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# Effects of pH and chelating agents on iron binding by dietary fiber: implications for iron availability<sup>1-3</sup>

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**ABSTRACT** Iron binding by isolated dietary fiber in the presence of iron ligands was measured. Iron-ligand solutions were mixed with isolated fiber and incubated. Bound and polymerized iron were estimated by centrifugation and ultrafiltration, respectively. Results showed that iron binding varied greatly with ligand used and with pH. Based on these studies, it was predicted that ligands would influence iron availability in the order ascorbate < citrate = nitrolotriacetic acid < EDTA. When added to a complex meal containing beef, bread, green beans, and milk, iron availability (measured by an in vitro method) was little affected by fiber, citrate, and ascorbate, but enhanced by nitrolotriacetic acid and EDTA. When added to a semisynthetic meal, fiber, ascorbate, citrate, nitrolotriacetic acid, and EDTA increased iron availability. It appears that the effect of dietary fiber on iron availability is greatly dependent on the ligands present in the meal. *Am J Clin Nutr* 1983;38:202-213.

**KEY WORDS** Iron iron availability, dietary fiber, in vitro, citrate, ascorbate, NTA, EDTA

## Introduction

It has been suggested that iron nutriture may suffer as a result of increased dietary fiber consumption (1, 2). The effect of dietary fiber on iron absorption has been examined in numerous in vivo studies (3-15). Several in vitro binding studies have examined the effect of dietary fiber on iron solubility (16-22). Results from these studies indicate that under some conditions, dietary fiber may reduce iron availability (3-7, 16-22), while under other conditions it appears to have no effect (8-15).

In vitro approaches to the estimation of iron availability rely on the assumption that iron must be in solution to be absorbed (23). Under some conditions, soluble iron complexes may be in the form of large molecular weight polymers consisting of an iron-hydroxy core surrounded by complexing species. These polymers have been observed in solutions containing iron and citrate (24, 25), iron and fructose (26, 27) and ferric nitrate (28). Polymerized iron, while soluble, is probably not available for absorption by the intestinal mucosa (29, 30). Thus, it may be argued that iron availability from a food or meal may be estimated by measuring the proportion of soluble, low molecular weight iron present (23).

Measurements of mineral binding to fiber offer an important tool for studying mineral-fiber interactions. However, the use of binding study results to predict in vivo effects on availability must be done carefully. An in vitro system can, at best, only approximate in vivo conditions. Purification of fiber components may cause chemical and physical alterations in the fiber thus influencing its behavior. Substances added to the in vitro system (buffers, reducing agents, etc) may affect binding.

The study described herein was designed to examine more closely the parameters of iron binding to dietary fiber and its possible nutritional consequences. Binding was determined in the presence of a number of ligands over the pH range 2.0 to 7.0. These conditions were chosen for two reasons: 1) Binding to dietary fiber is always relative to attraction of the iron to other complexing agents which could be anything from water

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molecules to iron-hydroxy polymers to soluble organic ligands. Thus, a qualitative measure of the affinity of iron for dietary fiber may be obtained by comparing binding in the presence of different ligands. 2) For very practical reasons, some type of complexing agent must be present with iron in solution at intestinal pH's, or all the iron present will appear to have been bound but will, in fact, have precipitated.

The dietary fiber used was enzymatically isolated, dephytinized wheat bran fiber. It was assumed to be very close chemically to native wheat bran fiber but separated from other potential iron binding substances such

as phytate, proteins, and starch. In order to determine whether conclusions drawn from these binding studies could predict the effect of dietary fiber on iron availability, a second set of experiments using an in vitro simulated digestion procedure (31) was performed. The data obtained from these experiments can be used to help interpret the varied results obtained in in vivo studies.

**Materials and methods**

*Binding studies*

*Fiber isolation.* A certified food grade wheat bran was obtained from the American Association of Cereal Chemists. It was treated using the procedure of Saun-

