Effect of ascorbic acid intake on nonheme-iron absorption from a complete diet[1,2]

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ABSTRACT

Background: Ascorbic acid has a pronounced enhancing effect on the absorption of dietary nonheme iron when assessed by feeding single meals to fasting subjects. This contrasts with the negligible effect on iron balance of long-term supplementation with vitamin C.
Objective: Our goal was to examine the effect of vitamin C on nonheme-iron absorption from a complete diet rather than from single meals.
Design: Iron absorption from a complete diet was measured during 3 separate dietary periods in 12 subjects by having the subjects ingest a labeled wheat roll with every meal for 5 d. The diet was freely chosen for the first dietary period and was then altered to maximally decrease or increase the dietary intake of vitamin C during the second and third periods.
Results: There was no significant difference in mean iron absorption among the 3 dietary periods despite a range of mean daily intakes of dietary vitamin C of 51–247 mg/d. When absorption values were adjusted for differences in iron status and the 3 absorption periods were pooled, multiple regression analysis indicated that iron absorption correlated negatively with dietary phosphate ($P = 0.0005$) and positively with ascorbic acid ($P = 0.0069$) and animal tissue ($P = 0.0285$).
Conclusions: The facilitating effect of vitamin C on iron absorption from a complete diet is far less pronounced than that from single meals. These findings may explain why several prior studies did not show a significant effect on iron status of prolonged supplementation with vitamin C.   *Am J Clin Nutr* 2001;73:93–8.

INTRODUCTION

Vitamin C is the only dietary constituent other than animal tissue that has been shown repeatedly to augment the absorption of nonheme iron in humans (1–5). The stimulating influence of ascorbic acid has been shown when ascorbic acid is given with inorganic iron alone and is even more pronounced when it is taken with food (6, 7). The facilitating effect of vitamin C is dose related. In one study in which increasing amounts of ascorbic acid ranging from 25 to 1000 mg were added to a liquid formula meal containing 4.1 mg nonheme iron, iron absorption increased progressively from 0.8% to 7.1% (3).

In contrast with the striking effect of ascorbic acid on iron absorption, the improvement in iron status when the diet is sup-

plemented with vitamin C has been minimal. In one study, the addition of 2000 mg vitamin C/d to the diet for ≤2 y did not alter iron stores significantly as measured by serum ferritin concentrations (8). Repeat iron absorption studies after 16 wk of supplementation showed that the lack of effect on iron status was not explained by adaptation of the gastrointestinal tract to a high intake of vitamin C. In another study, 100 mg ascorbic acid given 3 times daily with meals to menstruating women for 9 mo had no significant effect on iron status (9). Similarly, 25 healthy women aged 20–45 y with low iron stores as defined by a serum ferritin concentration <20 μg/L were given 500 mg ascorbic acid 3 times daily with meals for 10 wk. There was no significant alteration in biochemical indexes of iron status in these women, even in those consuming a diet low in bioavailable iron (10).

The explanation for the disparity between the pronounced effect of ascorbic acid on iron absorption and its meager influence on iron status after prolonged supplementation is not apparent. One possibility relates to the method used to measure the absorption of nonheme dietary iron. The traditional technique has been to administer single meals tagged extrinsically with radioactive iron to fasting subjects. However, this approach exaggerates the effect of dietary composition on iron absorption. For example, when iron absorption from a complete diet was measured over several days by having subjects consume a labeled bread roll with each meal of the day, the extremes in mean iron absorption between diets differing maximally in iron bioavailability ranged from only 3.2% to 8.0% compared with a significantly wider range of 2.3–13.5% when iron absorption was measured with single meals (11). The results of several other investigations using this method of total dietary labeling confirm the reduced influence of various dietary determinants of iron absorption when examined in the context of a complete diet (12–15). The present investigation was undertaken to reexamine the importance of dietary ascorbic acid in iron balance by measuring nonheme-iron absorption from a complete diet before and

after altering the diet to maximally increase or decrease the intake of dietary ascorbic acid.

SUBJECTS AND METHODS

Subjects

We performed 4 separate measurements of iron absorption in 10 women and 2 men. The subjects' ages averaged 25 y with a range of 20–38 y. All subjects were interviewed extensively before they were enrolled in the study to establish their willingness and capability to maintain detailed and accurate dietary records while consuming the labeled diets. The participants were shown an instructional video on methods for assessing portion sizes and maintaining dietary records. All subjects stated that they were in good health, were taking no iron medications, and had no history of recent infections or disorders known to influence iron absorption. The use of mineral or vitamin supplements was not allowed during the investigation. Iron status, on the basis of a serum ferritin concentration of >12 μg/L, was normal in all but one subject and none of the participants were anemic. Written, informed consent was obtained from all subjects before the investigation. The experimental protocol was approved by the Human Subjects Committee at the University of Kansas Medical Center.

