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N,N′-BIS-DIBENZYL ETHYLENEDIAMINEDIACETIC ACID (DBED): A SITE-SPECIFIC HYDROXYL RADICAL SCAVENGER ACTING AS AN "OXIDATIVE STRESS ACTIVATABLE" IRON CHELATOR *IN VITRO*

JEAN-BAPTISTE GALEY*, JACQUELINE DUMATS, IRÉNA BECK, BONA FERNANDEZ and MICHEL HOCQUAUX

L'Oréal Basic Research Center, 1 avenue Eugène Schueller, 93600 Aulnay sous bois, France

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During oxidative stress, iron traces are supposed to be released from normal storage sites and to catalyse oxidative damage by Fenton-type reactions. This type of damage is difficult to prevent *in vivo* except by the use of strong iron chelators such as deferoxamine (affinity constant for Fe(III): log K = 30.8). However, strong iron chelating agents are also suspected to mobilize iron from various storage and transport proteins thereby leading to toxic effects. In contrast, N,N′-bis-dibenzyl ethylenediaminediacetic acid (DBED) is an iron chelator with relatively low affinity for iron (affinity constant for Fe(III): log K < 15). In the present paper, we show that, in situations mimicking oxidative stress *in vitro*, DBED is site-specifically oxidized into new species with strong iron binding capacity. Indeed, in the presence of ascorbate as a reductant, the iron chelate of DBED reacts with H_2O_2 in aqueous solution to yield a purple chromophore with minor release of free HO· in the medium, as measured by aromatic hydroxylation assay. The formation of these purple species is not suppressed by the presence of HO· scavengers at high concentration. The visible spectrum of these species is consistent with a charge transfer band from a phenolate ligand to iron. N-2-hydroxybenzyl N′-benzyl ethylenediaminediacetic acid (HBBED) was identified in the medium as one of the oxidation products of DBED. Therefore, these results suggest that the iron chelate of DBED undergoes an intramolecular aromatic hydroxylation by HO· leading to 2-OH derivatives and hence that DBED is a site-specific HO· scavenger. Moreover, since the measured affinity for Fe(III) of HBBED (log K = 28) is at least 13 orders of magnitude higher than that of DBED and since ferric HBBED chelate is not a catalyst of Fenton chemistry, DBED may be looked as an "oxidative stress activatable" iron chelator, e.g. which increase in affinity for iron is triggered in the presence of H_2O_2 and an electron donor. Therefore it is proposed that DBED and related derivatives may be interesting as protective compounds against oxygen radicals toxicity, especially for chronic use.

KEY WORDS: Oxidative stress, iron chelator, hydroxyl radical, site specific reaction, intramolecular hydroxylation, hydrogen peroxide.

INTRODUCTION

Reactive oxygen species such as superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) are now widely recognized as an important source of tissue damage in various human diseases.[1-6] Their generation can be purposeful[1] e.g. by activated phagocytic cells, or

* To whom correspondence should be addressed.

accidental, e.g. by leakage from electron transport chain,[3] ischemia reperfusion injury, ultraviolet light exposure[7] or during the metabolism of various xenobiotics. Superoxide is converted by superoxide dismutase (SOD)[8] or by spontaneous dismutation into hydrogen peroxide [reaction (1)]. Hydrogen peroxide is detoxified either by catalase or by glutathione peroxidase with the expense of glutathione so that the steady state level of H_2O_2 is maintained[3] in the range $10^{-9} - 10^{-7}$ M. However, during oxidative stress, their is a disturbance in the prooxidant-antioxidant balance.[6] Traces of redox active metal ions, especially iron, are supposed to be released from their normal storage protein such as ferritin, and then participate into Fenton chemistry.[9,10] Most of damage done by superoxide and hydrogen peroxide is in fact thought to be due to their conversion into hydroxyl radical (HO·) by iron catalysed reactions such as Haber-Weiss reaction[11-13] [(reactions (2) and (4)]. Reductants present in cells, such as ascorbate (AH^-) are also suspected to be able to replace superoxide in the reaction (2) and therefore to drive Fenton chemistry[14,15] [reactions (3) and(4)].

$$2\,O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (1)$$

$$Fe^{3+} + O_2^{\cdot -} \longrightarrow Fe^{2+} + O_2 \quad (2)$$

$$Fe^{3+} + AH^- \longrightarrow Fe^{2+} + A^{\cdot -} \quad (3)$$

$$H_2O_2 + Fe^{2+} \longrightarrow HO^- + Fe^{3+} + HO\cdot \quad (4)\ \text{(Fenton reaction)}$$

HO· reacts with biological molecules by hydroxylation, hydrogen abstraction or electron transfer reaction at diffusion limited rates. Furthermore, most reactions involving oxidative damage to proteins and DNA are thought to be site specific e.g. the metal ion is bound to the target[11,15,16] and HO· directly reacts at the place where it is formed.

During oxidative insults, both cell structure and function are dramatically affected, including DNA strand breakage, lipid peroxidation, damage to proteins, and rise in intracellular calcium leading to activation of proteases and nucleases.[1]

A wide range of both natural and synthetic antioxidants have been proposed for use in the treatment of human diseases. Direct scavenging of free HO· is however a very unlikely mechanism of antioxidant action *in vivo* since hydroxyl radical reacts with almost all organic molecules[17] with second order rate constants of $10^9 - 10^{10}\,M^{-1}.s^{-1}$. Therefore very high concentration of scavenger would be required[2] to compete with biological molecules for any HO· generated. Most administered antioxidants never achieve such high concentration *in vivo*, so they cannot scavenge free HO·. In other respects, iron chelating agents may afford protection by at least three mechanisms: first by altering redox potential of iron,[18] second by suppressing its accessibility to superoxide and/or hydrogen peroxide[19] and third by directing redox reaction on to the chelator itself, thereby sparing more important targets.[11]

Some chelating agents, such as EDTA (ethylenediaminetetraacetic acid) or NTA (nitrilotriacetic acid), do not inhibit hydroxyl radical formation, but they may, in some situations, have an indirect protective effect by avoiding site specific reactions to especially sensitive targets which bind iron such as DNA e.g. by withdrawing iron from it and directing damage to other less important targets.

Various examples of inactivating iron chelators are known: deferoxamine for instance mainly restricts the accessibility of iron,[20,21] whereas o-phenantroline modifies its redox potential.[18] On the other hand, although some molecules are

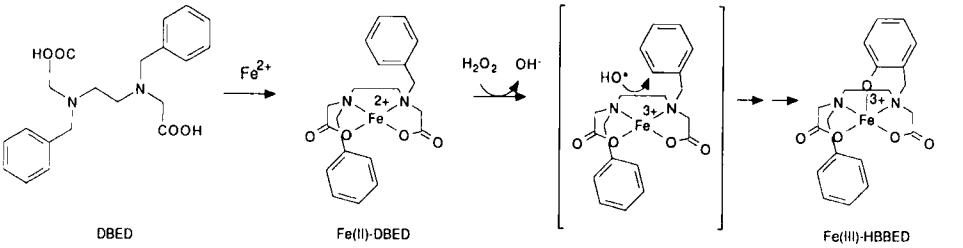

FIGURE 1 Proposed reaction scheme between Fe(II)-DBED and H_2O_2 (axial ligands have been omitted for clarity).

reported to direct radical attack on to themselves,[2,22] their is, to our knowledge, no rational design for such "scavenging chelators."

At the opposite, the iron complexes of EDTA and related molecules such as EDDA (ethylenediaminediacetic acid) are very good catalysts of ascorbate or superoxide-driven Fenton chemistry.[23,24,25] Although this reaction is not understood in its details,[26,27] it is likely that free HO· is formed under certain conditions and that most HO· escapes from the coordination sphere and few reacts with the ligand itself.

