Ascorbate Offsets the Inhibitory Effect of Inositol Phosphates on Iron Uptake and Transport by Caco-2 Cells (43924)

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Abstract. **Differentiated monolayer cultures of Caco-2 human intestinal cells were used as a model to examine interactions between various dietary factors related to the intestinal uptake and absorption of nonheme Fe. Caco-2 cells accumulated 91–98 pmol Fe/mg protein from uptake buffer containing 12 nmol of Fe(III)-nitrilotriacetate during a 1-hr incubation at 37°C. Addition of a 10-fold molar excess of inositol hexaphosphate (IP6) and its lesser phosphorylated derivatives (IP3, IP4, and IP5) decreased cellular uptake and transport of Fe from the lumenal compartment. Addition of ascorbic acid (AA) to the solution containing IPs stimulated Fe uptake and transport in a manner dependent upon the ratio of AA to IP and inversely proportional to the degree of phosphorylation of inositol (i.e., IP3 > IP4 > IP5 > IP6). A mixture of essential amino acids had minimal impact on Fe uptake in either the absence or presence of IPs. Cellular acquisition of Fe from solutions containing IPs was further enhanced by simultaneous addition of essential amino acids and AA. The stimulatory influence of ascorbic acid on Fe uptake from solutions containing IP6 was associated with an increase in the level of ferrous ion. These data further support the usefulness of Caco-2 cells as a model for investigating the effects of various dietary factors on mineral bioavailability.** [P.S.E.B.M. 1995, Vol 210]

Various dietary components are known to enhance or inhibit apical uptake of nonheme iron by enterocytes, whereas physiological factors such as iron status, chronic infection and age influence relatively undefined processes associated with the transfer of Fe from within the intestinal cell to the portal circulation (1–3). Phytate (i.e., inositol hexakisphosphate (IP6)) is present in high concentrations in cereal grains and legumes, and can significantly inhibit the absorption of nonheme Fe (4, 5). Lesser phosphorylated derivatives of inositol, (viz., IP3, IP4, and IP5) also have been reported to limit dietary Fe uptake both *in vivo* (6) and *in vitro* (7). In contrast, ascorbic acid can be a potent enhancer of nonheme Fe absorption in humans and animals (8–10). The extent to which ascorbic acid stimulates the absorption of dietary Fe largely depends on both an individual's iron status and diet composition. For example, some investigators have reported that supplementation of the diet with ascorbic acid offsets the inhibitory influence of phytic acid and polyphenols on Fe absorption in human subjects (11–13). However, long-term supplementation with ascorbic acid does not increase iron stores in normal adults (14, 15). These observations suggest that the primary effect of ascorbic acid is to enhance Fe uptake across the brush border membrane surface of the enterocyte, whereas the subsequent transfer of this newly acquired Fe to the portal circulation is primarily controlled by iron status.

We have adopted Caco-2, a highly differentiated, enterocyte-like intestinal cell of human origin, as a model for investigating the characteristics and regulation of uptake and transepithelial transport of dietary nonheme Fe. Previous studies revealed that IP6 and

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lesser phosphorylated derivatives (IP3, IP4, and IP5) inhibit the uptake of Fe from the apical (i.e., lumenal) compartment and its transport across the basolateral surface (7). In contrast, ascorbic acid enhanced apical uptake and transport of nonheme Fe by Caco-2 cells by reducing Fe(III) to Fe(II) (16).

In the present study, the effect of addition of different inositol phosphates combined with various concentrations of ascorbic acid on Fe uptake and transport by Caco-2 cells has been examined. In addition, the influence of a mixture of amino acids alone or in combination with inositol phosphates and ascorbic acid also has been investigated, since the literature contains contradictory reports on the effects of amino acids and peptides on the absorption of dietary nonheme Fe (17, 18).

Materials and Methods

Reagents. Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose, fetal calf serum, nonessential amino acids, L-glutamine, and phytic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Gentamicin, fungizone and 100x stock solution of essential amino acid mixture for Eagle's Basal Medium were purchased from GIBCO BRL (Grand Island, NY). ^{55}Fe (132 GBq/mmol $FeCl_3$) and D-[1-^{14}C] mannitol (2.04 GBq/mmol) were obtained from Dupont New England Nuclear (Boston, MA). The procedures for purification of inositol triphosphate (IP3), inositol tetraphosphate (IP4), inositol pentaphosphate (IP5), and inositol hexaphosphate (IP6) have been described previously (7).