Study design

Four separate iron absorption tests were performed in each subject by using dual radioactive iron labels sequentially. One of the initial pair of absorption tests included a standard hamburger meal that was used in prior studies in our laboratory (11). Iron absorption from this meal was used as a reference to facilitate comparisons with published studies of iron absorption. Expression of iron absorption from the complete diet as a percentage of the iron absorption from the standard meal in each subject minimized the effect of differences in iron status on iron absorption.

For the 3 remaining iron absorption tests, the complete diet was tagged by having the subjects consume radioactive bread rolls with each of the 3 main meals of the day for 5 d. Snacks between meals were not allowed. The subjects were instructed to maintain detailed daily records of all food items consumed during the 5-d labeling period. During the initial period of dietary tagging, termed the self-selected (SS) diet, the participants were allowed to consume their regular diets without restrictions on composition. For the remaining 2 dietary periods, the subjects were required to modify their usual diets to maximally increase (HC diet) or decrease (LC diet) their dietary intake of vitamin C. This was accomplished by providing a list of vitamin C–rich foods to either include or exclude when the subjects consumed the HC and LC diets, respectively. The amount of ascorbic acid in each food was provided as points, with 1 point corresponding to 15 mg ascorbic acid. Subjects were asked to consume 12–13 points during the HC dietary period and ≤2 points during the LC dietary period. One-half of the subjects were assigned randomly to the HC diet as the initial absorption test and the remainder to the LC diet.

The dietary records were analyzed for nutritional content by using the NUTRITIONIST IV program (N-squared Computing; First Data Bank Division, Hearst Corporation, San Bruno, CA). Tea consumption was estimated based on black tea equivalents as follows: 2 cups (480 mL) of iced tea and 1.5 cups (360 mL) of herbal tea or coffee were coded as 1 cup (240 mL) of black tea (16). Animal tissue content was estimated by determining the intake of beef, pork, fish, poultry, and seafood with each meal. Heme iron was calculated on the assumption that 40% of the iron in animal tissue is heme iron (17). Nonheme iron was calculated as the difference between total and heme-iron intake.

Iron absorption tests

Two days before the initial absorption test, 30 mL blood was drawn from each subject for measurement of background ^{59}Fe and ^{55}Fe in blood, serum ferritin concentration (18), and packed cell volume. Additional blood was obtained for these measurements at the beginning of the fifth week and a final sample 2 wk after the last absorption test.

For the initial absorption test, iron absorption was measured from the SS diet by using wheat rolls labeled with ^{55}FeCl$_3$. The subjects reported to the laboratory each weekday morning, at which time their dietary records for the previous day were reviewed in detail. The subjects were then given bread rolls labeled with ^{55}Fe to be consumed with each meal of the day. The 15 wheat rolls used for each dietary labeling period were tagged extrinsically by mixing 0.1 mg Fe as FeCl$_3$ with either ^{59}FeCl$_3$ or ^{55}FeCl$_3$ (Du Pont, Wilmington, DE) with the dough before baking (19). Each roll weighed 12–13 g and contained 1/15 of the total amount of administered radioactivity for each test. The latter was 37 kBq for ^{59}Fe and 74 kBq for ^{55}Fe.

The following week, absorption was measured from the standard meal that was fed on 2 successive mornings to minimize day-to-day variations in iron absorption. These meals were eaten between 0700 and 1000 by subjects who had fasted for ≥10 h. The standard meal contained 113 g ground beef, 53 g bun, 68 g French fries, and 148 g milk shake. The total iron content was 4.8 mg Fe. Each meal was tagged extrinsically by pipetting 1 mL 0.01 mol HCl/L containing 0.1 mg Fe and 18.5 kBq ^{59}FeCl$_3$ onto the hamburger bun. Two weeks later, 30 mL blood was drawn from each subject to measure ^{59}Fe and ^{55}Fe in blood.

