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Iron binding by tannic acid: effects of selected ligands

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Foods with high tannin content inhibit Fe absorption from meals. Presumably, tannins form complexes with Fe in the intestinal lumen reducing Fe bioavailability. Our objective was to assess the influence of various ligands on Fe binding by tannic acid *in vitro*. Different mixing sequences were employed to determine whether the ligands could prevent Fe from binding to the tannin or could remove Fe already bound. Solutions of Fe^{+3} ($FeCl_3$ in 0.1 N HCl), ligand (ethylenediamine-tetraacetic acid (EDTA), ascorbic acid, nitrilotriacetic acid (NTA) or citric acid) and tannic acid (200 $\mu g\,ml^{-1}$) in pH 4.4 acetate buffer were combined to obtain a final ligand:Fe molar ratio of 1:1 (89 μM). Three mixing sequences were followed: sequence I (Fe and ligand combined and added to tannin); sequence II (tannin and ligand combined and added to Fe); and sequence III (Fe and tannin combined and added to ligand). Fe–tannin binding was assessed by measuring absorbance at 560 nm (visible absorbance maximum) at 15 s intervals for 5 min. An Fe–tannin mixture without ligand served as the control. With EDTA, sequence I resulted in no binding. In sequence II and III, there was some binding initially, but it decreased with time. With ascorbic acid, sequence I resulted in no binding. In sequence II and III, initial binding was slightly lower than the control. Binding did not change with time. With NTA, initial binding varied with the sequence, but converged with time to a value slightly lower than the control. Citric acid did not affect binding regardless of addition sequence. These findings suggest that ligands with high affinity for Fe (e.g. EDTA) can prevent Fe from binding tannin and can remove Fe already bound. Ligands with lower affinity (e.g. citric acid) have little effect. The implications are that EDTA, ascorbic acid and NTA may affect Fe bioavailability from meals containing tannins.

INTRODUCTION

Iron absorption is affected by both promoters and inhibitors present in the diet. Two potent promoters of Fe absorption are ascorbic acid (Disler *et al.*, 1975a) and meat (Layrisse *et al.*, 1984). Ascorbic acid and meat enhance Fe absorption by reducing Fe^{+3} to Fe^{+2} and/or chelating Fe^{+3} (Gillooly *et al.*, 1983; Kapsokefalou and Miller, 1991). Fe^{+2} is more bioavailable than Fe^{+3} because it is more soluble at physiological pHs and because it has a lower affinity for ligands that may inhibit absorption. Two common inhibitors of Fe absorption are tannins and phytate. These components form complexes with Fe within the lumen, reducing Fe bioavailability (Disler *et al.*, 1975b). It is well established that tannin, a polyphenolic compound, reduces Fe absorption when included in the diet at high levels (Disler *et al.*, 1975a; Brune *et al.*, 1989).

Tannic acid, a hydrolyzable tannin, is hydrolyzed by acid to form gallic acid and glucose. Each molecule of gallic acid contains one galloyl group (Fig. 1). Presumably, Fe binds to the adjacent hydroxyls on the galloyl group. The galloyl group has been implicated as the structure responsible for the inhibition of Fe absorption by phenolic compounds in foods (Brune *et al.*, 1991). The Fe–galloyl complex forms a violet color with an absorbance maximum at approximately 550 nm (Mejbaum-Katzenellenbogen and Kudrewicz-Hubicka, 1966).

Iron ethylenediamine-tetraacetic acid (EDTA) has been shown to be an effective fortificant in meals of low Fe bioavailability when the molar ratio of EDTA to Fe is ≤ 1 (MacPhail *et al.*, 1994). Presumably, Fe complexed with EDTA will not bind to tannins or other inhibitors that may be present. Ascorbic acid has also

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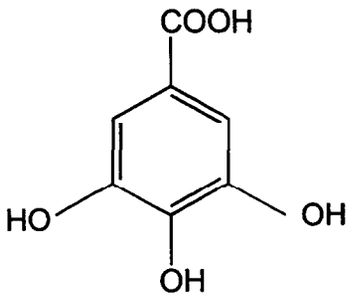

Fig. 1. Chemical structure of gallic acid (3,4,5-trihydroxybenzoic acid) containing a galloyl group.

been shown to chelate and reduce Fe, counteracting the negative effect tannin has on Fe absorption (Siegenberg *et al.*, 1991). Nitrilotriacetic acid (NTA) and citrate are other low molecular weight ligands known to chelate Fe (Bates *et al.*, 1967).