In our search for protective drugs against oxygen radicals toxicity, we have been interested in EDDA derivatives bearing benzyl moieties that could conceptually be effective site specific HO· scavengers. As a matter of fact, it is well known that HO· is a very efficient hydroxylation reagent of aromatic rings.[16] We have therefore selected N,N′-bis-dibenzyl ethylenediaminediacetic acid (DBED) as a promising scavenging chelator. Considering steric features of the iron (III) DBED chelate, intramolecular aromatic hydroxylation in position 2 of the phenyl moiety was a very likely process (Figure 1). Furthermore, this reaction would lead to phenolate ligand able to occupy a fifth or even a sixth coordination site of iron. Owing to the high affinity of Fe(III) for phenolate ligands,[28] it was hoped that the resulting chelate would have an increased association constant. Indeed, it is known that the ferric chelate of HBED [N,N′-bis-di(2-hydroxybenzyl) ethylenediaminediacetic acid (Figure 2)], has a much higher association constant than EDTA (log K of respectively 39.7 and 25).[29] Moreover, since iron(III)-HBED chelate is not a catalyst of Fenton chemistry (vide infra), it was likely that the resulting chelate would also lack catalytic properties.

This paper describes the first results obtained with this scavenging chelator. To our knowledge, this strategy has not been reported so far as a protective approach against oxidative stress.

MATERIAL AND METHODS

Material

Sodium ascorbate, ferrous ammonium sulfate, potassium nitrate, bromoacetic acid, ethylenediaminediacetic acid, potassium dihydrogen phosphate, and dipotassium hydrogen phosphate were purchased from Fluka. Ferric chloride (hexahydrate), hydrogen peroxide (3% solution), ethylenediaminetetraacetic acid tetrasodium salt,

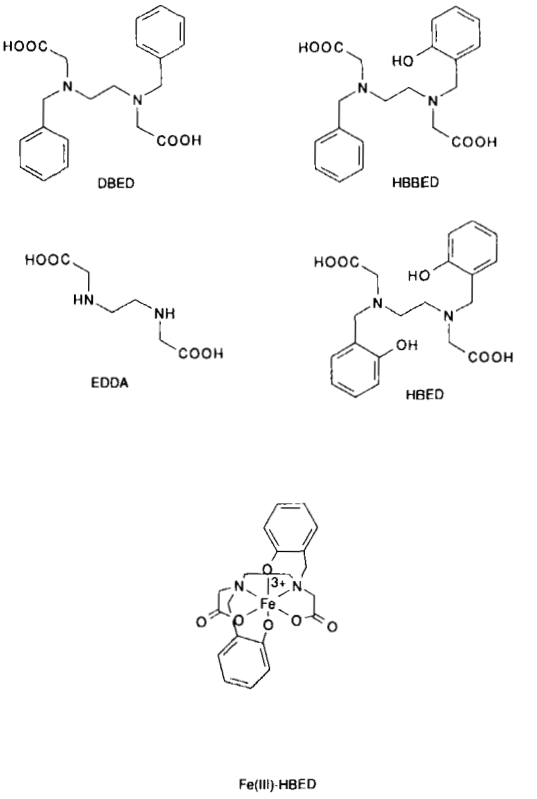

FIGURE 2 Structures of the iron chelate of N,N′-bis-di(2-hydroxybenzyl) ethylenediamine diacetic acid (HBED) and of the ligands cited: N,N′-bis-dibenzyl ethylenediamine diacetic acid (DBED), N-(2-hydroxy)-N′-benzyl ethylenediamine diacetic acid (HBBED), and ethylenediamine diacetic acid (EDDA).

and catalase from bovine liver were purchased from Sigma, ethylenediamine from Prolabo, N-benzylethylenediamine from Lancaster and 2-hydroxy benzaldehyde and sodium borohydride were purchased from Aldrich. Water used was bidistilled water for injection use from Biosedra (Paris, France). Other solvents were of the highest purity available and were used without further purification. UV visible spectra were recorded with a Varian Cary13 spectrophotometer. HPLC analysis were performed with a Waters Model 510 pump equipped with a Waters 990 photodiode array detector or a Waters Lambda Max Model 480 detector.

Synthesis of Ligands

DBED and HBED were synthetised by adaptation of reported procedures.[29,30,31] HBBED was synthetized as outlined in Figure 3.

N-(2-hydroxybenzyl)N′-benzylidene ethylenediamine. In a three necked flask equipped with a magnetic stirrer, 8.13 g (66.6 mmoles) of 2-hydroxybenzaldehyde

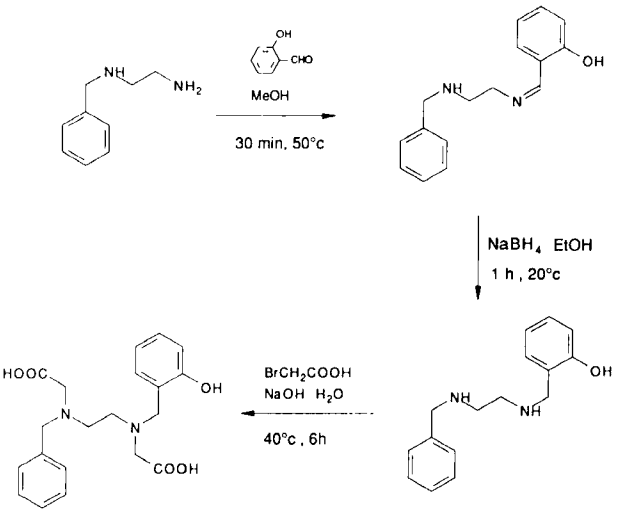

FIGURE 3 Reaction scheme used for the synthesis of HBBED.

was dissolved in 100 ml of methanol, to which 10.0 g (66.6 mmoles) N-benzyl ethylenediamine was added. The reaction mixture was stirred 30 min at 50°C and then allowed to stand at room temperature. Methanol was then evaporated under reduced pressure to yield 17 g of crude N-(2-hydroxybenzylidene),N′-benzyl ethylenediamine as a yellow oil. El-MS m/e 254 (M^+), 91 ($C_7H_7^+$).

N-(2-hydroxybenzyl) N′-benzyl ethylenediamine. 17.0 g (66.6 mmoles) N-(2-hydrobenzylidene) N′-benzyl ethylenediamine was suspended in 150 ml ethanol to which 1.3 g (34 mmoles) of $NaBH_4$ was added portionwise. The reaction mixture was stirred at room temperature for 1 hr. The solvent was removed by distillation. To the residue, 100 ml of water was added and concentrated HCl was used to adjust the pH of the solution to 1. The crystalline dihydrochloride was collected by filtration, washed with cold ethanol and vacuum dried over P_2O_5 to yield 13.85 g (81%) of N-(2-hydroxybenzyl) N′-benzyl ethylenediamine. El-MS m/e 256 (M^+), 91 ($C_7H_7^+$).

N-(2-hydroxybenzyl) N′-benzyl ethylenediamine diacetic acid. 13.5 g (52 mmoles) of N-(2-hydroxybenzyl) N′-benzyl ethylenediamine was dissolved in 150 ml of water containing 2.1 g (52 mmoles) of sodium hydroxide. Bromoacetic acid, 14.6 g (105 mmoles), was dissolved in 100 ml water in an ice water bath. To this solution, 6.9 g of $NaHCO_3$ was added portionwise. The two solutions were mixed and the reaction mixture was heated to 40°C. NaOH (30% solution) was used to maintain the pH of the solution in the range 11.5–12.5 for 6 hr. The reaction mixture was then allowed to stand at room temperature overnight. Concentrated HCl was added to the reaction solution until pH was reduced to 2. The white precipitate was filtered and recrystallised from isopropanol to yield 10 g of N-(2-hydroxybenzyl) N′-benzyl ethylenediamine diacetic acid hydrochloride as a white powder MP = 190°C (Yield = 46%).

^{1}H NMR (in d^6 DMSO): δ 3.14 (t, 2H), 3.31 (t, 2H), 3.42 (s, 2H), 3.90 (2s, 4H), 4.27 (s, 2H), 6.85 (td, 1H), 6.95 (d, 1H), 7.23 (m, 7H).

Anal. Calcd. for $C_{20}H_{25}N_2O_5Cl$ (+ 0.05 NaCl): C, 58.33; H, 6.08; N, 6.80; O, 19.45; Cl, 9.06. Found: C, 58.21; H,6.09; N6.53; O, 19.86; Cl, 9.20.