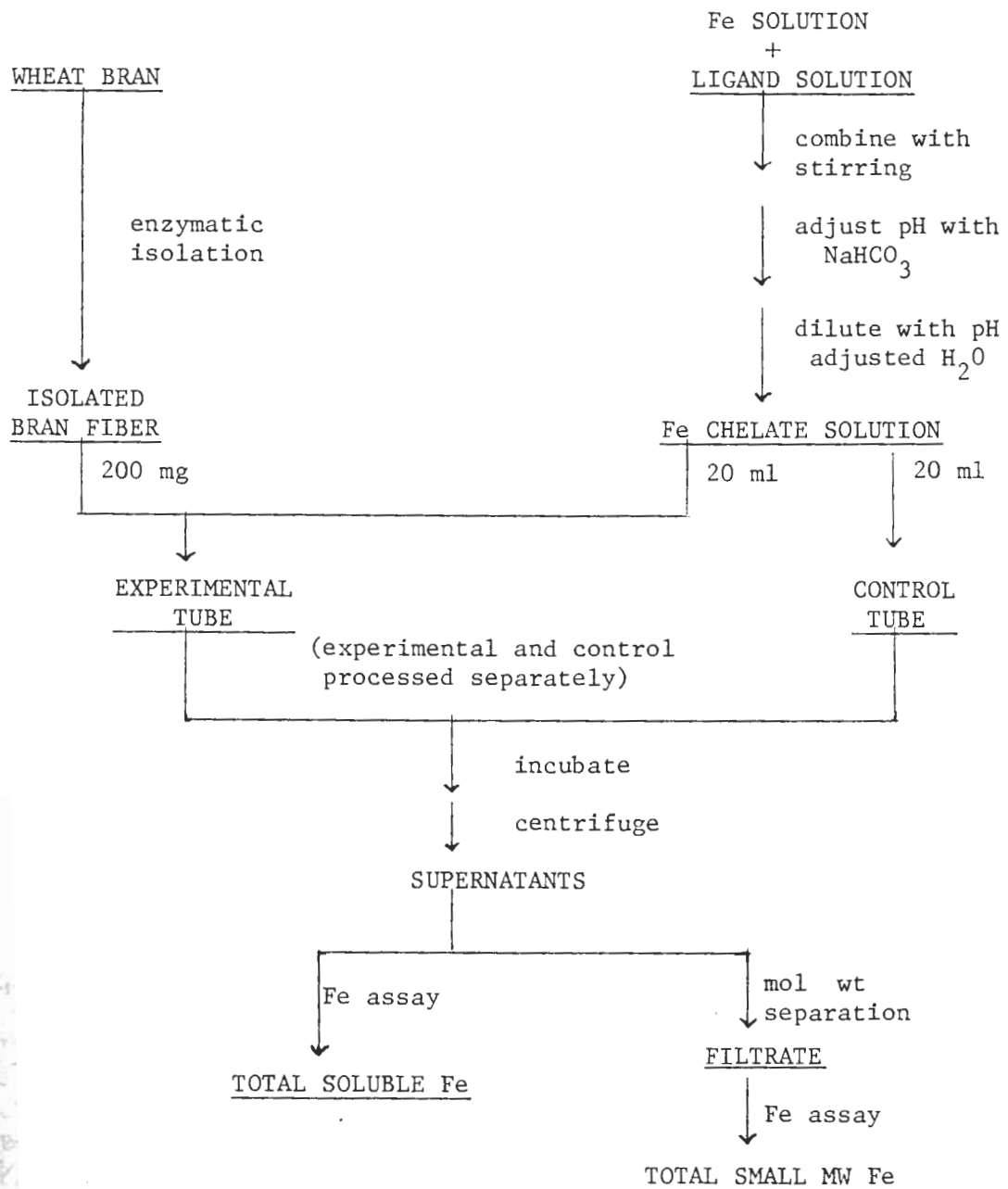


FIG 1. Flow chart showing steps in the procedure for the estimation of iron binding by dietary fiber.

ders and Hautala (32) to isolate a wheat bran dietary fiber. Before final air drying, the isolated fiber was incubated for 1 h in 1.8% disodium EDTA solution at room temperature to remove phytate and any residual endogenous iron. The resulting fiber was washed very thoroughly in distilled water and air dried.

**Preparation of iron chelate solutions.** Iron chelate solutions were prepared using ferric chloride and one of the following chelating agents in the indicated molar ratio of iron:ligand; disodium EDTA (Fisher Scientific Co, Pittsburgh, PA), 1:1; L-ascorbic acid (Fisher), 1:5 and 1:25; lactobionic acid, hemicalcium salt (Sigma Chemical Co, St Louis, MO), 1:1; citric acid monohydrate (Sigma), 1:0.4, 1:1, 1:25; or nitrolotriactic acid (NTA) (Sigma), 1:1. The final concentration of iron in these solutions was 2 ppm. The solutions were prepared by mixing small amounts of concentrated solutions of  $\text{FeCl}_3$  (in 0.1 N HCl) and chelating agent in a beaker, adding distilled, deionized water, titrating with 0.02 M sodium bicarbonate solution with constant stirring until the desired pH was reached, and bringing to volume with pH adjusted, distilled, deionized water. Solutions were prepared at the following initial pH levels: 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 6.8. Buffers were not used in order to prevent uncontrolled interactions with iron. Since the pH changed during incubation, statistical analyses between different treatments were impossible, and comparisons between groups can be qualitative in nature only.

**Incubation and estimation of binding.** The procedure followed is best described by the flow chart in **Figure 1**. Experimental incubations contained both isolated fiber and iron chelate solution. Control incubations contained only the iron chelate solution. After a 90-min incubation at 37°C with shaking, the pH was measured and the solutions were centrifuged to remove both suspended fiber and iron polymers. Small aliquots of the supernatants were removed for iron assay by atomic absorption spectrophotometry (Perkin-Elmer Co, Norwalk, CT) and for estimation of the percentage of small molecular weight iron species using Amicon ultrafiltration cones (Amicon Corp, Lexington, MA) with a 25,000 molecular weight cutoff. In all treatments reported here, no significant precipitation of iron from controls occurred.

**In vitro estimation of available dietary iron.** An in vitro procedure published by Miller et al (31) was used to estimate the bioavailability of iron in meals containing dietary fiber or dietary fiber and chelating agents, compared to meals containing neither. **Table 1** shows the composition of the basal meals. **Table 2** defines treatment meals prepared by deletion of fiber or addition of chelating agents. Except as indicated, treated meals had the same composition as the respective basal meals. NTA and EDTA were added to give a nonheme iron/ligand molar ratio of 1:1. Ascorbic and citric acids were added to give a 1:5 molar ratio of iron/ligand. The amount of fiber used in the basal meals was chosen to give approximately the same weight ratios of iron to fiber as were present in the binding studies.

The in vitro method essentially measures the soluble, small molecular weight iron species present in a meal under simulated gastrointestinal conditions. The percentage of iron which passes into 6000 to 8000 molec-

ular weight cutoff dialysis tubing (Spectrum Medical Industries, Inc, Los Angeles, CA) relative to the total iron present is defined as the percentage dialyzable iron. This value is assumed to represent an estimate of available iron in the sample compared to other samples evaluated at the same time.

The method as described by Miller et al (31) was varied slightly. No radioactive labels were used, and only atomic absorption spectrophotometry was used for iron assay on each sample. The general procedure is outlined in the flow chart in **Figure 2**. In the semisynthetic meals, total iron was assumed to represent total nonheme iron values. This was determined by ashing the samples, dissolving in acid, and assaying iron content using atomic absorption spectrophotometry. In standard meals, nonheme iron content was determined as described by Miller et al (31).

