



Voltammetric studies of Zn and Fe complexes of EDTA: Evidence for the push mechanism

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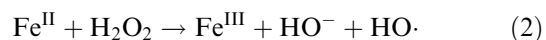
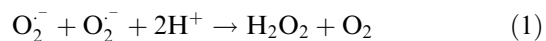
Abstract

The 'push' hypothesis for the antioxidant action of Zn^{2+} is based on its displacement of iron from a low molecular weight pro-oxidant complex. In this study, the chemical plausibility of that proposed function is investigated by cyclic voltammetry. As a model for a pro-oxidative low molecular weight iron complex the $\text{Fe}^{\text{II/III}}$ EDTA couple was examined. This complex was selected for its well-defined electrochemical, iron stability constants, and similarity to other low molecular weight chelates in physiological fluids in terms of logical binding sites, i.e. amino, and carboxylate groups. Also investigated were iron complexes of nitrilotriacetic acid and DL-glutamic acid. Results demonstrate that approximately 90% of the cyclic voltammetric peak current for Fe^{III} EDTA reduction and the EC' current for the mediated reduction of H_2O_2 by $\text{Fe}^{\text{II/III}}$ EDTA (Fenton Reaction) are lost when Zn^{2+} is introduced to a 1:1 molar ratio relative to iron. All experiments were conducted in HEPES buffered solutions at pH 7.4. Iron (II/III) complexes of nitrilotriacetic acid and DL-glutamic acid followed the same trends. Cyclic voltammetric experiments indicate that Zn^{2+} displaces Fe^{III} from EDTA despite the much larger stability constant for the iron complex ($10^{25.1}$) versus zinc ($10^{16.50}$). The hydrolysis aided displacement of Fe^{III} from EDTA by Zn^{2+} is considered by the equilibria modeling program, HySS. With Fe^{III} hydrolysis products included, Zn^{2+} is able to achieve 90% displacement of iron from EDTA, a result consistent with cyclic voltammetric observations.

Introduction

Zinc is increasingly being recognized as an important antioxidant. Besides its well-known role in Cu–Zn superoxide dismutase, zinc ions have been hypothesized as having a role in the control of biological oxidations (Chvapil *et al.* 1974; Chevion *et al.* 1990; DiSilvestro 2000; Powell 2000; Zago & Oteiza 2001; Rostan *et al.* 2002; Minqin *et al.* 2003). This property is not yet clearly defined, and puzzling since Zn^{2+} is a redox inactive ion. Present hypotheses have focused on the prevention of the sulhydryl group oxidation of certain enzymes, but also important is the moderation of the activity of transition metals involved with the generation of $\text{HO}\cdot$ from other reactive

oxygen species (Powell 2000). Of these metals, Fe because of its relative abundance in biological systems is perhaps the most prominent contributor to free radical damage. Such danger lies in the superoxide ion, H_2O_2 and the hydroxyl radical produced by the Fenton reaction (Babior 2000):



The hydroxyl radical has the ability to oxidize almost any organic species with diffusion-limited kinetics and is a significant agent in the pathogenesis of many diseases (Arouma & Halliwell 1988; Shi *et al.* 1991; Zhao *et al.* 1994; Smith *et al.* 1997; Crichton & Pierre 2002; Welch *et al.* 2002; Blokhina

et al. 2003). Most iron-containing enzymes are capable of Fenton reaction. With the release of HO radicals, oxidation of the organic ligand systems in these enzymes follows with release iron into a labile low molecular weight pool (Wiseman & Halliwell 1996; Avila *et al.* 2000; Collins 2002; Comporti *et al.* 2002; Kakhlon *et al.* 2002; Lehnen-Beyel *et al.* 2002; Petrat *et al.* 2002; Wand and De Montellano 2003). Because of a strong tendency towards chelation by a broad array of agents present in biological fluids such as nucleotides, DNA, peptides, and proteins free iron ions do not exist under physiological conditions (Weaver & Pollack 1989; Weaver & Pollack 1990; Zhan *et al.* 1990; Fahn & Cohen 1992; Sergent *et al.* 1997; Lipscomb *et al.* 1998; Powell 2000; Petrat *et al.* 2002). The ensuing metal complex releases HO· to the local environment, which causes site-specific damage near the immobilized metal (Chevion 1988). The 'push and pull' hypothesis for the mechanism of antioxidant actions is based on the abstraction of the transition metal by a high-binding constant chelator from its Fenton reaction active binding site (pull), or the displacement of the pro-oxidant metal by a redox inactive one (push) (Chevion 1991; Powell & Tortolani 1992; Powell 2000; Karch *et al.* 2001; Banin *et al.* 2003). In the case of Zn²⁺ the latter mechanism as hypothesized by Chevion would be applicable (Chevion 1991; Karch *et al.* 2001). Antagonism of Zn towards HO· production has been observed in biochemical and model systems (Searle & Tomasi 1982; Girotti *et al.* 1985).