Cells. Caco-2 cells were obtained from ATCC (Rockville, MD) and used between Passage 22 and 40. Details concerning the growth and maintenance of the stock and experimental cultures have been reported (7, 16). Cultures were used to study Fe uptake and transport 10–12 days after reaching confluency. Cells were fully differentiated as evidenced by maximal activities of sucrase and alkaline phosphatase (19) and the minimal rate (0.06%/hr · cm^2) of paracellular flux of ^{14}C-mannitol from the apical (i.e., lumenal) to the basolateral chamber (16). Addition of ascorbic acid, IPs and amino acids to the apical compartment did not alter the rate of passage of ^{14}C-mannitol from the apical to the basolateral compartments.

Fe Uptake and Transport. The measurement of cellular uptake of ^{55}Fe and transport of the metal from the apical to the basolateral chamber of cultures containing confluent monolayers of Caco-2 cells on microporous membrane inserts (3 μm pores) has been described (7, 16). Briefly, a stock solution containing 10 m*M* $FeCl_3 \cdot 6H_2O$ and 20 m*M* sodium nitrilotriacetate (NTA) in 1 m*M* HCl was prepared fresh and diluted 10-fold with deionized water. An appropriate aliquot of the acidified solution containing Fe and NTA was added to $^{55}FeCl_3$ and incubated at room temperature for 30 min. Then, uptake buffer (130 m*M* NaCl, 10 m*M* KCl, 1 m*M* $MgSO_4 \cdot 7H_2O$, 5 m*M* glucose and 50 m*M* Hepes, pH 7.0) without or with given concentrations of ascorbic acid was added. The final solution contained 10 μ*M* Fe(III) and indicated concentrations of the other components. The effects of single additions and combinations of 100 μ*M* IP6, 1000 μ*M* ascorbic acid, and 1% (v/v) essential amino acid mixture on the solubility and valency of Fe in uptake buffer were examined after incubation of test solutions for 1 hr at 37°C. Fe solubility was determined as previously reported (7). Test samples were vortexed and aliquots were removed.^{55}Fe content of one aliquot was measured directly, whereas another aliquot was centrifuged at 8160*g* for 10 min at 20°C and the level of ^{55}Fe in the supernate determined. Solubility was defined as the percentage of total ^{55}Fe present in the supernate. When added to uptake buffer as the Fe(III)-NTA complex, the solubility of Fe was 98% ± 2%; this was not affected by the addition of ascorbic acid and/or essential amino acids. The valency of Fe in uptake buffer without and with test compounds was determined by spectrophometric analysis of Fe(II)-ferrozine complex at 562 nm as described by Stookey (20). Fe(II) was not detected in uptake buffer containing Fe(III)-NTA only. The level of Fe(II) was <10% of the total after addition of essential amino acids and/or IP6. Addition of 100 and 1000 μ*M* ascorbic acid to uptake buffer containing only Fe(III)-NTA resulted in complete conversion of Fe to Fe(II).

For uptake studies, cultures were washed three times with Hanks' balanced salt solution (HBSS) at 37°C before the addition of the test solution (1.2 ml with 37 kBq ^{55}Fe) and incubated at 37°C in a humidified atmosphere of 95% air/5% CO_2 for 60 min. Then, the uptake buffer was removed, and the monolayer was washed three times with 2.0 ml ice-cold buffer containing 150 m*M* NaCl, 1 m*M* EDTA, and 10 m*M* Hepes, pH 7.0, to remove nonspecifically bound ^{55}Fe. Cellular material was collected in a 1.0-ml volume of 1.7 m*M* SDS and 1 m*M* EDTA, pH 7.0, and sonicated. Aliquots were placed in minivials and solubilized (Bio-Safe II; RPI, Mount Prospect, IL) before quantifying ^{55}Fe by liquid scintillation spectrophotometry (Model LS 6000 SE; Beckman Instruments, Fullerton, CA). Transport studies were initiated similarly. Spent medium was removed from the apical and basolateral chambers and the monolayer was washed three times with HBSS at 37°C. Test solutions were then added to the apical chamber (1.5 ml containing 78 kBq ^{55}Fe) and DMEM with 5% fetal calf serum (2.5 ml) was added to the basolateral chamber. Aliquots (0.2 ml) were removed from the basolateral chamber each hour and replaced with an equivalent volume of DMEM with 5% fetal calf serum. Data were corrected to account

for sample replacement. The rate of transfer of ^{55}Fe from the apical to the basolateral compartment was linear ($r > 0.98$) between 1 and 5 hr. Transport rates were calculated by linear regression analysis.

