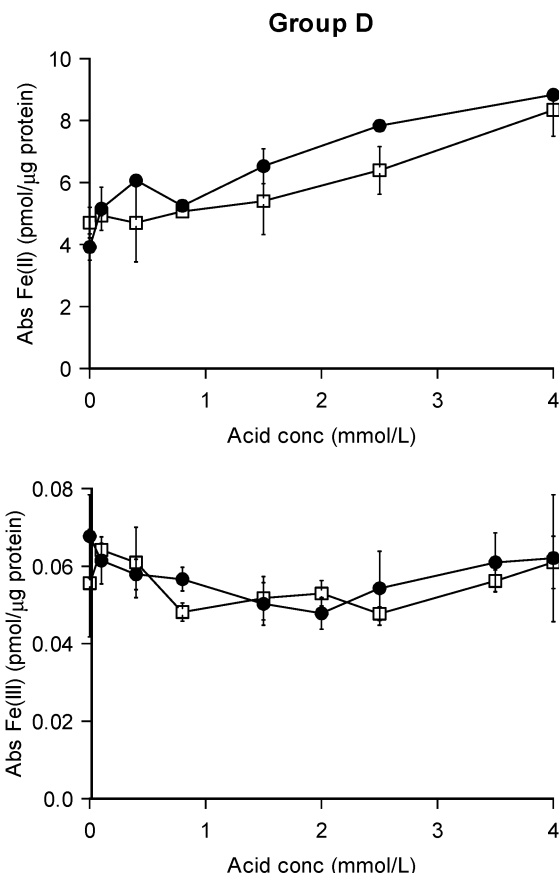
for lactic acid (0.5 mmol/L) and  $0.71 \pm 0.13$  and  $3.19 \pm 0.39$  pmol/μg protein for citric and oxalic acid (0.1 mmol/L), respectively; i.e., the decrease was 85, 83, and 21%. The lowest absorption values were observed at 4 mmol/L lactic and oxalic acid and 0.8 mmol/L citric acid, displaying a decreased absorption of 96, 88, and 94%. The Fe(III) uptake indicated an increase at low acid concentrations, which was particularly clear for oxalic acid. However, this increase disappeared at concentrations above approximately 0.5 mmol/L. At citric acid concentrations exceeding 2.5 mmol/L the absorption of ferric iron increased again, and reached a maximum absorption of  $0.21 \pm 0.03$  pmol/μg protein at 4 mmol/L of citric acid, corresponding to a 3.6-fold increase.

Acetic and propionic acid constitutes group D. These are both monocarboxylic acids with either a methyl or ethyl group attached (Figure 2). Both of these acids exhibited an  $\sim 2$ -fold linear increase in the uptake of ferrous iron as a function of acid concentration. No effect on ferric iron absorption was detected (Figure 6).

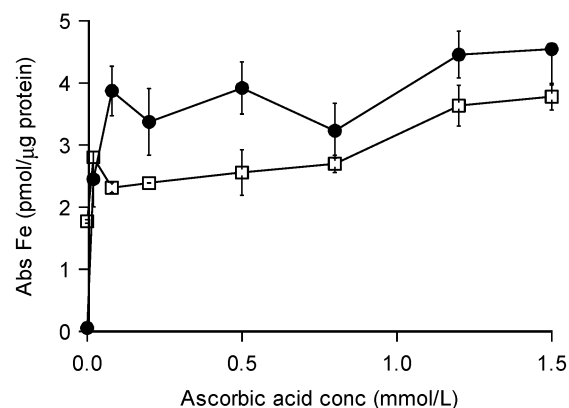
Ascorbic acid was also studied, although mainly as a reference acid. The results are shown in Figure 7. The concentration range studied (20–1500 μmol/L) differed from that of the other acids. The effect on ferrous iron absorption was a moderate 2-fold increase in an almost linear manner. On ferric iron uptake, the impact of ascorbic acid was extensive at low acid concentration, with a 70-fold increase already at 80 μmol/L.

## DISCUSSION

Several organic acids have previously been shown to influence the availability and absorption of nonheme iron. For



**Figure 6.** Absorption of Fe(II) (upper panel) and Fe(III) (lower panel) as a function of acid concentration: acetic (□) and propionic acid (●). Values represent mean  $\pm$  SD;  $n = 3$ .