## Results and Discussion

### Binding studies

The statistics reported were computed as follows:

% iron bound = 100

$$= \frac{\text{iron concentration in experimental supernatant}}{\text{iron concentration in control supernatant}} \times 100$$

% small molecular weight iron

in control supernatant

$$= \frac{\text{iron concentration in control filtrate}}{\text{iron concentration in control supernatant}} \times 100$$

% small molecular weight unbound

iron in experimental supernatant

$$= \frac{\text{iron concentration in experimental filtrate}}{\text{iron concentration in control supernatant}} \times 100$$

**Figure 3** shows the percentage iron bound for five of the treatments used as a function of pH. It is apparent from this graph that binding of iron to dietary fiber may vary widely depending on the pH and the iron ligands present. **Figure 4** shows the percentage small molecular weight species present in the control solutions for the same five treatments. As might be expected, the percentage of small molecular weight species decreases as pH increases for most treatments. Ligands such as EDTA and NTA are

TABLE 1  
Composition of basal meals\*

Standard meal		Semisynthetic meal	
Component	g/100 g of meal	Component	g/100 g of meal
Ground beef, cooked	15.70	Egg albumin	6.24
Bread, white, enriched	8.52	Glucose, anhydrous	7.80
Green beans, raw	10.52	Corn oil	5.90
Milk, whole	47.51	CaHPO <sub>4</sub>	0.11
Water, distilled	15.60	KH <sub>2</sub> PO <sub>4</sub>	0.22
Isolated bran fiber	2.15	FeCl <sub>3</sub> solution (1000 ppm in Fe)	0.625
		Water	75.48
		Isolated bran fiber	3.625

\* The pH of the meals was adjusted to 2 before bringing to the final weight with water.

TABLE 2  
Percentage dialyzable iron in treated standard and semisynthetic meals\*†

Standard meal		Semisynthetic meal	
Treatment	% Dialyzable Fe	Treatment	% Dialyzable Fe
None (basal meal)	7.12 ± 0.48 <sup>a</sup>	None (basal meal)	6.40 ± 0.17 <sup>a</sup>
No fiber‡	7.78 ± 0.38 <sup>a</sup>	No fiber§	3.27 ± 0.51 <sup>b</sup>
Added citric acid (39 mg/100 g)	6.57 ± 0.28 <sup>a</sup>	Added citric acid (66 g/100 g)	10.50 ± 0.41 <sup>c,d</sup>
Added ascorbic acid (34 mg/100 g)	7.74 ± 0.36 <sup>a</sup>	Added ascorbic acid (57 g/100 g)	8.35 ± 1.13 <sup>d,e</sup>
Added NTA (10.5 mg/100 g)	10.71 ± 0.05 <sup>b</sup>	Added NTA (17.5 g/100 g)	12.06 ± 0.35 <sup>c</sup>
Added EDTA (14 mg/100 g)	31.16 ± 0.73 <sup>c</sup>	Added EDTA (24 g/100 g)	36.02 ± 1.07 <sup>f</sup>

\* Values represent means of triplicates ± SE.

† Means within a column followed by different superscripts are significantly different ( $p < 0.01$ ).

‡ Bread was increased by 2.15 g/100 g.

§ Glucose was increased by 3.63 g/100 g.

seen to prevent polymerization, probably due to the great stability of the complexes formed.

Figures 5 and 6 show that the relative amounts of citrate in different solutions have little effect on binding of iron to isolated fiber, but that higher citrate concentrations result in higher concentrations of low molecular weight iron species. The binding curves for solutions containing NTA or lactobionate are very similar to the 1:1 citrate:iron curve as seen in Figure 3, but Figure 4 indicates that the molecular weight of the iron species in these three groups differ. It appears that some factors in addition to molecular weight must affect binding over the pH range examined. In the presence of citrate, NTA, or lactobionate, these factors probably vary depending on pH and other conditions.

At low pH, complex stability between iron and the ligand used may be low due to

incomplete ionization of the chelating agent. Also, most iron species present will have small molecular weights and be very soluble. Thus, the iron species are very available for binding, and some binding is observed. That this binding is not greater may be attributed to the incomplete ionization of binding groups on the fiber or to the stability of the iron species in acid solution.

As the pH increases to 3.5, binding increases to a maximum. This may be due to changes in the fiber such as steric changes or increased ionization of functional groups. Above pH 4.0, binding decreased in the presence of all three chelating agents. Concurrently, polymerization increased greatly for lactobionate and citrate but not for NTA. For all three, iron-ligand complex stability should increase as neutrality is approached and, for polymerized iron, complex stability is fairly strong (26). Although the ability of fiber groups to complex iron is probably

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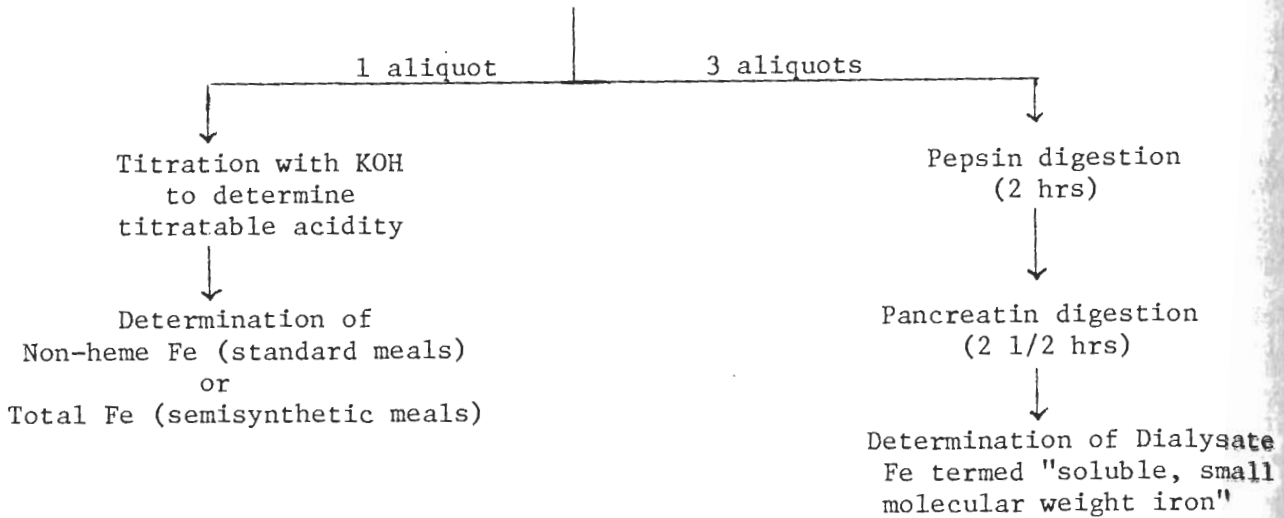


FIG 2. Scheme used in the in vitro estimation of iron availability.