In terms of ability to control iron-based oxidations, Zn²⁺ has been described as having the ability as to undergo a 'push' mechanism with iron complexes. Zinc ion is of intermediate hardness and has the ability to bind to ligands containing O,

N, and S donor atoms. Added to this flexibility is the ability to assume coordination numbers of 4, 5, and 6 (Bertini & Luchinat 1994). When compared with Fe²⁺, which is also of borderline hardness, the tendencies for binding are similar. Table 1 lists the binding constants for zinc and iron ions with a ligand that is a pure N donor (TREN), a pure O donor (citric acid), and a mix O, and N donor (EDTA). Only in the case of the pure N donor is there a significant difference in Zn²⁺ and Fe²⁺ binding. Ferric ions are hard in nature and under physiological conditions tend to bind O donor atoms. Ferric ions show a much greater tendency to bind to citrate and EDTA in comparison to Fe²⁺ and Zn²⁺.

Physiological fluids present an array of possibilities for the binding of metal ions e.g., proteins, nucleic acids, nucleotides, and citrate (Weaver & Pollack 1989; Weaver *et al.* 1990; Zhan *et al.* 1990; Fahn and Cohen 1992; Sergent *et al.* 1997; Lipscomb *et al.* 1998). The majority of these chelating agents contain the hard O donor atom with some contribution from the softer N atom. It is for this reason that EDTA was used as a model in this investigation (see Table 1 for EDTA structure).

When examining Table 1 it is not all clear that Zn²⁺ would be able to push either Fe^{II} or Fe^{III} from its Fenton reaction active chelation sphere. Only the TREN complex stands as a clear example where the Zn²⁺ would be predicted by binding constant considerations to displace Fe^{II}. However, another consideration is the hydrolysis of both Fe²⁺ and Fe³⁺. We shall see that even in the case of EDTA (10^{16.5} versus 10^{25.1}) complexes, where there is 9 order of magnitude preference for iron it is still possible for Zn²⁺ to push Fe^{III} from its chelation sphere

Table 1. Stability constants for the complexes considered in this study.

Log β_1	Zn ²⁺	Fe ²⁺	Fe ³⁺
Nitrilotris (2-ethylamine) (TREN) N(CH ₂ CH ₂ NH ₂) ₃	14.6	8.8	—
Glutamic Acid (Glu) (HOOC)CH ₂ CH ₂ CH(COOH, NH ₃)	4.72	3.52	12.1
Citric acid HOOCCH ₂ CH(OH, COOH)-CH ₂ COOH	4.27	4.4	11.5
Ethylenediaminetetraacetic acid (EDTA) (HOOCCH ₂) ₂ NCH ₂ CH ₂ N(CH ₂ COOH) ₂	16.5	14.32	25.1
Nitrilotriacetic acid (NTA) N(CH ₂ COOH) ₃	10.45	8.90	15.9
DNA	N/A	4*	14**

*(Bingham *et al.* 1994)

** (Marzilli 1981; Netto *et al.* 1991)

Experimental

All chemicals were of the highest purity available and obtained from the following sources: $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$, anhydrous citric acid, $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, 30% aqueous H_2O_2 , were from Fisher Scientific, USA. Ferrous sulfate heptahydrate was obtained from J.T. Baker (Phillipsburg, NJ). Ethylenediaminetetraacetic acid tetrasodium salt (99%, EDTA), nitrilotriacetic acid (NTA, 99%) DL-glutamic acid monohydrate (glu, 99%), were purchased from Acros, USA.