**Figure 7.** Absorption of Fe(II) (□) and Fe(III) (●) as a function of ascorbic acid concentration. Values represent mean  $\pm$  SD;  $n = 3$ .

instance, Hazell and Johnson (4) showed that 300 mg of ascorbic, citric, and malic acid increased the diffusible iron in white wheat flour from 4.5% to 17, 18.6, and 10.6%, respectively. Gillooly and co-workers (18) observed a significant increase in intestinal iron absorption from a rice meal supplemented with 1 g of ascorbic, citric, tartaric, or malic acid. In addition, lactic acid has been proposed to positively influence the iron absorption (19). Contradictory data, such as negative impact by lactic as well as citric acid on iron absorption, have also been published (20, 21). It seems clear that organic acids influence iron absorption; however, how and to what extent is not always clear. The presence of various inhibitory and promoting factors in complex meals, as well as differences in the experimental setup, may lead to conflicting data. As an initial



contribution, we therefore decided to investigate the influence of organic acids on iron absorption in a pure and well-defined system, varying only the acid and its concentration. The concentration range was chosen to match levels that might be found in meals with fruits, vegetables, and lactic fermented foods. Naturally occurring levels of organic acids are, for example, 0.35 mmol tartaric acid/100 g of grapes, 10.3 mmol malic acid/100 g of plums, or 17.8 mmol lactic acid/100 g of sauerkraut (for comparison, roughly corresponding to 3.5, 103, and 178 mmol/L, respectively).

The nine organic acids tested in our study were grouped according to their impact on iron absorption. A group-specific response was evident when plotting the uptake as a function of acid concentration. With few exceptions, the acids within each of these groups naturally fell into structurally related groups, strongly suggesting that similar properties and mechanisms, at relatively similar strengths, take place within each group. A linear response for instance, such as for the two acids of group B, suggests that only one mechanism is dominating. For these, a straight-line equation can nicely predict the iron uptake-level at a specific acid concentration in our assay. A nonlinear response, in which distinct phases are detectable, however, probably indicates that more than one mechanism is involved over the concentration range used. Formation of soluble acid-Fe complexes has been proposed to be a crucial mechanism rendering iron accessible for uptake.

The iron uptake is also dependent on the pH. Addition of organic acids to the uptake buffer used in our assay had a small effect on the pH of the sample solution applied to the cells. Typically, the pH at the highest acid concentration was 6.0 ( $\pm 0.2$ ). However, within the small pH range, the pH per se can account for only a small part of the effect seen and cannot explain the differences between acids. For instance, tartaric and malic acid (group A) resulted in equal changes in pH, but the iron uptake clearly differed between them. In group D, (propionic and acetic acid), the pH change between 2 and 4 mmol/L was negligible or very small (pH 6.6 to 6.5); however, within this interval, the Fe(II) uptake increased approximately 30%. Furthermore, at pH above 4, Fe(III) is well-known to be insoluble, making uptake very poor. In combination with the anion of the organic acids, however, Fe(III) appears to be accessible for uptake, most likely demonstrating increased solubility by chelation rather than a pH effect.

Solubility is frequently used as an estimation of iron availability or accessibility. The ability of a chelating compound to keep iron in solution, in contrast to precipitates of, for example, iron hydroxides or iron-phytate, is of major importance. Still, the solubility cannot be the only factor involved. The affinity of the acid-Fe complex for the iron uptake proteins as well as the tendency of the chelator to release the iron to the proteins must also determine the efficiency of uptake. In addition, a biological factor is also involved. This means that the status of the cell or the organism may influence the expression of proteins involved in the uptake in response to demands. This may be exerted by internal signals and potentially also by external signals, such as components present in the diet. Organic acids are clearly involved in the complex formation and delivery to the uptake proteins; however, it is not known whether the presence of an organic acid may affect the expression of uptake proteins.

