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MODEL STUDIES OF THE IRON-CATALYSED HABER-WEISS CYCLE AND THE ASCORBATE-DRIVEN FENTON REACTION

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Complementary hydroxylation assays and stopped-flow e.s.r. techniques have been employed in the investigation of the effect of various iron chelators (of chemical, biological and clinical importance) on hydroxyl-radical generation via the Haber-Weiss cycle and the ascorbate-driven Fenton reaction.

Chelators have been identified which selectively promote or inhibit various reactions involved in hydroxyl-radical generation (for example, NTA and EDTA promote all the reactions of both the Haber-Weiss cycle and the ascorbate-driven Fenton reaction, whereas DTPA and phytate inhibit the recycling of iron in these reactions). The biological chelators succinate and citrate are shown to be relatively poor catalysts of the Haber-Weiss cycle, whereas they are found to be effective catalysts of ·OH generation in the ascorbate-driven Fenton reaction.

It is also suggested that continuous redox-cycling reactions between iron, oxygen and ascorbate may represent an important mechanism of cell death in biological systems.

KEY WORDS: Iron, Haber-Weiss cycle, ascorbate, hydroxylation assays, stopped-flow e.s.r. spectroscopy.

ABBREVIATIONS USED: $AscH^-$ ascorbate anion; Asc^{2-} ascorbate dianion; AscH· ascorbyl radical; Asc · ascorbyl radical anion; Asc dehydroascorbate; DFO desferrioxamine (mesylate); 2,3-DHB 2,3-dihydroxybenzoate; DTPA diethylenetriaminepentaacetic acid; EDTA ethylenediaminetetraacetic acid; e.s.r. electron spin resonance; GSH glutathione (reduced); GSSG glutathione (oxidised); $NADP^+$ nicotinamide adenine dinucleotide phosphate (oxidised); NADPH nicotinamide adenine dinucleotide phosphate (reduced); NTA nitrilotriacetic acid; SOD superoxide dismutase; tris tris(hydroxymethyl)-methylamine;

INTRODUCTION

The possibility that the mammalian cell possesses the ability to generate potentially cytotoxic oxygen-centred free radicals (as also generated via the interaction of ionising radiation with living systems) was recognised by McCord and Fridovich, who reported the discovery of the superoxide dismutase (SOD) family of proteins.[1] The function of SOD is evidently to catalyse the disproportionation of the superoxide radical ($O_2^{\cdot-}$), and hence to remove this species from the system [reaction (1)].

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (1)$$

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It is also believed that the single-electron reduction of molecular oxygen to superoxide occurs during the metabolism of many xenobiotics (e.g. adriamycin[2] and menadione[3]) and also as a consequence of the normal cellular metabolism of oxygen (for example, it has been reported[4] that beef heart submitochondrial particles can generate 5.3 nmol superoxide min^{-1} mg^{-1} protein).

Because of the relatively low reactivity of $O_2^{\cdot-}$ towards biological molecules, it is also recognised that the apparent cytotoxicity of this species may be due to its reactions with redox-active metal ions, such as iron and copper, which leads to the generation of the more reactive hydroxyl radical ($\cdot OH$).[5,6] Such reactions with iron constitute the iron-catalysed Haber-Weiss cycle [reactions (2) and (3), in which reaction (3) is known as the Fenton reaction].

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

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^- \quad (3)$$

It is believed that only iron bound to low-molecular weight chelators (e.g. citrate and the adenine nucleotides) is able to participate in the Haber-Weiss cycle.[7] The evidence for the existence of a cellular pool of "catalytic" iron remains tentative (largely on account of the difficulties associated with the determination of iron complexes in biological samples), and it is possible that iron is only available in this form in iron-overload conditions (e.g. haemochromatosis) in which the normal capacity of the system to sequester the metal ion is saturated.[8,9] However, superoxide has been demonstrated to be capable of acting as the reductant necessary for the release of iron from its storage protein, ferritin,[10] and it is therefore possible that increased levels of $O_2^{\cdot-}$ generation (associated, for example, with ischaemia-reperfusion injury[11]) may well be accompanied by the mobilisation of iron. Furthermore, the presence of low molecular weight iron-complexes (capable of participating in the Fenton reaction) has been demonstrated at micromolar concentrations in extracellular fluids, such as cerebrospinal fluid and synovial fluid taken from rheumatoid patients.[12]

Although the $\cdot OH$ radical is believed to be generated in the Fenton reaction [reaction (3)], it should also be noted that if the rate of this reaction (for a given iron-complex) is fast, then, in the presence of excess $O_2^{\cdot-}$ and H_2O_2 (as may occur during the metabolism of many redox-cycling drugs[2,3]), the recycling of Fe(III) to Fe(II) [reaction (2)] may become the limiting factor in (overall) $\cdot OH$ generation.