During the fifth week of the study, the HC or LC diet was tagged for 5 d according to the same protocol used for the SS diet. The alternate diet was tagged the following week. Bread rolls labeled with ^{55}Fe were used for the first dietary period (LC or HC) and bread rolls labeled with ^{59}Fe for the second. As with the SS diet, dietary records were reviewed daily with each subject. Two weeks after the final dietary labeling period, 30 mL blood was drawn to measure the increase in ^{55}Fe and ^{59}Fe in blood.

Radioactivity was measured in duplicate 10-mL blood samples by a modification of the method of Eakins and Brown (20). Percentage absorption was calculated based on the total blood volume estimated from the height and weight of each subject (21, 22). The red cell incorporation of absorbed radioactivity was assumed to be 80% in all subjects (23).

Statistical analysis

The mean daily intakes of nutrients with the SS, LC, and HC diets were compared by using analysis of variance (ANOVA) followed by Duncan's new multiple-range test. Iron absorption percentages were transformed to logarithms for statistical comparisons and the results recovered as antilogarithms (24). Two-way ANOVA was used to compare the effect of the order in which the HC and LC diets were consumed. Student's *t* tests were used to compare the absorption ratios between any 2 dietary periods by determining whether the mean log absorption ratio differed significantly from zero. The study design had an 80% chance of detecting a 50% shift in the ratio with a significance level of

TABLE 1
Daily nutrient intake during the 3 labeling periods[1]

Nutrient	Self-selected diet	High–vitamin C diet	Low–vitamin C diet
Macronutrients			
Energy (kJ)	7678 (4452–13472)	8427 (4753–12121)	7355 (5473–9924)
Carbohydrate (g)	239 (142–353)	294 (154–415)	234 (160–315)
Protein (g)	73 (40–174)	78 (44–110)	75 (54–108)
Fat (g)	68 (38–138)	63 (32–115)	61 (39–87)
Iron (mg)			
Nonheme	11.4 (65–18)	16.6 (8.0–31.9)[2]	12.8 (7.2–21.8)
Heme	0.7 (0.2–2.22)	1.0 (0–2.7)	0.8 (0.3–1.8)
Total	12.1 (7.2–20.2)	17.6 (9–35)[2]	13.6 (9–2237)
Other			
Vitamin C (mg)[3]	90 (23–201)	247 (80–388)	51 (21–98)
Animal tissue (g)	109 (22–246)	84 (0–190)	103 (28–98)
Calcium (mg)	919 (408–2192)	1300 (316–6841)	811 (393–1151)
Phosphorus (mg)	1247 (606–2698)	1125 (609–1495)	1106 (476–1595)
Fiber (g)	13.4 (5.8–34.0)	16.0 (10.7–22.5)	12.5 (6.5–33.7)
Tea			
(cups)	0.43 (0–1.32)	0.38 (0–1.35)	0.42 (0–1.1)
(mL)	103 (0–317)	91 (0–324)	101 (0–264)

[1] $\bar{x}$; range in parentheses.
[2] Significantly different from self-selected, $P = 0.05$.
[3] All diets significantly different from one another, $P = 0.0001$.

0.05. Pearson's correlation coefficients between nutrient intake and log absorption data were calculated. Multiple regression analysis with stepwise selection was used to test the combined influence of dietary factors on absorption. When the results of all 3 dietary periods were pooled, the variation within subjects was removed by forcing the subjects to be part of the regression model along with the dietary variables. The effect was to eliminate the correlation between observations on the same subject. All statistical analyses were performed by using the SAS program (SAS Institute, Inc, Cary, NC).

RESULTS

The daily nutrient intakes during the 3 labeling periods are listed in **Table 1**. Energy, carbohydrate, protein, and fat intakes did not differ significantly among the 3 dietary periods. The mean daily intake of heme iron was not significantly different among the dietary periods but nonheme and total iron intakes differed as determined by one-way ANOVA. This reflected different intakes with the SS and HC diet of 11.4 and 16.6 mg for nonheme iron and of 12.1 and 17.6 mg for total iron. These differences were related in part to a higher mean energy intake with the HC diet because the difference in total iron intake was no longer significant when iron intake was expressed as a percentage of energy intake.

By design, there was a highly significant difference in the mean daily intake of ascorbic acid among the 3 dietary periods. The mean intake of 90 mg/d for the SS diet was nearly identical to the median value of 87 mg/d reported for women aged 20–29 y in the third National Health and Nutrition Examination Survey (25). The mean vitamin C intake increased to 247 mg/d with the HC diet and fell to 51 mg/d with the LC diet, resulting in a 5-fold difference in ascorbic acid intake between these 2 dietary periods.