Electrochemical studies were conducted on a Bioanalytical Systems CV-50w potentiostat. A 5 mm glassy carbon working electrode, and a Ag/AgCl reference electrode were also from Bioanalytical Systems (West Lafayette, IN). The counter electrode was a spectroscopic grade graphite rod.

Solutions of $\text{Fe}^{\text{II}}\text{EDTA}$, $\text{Fe}^{\text{III}}\text{EDTA}$ and $\text{Fe}^{\text{III}}\text{citrate}$, were made by mixing 1:1 molar solutions of the chelate and metal ion solutions. Solutions of Fe^{II} complexes were made freshly in N_2 sparged solutions just prior to their use in experiments. All stock and test solutions were made up in 0.10 M HEPES(aq) buffer adjusted to pH 7.4. For $\text{Fe}^{\text{III}}\text{NTA}$, all stock and test solutions were made up in 0.010 M HEPES(aq) buffer adjusted to pH 7.4 and 0.10 M NaNO_3 (aq).

Electrochemical experiments: A 1 ml aliquot of 10 mM $\text{Fe}^{\text{II}}\text{EDTA}$ or $\text{Fe}^{\text{III}}\text{EDTA}$ (1:1) and 1 ml or less of 10 mM $\text{Zn}(\text{NO}_3)_2$ (aq) were added

to 8 ml of aqueous HEPES buffer and purged with N_2 for 10–15 min. The final volume was adjusted to 10 ml with distilled water. Cyclic voltammetry was then conducted at 25, 10, and 5 mV/s from 400 mV to –700 mV ($\text{Fe}^{\text{III}}\text{EDTA}$ solutions) and –700 mV to 400 mV ($\text{Fe}^{\text{II}}\text{EDTA}$ solutions).

Electrocatalytic experiments: A 1 ml aliquot of 1 mM $\text{FeEDTA}(\text{aq})$ (1:1) and 1 ml or less of 1 mM $\text{Zn}(\text{NO}_3)_2(\text{aq})$ were added to 7 ml of aqueous buffer solution and purged with N_2 for 10–15 min. After which 1 ml of 230 mM $\text{H}_2\text{O}_2(\text{aq})$ (purged) was added. The final volume was adjusted to 10 ml with distilled water. Cyclic voltammetry was then conducted at 25, 10 and 5 mV/s from 400mV to –700 mV.

Generation of pH Distribution Diagram: All metal complex speciation diagrams were generated by the computer program Hyperquad Speciation and Simulation, HySS (Alderighi et al. 1999).

Results

Figure 1 demonstrates that the cyclic voltammetric characteristics for the $\text{Fe}^{\text{II/III}}\text{EDTA}$ are quasi-reversible with a difference in peak current potentials of 65 mV and a half-wave potential of –120 mV *versus* Ag/AgCl. At a molar ratio of 1:1 for Zn^{2+} : $\text{Fe}^{\text{III}}\text{EDTA}$ the peak currents are reduced by approximately 90% for the $\text{Fe}^{\text{II/III}}$

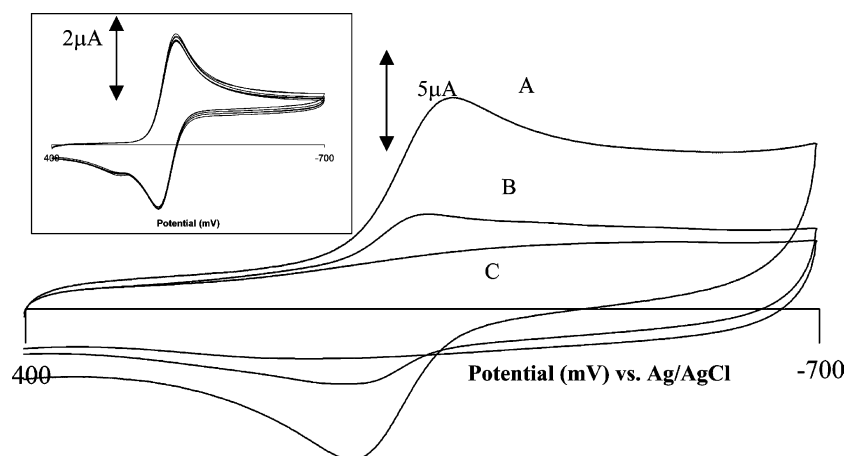
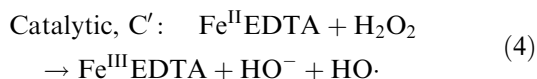
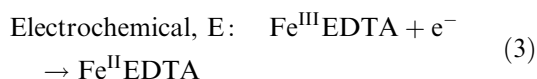