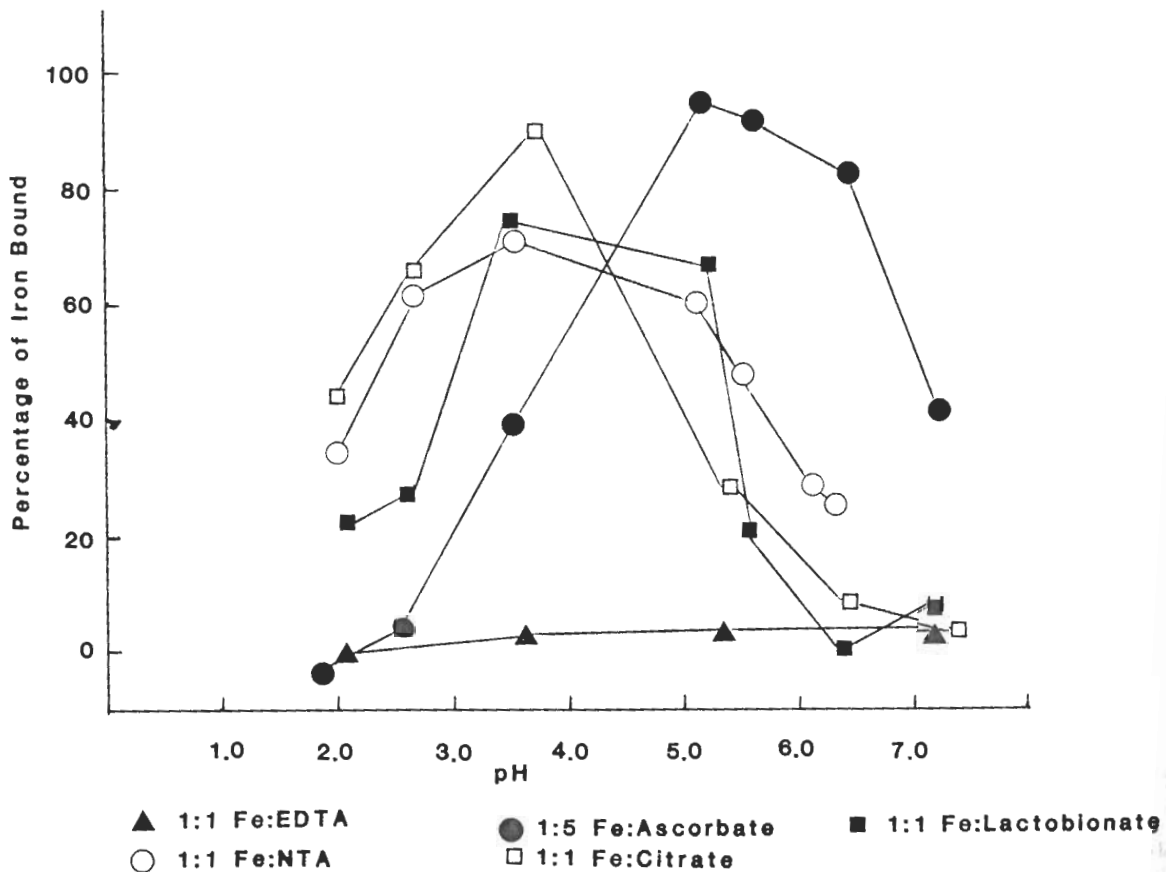


FIG 3. Effect of pH and iron ligand on iron binding by dietary fiber.

greatest at neutral pH due to maximum ionization of functional groups, these groups may be unable to compete with the ligands used or with polymer formation.

In the presence of EDTA, little binding of iron occurs and little or no polymerization

occurs. This expected result may be assumed to be due to the great stability of the iron/EDTA complex at all pH levels.

Binding to dietary fiber was examined in the presence of two different levels of ascorbate. The results obtained for 1:5 Fe/ascor-