Methods

(1) *Preparation of the iron chelates.* Ferric DBED and HBBED 1:1 chelates were prepared according to the following procedure: 0.11 mmoles of ligand were dissolved in 1 ml 0,1N NaOH and the pH of the resulting solution was adjusted to 7 with diluted HCl. A solution of 27 mg (0.1 moles) of $FeCl_3.6H_2O$ in 1 ml water was then added. Acetate or phosphate buffer saline, was then added to give a final volume of 100 ml (pH was readjusted to desired value with diluted NaOH or HCl). The resulting 1 mM Fe(III) HBBED solution is purple-red whereas Fe(III) DBDE is pale yellow in phosphate buffer and deep yellow in acetate buffer. Fe(III) DBED solutions slowly precipitate after a few hours. Ferrous DBED chelate was prepared according to the same method with replacement of $FeCl_3$ by $Fe^{2+}(NH_4)_2(SO_4)_2$ (Mohr's salt). The resulting solution is very pale yellow and is stable in the absence of air. In aerated solutions it very slowly autoxidizes to yield a purple solution.

(2) *Assay of hydroxyl radical generation in the ascorbate driven Fenton reaction.* Generation of HO· was followed using a method adapted from Hider *et al.*[32] This method was originally developed for the colorimetric detection of HO· generated during the incubation of various iron complexes with hydrogen peroxide. It is based on the spectrophotometric detection at 430 nm of the hydroxylation of an

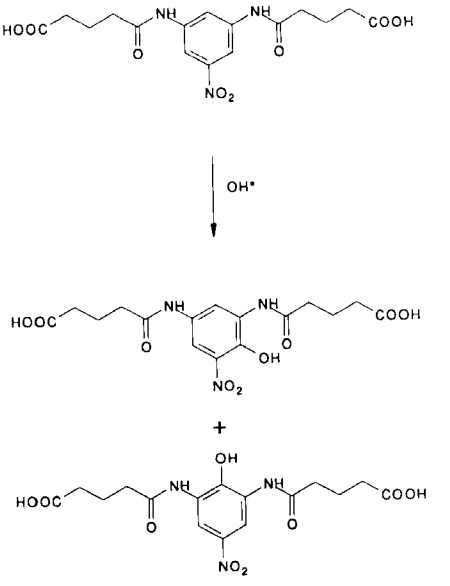

FIGURE 4 Reaction of N,N′-(5-nitro-1,3-phenylene)bisglutaramide (NPG) with hydroxyl radicals leading to hydroxylated derivatives allowing spectrophotometric continuous follow at 430 nm of HO· generation.

aromatic substrate: N,N′-(5-nitro 1,3-phenylene) bis-glutaramide (NPG, Figure 4), that was synthetised for the purpose of the experiment. Neither the substrate nor its hydroxylation products binds either iron (II) or iron (III).

NPG was prepared in our laboratory according to the reported procedure[32] and the method was adapted to follow continuously HO· generation from ascorbate driven Fenton reaction. This is an important advantage over other classical assay for HO· such as deoxyribose test or salicylate hydroxylation[33] which do not allow a continuous monitoring.

Under typical experimental conditions, incubations were performed at 37°C in 50 mM phosphate buffer containing NPG, ascorbate, hydrogen peroxide and iron(III) chelates at final concentrations of respectively 15 mM, 2 mM, 2 mM and 0.1 mM, at a final readjusted pH of 7,4. Optical density at 430 nm was recorded for 5–10 min after initiation by the addition of iron chelate. In the range of concentration used, the contribution to OD^{430} of purple chelates formed during the reaction was negligible.

(3) *Formation of purple chelates.* Under typical experimental conditions, incubations were performed at room temperature in 3 ml cuvettes containing either iron (II) or iron (III) complexes at a final concentration of 0.1–1 mM, in 20 mM acetate or phosphate buffer at various pH. When iron (II) complexes were studied, reactions were initiated by the addition of H_2O_2 from a concentrated solution to give a final concentration of 1–20 mM. When iron (III) complexes were used, sodium ascorbate was added first to give a final concentration in the range 1–20 mM. Reactions were then initiated by the addition of H_2O_2 to give a final concentration in the range 1–20 mM.

TABLE 1

Random ordered worksheet of RSM experimental design used to investigate the influence of concentrations of Fe(III)-DBED, ascorbate and hydrogen peroxide on the percentage conversion of DBED to purple chelates

run	[Fe-DBED] mM	[ascorbate] mM	[H_2O_2] mM	conversion %
1	0.316	7	7	46
2	0.316	7	7	45
3	1	50	50	45
4	0.316	190	7	81
5	0.316	7	7	46
6	0.316	7	7	44
7	0.1	50	1	81
8	2.187	7	7	12
9	0.046	7	7	80
10	0.1	1	1	35
11	1	50	1	10
12	1	1	1	19
13	0.1	50	50	80
14	1	1	50	18
15	0.316	7	7	43
16	0.316	7	190	24
17	0.1	1	50	25
18	0.316	7	0.26	21
19	0.31	7	7	44
20	0.316	0.26	7	17

(4) *Influence of the concentrations of reactants on the formation of purple chelates.* Response Surface Methodology (RSM)[34] was used to study the conversion of Fe(III) DBED to purple species. The effect of three concentrations at pH 6 was examined: ascorbate (1–50 mM), H_2O_2 (1–50 mM) and Fe(III) DBED (0.1–1 mM). A Central composite circumscribed RSM experimental design for three variables at 5 levels with 4 centerpoints was adopted. For each set of concentrations choosen (Table 1), the percentage of conversion of DBED to purple species was measured after 1 h incubation at room temperature, assuming a mean extinction coefficient at 500 nm of $1800\,M^{-1}.cm^{-1}$ (vide infra). Statistical analysis (least square method) was then performed to evaluate the relative contribution of the reactants to DBED conversion.

(5) *HPLC analysis of the products formed.* To a solution of 1:1 DBED Fe(III) chelate was added ascorbate and H_2O_2 to final concentrations of respectively 0.1, 10 and 10 mM. After various incubation times at room temperature, the reaction was quenched by addition of catalase and excess EDTA was added to give a final concentration of 100 mM. The mixture was gently heated at 50–60°C for a few minutes until the purple solution was completely bleached which shows displacement of iron from chelates. 20 µl of the solution was then analysed by HPLC. Separation of DBED, HBBED and HBED was achieved using a Supelcosil™ 5 µm C18 column (15 cm × 4.6 mm) with a mobile phase containing 48% methanol, 52% water, 0.1% orthophosphoric acid and 0.05% sodium dodecyl sulfate at a flow rate of 1 ml/min. Chromatograms were recorded at 210 nm and the UV spectrum of each peak was recorded in order to allow identification by comparison with synthetic samples. In the conditions used ascorbate and EDTA are eluted far before DBED, HBBED and HBED but may obstruct detection of more polar DBED oxidation products.

(6) *Affinity constant evaluation.* Since HBBED ferric chelate has an intense red-purple color, the affinity constant of Fe(III) for HBBED was evaluated spectrophotometrically by competitions studies with EDTA according to classical methods.[29,36] Equilibrium conditions were obtained by stirring at 37°C for 24 h a 200 µM Fe(III) HBBED solution (μ = 0.1 M KNO_3) in the presence of various concentrations of EDTA so that the molar ratios of EDTA varied from 1 to 10 with respect to HBBED (Table 2). For the calculation of the Fe(III) HBBED overall equilibrium constant, ligand pKa values were estimated from potentiometric titration. EDTA protonation constants and affinity constant of Fe(III) for EDTA used were those listed in Table 3. DBED pKa and affinity constant of DBED for Fe(III) were evaluated potentiometrically according to Chaberek and Martell.[35]

TABLE 2
Spectrophotometric determination of the affinity constant for iron(III) of HBBED by competition studies with EDTA (μ = 0.1 M KNO_3). Concentration of ferric HBBED chelate at equilibrium ($[Fe\text{-}HBBED]_{eq}$) was estimated from OD^{500} after 24h stirring at 37°C[29,36]

[HBBED] µM	[EDTA] µM	$[Fe^{3+}]$ µM	pH	$\frac{[Fe\text{-}HBBED]_{eq}}{[HBBED]}$	log K
200	200	200	7.0	0.618	28.4
200	400	200	7.1	0.417	28.1
200	600	200	7.2	0.295	28.0

TABLE 3
Ligand pKa values and stability constants for Fe(III) (log K) of various iron chelates

	EDDA[a]	EDTA[b]	DBED[c]	HBBED[c]	HBED[b]	transferrin[a]
pK_1	6.4	2.0	4.8	4.7	4.6	-
pK_2	9.5	2.7	9.6	9.2	8.3	-
pK_3		6.2		11.0	11.0	-
pK_4		10.3			12.5	-
log K	17	25	<15	28	39.7	21

[a] From ref 41. [b] From ref 29.
[c] This study, for log K values, error are estimated as ± 1 log unit.