Differences in the mean absorption of dietary nonheme iron among the 3 dietary periods were modest (**Table 2**). For the SS diet, percentage absorption averaged 4.57% with a range of 1.18–24.27%. When vitamin C intake was increased during the

HC diet, mean absorption increased to 7.69%, although the mean absorption ratio for the HC to SS diet of 1.67 (± 1 SE: 1.25, 2.24) was not significant. Compared with the HC diet, there was a modest reduction in iron absorption with the LC diet to a mean of 5.69%, which was higher than that observed with the SS diet. The differences in mean absorption for the 3 dietary periods were small in relation to the characteristic wide variation in absorption between subjects (**Figure 1**).

The order in which absorption from the HC and LC diet was measured did not affect mean absorption significantly as determined by two-way ANOVA. Mean absorption from the HC averaged 6.1% when measured during the fifth week with ^{55}Fe and 9.68% when measured during the sixth week with ^{59}Fe. Corresponding mean absorption values of 5.81% and 5.58% were obtained with the LC diet. The mean absorption ratios for the 3 dietary periods relative to the mean of 8.00% for the standard meal were 0.57 (± 1 SE: 0.45, 0.72) for the SS diet, 0.96 (± 1 SE: 0.78, 1.18) for the HC diet, and 0.71 (± 1 SE: 0.57, 0.89) for the LC diet.

It was of interest to assess the relation between iron absorption from a complete diet and the diet's nutrient composition. When the mean ascorbic acid intakes for the 3 dietary periods were compared with iron absorption in each subject, no relation was apparent (**Figure 2**). To minimize the effect on absorption of differences in iron status, iron absorption in subsequent analyses was adjusted to a serum ferritin concentration of 30 μg/L as described previously (11, 26). With this adjustment, iron absorption was decreased if the serum ferritin concentration was <30 μg/L and increased if >30 μg/L. When iron absorption from the SS diet was evaluated, none of the correlation coefficients between iron absorption and the nutrient intakes listed in Table 1 were statistically significant. When all 3 dietary periods were included, only phosphorus was significantly correlated with iron absorption ($r = -0.45$, $P < 0.001$).

The relation between the nutrient composition of the diet and iron absorption was further examined by using multiple regression. With use of log absorption values adjusted to a serum fer-

TABLE 2
Age, sex, iron status, and absorption data in human subjects

Subject	Age	Packed cell volume	Serum ferritin	Iron absorption			
				Standard meal	Self-selected diet	High–vitamin C diet	Low–vitamin C diet
	y	%	μg/L	% of dose			
Male	22	41	85	4.81	1.18	3.95	3.18
Female	24	45	65	2.15	1.86	1.77	1.62
Female	22	38	54	21.51	24.27	17.15	9.18
Female	29	42	48	6.18	2.16	3.16	8.78
Female	27	39	43	2.90	2.42	3.36	4.35
Female	21	38	42	13.96	5.20	7.21	4.01
Male	22	44	31	10.61	2.70	23.07	12.72
Female	23	38	29	11.22	6.86	9.21	6.15
Female	38	36	18	11.13	15.21	3.63	4.12
Female	27	43	17	10.63	1.52	14.11	4.22
Female	20	43	14	3.32	6.62	16.65	12.00
Female	23	37	11	26.27	20.41	25.91	9.70
Mean[1]	25	40	31	8.00	4.57	7.69	5.69
−1 SE	—	—	37	10.06	6.17	9.99	6.80
+1 SE	—	—	26	6.36	3.39	5.93	4.76

[1] Geometric mean for serum ferritin and absorption values.

ritin concentration of 30 μg/L as the dependent variable, the relation with the intake of nutrients that were reported to influence the absorption of nonheme iron (phosphorus, ascorbic acid, fiber, calcium, tea, animal tissue, and heme and nonheme iron) was examined. Evaluation of the SS diet alone did not identify any significant dietary determinants of iron absorption. We then examined the relation between dietary factors and iron absorption in all 3 dietary periods. The results indicated that the total variation in absorption was 85%, of which 54% was due to subject variability and 31% was due to dietary factors (**Table 3**). The strongest association was with phosphorus (19%), followed by ascorbic acid (8%) and animal tissue (4%).