There have been numerous reports on the ability of various iron-complexes (of both biological and medical interest) to catalyse the *individual* steps of the Haber-Weiss cycle (see e.g. refs.[13–16]). For example, it has been established that both Fe(II)-EDTA and Fe(II)-DTPA react readily with H_2O_2 to form $\cdot OH$ [reaction (3)].[5,15] On the other hand, pulse-radiolysis studies lead to the suggestion that superoxide can reduce only the EDTA-complex of Fe (III), and not the corresponding DTPA-complex [via reaction (2)]; hence it is believed that Fe-EDTA is a good catalyst of the Haber-Weiss cycle, whereas Fe-DTPA is not.[14,16] In contrast, it has been claimed that the phytate-complex of iron fails to support the Haber-Weiss cycle, not because the Fe(III)-reduction step is inhibited, but because the Fenton reaction is prevented by chelation of iron by phytate.[17,18]

The ease with which Fe(III) is reduced by superoxide is expected to depend upon the difference in redox potentials (and hence the free-energy change of the reaction) of the $O_2/O_2^{\cdot-}$ and Fe(III)/Fe(II) couples for a given chelator: thus, the standard-redox

potential (pH 7) of the hexaquo-complex of iron [for the Fe(III)/Fe(II) couple] is 0.77 V,[19] whereas that of, for example, the EDTA-chelated metal ion is 0.12 V.[19] The redox potential for the $O_2/O_2^{\cdot-}$ couple under standard confitions (i.e. 1 atm. O_2 and 1 M $O_2^{\cdot-}$) is -0.33 V[20] (a value of -0.16 V[21] is often quoted for conditions of 1 M O_2 and 1 M $O_2^{\cdot-}$); however, under *physiological* conditions standard concentrations are not achieved, resulting in an estimated physiological redox potential for the couple of 0.22 V (calculated assuming an intracellular $O_2^{\cdot-}$ concentration of 10^{-11}–10^{-12} M and oxygen concentration of[22] 3.5×10^{-5} M). Therefore, because of the failure of the cell to achieve a high $[O_2^{\cdot-}]/[O_2]$ ratio, the ability of superoxide to act as a reductant of Fe(III) complexes under physiological conditions may be relatively poor.

On the other hand, other reductants present in cells [such as ascorbic acid (vitamin C) and glutathione (GSH)] may be able to replace superoxide in the Haber-Weiss cycle, and therefore promote the toxicity of the iron/hydrogen peroxide system. The reduction of Fe(III) by ascorbic acid has been studied extensively, and the pH-dependence of the reaction rate is believed to reflect the relative rates of reduction of iron by the various ionised forms of ascorbic acid present at a given pH.[23,24] Ascorbic acid ($AscH_2$) has two acidic protons, with pK_a 4.04 and 11.34,[25] so that the predominant species present in solution at pH 7.4 is the monoionised ascorbate anion ($AscH^-$ *ca.* 99.9%). The reduction of Fe(III) by $AscH^-$ is expected to generate the ascorbyl radical anion ($Asc^{\cdot-}$) [reaction (4)]:[24] only at very high proton concentrations is the less delocalized ascorbyl radical ($AscH^{\cdot}$: pK_a –0.45[25]) expected to be formed to a significant extent.

$$Fe^{3+} + AscH^- \rightarrow Fe^{2+} + Asc^{\cdot-} + H^+ \quad (4)$$

However, several investigators have suggested that the dianion of ascorbate (Asc^{2-}) is the kinetically important reductant of iron (and other metal complexes) at physiological pH values,[24–26] despite it representing only *ca.* 10^{-2}% of the total ascorbic acid present [reaction (5)].