Protein. Cell protein was determined according to Nerurkar *et al.* (21) using bovine serum albumin as standard. Variability of protein content per well both within and between experiments was less than 5%.

Analysis of Data. Presented data (means ± SE) represent results from at least two separate experiments using three wells for each parameter. Results were analyzed using the General Linear Models (SAS System, Cary, NC) and Tukey's multiple range test was used to determine significant differences among means ($P < 0.05$) for uptake and transport data (22).

Results

Differentiated cultures of Caco-2 accumulated 91–98 pmol Fe/mg protein (equivalent to 27–29 pmol/cm^2 and 255–275 pmol/well) from uptake buffer containing 12 nmoles of Fe(III)-NTA and no ascorbic acid during a 1-hr incubation at 37°C (Table I). Addition of a 10-fold molar excess of phytic acid (i.e., 100 μ*M* inositol hexaphosphate; IP6) decreased Fe solubility by 19% and cellular uptake by 50%; these changes are similar to that previously reported (7). Because ascorbic acid (≥8 μ*M*) significantly enhances uptake of Fe across the apical surface of Caco-2 cells in a dose-dependent manner (16), the impact of simultaneous addition of both the enhancer (ascorbic acid) and the inhibitor (IP6) on Fe uptake was tested. When the molar ratio of ascorbic acid to phytic acid was ≤0.6, Fe uptake remained 50%–60% as high as that in cultures containing only uptake buffer (Fig. 1). At a molar ratio of ascorbic acid to IP6 of 1.25, cellular uptake of Fe was increased to the control level. Further increases in the ratio of ascorbic acid to IP6 in the uptake solution (i.e., 2.5–10) were associated with a proportional elevation of Fe uptake by the cells. The addition of 1000 μ*M* ascorbic acid to uptake buffer containing 100 μ*M* IP6 increased the solubility of Fe from 81% ± 3% to 91% ± 2% ($P < 0.05$) and the percentage of Fe(II) from 0 to 47% ± 2% ($P < 0.05$).

Fe uptake by Caco-2 cells was also attenuated by the presence of a 10-fold molar excess of the lesser phosphorylated derivatives of IP6 (viz., IP3, IP4, and IP5) (Table I). As previously reported (7), the inhibitory effect of IP4 and IP5 on Fe uptake was greater than that observed with IP6. This may be due to the incomplete removal of Fe-IP6 precipitate from the cell surface (23). The inhibitory influence of IPs on Fe uptake was offset by addition of 1000 μ*M* ascorbic acid to uptake buffer (Table I). Ascorbic acid stimulated Fe uptake from solutions containing IPs 1.8- to 8.2-fold above that of cultures containing Fe(III)-NTA alone. The degree to which ascorbic acid enhanced Fe uptake from solution containing IPs was inversely proportional to the degree of phosphorylation of inositol.

Fe transport from the apical to the basolateral compartment by differentiated cultures of Caco-2 cells was linear between 1 and 5 hr of incubation (data not shown) with a mean rate of 1.45 pmol/(hr · cm^2). Addition of IPs to the uptake buffer decreased the rate of Fe transport across the cell monolayer and the degree