All of the acids in group A (malic and tartaric acid), and group B (succinic and fumaric acid) are four-carbon dicarboxylic acids. The only structural difference between the two groups is that the acids in group A also have hydroxyl groups. The

positive influence on both ferrous and ferric iron uptake is probably due to formation of a soluble acid-iron complex. The pH in the uptake assay was always far above the  $pK_a$  (with the exception for citric acid), and hence, all carboxyl groups were deprotonated, enabling the binding of the iron ions. The effect on ferric iron is quite similar for the two groups, except for the primary rapid increase of iron uptake for the acids of group A (see inserted detail in **Figure 3** showing the lowest acid concentrations). For a number of reasons, we believe that the hydroxyl groups, in addition to the carboxyls, may interact with iron and the subsequent uptake: (i) Tartaric acid with two hydroxyl groups was superior to malic acid, which has only one. (ii) The rapid response on acid addition already at low concentrations was largely similar to our data on ascorbic acid (**Figure 7**). Ascorbic acid is known to have a strong positive influence on iron absorption, primarily caused by its reduction capacity via its hydroxyl groups (15). (iii) Furthermore, the ascorbate-like increase at low concentrations was detected only on Fe(III), not on Fe(II). (iv) Finally, the effect was not obtained for acids of group B, which lack hydroxyl groups. The rapid increase of ferric iron uptake at low acid concentrations may indicate either of two possibilities: (i) a reduction of Fe(III) to the Fe(II) valency state, which is the most readily absorbed form of iron (15, 22); or (ii) iron interaction with the hydroxyl group in the complex formation. Königsberger and co-workers (23) have proposed that in neutral or alkaline solutions, a metal ion may interact with hydroxyl as well as carboxyl groups. We have also tested maleic acid (data not shown), which is the geometrical isomer of fumaric acid. Maleic acid displayed exactly the same response as fumaric acid.

The organic acids in group C (citric, lactic, and oxalic acid) are structurally heterogeneous but show a similar response, however not in intensity, on iron absorption. Very strong complexes formed between ferrous iron and the acid may cause a substantial blocking of ferrous iron uptake. For citric acid, these are probably in the form of dicitrate complexes, which according to Königsberger and co-workers (23), is the dominating association between Fe(II) and citric acid. The formation of strong chelates can cause a reluctance to donate the iron to the epithelial cells, as also observed by Narasinga Rao and Subba Rao (7). Our data, showing an initial increase in ferric iron absorption with citric acid present followed by a drop, was consistent with the work of Bates and co-workers (24). They proposed that at citric acid concentrations above 0.2 mmol/L, ferric dicitrates complexes are formed, which slow the transfer of iron to uptake proteins. This is also supported by Königsberger and co-workers (23). Oxaloacetic acid, which is a four-carbon dicarboxylic acid with a keto group, showed a similar response on iron absorption as the other acids in group C (data not shown). The more positive effect on ferric iron, as compared with ferrous, by the organic acids in group C is consistent with data by Nadeau and Clydesdale (25, 26), who studied the solubility of iron in combination with citric and lactic acid together with wheat flakes. The large difference between lactic and propionic acid regarding level of impact on iron absorption suggests that the hydroxyl group of lactic acid again plays a role in the complex formation.

The acids within group D (acetic and propionic acid) are both monocarboxylic acids with either a methyl or an ethyl group attached. These exerted an impact on ferrous iron similar to that of the acids in group B, that is, a linear correlation indicating that one major mechanism dominates. The deprotonated carboxyl ions will probably bind Fe(II), yielding soluble complexes that appears to be effective donors of iron to the uptake system

of the epithelial cells. The more acid, the more iron will exist in dissolved complexes and probably the less iron will be in poorly dissolvable competing iron hydroxide complexes. The uptake-promoting effect was stronger for acids in group B than for those in group D, suggesting that two carboxyls are more effective than one. The effect was not detectable for ferric iron, indicating that ferric iron does not form complexes with the carboxyl ion as readily as the ferrous iron.

To conclude, the impact of chelating agents, such as organic acids, on iron uptake is a complex combination of different forces and mechanisms. We suggest that the following events are important: (i) degree of complex formation, (ii) types of complexes, (iii) solubility of complex, (iv) strength of bonds in complex, (v) reduction of Fe(III) to Fe(II), (vi) affinity of complex to the uptake system, and (vii) tendency of complex to deliver iron to uptake proteins. These factors are closely linked with the chemical structure of the organic acids. In addition, the physiological state of the cell is important for the result. However, it is still uncertain what happens with the organic acids and iron in a complex meal, containing other confounding factors, that has to pass through the digestive system before it reaches the site of absorption. More studies are needed for a broader understanding of the effects from adding specific acids in a meal.

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