Figure 1. Cyclic voltammogram of 1.0 mM $\text{Fe}^{\text{III}}\text{EDTA}_{(\text{aq})}$ in the presence of (A) 0 mM Zn^{2+} , (B) 1 mM Zn^{2+} , (C) Background current, buffer only. Other conditions 0.1 M HEPES and pH 7.4.

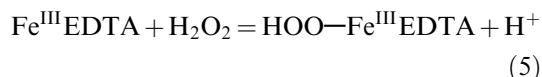
Inset: Multiple cyclic voltammograms of 1 mM $\text{Fe}^{\text{III}}\text{EDTA}$ (1:1) in the presence of 0.5 mM Zn^{2+} . Continuous scan taken on same sample over the course of 2 h. Other conditions pH 7.4 0.1 M HEPES, 5 mV/s sweep rate.

EDTA CV waves (Curves 1A and 1B). The apparent loss of the iron complex was observed when Zn^{2+} is added to either a solution of pure $\text{Fe}^{\text{III}}\text{EDTA}$ or one of $\text{Fe}^{\text{II}}\text{EDTA}$ (not shown). In the case of the former, brown precipitates, presumably insoluble iron hydroxides formed after 30 min. Nearly identical CV results were found for NTA, citrate, and glutamate ligands. The inset for Figure 1 demonstrates that the kinetics of Fe^{3+} displacement (push) from EDTA by Zn^{2+} is facile and is almost immediate between the additions of Zn^{2+} to the first CV experiment (< 5 min). The molar ratio of $\text{Zn}^{2+}:\text{Fe}^{\text{III}}\text{EDTA}$ is 0.5:1 in the inset. The peak current for the reduction for $\text{Fe}^{\text{III}}\text{EDTA}$ reduction in Figure 1 inset is reduced by 30% when compared to Figure 1a. The amplitude of the cyclic voltammetric peak currents is constant over the course of 2 h indicating very little loss of the $\text{Fe}^{\text{III}}\text{EDTA}$ complex after the initial addition of Zn^{2+} . No precipitates were observed to form during this time.

Figure 2 demonstrates that the of the Fenton reaction activity of $\text{Fe}^{\text{II}}\text{EDTA}$ is lost with increasing mole ratio of $\text{Zn}^{2+}:\text{Fe}^{\text{III}}\text{EDTA}$. The inset exhibits an amplified cathodic wave, which is produced by the electrochemical-catalytic (EC') electrode reaction show below.



Reaction 3 produces the Fe^{II} complex, which is consumed in the Fenton reaction (Reaction 4). The production of the reactant $\text{Fe}^{\text{III}}\text{EDTA}$ near the electrode surface enhances the cathodic current observed by Reaction 3. The absence of an anodic wave is due to the complete consumption of $\text{Fe}^{\text{II}}\text{EDTA}$ in reaction 4. The $\text{Fe}^{\text{II/III}}\text{EDTA}$ couple acts as the redox cycling intermediate for the reduction of H_2O_2 . Curves A and B in Figure 2 are in a region where the peroxy- $\text{Fe}^{\text{III}}\text{EDTA}$ ($\beta \approx 10^{22}$) intermediate predominates from the following equilibrium:



The formation of the $\text{HOO}-\text{Fe}^{\text{III}}\text{EDTA}$ greatly enhances the EC' current as evidenced by the comparison of the inset and Figure 2 Curve A (Babko & Loriya 1968; Smith & Martell 1975; Francis *et al.* 1985; Koch & Ackermann 1985;