In the present study, a spectrophotometric method was used to study the effects of different ligands on the formation and stability of the Fe–galloyl complex. Different mixing sequences were employed to determine whether the ligands could prevent Fe from binding to tannin or could remove Fe already bound. Like all *in vitro* systems, our methods only approximate *in vivo* conditions and may not predict Fe bioavailability in humans. However, an understanding of chemical interactions between Fe and ligands can be helpful for explaining observed effects of these ligands in *in vivo* situations.

MATERIALS AND METHODS

Materials and equipment

Distilled, deionized water was used throughout. All glassware was washed with detergent, rinsed with water, soaked overnight in 1 N HCl, rinsed again and dried. All chemicals were obtained from Sigma Chemical (St. Louis, MO, USA). An $FeCl_3$ solution (1020 μg Fe ml^{-1} in 1% HCl) (Iron Atomic Absorption Standard Solution) was used as the Fe source. Solutions of $FeCl_3$ (in 0.1 N HCl), the various ligands and tannic acid (in pH 4.4 acetate buffer) were prepared so that the concentrations of Fe, ligand and tannic acid in the final mixture were 89 μM, 89 μM and 200 μg ml^{-1}, respectively. Each data point plotted in Figs 2–6 represents a single measurement. A Perkin–Elmer Lambda 3B scanning spectrophotometer was used for all absorbance measurements.

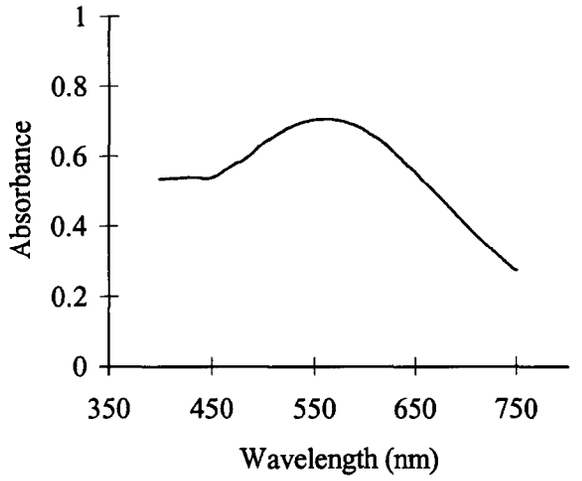

Fig. 2. Absorbance spectra for tannic acid (200 μg ml^{-1}) and ferric chloride (89 μM).

Absorbance spectra

The absorbance spectra of Fe (89 μM) in a tannic acid solution (200 μg ml^{-1}) at pH 4.4 was recorded over a wavelength range of 350–750 nm versus a reagent blank containing the tannic acid solution without the Fe.

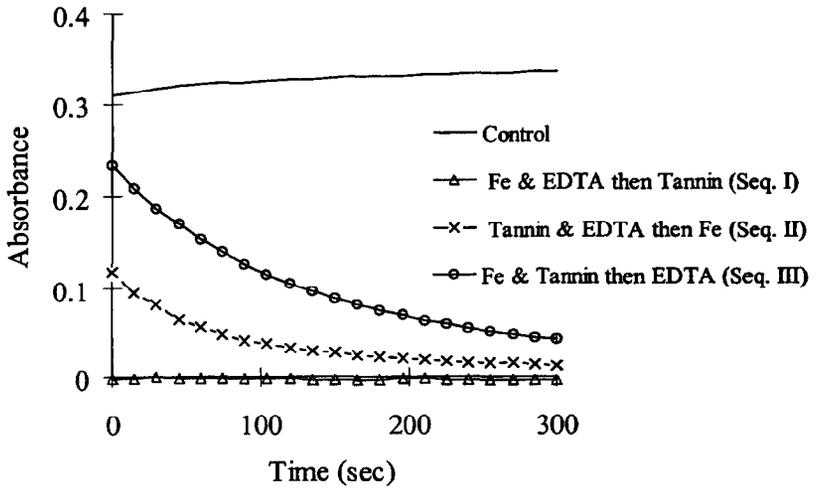

Fig. 3. Absorbance at 560 nm for tannic acid (200 μg ml^{-1}), ethylenediamine-tetraacetic acid (EDTA; 89 μM), and ferric chloride (89 μM) mixtures prepared with different addition sequences.