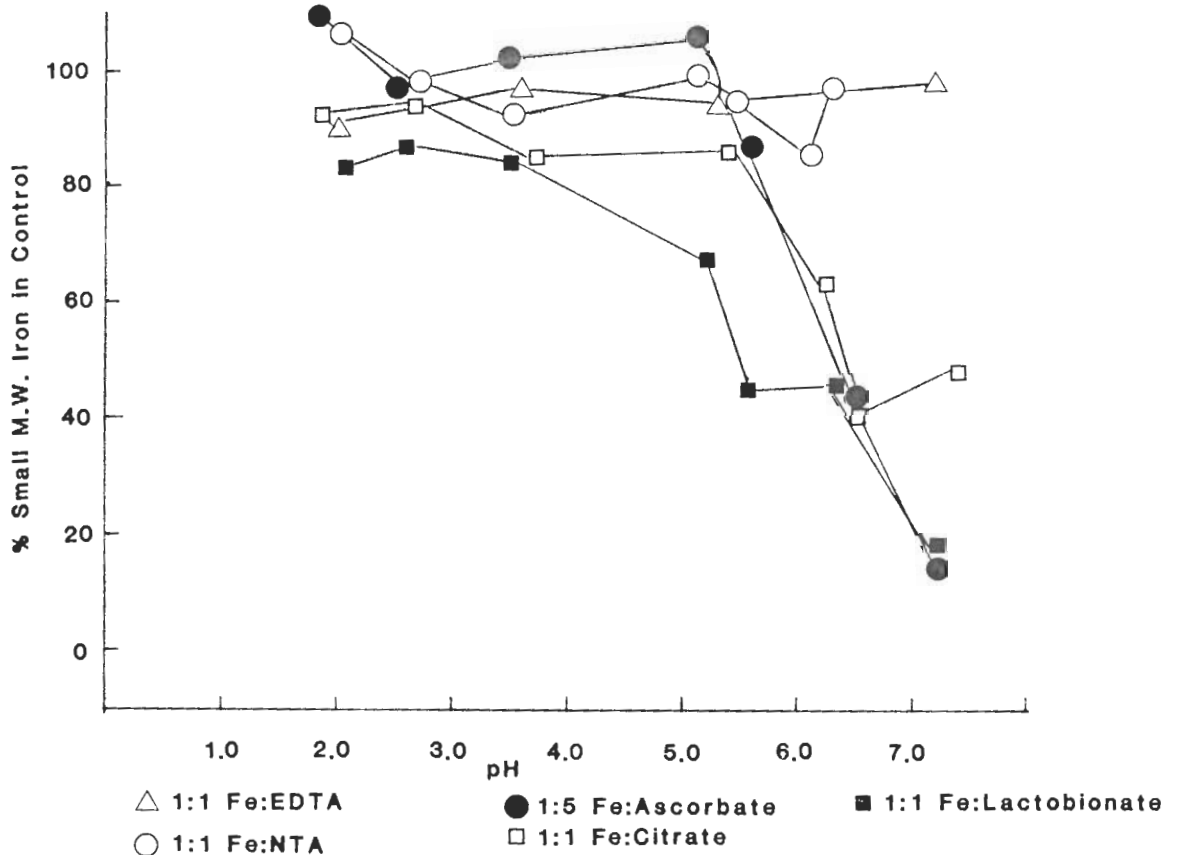


FIG 4. Effect of pH and iron ligand on iron polymerization in control solutions.

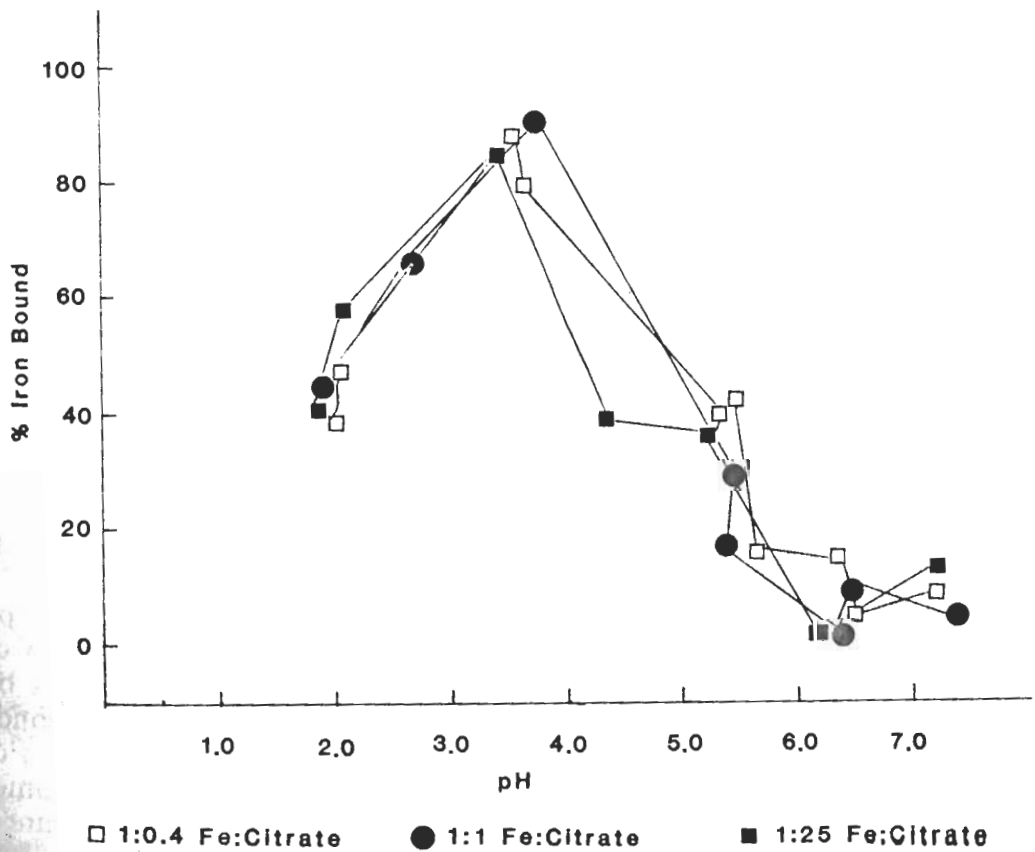


FIG 5. Effect of pH and citrate concentration on iron binding by dietary fiber.