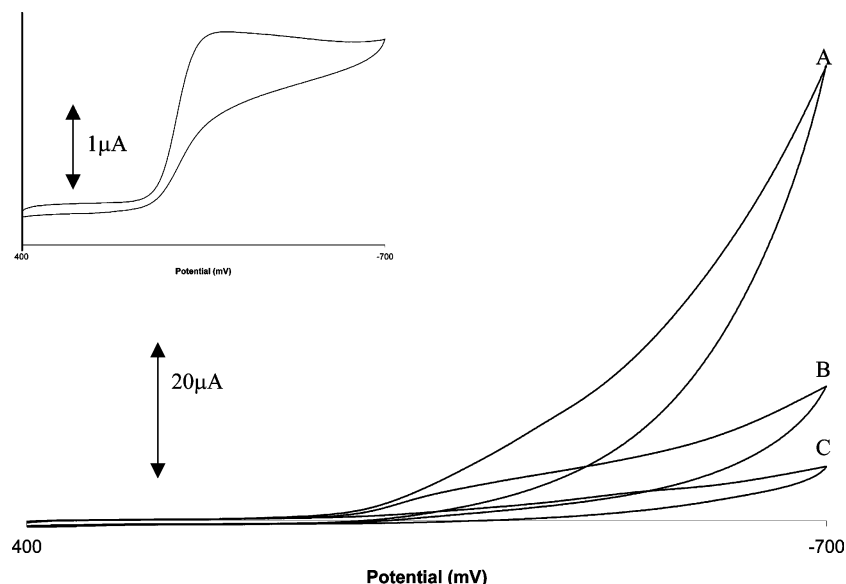


Figure 2. Electrocatalytic (EC') reduction of H_2O_2 in the presence of various $(\text{Fe}^{3+}):(\text{EDTA}):(\text{Zn}^{2+})$. (A) (1):(1):(0), (B) (1):(1):(1), (C) blank (0):(0):(0). Other conditions 22.8 mM H_2O_2 , and pH 7.4, 10 mV/s sweep rate. Inset: Electrocatalytic reduction of 1 mM H_2O_2 in the presence of 0.1 mM $\text{Fe}^{\text{III}}\text{EDTA}$.

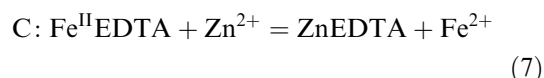
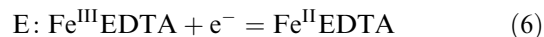
Engelmann *et al.* 2003). Figure 2 Curve B demonstrates that the Fenton reaction characteristics are nearly lost in the presence of an equivalent amount of Zn^{2+} . The observed catalytic current drops by 80%. Curve 2C is control current for the direct reduction of H_2O_2 in the absence of the $\text{Fe}^{\text{II/III}}$ /EDTA couple. Again, this is consistent with the push mechanism for antioxidant behavior proposed in Chevion's hypothesis (Chevion 1991; Karck *et al.* 2001).

Three other chelates were examined, citrate, NTA, and Glu. All three $\text{Fe}^{\text{III/II}}$ complexes exhibited EC' characteristics very similar to $\text{Fe}^{\text{II/III}}$ EDTA. The EC' currents for each complex was measured at -700 mV *versus* Ag/AgCl are shown in Figure 3 which demonstrates that Zn^{2+} again suppresses the EC' current that arises from the Fenton reaction (Equation 4).

Discussion

The possibility for the displacement of Fe^{III} from EDTA is not predicted by simply considering the EDTA binding constants for Zn^{2+} and Fe^{3+} (see Table 1). Conceivably, there may be two logical mechanisms by which Zn^{2+} is able to interfere with the EC' voltammetric waves produced by the

Reactions 3 and 4 sequence. The first would be the displacement of Fe^{II} from EDTA by Zn^{2+} as predicted by the stability constants of Table 1. This would manifest itself as an EC (electrochemical-chemical) type of mechanism (Bard & Faulkner 2001).



However, none of the applied voltammetric diagnostics indicated this mechanism (Bard & Faulkner 2001). Perhaps the most significant indication that this was not an EC mechanism is that CV wave for the reduction of $\text{Fe}^{\text{III}}\text{EDTA}$ (Reaction 6) is lost immediately upon addition of Zn^{2+} . The displacement of both Fe^{III} and Fe^{II} from EDTA is demonstrated by experiments described by Figures 1 and 2. Moreover, this effect was observed with the iron complexes of citrate and NTA (Figure 3). The possibility for the displacement of Fe^{III} from EDTA by Zn^{2+} may lie in the hydrolysis aided replacement reaction:

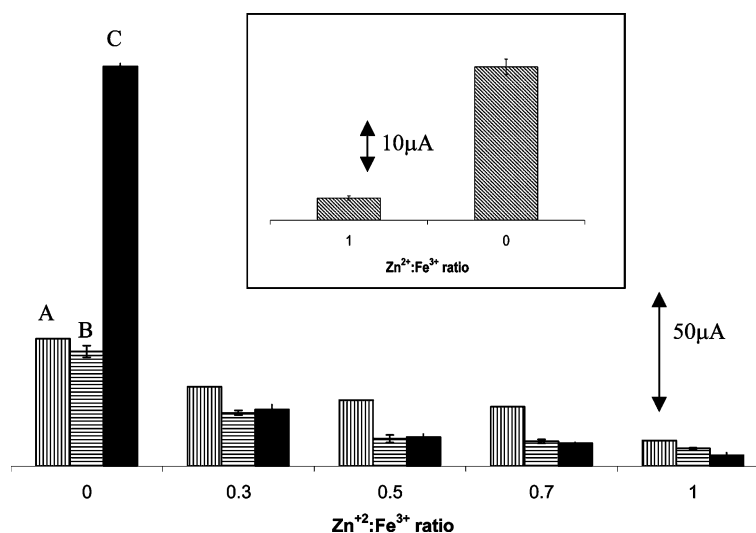
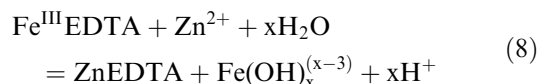


Figure 3. Suppression of Fenton reactivity of Fe complexes by addition of Zn^{2+} . The electrocatalytic reduction current, of H_2O_2 , was measured at -700 mV *versus* Ag/AgCl. (A) 1:1 [Fe^{3+}]:[EDTA], (B) 1:1 [Fe^{3+}]:[citrate], (C) 1:2 [Fe^{3+}]:[NTA]. Inset: 0.1 mM Fe^{III} Glu in the presence of various amounts of Zn^{2+} . Other common conditions; 0.1 mM Fe^{3+} , 0.1 M HEPES pH 7.4, and 10 mV/s. Each bar represents the average of three runs. The error bars represent one standard deviation.

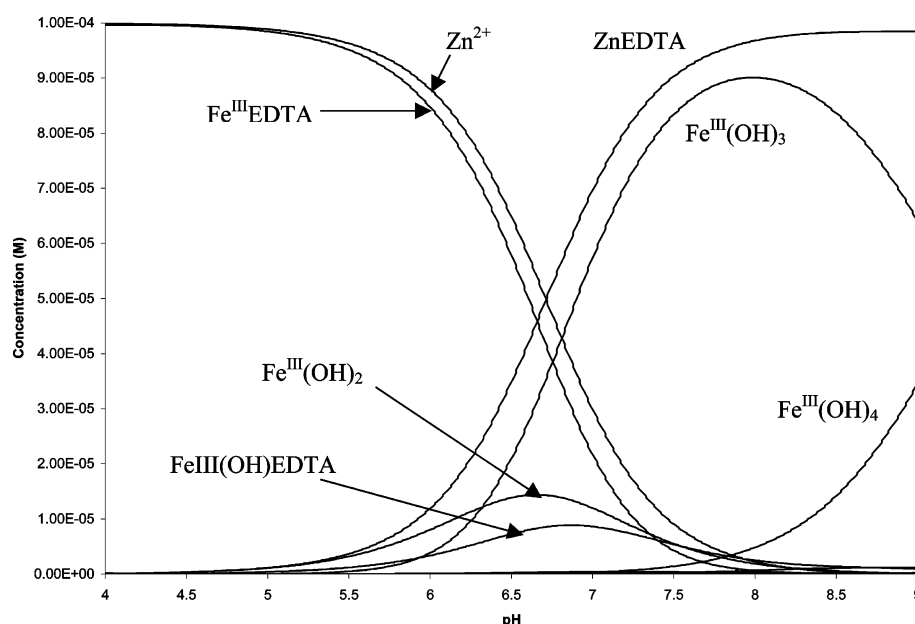


Figure 4. Speciation diagram of 0.1 mM $\text{Fe}^{\text{III}}\text{EDTA}_{(\text{aq})}$ in the presence of 0.1 mM $\text{Zn}^{+2}_{(\text{aq})}$. Insoluble species are omitted due to slow kinetics. The diagram was produced with HySS. See Table 2 for the species considered in the model.