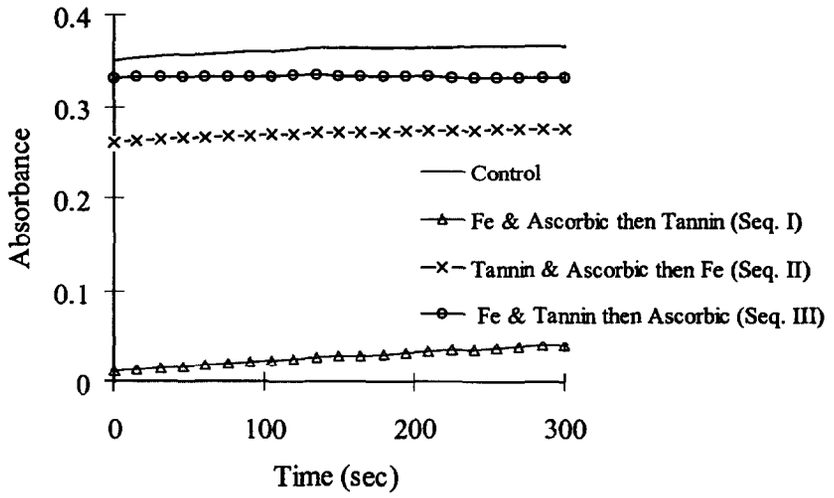

Fig. 4. Absorbance at 560 nm for tannic acid (200 μg ml^{-1}), ascorbic acid (89 μM), and ferric chloride (89 μM) mixtures prepared with different addition sequences.

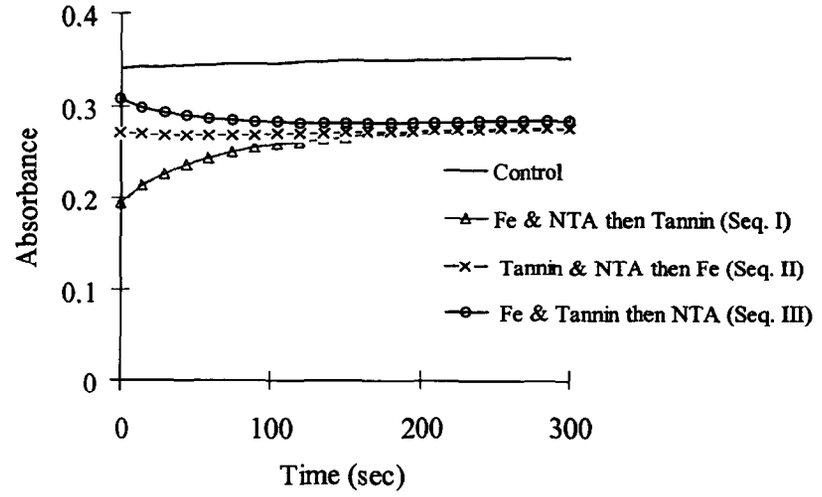

Fig. 5. Absorbance at 560 nm for tannic acid (200 μg ml^{-1}), nitrilotriacetic acid (NTA; 89 μM), and ferric chloride (89 μM) mixtures prepared with different addition sequences.