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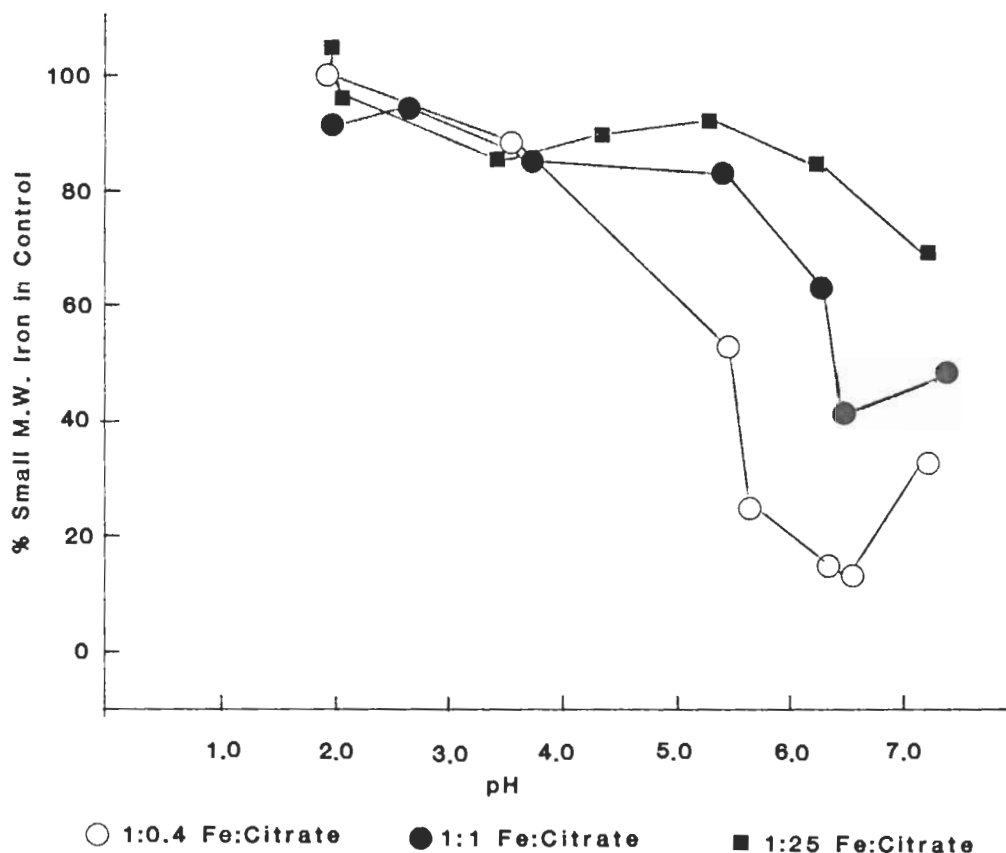


FIG 6. Effect of pH and citrate concentration on iron polymerization in control solutions.

bate differ greatly from the above studies as is shown in Figure 3. Results were similar when ascorbate was increased to 1:25 Fe/ascorbate as shown in Figure 7. These results could have been affected by all the factors mentioned above for solutions containing NTA, citrate, and lactobionate, but also by changes in the oxidation state of the iron present. It is likely that below pH 5.0 iron is in the ferrous form in the presence of ascorbate (33). Above pH 5.0 some of the iron could be oxidized during the course of the incubation period. At neutral pH, in the presence of a higher concentration of ascorbate, polymer formation was suppressed (Fig 8). This is probably responsible for the higher amounts of binding observed in the 1:25 iron/ascorbate solution as more small molecular weight iron would be available relative to solutions in which polymer formation was greater.

Since it is probable that iron is only available if it is both in solution and of small molecular weight, it is instructive to examine the proportion of small molecular weight iron left in solution in experimental super-

natants as a percentage of control solution. This information is shown graphically in Figure 9. Since gastric contents are probably at pH 2.0 or lower and are brought up to neutral pH fairly gradually in the intestine, it seems plausible that a range of pH levels exists in the duodenum where most iron absorption occurs. Assuming that most iron is absorbed in the pH region 5.0 to 7.5, it appears from Figure 9 that a system containing EDTA will have the most absorbable iron although this iron may not be available to body tissues. Iron availability from iron-fiber mixtures containing NTA, ascorbate, and citrate should be considerably less, and although differences appear to be small, the expected ranking would be NTA = citrate > ascorbate.

Recently, there have been three reports of in vitro iron-fiber binding studies. Comparisons between studies are difficult because of differences in experimental conditions, types of fibers, and concentrations of iron and chelating agents; however, some similarities and differences in results are apparent.

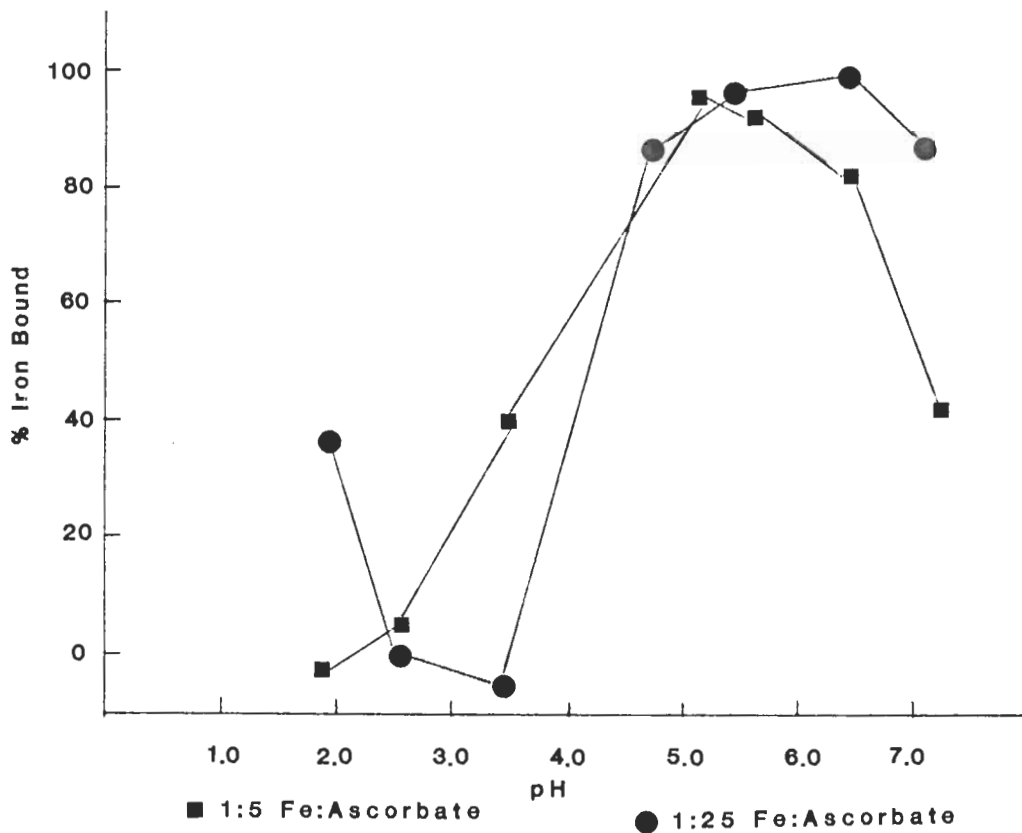


FIG 7. Effect of pH and ascorbate concentration on iron binding by dietary fiber.