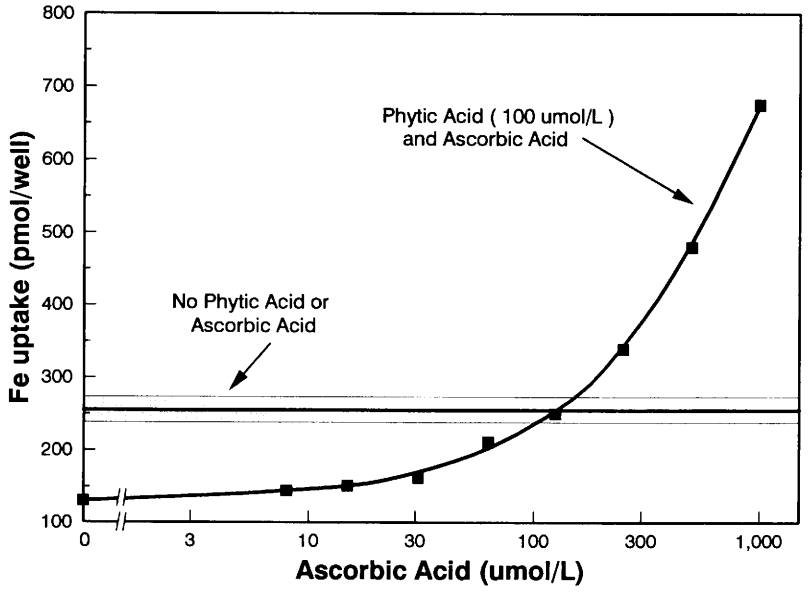

Figure 1. Effect of the concentration of ascorbic acid on phytic acid-mediated inhibition of Fe uptake by Caco-2 cells. Differentiated cultures of Caco-2 cells were incubated for 1 hr at 37°C with uptake buffer (1.2 ml) containing 37 kBq ^{55}Fe, 10 μ*M* Fe^{3+}, 20 μ*M* NTA, 100 μ*M* phytic acid (IP6), and 0–1000 μ*M* ascorbic acid. Fe uptake was measured after removing loosely bound ^{55}Fe from the cell surface by washing with ice-cold saline containing 1 m*M* EDTA, pH 7.0. Each indicated value represents the means from two separate experiments each using three replicate wells for a test parameter. Standard errors (not shown) were less than 5% of the mean for all values. In the absence of phytic acid, iron uptake was 255 ± 17 as indicated by the hatched region. Fe uptake was decreased significantly ($P < 0.05$) by the presence of phytic acid. Cellular uptake of Fe in the presence of phytate was enhanced significantly ($P < 0.05$) by addition of ≥64 μ*M* ascorbic acid.

Table I. Ascorbate-Mediated Stimulation of Fe Uptake by Caco-2 Cells Is Inversely Proportional to the Degree of Phosphorylation of Inositol

Apical solution	Fe uptake (pmol/mg cell protein)	
	No ascorbic acid	Ascorbic acid
Fe only	91 ± 4[a]	1000 ± 21[a]
Fe + IP3	52 ± 4[b]	751 ± 9[b]
Fe + IP4	39 ± 2[c]	243 ± 10[c]
Fe + IP5	36 ± 1[c]	171 ± 8[d]
Fe + IP6	56 ± 1[b]	166 ± 6[d]

Note. Uptake buffer containing 37 kBq ^{55}Fe, 10 μ*M* Fe^{3+}, 20 μ*M* NTA and 100 μ*M* of the indicated inositol phosphate (IP) and either 0 or 100 μ*M* ascorbic acid was added to 10- to 20-day postconfluent cultures of Caco-2. Cultures were incubated with test compounds for 1 hr at 37°C. Fe uptake was assessed as described in Materials and Methods. Data are means ± SE for two separate experiments each using three wells per test variable ($n = 6$). Significant differences ($P < 0.05$) for cultures incubated in the presence or absence of ascorbic acid are indicated by different letters as superscripts within a column. The presence of ascorbic acid significantly increased ($P < 0.05$) Fe uptake by cells incubated in the presence or absence of IP.

of inhibition was proportional to the extent of phosphorylation of inositol (Table II). Addition of ascorbic acid to solutions without and with IPs increased the rate of Fe transport between the two compartments. The stimulatory influence of ascorbic acid was directly proportional to its concentration and inversely proportional to the level of phosphorylation of inositol.

Fe uptake was increased 10%–30% ($P > 0.05$) by the addition of a mixture of essential amino acids to the uptake buffer either without or with IPs. The addition of essential amino acids to the uptake solution containing IP6 did not significantly alter the solubility (81% ± 3%) or the valency (7% ± 1% as Fe[II]) of Fe. Addition of 1000 μ*M* ascorbic acid alone or in combination with the mixture of essential amino acids enhanced Fe uptake 10- and 11.5-fold, respectively. Moreover, the addition of both essential amino acids and ascorbic acid to solution containing IPs stimulated cellular uptake of Fe synergistically since the degree of enhancement exceeded that predicted from the separate addition of amino acids or ascorbic acid (Table III). The solubility of Fe in uptake buffer containing IP6 and ascorbic acid was 91% ± 2%; addition of essential amino acids to this solution did not significantly increase Fe solubility (93% ± 2%). In contrast, the addition of ascorbic acid alone and in combination with essential amino acids increased the level of Fe(II) in the IP6-containing solution to 47% ± 2% and 59% ± 1%, respectively; these values differ significantly (<0.05) from each other and from those in uptake buffer containing IP6 without and with essential amino acids (see above).