RESULTS

(1) *Assay for HO· generation in the medium for the ascorbate driven Fenton reaction.* Figure 5 shows that incubation of Fe(III)-EDTA with ascorbate and H_2O_2 generates in the medium HO· or a similar species which is able to hydroxylate an aromatic ring. For 2 mM ascorbate, 2 mM H_2O_2 and 100 μM Fe(III)-EDTA, hydroxylation of NPG reaches a plateau within 5 min (all H_2O_2 is consumed, as can be checked by further addition of H_2O_2 – data not shown). With EDDA iron chelate, HO· generation is slower and linear within the time scale used. Iron(III)-DEBD is a comparatively poor catalyst of HO· formation, however a weak hydroxylation of NPG is measurable. This rate of HO· generation is very close to that of

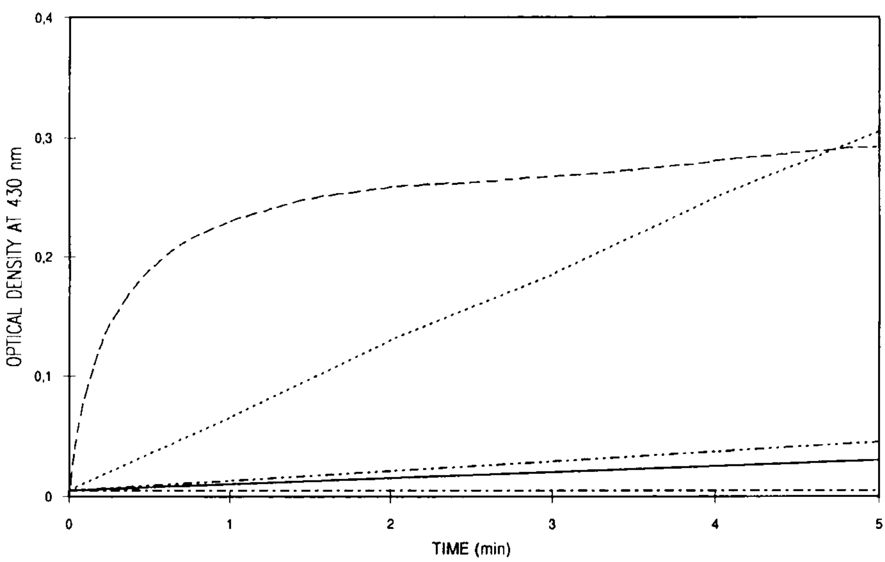

FIGURE 5 Variation of optical density at 430 nm with time for solutions of NPG (15 mM) in pH 7.4 50 mM phosphate buffer 136 mM NaCl containing ascorbate (2 mM), hydrogen peroxide (2 mM) and various iron chelates (0.1 mM).
EDTA: _ _ _ _ _ _ _ _ _, EDDA: ...., DBED: -··-··-··-··-··-··-··-··-··-··-,
control (no iron): ____________________, HBBED and HBED: -·-·-·-·-·-·-·-·-·-·-·-
Reactions were initiated by the addition of iron chelate.

the control experiment when no metal chelate is added which reflects catalysis by trace metal contamination of phosphate buffer. Therefore, these results show that iron DBED chelate does not catalyse important release of HO· in the medium compared to EDTA chelate.

On the other hand HBED and HBBED are not catalyst of Fenton chemistry in these conditions since no hydroxylation of NPG at all is observable for these iron complexes.

(2) *Formation of purple chelates.* Figure 6a shows that the ferrous chelate of DBED undergoes a dramatic change of color following addition of hydrogen peroxide. A similar spectrum is also obtained by incubation of the corresponding ferric complexes with excess ascorbate and hydrogen peroxide. When the same experiment is performed in the presence of excess ethanol (up to 0.5 M) or D-mannitol (up to 0.1 M), the apparition of the chromophore is not suppressed but only partially decreased (Figure 6b). The optical spectrum of the resulting chromophore strongly depends on the pH i.e. it reversibly disappears at pH < 1.5 and the maximum is shifted from 490 nm to 525 nm when the pH of the medium is varied from slightly acidic conditions to neutral conditions. On the other hand, when excess EDTA is

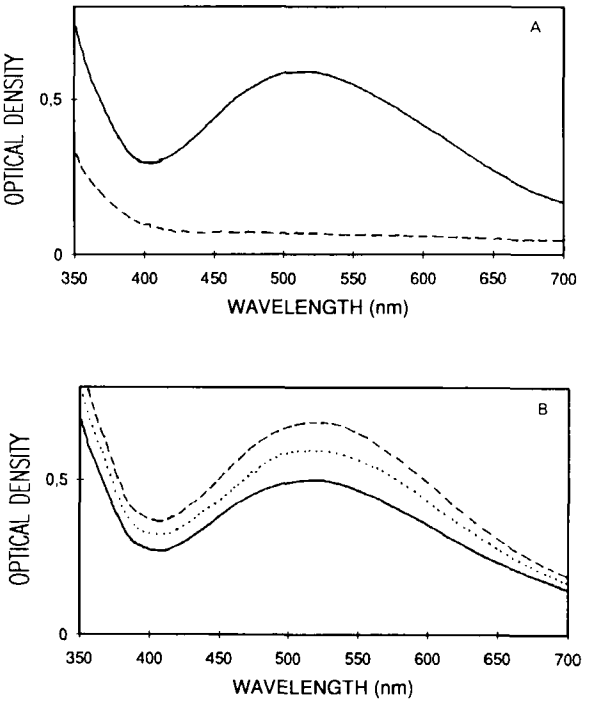

FIGURE 6 A. Visible spectrum of Fe(II) DBED (1 mM) in 136 mM NaCl pH5 before (----------) and after (——————) addition of hydrogen peroxide at a final concentration of 15 mM. Incubation time is 5 min.

B. Visible spectrum in 50 mM acetate buffer pH5 of 0.5 mM Fe(III) DBED incubated 5 min with 20 mM ascorbate and 20 mM hydrogen peroxide either in absence (----------) or in presence of hydroxyl radical scavengers: 0.5 M ethanol (——————) or 0.1 M D-mannitol (·····).

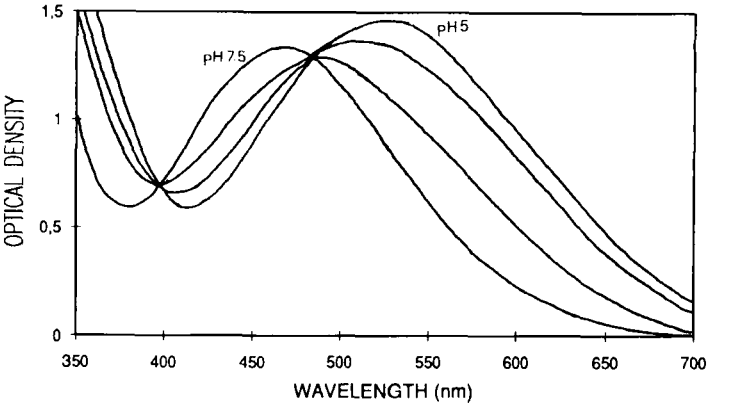

FIGURE 7 Visible spectrum of Fe(III) HBBED (0.76 mM) in 136 mM NaCl as a function of pH. pH was varied by addition of aliquots of concentrated sodium hydroxyde or hydrochloric acid.

added to the chromophore solution, and heated at 60°C for a few minutes, a rapid bleaching is observed. Hence, the relatively intense band in the visible region obtained by incubation of iron (II) DBED and H_2O_2 is to be assigned to a ligand-metal interaction. This spectrum is very close to that of Fe(III) HBBED (obtained by chemical synthesis, Figure 3), which shows characteristic charge transfer band from a p orbital on the ligand to a half-filled d orbital of iron[36] with two isosbestic points on pH variation (Figure 7, $\lambda_{max} = 525$ nm, $\epsilon = 1900\,M^{-1}.cm^{-1}$ at pH 5 and $\lambda_{max} = 480$ nm, $\epsilon = 1750\,M^{-1}.cm^{-1}$ at pH 7,5). Therefore, all these results are consistent with a Fenton type reaction in which intramolecular hydroxylation by HO· in position 2 of one of the two phenyl moieties of DBED leads to new (colored) iron chelates with at least one phenolate ligand according to Figure 1.