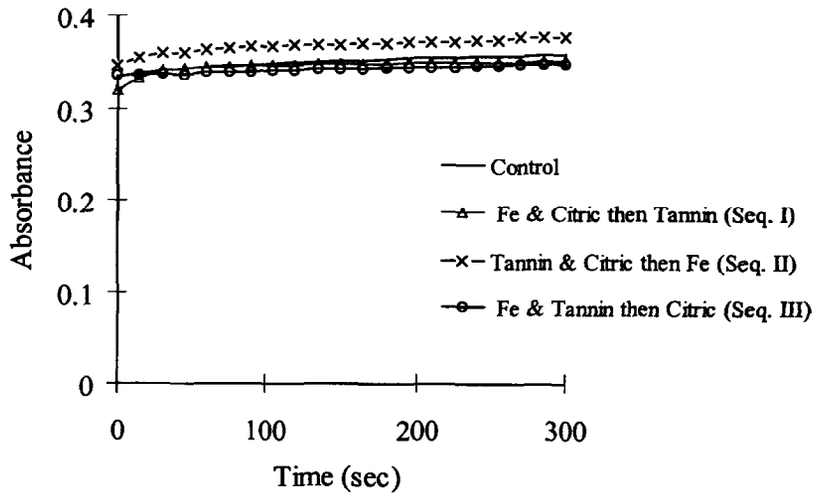

Fig. 6. Absorbance at 560 nm for tannic acid (200 μg ml^{-1}), citric acid (89 μM), and ferric chloride (89 μM) mixtures prepared with different addition sequences.

Sequence addition effects

Absorbances of the mixtures were determined at 560 nm immediately after mixing and at 15 s intervals for 5 min. The sequence of addition was varied as noted. Sequence I: 0.5 ml of the ligand solution was added to 0.5 ml of the Fe solution and the test tube was vortexed; 9.0 ml of the tannic acid solution was then added and the test tube was again vortexed. Sequence II: 0.5 ml of the ligand solution was added to 9.0 ml tannic acid and the test tube was vortexed; 0.5 ml of the Fe solution was then added and the test tube was again vortexed. Sequence III: 9.0 ml of the tannic acid solution was added to 0.5 ml of the Fe solution and the test tube was vortexed; 0.5 ml of the ligand solution was added and the test tube was again vortexed.

For all samples, absorbance was read versus a reagent blank containing 9 ml of the tannic acid solution, 0.5 ml of the corresponding ligand solution and 0.5 ml of 0.1 N HCl added in each respective sequence.

RESULTS AND DISCUSSION

Absorbance spectra

When ferric chloride and tannic acid solutions were combined, a violet color formed with an absorbance maximum of 560 nm (Fig. 2). Others have reported similar absorbance maxima for Fe–galloyl complexes (Disler *et al.*, 1975b; Brune *et al.*, 1991). Thus, the effect of different ligands on Fe–galloyl complex formation was determined by measuring absorbance at 560 nm.

Sequence addition effects

A Fe/tannic acid solution without added ligand served as the control for all treatments. The initial absorbances of the control were approximately 0.33 and increased slightly with time.

The addition of tannic acid to the Fe/EDTA solution (sequence I) resulted in no color formation over 5 min (Fig. 3). Presumably, the Fe present was chelated by EDTA and, thus, was unavailable to bind with tannic acid. In contrast, Fe added to the tannic acid/EDTA solution (sequence II) resulted in an initial absorbance of 0.11 with a gradual reduction in absorbance over time. Absorbance reduction indicated the disruption of the Fe–galloyl complex. The addition of EDTA to the Fe/tannic acid solution (sequence III) resulted in an initial absorbance of 0.23 with a gradual reduction in absorbance over time. Absorbance was higher than that found in sequence II, presumably because Fe–galloyl complexes were allowed to form prior to EDTA addition. In both sequences II and III the absorbance approached zero with time, indicating that the Fe–EDTA complex was more stable than the Fe–galloyl complex. Although initially there were large absorbance differences, after 5 min, absorbances for all mixing sequences were nearly zero.

When ascorbic acid was combined with Fe prior to tannic acid addition (sequence I), very little color formed, indicating complex formation was inhibited (Fig. 4). Presumably, ascorbic acid reduced complex formation by either chelating Fe, thereby making it unavailable to bind tannic acid, or by reducing Fe^{+3} to Fe^{+2}. Fe^{+2} complexes with ligands containing oxygen are less stable than Fe^{+3} complexes (Reddy *et al.*, 1986). When Fe was added to the ascorbic acid/tannic acid solution (sequence II), absorbance was initially 0.27. Again, ascorbic acid appeared to chelate or reduce Fe, inhibiting formation of the color complex. The addition of ascorbic acid to the Fe/tannic acid solution (sequence III) resulted in an absorbance of 0.33. Thus, Fe complexed with tannin appeared to be protected from reduction by ascorbic acid.