Table II. Ascorbate Enhances Fe Transport by Caco-2 Cells Exposed to Inositol Phosphates

Apical solution	Apical → basolateral transport of Fe pmol Fe transported/(hr · cm^2)		
	No ascorbic acid	100 μ*M* ascorbic acid	1000 μ*M* ascorbic acid
Fe only	1.45 ± 0.14[a]	14.90 ± 1.40[a]	43.7 ± 1.1[a]
Fe + IP3	1.22 ± 0.33[b]	8.61 ± 0.50[b]	34.5 ± 1.7[b]
Fe + IP4	0.66 ± 0.02[c]	2.15 ± 0.16[c]	25.7 ± 0.7[c]
Fe + IP5	0.50 ± 0.02[c,d]	1.60 ± 0.02[c]	18.4 ± 0.6[d]
Fe + IP6	0.05 ± 0.03[d]	0.30 ± 0.01[c]	10.1 ± 0.8[e]

Note. Uptake buffer containing 74 kBq $^{55}Fe^{3+}$ as 10 μ*M* Fe^{3+}, with 20 μ*M* NTA, given concentration of ascorbic acid, and either 0 or 100 μ*M* of indicated inositol phosphate (IP) was added to the apical compartment of 10- to 12-day postconfluent cultures of Caco-2 grown on microporous membrane inserts. Cultures were incubated for 5 hr at 37°C. The rate of Fe transport to the basolateral compartment was assessed as described in Materials and Methods. Data are means ± SE for at least three replicate wells from two separate experiments ($n = 6$). Significant differences ($P < 0.05$) for cultures exposed to the same level of ascorbic acid (i.e., either 0, 100, or 1000 μ*M*) are indicated by different superscripts within a column. The rate of Fe transport by cultures treated with each IP was significantly increased ($P < 0.05$) as the concentration of ascorbic acid as elevated.

Discussion

While intact laboratory rodents and intestinal segments and isolated cells from animals are often used to investigate factors influencing Fe bioavailability, the validity of extrapolating these findings from animals to humans has been challenged by Reddy and Cook (24). Consequently, we and other investigators have used the Caco-2 human cell line as an alterative model to examine problems related to mineral bioavailability (cited in Ref. 7). These cells are derived from a human colonic adenocarcinoma and have the unique characteristic of spontaneously differentiating at confluency into a phenotype with morphological and functional characteristics quite similar to polarized, absorptive intestinal epithelial cells (19, 25).

The inhibitory effect of phytic acid (IP6) on nonheme iron absorption has been extensively documented in humans (4, 5). Because phosphate groups can be cleaved from the inositol ring by both plant phytase activity and the processing of foods (26–28), there is interest in the impact of lesser phosphorylated derivatives of inositol on mineral bioavailability (7, 29–31). We previously reported that IP3, IP4, and IP5 also inhibit the uptake of Fe across the brush border surface and its transfer across the basolateral surface by differentiated cultures of Caco-2. Moreover, the degree of the inhibitory effects of IPs on Fe transport was highly correlated with the level of phosphorylation of inositol.

The ability of ascorbic acid to enhance the absorption of dietary nonheme Fe by humans and animals is widely recognized (10, 11, 32). For example, the level

Table III. Essential Amino Acids (EAA) and Ascorbic Acid Act Synergistically to Enhance Fe Uptake in the Presence or Absence of Inositol Phosphates (IP)

Apical solution	Fe uptake with addition to uptake buffer (pmol Fe/well)			
	None	+EAA	+ascorbate	+EAA +ascorbate
Fe only	92 ± 2^{a,*}	118 ± 1^{a,*}	920 ± 26^{a,†}	1051 ± 3^{b,‡}
Fe + IP3	49 ± 3^{b,*}	64 ± 4^{b,*}	756 ± 27^{b,†}	1220 ± 23^{a,‡}
Fe + IP4	37 ± 2^{c,*}	49 ± 6^{c,*}	388 ± 24^{c,†}	603 ± 13^{c,‡}
Fe + IP5	36 ± 1^{c,*}	41 ± 3^{c,*}	280 ± 6^{d,†}	501 ± 16^{d,‡}
Fe + IP6	56 ± 1^{b,*}	62 ± 1^{b,*}	264 ± 4^{d,†}	331 ± 7^{e,‡}