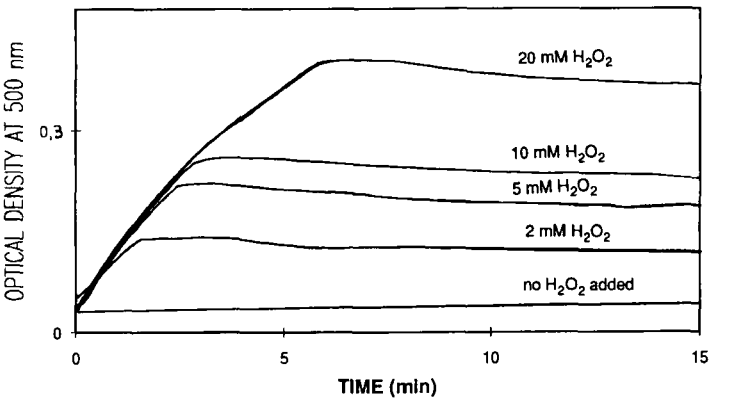

FIGURE 8 Time dependence of the apparition of purple chromophore at 500 nm during the incubation of 1 mM Fe(III) DBED in air saturated phosphate buffer saline pH 7 with 20 mM ascorbate and various concentrations of hydrogen peroxide. Reactions were initiated by the addition of H_2O_2.

(3) *Influence of the concentration of reactants on the formation of purple species*

- *time dependence of the formation of purple chromophore.* The evolution with time of OD^{500} was studied for various H_2O_2 concentration at fixed Fe(III) DBED and ascorbate concentrations in air saturated 20 mM phosphate buffer at pH 7 (Figure 8). A very slow rate of apparition of purple chromophore is also observable in the absence of added H_2O_2. This slow increase of OD highly depends on aeration of the incubation medium e.g. it is much faster when solutions are stirred in opened vials with large air exchange surface than in spectrophotometer cuvettes. In the absence of air and added H_2O_2, no increase of OD^{500} is observable (data not shown). Therefore, it is likely that ascorbate autoxidation leading to $O_2^{\cdot -}$ and H_2O_2 formation is responsible for this slow rate of apparition of purple species.

- *effect of reactants concentrations and pH.* Although at pH 7, the apparition of purple chromophore is relatively slow (Figure 8), it is very fast at lower pH, reaction being almost instantaneous at pH 5. On the other hand, the shape of optical spectra of the species formed is also dependent on the pH of the medium in which the incubation is carried. Figure 9 represents optical spectra obtained by incubation of Fe(III) DBED with excess ascorbate and hydrogen peroxide at various pH. Reactions performed at pH 8 or above became purple- brown, showing that other products that may be melanine-like compounds are also formed in this pH range.

The effect of varying the ascorbate concentration on the OD^{500} at plateau is shown in Figure 10 for various concentrations of H_2O_2 at pH 5.5. Optical spectra of the resulting solutions also showed differences as for pH dependence e.g. the solutions turned purple brown for excess H_2O_2 (not shown).

To study more completely the influence of the reactants concentrations on the conversion of iron DBED chelate to purple species, a RSM experimental design was used (see Material and Methods). Such a design allows a systematic scanning of the experimental domain by changing all the variables simultaneously. The set of conversion for the concentrations of ascorbate, H_2O_2 and Fe(III) DBED used is reported in Table 1. Conversion varies from 10 to 81% depending on the conditions. A second

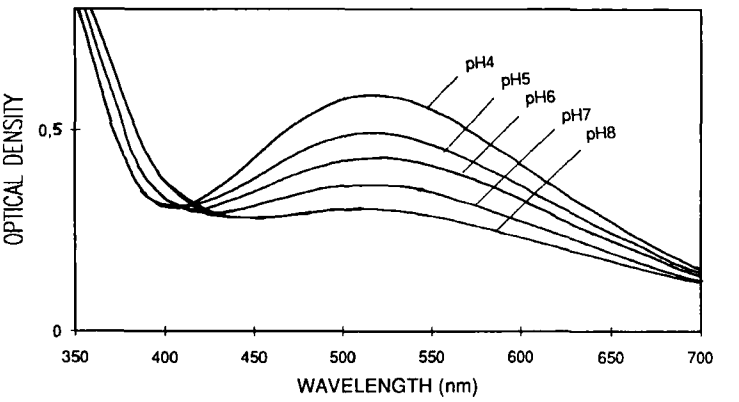

FIGURE 9 Optical spectra of solutions of 0.5 mM Fe(III) DBED incubated 15 min with 20 mM ascorbate and 20 mM H_2O_2 in acetate or phosphate buffer at various pH.

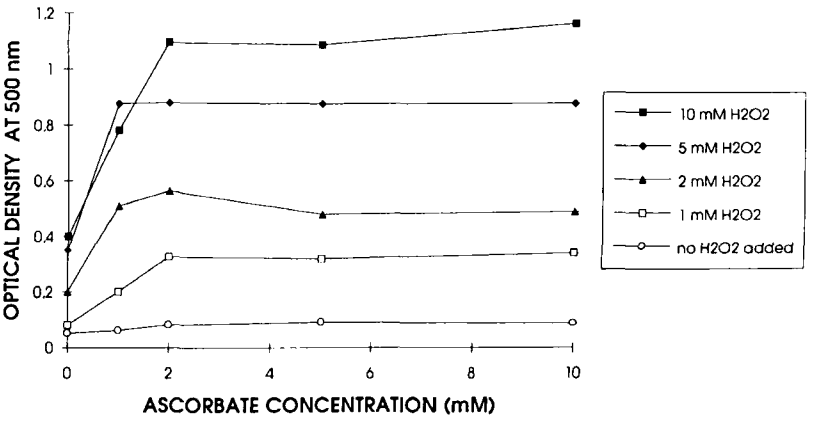

FIGURE 10 Formation of chromophore at 500 nm as a function of ascorbate concentration for 1 mM Fe(III) DBED in 50 mM acetate buffer pH 5.5 in the presence of various concentrations of H_2O_2. Solutions were incubated at room temperature for 15 min.

order regression equation was derived that explained 96% of the variability in the conversion of DBED to purple species. An example of a contourplot showing the conversion of DBED at various concentrations of ascorbate and Fe(III) DBED is shown in Figure 11. The curves in Figure 11 and those of the other plots showed that conversion of DBED is maximum at low chelate concentration (100 μM or lower) and excess ascorbate. High H_2O_2 concentration shows a negative effect on conversion.

(4) *Analysis of the products formed.* Figure 12 shows representative HPLC chromatograms obtained by injecting a solution of Fe(III)-DBED incubated in the presence of ascorbate and H_2O_2 to which excess EDTA was added to displace iron from chelates. It was checked that, in these conditions, iron displacement from HBBED chelate is quantitative.

DBED was found to be consumed during the time course of the reaction and HBBED to be formed, as shown by retention time and UV spectrum in the range 200–300 nm using diode array detection. However, no HBED was detected in the conditions studied. Similar profiles are obtained when direct reaction between Fe(II)-DBED and H_2O_2 is studied (data not shown). For this reaction, about 7 molar equivalents are needed to consume all DBED present in the conditions studied.

The amount of HBBED observable on the chromatogram is lower (10 to 20% depending on the conditions) than expected from total conversion. Therefore these results suggest that, in addition to HBBED, DBED is oxidized into other compounds. Since conversion of DBED estimated from OD^{500} of purple chelates is higher than 80% in the conditions studied (Figure 11), it can be concluded that some of these compounds also form colored iron chelates. They are likely to result from further hydroxylation of the aromatic ring (see discussion) and are therefore probably eluted at a shorter retention time e.g. masked by the presence of ascorbate and EDTA on the chromatogram.