$$Fe^{3+} + Asc^{2-} \rightarrow Fe^{2+} + Asc^{\cdot-} \quad (5)$$

A comparison of the standard redox potentials (at pH 7) for the $Asc^{\cdot-}/AscH^-$ (0.282 V) and $Asc^{\cdot-}/Asc^{2-}$ (0.019 V) couples[19] indicates that Asc^{2-} is a more powerful reductant than $AscH^-$. Since the standard redox potentials for both the $Asc^{\cdot-}/AscH^-$ and $Asc^{\cdot-}/Asc^{2-}$ couples are higher than the value for the $O_2/O_2^{\cdot-}$ couple (-0.33 V[20]), ascorbate is expected to be a poorer reductant than superoxide under standard conditions. However, because of the high $[AscH^-]$:$[Asc^{\cdot-}]$ and $[Asc^{2-}]$:$[Asc^{\cdot-}]$ ratios maintained in the cell by the action of various enzyme systems (including dehydroascorbate reductase, see below), the redox potentials for the $Asc^{\cdot-}/AscH^-$ and $Asc^{\cdot-}/Asc^{2-}$ couples at *physiological* concentrations will be lower than their standard values, therefore rendering ascorbate a better reductant than superoxide *in vivo*. For example, the concentration of ascorbic acid in liver is *ca.* 2×10^{-3} M, giving $[Asc^{2-}]$ *ca.* 2×10^{-7} M. If the concentration of $Asc^{\cdot-}$ is taken as, for example, *ca.* 10^{-11} M then the redox potential for the $Asc^{\cdot-}/Asc^{2-}$ couple is estimated to be -0.2 V; it can similarly be shown that the physiological redox potential for the $Asc^{\cdot-}/AscH^-$ is also *ca.* -0.2 V (cf. the *physiological* value for the $O_2/O_2^{\cdot-}$ couple calculated above).

Following the reduction of Fe(III) by ascorbate, the resultant $Asc^{\cdot-}$ is expected to disproportionate [reaction (6)][27] and the dehydroascorbic acid (Asc) formed is expected to be reduced to ascorbate by the action of dehydroascorbate reductase [reaction

(7)].[28] Since the enzyme dehydroascorbate reductase uses glutathione (GSH) as an electron donor [which is in turn reduced by the NADPH-requiring glutathione reductase, reaction (8)], it is possible that prolonged redox-cycling of iron by ascorbate may lead in turn to the extensive oxidation of the cellular GSH and NADPH pools. A decrease in the [GSH]:[GSSG] and [NADPH]:[$NADP^+$] ratios is recognised by toxicologists as being a major mechanism of cell death.[3,29,30] The toxic effects of such redox-cycling reactions will be only further exacerbated by the concomitant formation of hydroxyl radicals.

$$2Asc^- + H^+ \rightarrow AscH^- + Asc \quad (6)$$

$$2Asc + 2GSH \rightarrow 2AscH^- + GSSG + 2H^+ \quad (7)$$

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+ \quad (8)$$

Although ascorbate is known to increase profoundly the level of tissue damage experienced by individuals with iron-overload,[31] many other studies have demonstrated its antioxidant properties:[32] ascorbate is believed to function as a major free-radical scavenger of the aqueous-phase, and it is also believed to protect biological membranes from lipid peroxidation via its interaction with vitamin E.[33-37]

Indeed, in a previous study on the role of the Haber-Weiss cycle in promoting the toxicity of iron towards isolated mitochondria, we demonstrated both pro-oxidant and antioxidant properties of the vitamin.[38] The pro-oxidant action of ascorbate in iron-challenged mitochondria is attributed to its recycling of iron in an ascorbate-driven Fenton reaction, in competition with antioxidant radical-scavenging reactions (which are particularly important at high ascorbate concentrations).

The aims of the experiments reported here were both to compare the ability of several iron-complexes to participate in both reactions of the iron-catalysed Haber-Weiss cycle, and to investigate the ability of ascorbate to modify the level of hydroxyl-radical generation by these complexes in the ascorbate-driven Fenton reaction.