Note. Uptake was determined as in Table I. The mixture of essential amino acid (EAA) included 200 μ*M* L-isoleucine, L-leucine, L-lysine.HCl, L-threonine, and L-valine; 100 μmol/L L-arginine.HCl, L-phenylalanine, and L-tyrosine; 50 μ*M* L-cystine, L-histidine, and L-methionine; and 20 μ*M* L-tryptophan. When present, the concentration of ascorbic acid was 1000 μ*M*. Data are means ± SE for two separate experiments with three replicate wells for each test variable. Significant differences ($P < 0.05$) in Fe uptake in cultures exposed to IPs in the presence or absence of EAA and/or ascorbate are indicated by the presence of different letters as superscripts within a column. The presence of different symbols (*,†,‡) as superscripts within a row indicate that cellular uptake of Fe from the solutions without or with the indicated IP was significantly ($P < 0.05$) altered by the addition of EAA and/or ascorbate.

of Fe absorption from a semisynthetic meal was directly proportional to the amount of ascorbic acid added over the range of 25–1000 mg of ascorbic acid (32). However, the stimulatory influence of ascorbic acid on Fe absorption was attenuated when the diet contained substantial amounts of meats (14, 15) and in subjects with adequate stores of Fe (2, 33). We have demonstrated that ascorbic acid stimulates Fe uptake and transport by Caco-2 cell grown and maintained in medium containing standard levels of Fe (16). This stimulatory effect of ascorbic acid on Fe uptake and transport was found to be due to the reduction of Fe(III) to Fe(II) in the apical compartment.

Hallberg and associates (11, 12) have examined the influence of ascorbic acid supplementation on the absorption of Fe by humans consuming a meal containing relatively high levels of IP6. They reported that the absorption of Fe from test meals was dependent on the relative levels of IP6 and ascorbic acid present. Likewise, the present results with Caco-2 cells demonstrate that the stimulatory effect of ascorbic acid on cellular uptake and transport of Fe by Caco-2 cells was dependent on both the relative amounts of ascorbic acid and IPs and the degree of phosphorylation of inositol. Ascorbate-mediated stimulation of Fe uptake from the solution containing IP6 also was associated with and likely dependent on (16) the reduction of Fe(III) to Fe(II). Although the levels of Fe(III) and Fe(II) in uptake buffer containing ascorbic acid and either IP3, IP4 or IP5 were not determined, it is probable that the percentage of Fe(II) was as high as or higher than that in the solution containing ascorbic acid and IP6.

The roles of specific amino acids and peptides as enhancers of Fe bioavailability has received considerable attention, because meat and fish enhance the absorption of nonheme Fe (10). Investigations using intact rats (8) and ligated intestinal segments of the rat (17) suggested that histidine, lysine, and cysteine increased Fe absorption. In human studies, cysteine and glutathione, but not other amino acids, increased the absorption of Fe from black beans (18, 34). In the present study, we examined the effect of a mixture of amino acids which included lysine, histidine, and cystine instead of cysteine on acquisition of nonheme Fe from the apical compartment by Caco-2 cells. The amino acid mixture itself had minimal impact (10%–30% increase) on Fe uptake in the absence and presence of IPs. However, the addition of both the amino acid mixture and ascorbic acid increased Fe uptake to a greater extent than the sum of that with amino acids and ascorbic acid alone. Similarly, Fe absorption was stimulated to a greater extent in rats given ascorbic acid plus histidine compared with ascorbic acid alone (35).

It is generally accepted that specific amino acids enhance the absorption of Fe by either chelating Fe(III) and preventing its hydration, polymerization and precipitation (36), or by reducing Fe(III) to Fe(II) (37). Van Campen and Gross (35) suggested that cysteine, lysine and histidine enhanced Fe absorption in the rat by increasing the solubility of Fe(III). Thus, it is likely that the amino acids supplement had minimal impact on Fe uptake by the Caco-2 cells because the metal was introduced as the soluble NTA complex (7). This suggests that the synergistic influence of ascorbic acid plus amino acids is related to ascorbate-mediated reduction of Fe(III) to Fe(II) and the higher affinity of many amino acids for Fe(II) than Fe(III) (38). The amino acids may have retarded the reoxidation of ascorbate-generated Fe(II) by forming a stable complex with Fe(II). This possibility is supported by our observation that the relative level of Fe(II) in the solution containing IP6, ascorbic acid, and essential

amino acids was significantly ($P < 0.05$) higher than in that containing IP6 with only ascorbic acid (59% ± 1% vs 47% ± 2%, respectively).

In summary, our results further support the usefulness of Caco-2 cells as a model for investigating the influence of various dietary factors on mineral bioavailability. The present data also demonstrate that the inhibitory influence of IP6 and lesser phosphorylated derivatives of inositol on Fe bioavailability can be offset by equimolar or higher levels of ascorbic acid, but not by a mixture of amino acids alone.

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