Purple species obtained from incubation of ferrous DBED chelate with H_2O_2 were partially extracted with n-butanol. The resulting solution was evaporated to

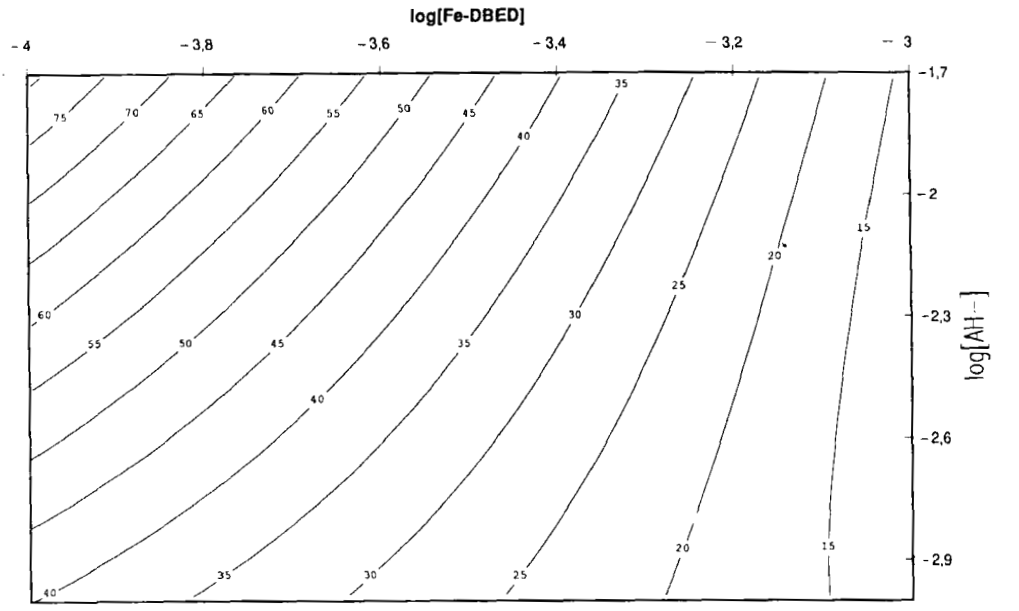

FIGURE 11 Contourplot showing % conversion of Fe(III) DBED to purple species (see Material and Methods) versus Fe(III) DBED and ascorbate concentrations at a fixed H_2O_2 concentration (1 mM).

dryness and the violet powder obtained treated with excess 8-hydroxy quinoline in dichloromethane to remove iron.[39] The white residue obtained after solvent evaporation is a mixture of compounds for which a separation was not achieved yet. However, addition of ferric chloride to an aqueous solution of this mixture leads to instantaneous purple coloration, showing again that purple color directly results from iron-ligand interaction.

(5) *Affinity constants for iron.* Affinity constant of HBBED for Fe(III) was measured spectrophotometrically by competition with EDTA (Table 2). An equilibrium constant of logK = 28 was obtained which is consistent with affinity of Fe(III) for structurally similar molecules.[28] The overall equilibrium constant for the mixture of purple species is likely to have the same order of magnitude since competition studies with EDTA gave similar results for HBBED chelate and for crude purple species obtained by reaction of ferric DBED chelate with ascorbate and H_2O_2. Affinity constant of DBED for iron(III) was evaluated potentiometrically to be logK < 15, which is a relatively low value compared to EDTA (logK = 25). The resulting set of equilibrium constant is listed in Table 3, which also include values for transferrin[41] as a reference.

DISCUSSION

Effective sequestration of iron is an important antioxidant defense. Hence, iron is never "free" in biological systems but carefully handled by transport and storage pro-

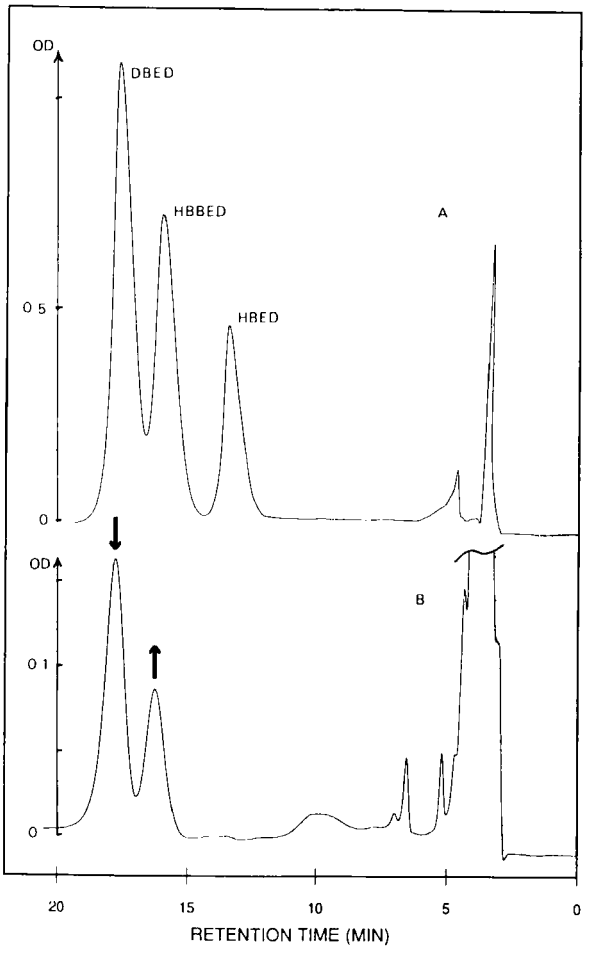

FIGURE 12 Representative HPLC chromatogram showing partial conversion of DBED to HBBED (UV detection at 210 nm).
(A) synthetic mixture of HBED, HBBED and DBED in phosphate buffer.
(B) 0.1 mM Fe(III) DBED incubated 15 min at room temperature with ascorbate (10 mM) and hydrogen peroxide (10 mM). 100 mM EDTA were added before injection to displace iron from purple chelates.

teins. On the other hand, a lot of literature is now available showing the release of catalytic iron during oxidative stress.[1,9,10] The use of strong iron chelators such as Deferoxamine therefore appears to be a promising way of protection against oxidative damage.[1] However, owing to the ubiquitous role of iron in cell functions, caution must be taken in administrating strong iron chelators for long periods in other situations than iron overload. Deferoxamine for instance, which is a widely used drug for the treatment of iron overload (log K = 30.8), has probably also an important therapeutic potential use against oxidative stress in acute situations but it shows side effects for prolonged periods of time, probably related partially to its

capacity to remove "safe" iron from various sites.[2] As well, HBED, which shows great promise as an orally active iron chelating drug[37] for the treatment of iron overload is likely to show similar side effects regarding its very high affinity (log K = 39.7) for iron(III).

Owing to the considerably lower affinity of DBED for iron (log K < 15), it can be hoped that the side effects related to "safe" iron chelation will be lower. It is indeed noteworthy that the affinity of DBED for iron(III) is much lower than that of transferrin (Table 3). Therefore DBED cannot compete thermodynamically with this protein. However, it is not meaningful to simply compare the magnitudes of the overall formation constants, which do not take into account ligand protonation constants and concentrations dependencies. The concentrations of free ferric iron in equilibrium at pH 7.4 in the presence of equimolar excess ligand (pM values[40]) provide a more relevant comparison of the abilities of various chelators to chelate iron under physiological conditions. The comparison of pM values for transferrin (pM = 21) and DBED still confirms that it is safe to assume that iron mobilisation from metalloproteins will be avoided. Moreover, interaction between DBED and calcium is reported to be weak,[30] thus possible side effects related to interaction with calcium metabolism may not be important. It remains however to be determined whether the affinity for iron of DBED and exchange kinetics are high enough to partially withdraw non specifically bound iron from target molecules during oxidative stress.

Our results show that, in the presence of excess ascorbate, the iron chelate of DBED reacts with hydrogen peroxide to yield by intramolecular hydroxylation a mixture of 2-hydroxy derivatives among which HBBED was detected. Such specific intramolecular hydroxylation of an arene has already been reported for copper binuclear complexes,[42] although the reaction pathway is somewhat different. Furthermore, aromatic hydroxylation of aromatic compounds by hydrogen peroxide in the presence of ascorbate and catalytic iron is very well known (Udenfriend catalyst[43]). In other respects, specific reactions with HO· generated by Fe-EDTA derivatives have also been intensively studied for use in affinity cleaving of DNA or proteins. In this case attachment of Fe-EDTA to a DNA or protein binding moiety creates a specific cleaving reagent[44,45] e.g. HO· reacts with DNA or protein backbone and not with the ligand.