The iron-chelators selected for investigation included those which are suspected as being biological catalysts of the Haber-Weiss cycle (for example, citrate and succinate[7]), examples which are used clinically (desferrioxamine[39]) or have been claimed to have antioxidant properties (phytate[17,18]) and non-biological examples which have been used widely in model-studies of the Haber-Weiss cycle (namely, EDTA and DTPA[5,14-16]). Xanthine oxidase was used to generate superoxide and H_2O_2 in experiments designed to model the iron-catalysed Haber-Weiss cycle, and salicylic acid (2-hydroxybenzoic acid) was employed as a trap for the detection of the hydroxyl radical [the addition of $\cdot OH$ to salicylate yields principally 2,3-dihydroxybenzoic acid (2,3-DHB), the concentration of which can be measured via a simple colorimetric assay (based on the formation of a tungstate-complex)[40]]. This system provides also a suitable model in which to identify and quantify the possible modifying effects of ascorbate. Since the reaction of ascorbate with iron (III) results in the formation of the relatively stable ascorbyl radical anion,[25,33] complementary stopped-flow e.s.r. experiments were designed in an attempt to observe directly the fate of the radical, if it is indeed formed in this reaction. It was hoped that by monitoring this reaction via the formation of the radical using e.s.r. spectroscopy, rather than using optical techniques (as used previously by other workers, see e.g. refs. [24,26]), additional information on the reactions of ascorbate with iron complexes might be gained.

MATERIALS AND METHODS

Materials

All chemicals purchased were of the highest quality available (i.e. Analar grade), and used without further purification. Catalase (thymol-free), 2,3-dihydroxybenzoic acid, ferrous chloride, xanthine and xanthine oxidase (grade IV) were purchased from Sigma Chemical Company (Poole, Dorset, England). L-Ascorbic acid and tris(hydroxymethyl)-methylamine were obtained from BDH Chemicals Ltd. (Poole, Dorset). Deferrioxamine mesylate was purchased from Ciba-Geigy. All other chemicals used were purchased from FSA Laboratory Supplies (Loughborough, England).

All reagent solutions were prepared in de-ionised water.

Methods

(i) Incubations with iron and the determination of 2,3-DHB formation Incubations were performed in 15 ml glass test-tubes (open to the atmosphere), and contained 50 mM NaCl, 10 mM K_2HPO_4/KH_2PO_4, 2.5 mM salicylate, 0.2 mM xanthine, 0.01 units ml^{-1}xanthine oxidase, 0.4 mM metal-chelator, plus 0.1 mM ascorbic acid (when indicated) at pH 7.4. Following the pre-incubation of reagents for 5 minutes in a shaking water-bath (37°C), reactions were initiated via the addition of a small aliquot (typically 25 μl) from a concentrated solution of either $FeCl_2$ (prepared immediately before use in nitrogen-purged water) or $FeCl_3$, to give a final iron concentration of 0.1 mM and total volume of 2 ml. Following a 90 min. incubation period, reactions were terminated via the addition of 60 μl 11.8 M HCl, and the level of 2,3-DHB formed measured using the improved salicylate-hydroxylation assay described in ref.[40].

(ii) Stopped-flow e.s.r. investigations Stopped-flow studies on the reaction between ascorbate and various iron (III)-complexes employed a twin-syringe stopped-flow apparatus in conjunction with a Bruker ESP 300 electron-spin resonance spectrometer (employing 100 kHz modulation and an X-band klystron), equipped with a glass aqueous-flow sample cell.

Under typical experimental conditions, one syringe of the stopped-flow apparatus contained 0.5 mM $FeCl_3$ (together with the chelator indicated, 4 mM) and the other contained 100 mM ascorbic acid. Reactant solutions were prepared in 50 mM NaCl plus either 10 mM KH_2PO_4/K_2HPO_4 (pH 7.4) or 2 mM tris-Cl and adjusted to pH 7.4 via the addition of NaOH or HCl. When indicated, solutions were either deoxygenated or oxygenated immediately prior to use via purging with nitrogen or oxygen. In experiments performed in the presence of either H_2O_2 or catalase, these reagents were added to the iron-containing syringe before mixing (H_2O_2 to 250 mM or catalase to 4×10^4 units ml^{-1}).