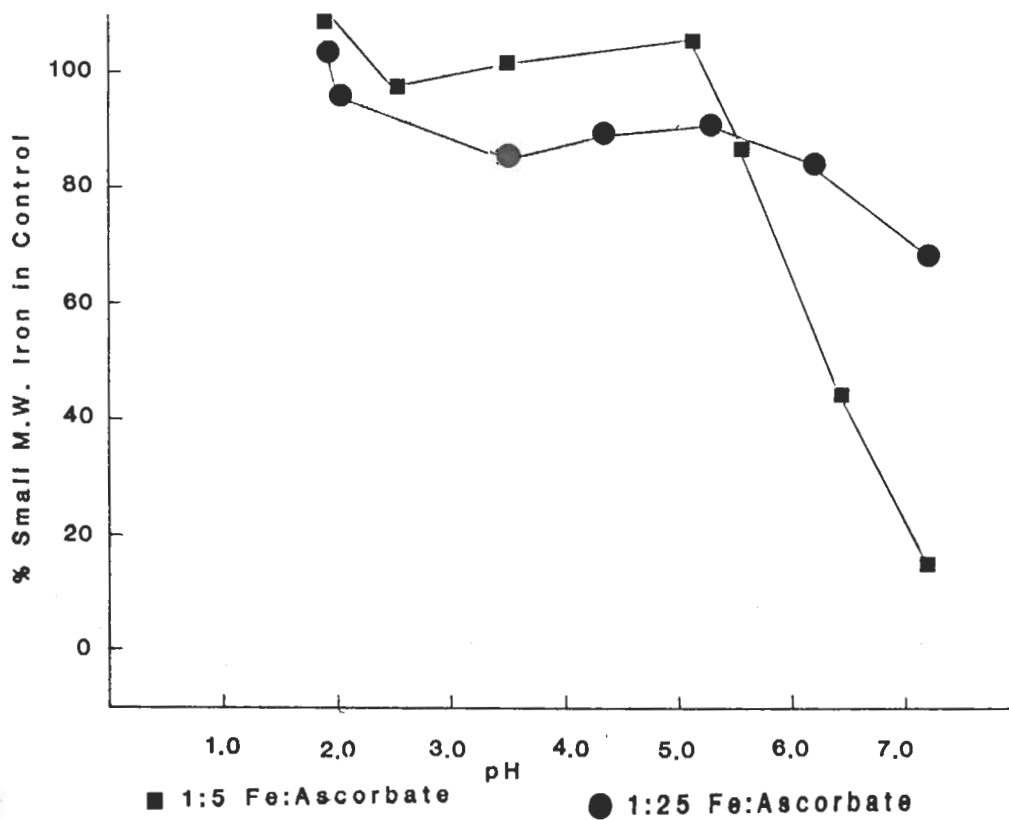


FIG 8. Effect of pH and ascorbate concentration on iron polymerization in control solutions.

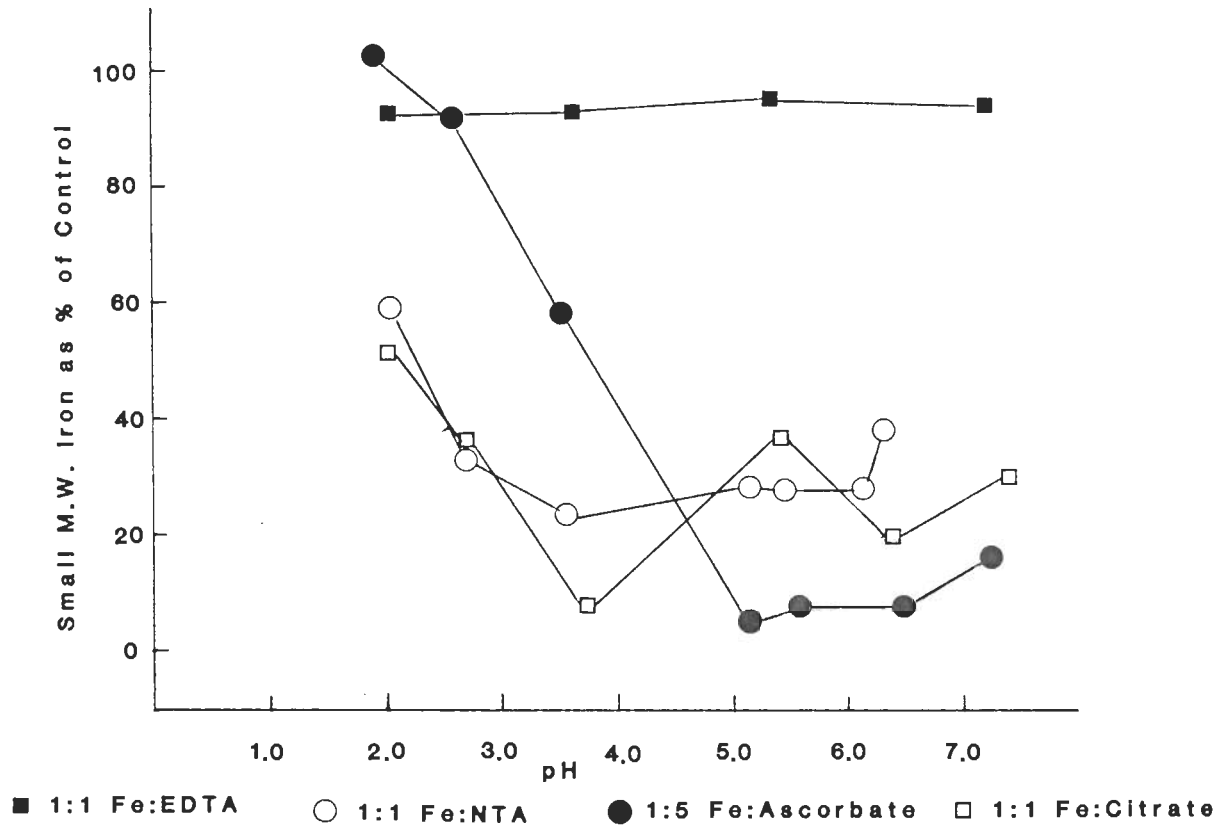


FIG 9. Effect of pH and iron ligands on unbound small molecular weight iron.