The present paper shows that HBBED iron chelate is not a catalyst of Fenton chemistry (Figure 5). The very large increase in log K (Δ log K > 13) between DBED and HBBED (Table 3) is not surprising regarding the affinity of iron(III) for phenolate ligand.[28] Neither is surprising the absence of catalysis of Fenton chemistry of Fe(III) HBBED chelate which is consistent with the low redox potential reported for mononuclear iron(III) phenolate complexes[37]

A possible mechanism of the reaction is tentatively depicted in Figure 13. The use of molecular models shows that hydroxylation of position 2 (or 6) is highly sterically favored. The presence of two benzyl moieties may increase this probability since H_2O_2 can approach from both sides of DBED chelate. Moreover, HO· addition to the aromatic ring in preference to abstraction of a benzylic hydrogen atom is consistent with literature.[46] The rate limiting step of the overall scheme is likely to be the reaction between H_2O_2 and ferrous DBED chelate or the reduction of ferric iron by ascorbate rather than addition of HO· to the aromatic ring since the former is known to occur with very high rate constants. Intramolecular reaction of HO· seems to be quite efficient since high concentrations of HO· scavengers only partially inhibits the reaction and since only a weak release of HO· is observed in the medium (Figure 5)

in the conditions studied. This HO· leak may come in part from path (a) of Figure 13, which reflects an escape from the coordination sphere. It may also originate from the reactivity of the intermediate hydroxy cyclohexadienyl radical (HCHD). Indeed it can be speculated that the HCHD can either directly react with excess H_2O_2 or reduce Fe(III) since some HCHD are known as powerfull one electron reductants.[16] Reaction with H_2O_2 of the resulting ferrous chelate would then lead to HO· by Fenton reaction. Thus an expected stoichiometry for the reaction leading to HBBED would be 2 H_2O_2 for 1 DBED.

However, the actual stoichiometry of H_2O_2 is about 7 for 1 according to HPLC evaluation of DBED consumption. The origin of this supplementary consumption of H_2O_2 is likely to be linked to the formation of further oxidized products and has not been fully investigated up to now. Addition of dioxygen to HCHD should also be

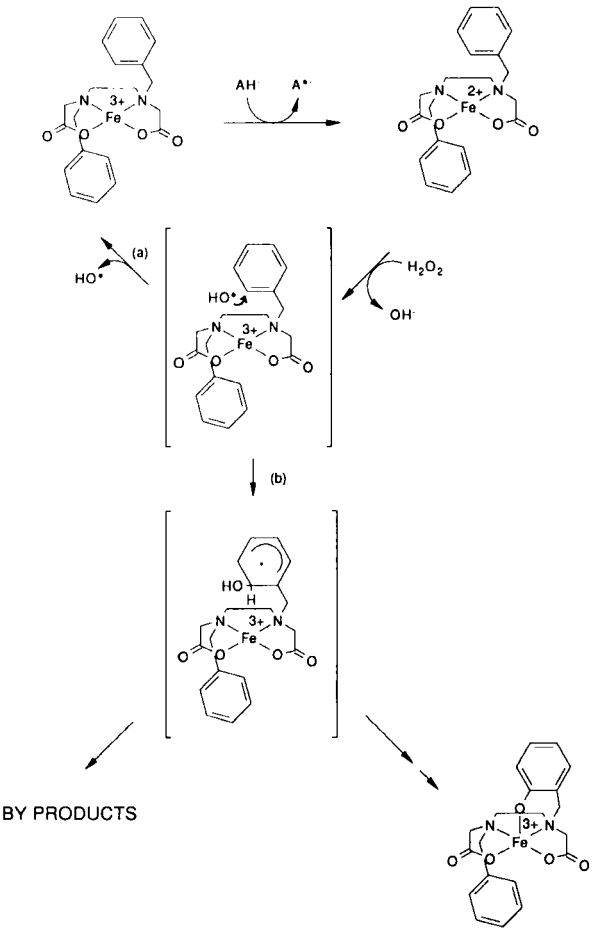

FIGURE 13 Proposed scheme showing reaction between Fe(III) DBED, hydrogen peroxide and ascorbate (AH^-). The structure of DBED chelate in solution is not known, however, this representation is a likely one considering coordination chemistry of ethylenediaminediacetic acid derivatives.[29] Axial ligands have been omitted for clarity. $A^{-\cdot}$ is ascorbyl radical.

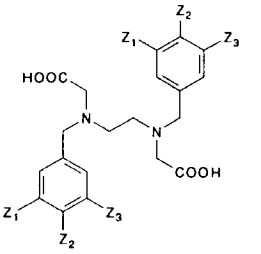

FIGURE 14 Structure of DBED derivatives under investigation.

considered. As well, the final oxidation state of iron in purple chelates has not yet been evaluated. However, considering the low redox potential of iron phenolate chelates, the ferric state of iron should be higly stabilised and therefore the final oxidation state of iron in purple chelates is likely to be ferric.

It is noteworthy that HBED (Figure 2) was not a product of the reaction although it could be expected to be formed from HBBED chelate according to a similar pathway. This may be explained by the decrease of redox potential of Fe(III)-HBBED which is likely to prevent further iron reduction by ascorbate.

Only DBED derivatives bearing a hydroxy moiety in position 2 can give colored 1:1 chelates for steric reasons. Indeed, it was checked that synthetic N,N′-bis di(3-hydroxybenzyl) ethylenediamine diacetic acid and N,N′-bis di(4-hydroxybenzyl) ethylene diamine diacetic acid do not give purple ferric 1:1 chelates but only pale yellow species (data not shown). Considering the high conversion of DBED ferric chelate to purple chelates in the conditions discussed above (Figure 11), it can be concluded that oxidation mainly leads to 2-hydroxy derivatives. These products, apart from HBBED, are therefore likely to be polyhydroxylated derivatives and their identification is currently under progress.

To resume, although the reaction pathway shown in Figure 13 seems plausible in the conditions studied, it remains to be determined whether it is relevant under physiological conditions. To this end, chemical and biological evaluation of DBED derivatives of structures depicted in Figure 14, as well as characterisation of the corresponding iron chelates, are currently under investigation. The choice of Z_1 and Z_3 as electron donating groups should favor intramolecular hydroxylation by increasing electron densities at position 2 and 6 and thereby favouring electrophilic addition of HO· while minimizing the formation of polyhydroxylated products. Moreover, according to Z_1, Z_2 and Z_3, physicochemical properties of these molecules may also be modulated so as to be for instance water or lipid soluble, excluded from or able to pass through membranes.

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References

1. B. Halliwell and J.M.C. Gutteridge (1989), *Free Radicals in Biology and Medecine*, second Edition, Clarendon Press Oxford.