DISCUSSION

The present investigation was undertaken to determine whether the effect of vitamin C on iron absorption has been exaggerated by measuring absorption from single meals rather than from a complete diet. As in other iron absorption measurements from a complete diet, the effect on iron absorption of known dietary determinants such as vitamin C was much less than reported with single-meal measurements. We found a 5-fold difference in mean dietary vitamin C intake between the LC and HC diets: 51 compared with 247 mg/d. In a previous iron absorption study in which a meatless meal was fed to fasting subjects, a 100% increase in iron absorption was observed when the vitamin C content was increased from 50 to 250 mg (3). This increase was far greater than the modest 35% higher absorption from the HC diet than from the LC diet in the present study. Even at these extremes in dietary vitamin C, the differences in mean iron absorption from a complete diet were small relative to the differences in absorption between subjects (Figure 1).

Multiple regression analysis showed that phosphorus had a significant inhibitory influence, an effect that was stronger than the enhancing effect of either ascorbic acid or animal tissue. The nature of the inhibitory effect of dietary phosphorus on iron absorption is unclear. One possibility is that this inhibitory

effect reflects the content of dietary phytate, which accounts for the potent inhibitory effect of wheat bran (27, 28) and soy protein (29–32) on iron absorption. Studies with various isolates of soy protein showed that even relatively small amounts of residual phytate can strongly inhibit iron absorption (33). In a recent investigation, nonheme-iron absorption from 25 complex meals correlated highly with phytic acid content (26). However, note that phosphate per se also has a strongly inhibitory effect on
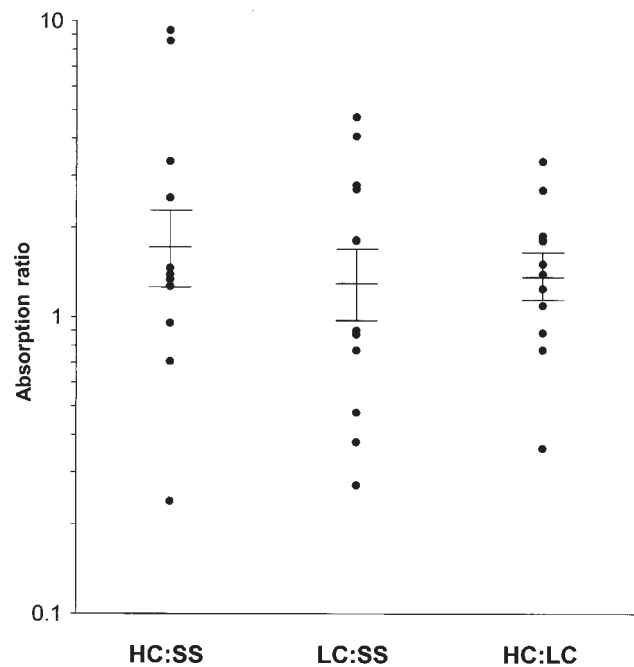

FIGURE 1. Effect of varying the dietary intake of ascorbic acid on nonheme-iron absorption. Absorption ratios are between self-selected (SS), high–vitamin C (HC), and low–vitamin C (LC) diets. The horizontal bars depict the mean ± 1 SE.
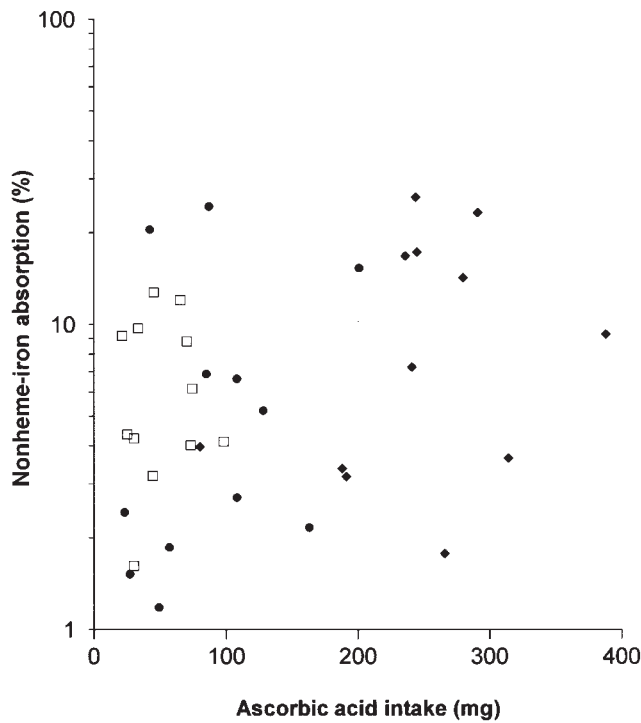
FIGURE 2. Relation between nonheme-iron absorption and ascorbic acid intake during 3 dietary periods: a self-selected diet (●), a low–vitamin C diet (□), and a high–vitamin C diet (◆).

iron absorption (34, 35). The negative effect of phosphorus could reflect dietary sources other than phytate, such as meat and dairy products, or could even be a spurious result of testing several dietary variables.