When tannic acid was added to an Fe/NTA solution (sequence I), absorbance was initially 0.19. Absorbance then increased and plateaued at an absorbance of 0.28 (Fig. 5). Fe added to a NTA/tannic acid solution (sequence II), had an initial absorbance of 0.28. Absorbance did not change with time. When NTA was added to an Fe/tannic acid solution (sequence III), absorbance was initially nearly equal to the control, but decreased with time, approaching the absorbances found in sequence I and II. After 2 min, all mixing sequences had an absorbance of 0.28.

Citric acid had little effect on absorbance (Fig. 6) regardless of the addition sequence.

EDTA, NTA and citrate are known Fe chelators. In our study, EDTA and NTA reduced absorbances of mixtures containing Fe and tannic acid, presumably by chelating Fe making it unavailable to bind with tannic acid. EDTA resulted in the greatest absorbance reduction, indicating it had a higher affinity for Fe than all other ligands employed. NTA reduced absorbance slightly, indicating it had much less affinity for Fe than EDTA. Citrate had little effect on absorbance indicating it had the lowest affinity for Fe. Stability constants for Fe–EDTA, Fe–NTA and Fe–citrate are $10^{25.10}$ (Hegenauer *et al.*, 1979), $10^{15.87}$ (Bates *et al.*, 1967) and $10^{9.46}$ (Warner and Weber, 1953), respectively. Therefore, our results are qualitatively consistent with previously reported stabilities of these complexes.

EDTA is a hexadentate ligand which forms stable chelates with metal ions. Sodium and calcium EDTA are recognized food additives and are used extensively to prevent oxidation and color changes in food. EDTA may also be employed in meals of low Fe bioavailability to increase Fe absorption. For example, Na_2EDTA added to meals of low Fe bioavailability at molar ratios of EDTA to Fe between 1.0 and 0.25 significantly increased Fe absorption (MacPhail *et al.*, 1994). In addition, Fe in the form of NaFeEDTA was found to be two to four times better absorbed than $FeSO_4$ in foods containing inhibitors of Fe absorption (Layrisse and

Martinez-Torres, 1977; Martinez-Torres *et al.*, 1979; MacPhail *et al.*, 1981). Thus, the use of EDTA to increase Fe absorption in diets of low Fe bioavailability has been proposed. There is concern, however, that increased consumption of EDTA could lead to depletion of other trace elements from the body. Studies have shown, however, that employing EDTA as NaFeEDTA has no detrimental effects on Zn, Cu, or Ca metabolism (Hurrell *et al.*, 1994).

Citric acid and NTA also chelate metal ions and both have been shown to increase Fe solubility (Kojima *et al.*, 1981; Leigh and Miller, 1983). The effect of citric acid on Fe absorption is unclear. For example, Hallberg and Rossander (1984) reported that citric acid decreased Fe absorption by one third, while Gillooly *et al.* (1983) found that citric acid increased Fe absorption over two-fold.

It is widely accepted that ascorbic acid increases Fe absorption (Monsen, 1988). For example, ascorbic acid was found to overcome the inhibitory effects of tannic acid addition to a meal (Siegenberg *et al.*, 1991). Ascorbic acid is both an Fe chelator and a strong reductant. As an Fe chelator, ascorbic acid increases Fe solubility. As a reductant, it reduces Fe^{+3} to the more soluble Fe^{+2} form. It may also prevent Fe^{+2} from oxidizing to Fe^{+3}. In addition, Fe^{+2} forms less stable complexes than Fe^{+3}. Thus, in our study, ascorbic acid presumably reduced Fe from Fe^{+3} to Fe^{+2} reducing the stability of the Fe–galloyl complex. We found, however, that ascorbic acid resulted in an absorbance reduction only when combined with Fe before mixing with tannic acid.

CONCLUSIONS

An effective promoter of Fe absorption should have a high affinity for Fe in order to compete with other ligands in the diet that decrease Fe absorption. Our findings suggest that EDTA might be an effective promoter of Fe absorption from meals containing inhibitors, because it can prevent Fe from complexing with inhibiting ligands and can remove Fe from complexes already formed.

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