2. B. Halliwell, J.M.C. Gutteridge and C.E. Cross (1992) Free radicals, antioxidants, and human disease: Where are we now?. *Journal of Laboratory and Clinical Medicine*, **119**(6), 598–620.
3. B. Chance, H. Sies and A. Boveris (1979) Hydroperoxide metabolism in mammalian organs. *Physiological Reviews*, **59**(3), 527–605.
4. B.S. Van Asbeck (1990) Oxygen toxicity: role of hydrogen peroxide and iron, *In Antioxidants in Therapy and Preventive Medecine, I. Emerit ed., Plenum Press, New York*, 235–246.
5. H. Sies (1986) Biochemistry of oxidative stress. *Angewdte Chemie (Internatinal edition in English)*, **25**, 1058–1071.
6. B.N. Ames and M.K. Shigenaga (1992) Oxidants are a major contributor to aging. *Annals of the New York Academy of Sciences*, **663**, 85–96.
7. R. Tyrrell (1991) UVA(320–380 nm) radiation as an oxidative stress. *In Oxidative Stress, oxidants and antioxidants, H. Sies ed., Academic Press Limited*, 57–83.
8. J. McCord and I. Fridovich (1969) Superoxide dismutase. An enzymic function for erythrocuprein. *The Journal of Biological Chemistry*, **244**, 6049–6055.
9. T.P. Ryan and S.D. Aust (1992).The role of iron in oxygen-mediated toxicities. *Critical Reviews in Toxicology*, **22**(1), 119–141.
10. D.W. Reif (1992) Ferritin as a source of iron for oxidative damage. *Free Radical Biology and Medecine*, **12**, 417–427.
11. B. Halliwell (1990) How to characterize a biological antioxidant. *Free Radical Research Communications*, **9**(1), 1–32.
12. R.A. Floyd and C.A. Lewis (1983) Hydroxyl free radical formation from hydrogen peroxide by ferrous iron-nucleotide complexes. *Biochemistry*, **22**, 2645–2649.
13. M. Chevion (1988) A site-specific mechanism for free radical induced biological damage: the essential role of redox-active transition metals. *Free Radical Biology and Medecine*, **5**, 27–37.
14. M.J. Burkitt and B.C. Gilbert (1990) Model studies of the iron-catalysed Haber- Weiss cycle and the ascorbate-driven Fenton reaction. *Free Radical Research Communications*, **10**, 265–280.
15. E.R. Stadtman (1990) Metal ion-catalysed oxidation of proteins: biochemical mechanism and biological consequenses, *Free Radical Biology and Medecine*, **9**, 315–325.
16. A. Samuni, J. Aranovich, D. Godinger, M. Chevion and G. Czapski (1983) On the cytotoxicity of vitC and metal ions. A site specific mechanism. *European Journal of Biochemisity*, **137**, 119–124.
17. S. Steenken (1987) Addition-elimination paths in electron-transfer reactions between radicals and molecules. *Journal of the Chemical Society. Faraday transactions I*, **83**, 113–124.
18. D.M. Miller, G.R. Buettner and S.D. Aust (1990) Transition metals as catalysts of "autoxidation" reactions. *Free Radical Biology and Medecine*, **8**, 95–108.
19. E. Graf, J.R. Mahoney, R. Bryant and J.W. Eaton (1984) Iron-catalysed hydroxyl radical formation. Stringent requirement for free iron coordination site. *The Journal of Biological Chemistry*, **259**(6), 3620–3624.
20. J.M.C. Gutteridge (1990) Superoxide-dependent formation of hydroxyl radicals from ferric-complexes and hydrogen peroxide: an evaluation of fourteen iron chelators. *Free Radical Research Communications*, **9**(2), 119–125.
21. J.B. Smith, J.C. Cusumano and C.F. Babbs (1990) Quantitative effects of iron chelators on hydroxyl radical production by the superoxide-driven Fenton reaction. *Free Radical Research Communications*, **8**(2), 101–106.
22. D. Gelvan and P. Saltman (1990) Small chelators and the nature of the metal ion in site specificity of HO· damage. *Free Radical Biology and Medecine*, **9(s1)**, 59.
23. M.J. Burkitt and B.C. Gilbert (1991) The autoxidation of iron(II) in aqueous systems: the effects of iron chelation by physiological, non physiological and therapeutic chelators on the generation of reactive oxygen species and the inducement of biomolecular damage. *Free Radical Research Communications*, **14**(2), 107–123.
24. J.D. Rush and W.H. Koppenol (1986) Oxidizing intermediates in the reaction of ferrous EDTA with hydrogen peroxide. *The Journal of Biological Chemistry*, **261**(15), 6730–6733.
25. J. Butler and B. Halliwell (1982) Reaction of iron-EDTA chelates with the superoxide radical. *Archives of Biochemistry and Biophysics*, **218**(1), 174–178.
26. I. Yamazaki and L.H. Piette (1991) EPR spin-trapping study on the oxidizing species formed in the reaction of the ferrous ion with hydrogen peroxide. *Journal of the American Chemical Society*, **113**, 7588–7593.
27. S. Croft, B.C. Gilbert, J.R. Lindsay Smith and A.C. Whitwood (1992) An ESR investigation of the reactive intermediate generated in the reaction between Fe(II) and H_2O_2 in aqueous solution. Direct evidence for the formation of the hydroxyl radical. *Free Radical Research Communications*, **17**(1), 21–39.
28. A.E. Martell, W.F. Anderson and D.G. Badman (eds). Development of iron chelators for clinical use, Elsevier North Holland, New York, 1981.

29. F. L'Eplattenier, I. Murase and A.E. Martell (1967) New mutidentate ligands. VI. Chelating tendencies of N,N′-Di(2-hydroxybenzyl)ethylene diamine-N,N′-diacetic acid. *Journal of the American Chemical Society*, **89**(4), 837–43.
30. A.E. Frost Jr. and A.E. Martell (1950) The preparation and properties of some N,N′-dialkylethylenediaminediacetic acids. *Journal of the American Chemical Society*, **72**, 3743–46.
31. Y. Sun, A.M. Martell, J.H. Reibenspies and M.J. Welch (1991) Synthesis of multidentate ligands containing hydroxypyridyl donor groups. *Tetrahedron*, **47**(3), 357–64.
32. S. Singh and R.C. Hider (1988) Colorimetric detection of the hydroxyl radical: comparison of the hydroxyl-radical-generating ability of various iron complexes. *Analytical Biochemistry*, **171**, 47–54.
33. B. Halliwell, M. Grootveld and J.M.C. Gutteridge (1989) Methods for the measurement of hydroxyl radical in biochemical systems: deoxyribose degradation and aromatic hydroxylation. *Methods of Biochemical Analysis*, **33**, 59–90.
34. G.E.P. Box and W.G. Hunter (1978) *In statistics for experimenters*, Wiley, New York.
35. S. Chaberek Jr. and A.E. Martell (1952) Stability of metal chelates. IV. N,N′-ethylenediaminediacetic acid and N,N′-ethylenediaminediacetic- N,N′-dipropionic acid. *Journal of the American Chemical Society*, **74**, 6228–6231.
36. M. Streater, P.D. Taylor, R.C. Hider and J. Porter (1990) Novel 3-hydroxy-2(1H)-pyridinones. Synthesis, iron(III)-chelating properties and biological activity. *Journal of Medicinal Chemistry*, **33**, 1749–1755.
37. K. Ramesh and R. Mukherjee (1992) Trends in the spectral and redox potential data of mononuclear iron(III) (S = 5/2) phenolate complexes. *Journal of the Chemical Society. Dalton Transactions*, 83–89.
38. R.W. Grady and C. Hershko (1990) HBED: a potential oral iron chelator. *Annals of the New York Academy of Sciences*, **612**, 361–68.
39. A.E. Martell, R.J. Motekaitis and E.T. Clarke (1986) Synthesis of N,N′-di(2-hydroxybenzyl) ethylenediamine-N,N′-diacetic acid (HBED) and derivatives. *Canadian Journal of Chemistry*, **64**(3), 449–456.
40. R.J. Motekaitis, A.E. Martell and M.J. Welch (1990) Stabilities of trivalent metal complexes of phenolic ligands related to N,N′-bis(2-hydroxybenzyl) ethylenediamine-N,N′-diacetic acid (HBED). *Inorganic Chemistry*, **29**, 1463–67.
41. W.R. Harris, C.J. Carrano and K.N. Raymond (1979) Coordination chemistry of microbial iron transport compounds. 16. Isolation, characterisation, and formation constants of ferric aerobactin. *Journal of the American Chemical Society*, **101**(10), 2722–27.
42. K.D. Karlin, J.C. Hayes, Y. Gultneh, R.W. Cruse, J.W. McKown, J.P. Hutchinson and J. Zubieta (1984) Copper-mediated hydroxylation of an arene: model system for the action of copper monoxygenases. Structures of a binuclear Cu(I) complex and its oxygenated product. *Journal of the American Chemical Society*, **106**, 2121–28.
43. S. Udenfriend, C.T. Clark, J. Axelrod and B.B. Brodie (1954) Ascorbic acid in aromatic hydroxylation 1. A model system for aromatic hydroxylation. *The Journal of Biological Chemistry*, **208**, 731–750.
44. R.P. Herzberg and P.B. Dervan (1982) Cleavage of double helical DNA by (methidiumpropyl-EDTA)iron(II). *Journal of the American Chemical Society*, **104**, 313–315.
45. M.A. Price and T.D. Tullius (1992) Using hydroxyl radical to probe DNA structure. *Methods in Enzymology*, **212(Pt. B)**, 194–219.
46. C. Walling and R.A. Johnson (1975) Fenton's reagent. V. Hydroxylation and side chain cleavage of aromatics. *Journal of the American Chemical Society*, **97**(2), 363–367.

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