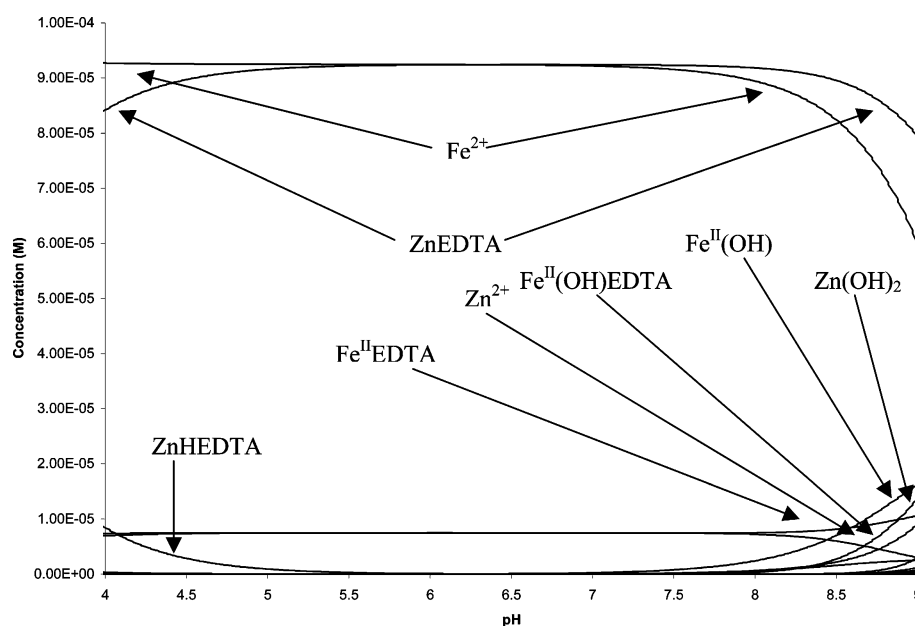


Figure 5. Speciation diagram of 0.1 mM $\text{Fe}^{\text{II}}\text{EDTA}_{(\text{aq})}$ in the presence of 0.1 mM $\text{Zn}^{+2}_{(\text{aq})}$. Insoluble species are omitted due to slow kinetics. The diagram was produced with HySS. See Table 2 for the species considered in the model.

The pH speciation diagrams using accepted stability constants are presented for Zn^{2+} in the presence of $\text{Fe}^{\text{III}}\text{EDTA}$ and $\text{Fe}^{\text{II}}\text{EDTA}$ are presented in Figures 4 and 5, respectively (Smith & Martell 1975). Table 2 lists the compounds considered and their respective stability constants.

Formation of insoluble iron species were omitted because of the observation that no precipitates were observed to form during the time scales of the CV experiments ($RT/vnF = 5$ sec) illustrated in Figures 1–3 (Bard & Faulkner 2001). A 1:1 molar ratio of $\text{Zn}^{2+}:\text{Fe}^{\text{III}}\text{EDTA}$ indicates that at pH 7.4

Table 2. Species considered for the Zn^{2+} displacement of Fe from EDTA with HySS in Figures 4 and 5.