Reinhold et al (18) studied the pH dependence of iron II binding to cellulose, to wheat and maize NDF, and to wheat and maize ADF using iron/ascorbate molar ratios of about 1:9. They found little binding at pHs between 1 and 4 and a steady rise in binding as the pH was increased from 4 to 7. Percentage binding was greatest for NDF with ADF and cellulose showing much less binding. The compositions of NDF and the isolated fiber used in this study should be similar, although conditions for isolation of NDF are harsher and less physiological than those used in this preparation of isolated fiber. The binding curve for NDF reported by Reinhold et al (18) is quite similar to the curves in Figure 7 except that the NDF curve is shifted toward higher pHs and the decrease in binding above pH 6 did not occur. Reinhold et al (18) also looked at the effects of EDTA and citrate. Their results are not directly comparable to ours because ascorbic acid was present in all of their treatments. They did observe that binding at pH 6.45 in the presence of EDTA and citrate was substantially lower compared to binding with-

out these ligands. Figure 3 shows that binding at pH 6.5 in the presence of citrate and EDTA is quite low.

Fernandez and Phillips (34) studied iron binding to highly purified fiber fractions (lignin, hemicellulose, pectin, and cellulose) using a 1:1 molar ratio of iron/ascorbate. Conditions in this study were so different from those reported here that any comparisons are risky, at best. Fernandez and Phillips (34) did observe an increase in binding with increasing pH and did not show a decline in binding at higher pHs.

Kojima et al (35) measured the solubilization of iron from cooked pinto beans by several different chelating agents. They found maximum solubilization (minimum binding) by citrate at pH 6 and minimum solubilization at pH 3, findings which agree quite closely with the results depicted in Figure 5. Ascorbic acid was most effective in iron solubilization at low pHs and least effective at high pHs, results which are similar to those shown in Figure 7.

*In vitro estimation of iron availability in meals.* The percentage dialyzable iron in

different prepared meals as determined by the in vitro method of Miller et al (31) is reported in Table 2. It is important to remember that comparisons can only be made within each group of samples. Statistical significance was evaluated using Duncan's multiple range test (36).

*Standard meals.* Results shown in Table 2 indicate that there is little effect of using low concentrations of citric and ascorbic acids or moderate amounts of dietary fiber when the diet consists of meat, milk, and vegetables. The lack of effect of citric and ascorbic acids may be explained by the presence of other ligands released during the digestion which masked their effect. The enhancing effects of NTA and EDTA are probably related to the high affinity of these ligands for iron, allowing them to exert an effect even in the presence of other ligands.

*Semisynthetic meals.* Effects of the various treatments were more pronounced in the semisynthetic meals compared to the standard meals. This is an expected result since fewer competing ligands would be present in the semisynthetic meals. The magnitude and ranking of the ligand effects shown in Table 2 are predictable from Figure 9. The apparent enhancing effect of isolated fiber is quite striking. A possible explanation is that isolated dietary fiber serves as a weak ligand for iron in solution and thus prevents its polymerization and precipitation (18). In this respect, it may act similarly to the other ligands used but is probably weaker and, while binding it, prevents iron from crossing the dialysis tubing. However, both this in vitro system and the gastrointestinal tract are not static systems at equilibrium. They are instead dynamic systems that never reach equilibrium due to constant changes in the concentrations of the components available for dialysis or absorption. In both situations, dietary fiber may bind soluble, low molecular weight iron, thus preventing polymerization and precipitation. When the iron concentration of the solution is reduced through absorption or dialysis, mass action will cause iron weakly bound to insoluble ligands such as fiber to be released into solution and become available for absorption or, in the in vitro method, dialysis.

## Summary

Overall, these results suggest that the availability of iron may be very much dependent on the combination of foods and food constituents present in the gastrointestinal tract. Results of studies that have concluded that dietary fiber depresses iron availability may have been influenced by a variety of factors including nonfiber components of whole grains, such as phytates, which may depress iron availability, and nonbinding effects of fiber such as decreased transit times which may reduce the time during which the iron is in contact with the intestinal mucosa. It is evident from the binding and in vitro studies reported here that common dietary iron ligands may have a large effect on iron availability in the presence of fiber. The different effects of these iron ligands in standard and semisynthetic diets indicates that they may be negated or enhanced by the overall dietary makeup.

These results caution against making simple assumptions in assigning to certain food constituents a major role in influencing nutrient homeostasis. Food constituents are generally heterogeneous in nature, seldom present in predictable proportions, and generally all too labile in very common processing and gastrointestinal conditions. Foods are extremely complex in makeup and have not only chemical properties but also biological structure and properties as well. The binding studies reported here indicate that food constituents, even when well defined and controlled, can interact with unpredictable results.

It is clear from the research presented here that the solubility and character of iron in the diet is greatly affected by the nature of the dietary ligands present and the chemical conditions to which the system is subjected. Diets rich in meats and vegetables may have higher availability of nonheme iron compared to dietaries rich in cereal foods, due to the presence of factors which enhance iron absorption, rather than the relative absence of dietary fiber. This may account for some data from the in vivo studies reviewed above. Studies such as that of Bjorn-Rasmussen (7) and epidemiological evidence of Mameesh et al (5) indicated that in diets or

meals low in meat and vegetables and high in cereal foods, dietary fiber may depress iron absorption. Most of the studies which found no effect of dietary fiber used meals containing meat and/or vegetables (11-13). Although the results of the in vitro method studies reported here indicate that fiber enhances iron availability in semisynthetic meals, it is possible that in vivo the biological effects of fiber ingestion (ie, decreased transit time) take precedence over its chemical effects on the gastrointestinal contents. When consumed with meat and vegetables, the chemical effects of the additional iron ligands may take precedence over the biological effects of the fiber.

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