Vitamin C accounted for only 8% of the variation in iron absorption as compared with 19% for phosphorus (Table 3). This relatively small influence is surprising in view of the wide range of vitamin C intakes examined; the average vitamin intakes during the LC and HC dietary periods were similar to the mean dietary intakes of 45 and 203 mg/d reported for the 10th and 90th percentiles, respectively, in middle-aged women (36). The rather limited effect of vitamin C agrees with the observations by Hunt et al (10), who measured iron absorption from a complete diet by using the fecal balance method. These

TABLE 3
Predictors of nonheme-iron absorption as determined by multiple regression analysis[1]

Independent variable	Parameter estimate	Percentage variance[2]	P
		%	
Intercept	2.98	—	—
Phosphorus	−0.001	18.91	0.0005
Vitamin C	0.003	7.67	0.0069
Animal tissue	0.17	3.96	0.0285

[1] Percentage iron absorption was adjusted to a serum ferritin concentration of 30 μg/L. Model $R^2 = 0.8495$, adjusted $R^2 = 0.7491$, $F = 8.47$, $P \leq 0.0001$.
[2] Squared semipartial correlation (31% of the variation was explained by the 3 dietary variables as compared with 54% by subject variability).

investigators were unable to detect a significant increase in absorption when 1500 mg vitamin C/d was added to either a diet low in bioavailable iron or to a typical Western diet in women with low iron stores. Taken together, the results of the study by Hunt et al and the present findings indicate that the influence of ascorbic acid on iron absorption from a complete diet is less than commonly assumed.

An alternative approach to evaluating the influence of ascorbic acid intake on iron balance is to examine the relation in a population between the consumption of dietary factors known to influence iron absorption and iron status. In a recent epidemiologic investigation including 634 elderly individuals aged 67–93 y, iron stores as measured by serum ferritin were compared with dietary intake during the previous year as assessed by a food-frequency questionnaire (37). Individuals with a pathologic elevation in serum ferritin were excluded and multiple regression analysis was used to control for sex, age, body mass index, total energy intake, smoking, and the use of medications known to affect blood loss. A significant positive association was observed between iron stores and dietary intake of vitamin C ($P = 0.04$), heme iron ($P = 0.0001$), supplemental iron ($P = 0.0001$), and alcohol; coffee had a negative association. It is of interest that a significant association was observed only with dietary vitamin C, which averaged 153 ± 79 mg/d, but not with supplemental vitamin C, which provided an additional 98 mg/d. Although the strength of the relation between iron status and the dietary intake of vitamin C was modest, most epidemiologic studies have failed to show an association between ascorbic acid intake and iron status.

The effect of phosphorus, ascorbic acid, and animal tissue on iron absorption observed in the present study agrees remarkably well with a recently published model for predicting food iron absorption in humans from the biochemical composition of the meal (26). In this study, animal tissue, phytic acid, and ascorbic acid were significantly related to iron absorption, suggesting that phytic acid accounts for the effect of dietary phosphorus observed in the present report. Additionally, in the previous study, the influence of both animal tissue and phytic acid ($P = 0.0001$) was greater than that of ascorbic acid ($P = 0.0441$), whereas animal tissue appeared to be less important than ascorbic acid in the present investigation. Nevertheless, the identification of similar dietary factors in the 2 reports underscores their importance as dietary determinants of nonheme-iron absorption in humans.

The reasons for the diminished influence of dietary factors when iron absorption is measured from a complete diet rather than from individual meals are unknown. One possibility is that residual gastric contents from meals eaten throughout the day dampen the influence of dietary factors compared with that in fasting subjects. Another is that the range of meals consumed over a 5-d period is much greater than with an isolated meal and consequently the biochemical composition of the total diet is more varied. The negative influence of phosphorus intake on dietary absorption shown in this study supports the idea that the facilitating effect of ascorbic acid on iron absorption from a complete diet is at least partly offset by dietary inhibitors. For whatever reason, the influence of dietary ascorbic acid on iron absorption is substantially less than indicated by absorption studies with single meals. There is a need for additional information on the precise role of dietary vitamin C in iron nutrition.

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