Species considered for the 1:1 molar ratio of Zn^{2+} with $\text{Fe}^{\text{III}}\text{EDTA}$	Log β	Species considered for the 1:1 molar ratio of Zn^{2+} with $\text{Fe}^{\text{II}}\text{EDTA}$	Log β	Species considered for both studies	Log β
$\text{Fe}^{\text{III}}(\text{OH})^{2+}$	-2.6	$\text{Fe}^{\text{II}}(\text{OH})^+$	-9.27	OH^-	-13.77
$\text{Fe}^{\text{III}}(\text{OH})_2^+$	-5.34	$\text{Fe}^{\text{II}}(\text{OH})_2$	-20.34	HEDTA^{3-}	10.17
$\text{Fe}^{\text{III}}(\text{OH})_3$	-11.51	$\text{Fe}^{\text{II}}(\text{OH})_3^-$	-31.31	$\text{H}_2\text{EDTA}^{2-}$	16.28
$\text{Fe}^{\text{III}}(\text{OH})_4^-$	-20.88	$\text{Fe}^{\text{II}}(\text{OH})_4^{2-}$	-45.48	H_3EDTA^-	18.96
$(\text{Fe}^{\text{III}})_2(\text{OH})_3^{3+}$	-2.84	$\text{Fe}^{\text{II}}\text{EDTA}^{2-}$	14.32	H_4EDTA	20.96
$(\text{Fe}^{\text{III}})_4(\text{OH})_3^{9+}$	-5.38	$\text{Fe}^{\text{II}}\text{HEDTA}^-$	17.07	H_5EDTA^+	22.46
$\text{Fe}^{\text{III}}\text{EDTA}^-$	25.1	$\text{Fe}^{\text{II}}\text{H}_2\text{EDTA}$	19.54	$\text{H}_6\text{EDTA}^{2+}$	22.46
$\text{Fe}^{\text{III}}\text{HEDTA}$	26.4	$\text{Fe}^{\text{II}}(\text{OH})\text{EDTA}^{3-}$	5.25	$\text{Zn}(\text{OH})^+$	-9.07
$\text{Fe}^{\text{III}}(\text{OH})\text{EDTA}^{2-}$	17.61	$\text{Fe}^{\text{II}}(\text{OH})_2\text{EDTA}^{4-}$	-4.59	$\text{Zn}(\text{OH})_2$	-15.34
$\text{Fe}^{\text{III}}(\text{OH})_2\text{EDTA}^{3-}$	8.2			$\text{Zn}(\text{OH})_3^-$	-27.18
				$\text{Zn}(\text{OH})_4^{2-}$	-39.58
				$\text{Zn}_2(\text{OH})^{3+}$	-8.27
				$\text{Zn}_4(\text{OH})_4^{4+}$	-27.18
				ZnEDTA^{2-}	16.5
				ZnHEDTA^-	19.5
				ZnH_2EDTA	20.7
				$\text{Zn}(\text{OH})\text{EDTA}^{3-}$	4.83

approximately 90% of the iron complex is lost due to Reaction 8. This matches the CV study of Figure 1 where the peak current for $\text{Fe}^{\text{III}}\text{EDTA}$ reduction is reduced by 90% and the study EC' voltammetric study of Figure 2 where that current was reduced by 80%. In the case of $\text{Fe}^{\text{II}}\text{EDTA}$ hydrolysis of iron is not a major contribution to the displacement of iron by Zn^{2+} . At pH 7.4, Figure 4 illustrates that the iron complex is completely lost in a 1:1 molar ratio of Zn^{2+} : $\text{Fe}^{\text{II}}\text{EDTA}$ producing free Fe^{2+} .

The experiments conducted for this investigation add evidence to the 'push' mechanism for Zn^{2+} antioxidant action as hypothesized by Chevion (Chevion 1988; Chevion *et al.* 1990; Chevion 1991; Karck *et al.* 2001). However for the push mechanism to have physiological pertinence it must be part of an ensemble of actions that lead to an overall antioxidant effect. As iron is displaced by Zn^{2+} , from the pro-oxidant complex another chelate with an overall antioxidant effect must take up the iron, otherwise it will simply re-enter the pro-oxidant low molecular weight complexation pool (Petrat *et al.* 2002; Welch *et al.* 2002). Such antioxidant effect may affect the oxygen-activating, or Fenton reaction characteristics of the $\text{Fe}^{\text{II/III}}$ center through kinetic or thermodynamic means (Babko & Loriya 1968; Cheng

et al. 1996; Cheng & Breen 2000). The possibilities for this effect may lie in the physiologically occurring iron transferring protein, transferring, which has antioxidant characteristics (Hubel *et al.* 1996; Loban *et al.* 1997; Sahlstedt *et al.* 2002; van Campenhout *et al.* 2003). Another possibility, is that iron may be complexed by non-nutritive dietary or medicinal chelates such as the flavonoids, which are now being recognized as an important class of antioxidant (Arouma & Halliwell 1988; Cheng *et al.* 1996; van Acker *et al.* 1996; Yoshino & Murakami 1998; Cheng & Breen 2000; Mellican *et al.* 2003).

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