In a typical experiment a base-line was recorded initially (by recording the spectrum of the ascorbyl radical anion present in the cell from a previous run) and then the magnetic field of the e.s.r. spectrometer was locked onto the first peak of the ascorbyl radical spectrum (a sharp doublet). The e.s.r. spectrometer was then allowed to scan (in the time-mode) and changes in the signal height recorded following the mixing of $FeCl_3$ and ascorbate using the stopped-flow apparatus. Absolute radical concentra-

tions were determined by calibration of the spectrometer under identical conditions with a known concentration of the stable radical 2,2,6,6-tetramethyl-1-piperidyl-N-oxyl.

Simulations of the time-dependence of ascorbyl radical anion concentrations were performed using a computer programme kindly provided by Dr. J.E. Bennett.

RESULTS AND DISCUSSION

The salicylate-hydroxylation assay of hydroxyl-radical generation in the Haber-Weiss cycle

Experiments were performed via the incubation of both iron (II)-and iron(III)-complexes with superoxide (xanthine/xanthine oxidase). Table I shows the amount of 2,3-DHB formed following the incubation of various iron-complexes in this system. These results immediately indicate a marked difference in the relative abilities of different iron-complexes to generate the hydroxyl radical, and also clearly establish that iron(II)-complexes are more effective than the corresponding iron(III)-complexes in catalysing generation.

Demonstrable hydroxyl-radical generation (as measured in this assay) in incubations with iron(II) is believed to confirm the operation of the Fenton reaction, whereas generation with iron(III) indicates that both the reduction of iron(III) by superoxide *and* the Fenton reaction (i.e. the complete Haber-Weiss cycle) must have occurred. Our results suggest that certain iron-chelators are effective catalysts of both steps of the Haber-Weiss cycle [for example, relatively high levels of ·OH generation were detected in incubations with both the iron(II)- and iron(III)-complexes of EDTA and NTA], whereas others are good catalysts of the Fenton reaction, but do not permit a sufficient rate of reduction of the iron(III) formed to support overall high levels of generation (e.g. DTPA and citrate). A further category of chelators may be identified which generally do not stimulate high levels of ·OH generation (notably DFO).

These general observations appear to correlate well to what is already known about the behaviour of the iron-complexes studied in reactions with reduced-oxygen species. For example, DFO is believed to inhibit hydroxyl-radical generation via the Haber-

TABLE I

The relative ability of various Fe(II)/Fe(III)-complexes to generate the hydroxyl radical in the Fenton reaction and Haber-Weiss cycle, as determined via the hydroxylation of salicylate.

	nmol 2,3-DHB formed[b]	
Chelator[a]	Fe(II)	Fe(III)
Succinate	3.5 ± 0.9	1.9 ± 2.1
Citrate	16.5 ± 1.2	4.4 ± 5.4
NTA	50.0 ± 0.9	19.8 ± 2.3
Phytate	8.6 ± 4.1	1.1 ± 1.0
DFO	3.7 ± 0.6	4.1 ± 0.8
EDTA	58.8 ± 3.6	31.1 ± 3.1
DTPA	49.2 ± 2.0	10.4 ± 2.8

[a]Incubations contained 50 mM NaCl, 10 mM K_2HPO_4/KH_2PO_4, 2.5 mM salicylate, 0.2 mM xanthine, 0.01 unit ml^{-1} xanthine oxidase, 0.4 mM iron-chelator, and 0.1 mM $FeCl_2$ or $FeCl_3$.

[b][2,3-DHB] was determined following 90 min. incubation at 37°C. [Values are corrected against control incubations (in which iron was omitted)] Errors represent ± 1 S.D.[n = 2, iron(II); n = 3, iron(III)].

Weiss cycle,[13,14] and the findings of pulse radiolysis studies (and product-based tests) suggest that EDTA is a good catalyst of the Haber-Weiss cycle, whereas DTPA blocks the reduction of iron(III) by superoxide but not the Fenton step of the Haber-Weiss cycle[5,15,16] Less is known about the effects of biological chelators of iron on the Haber-Weiss cycle, though they are believed to be less effective catalysts than the non-biological agent, EDTA.[13]

The conclusion that the redox-potential of an iron-complex is the primary factor in determing its ability to catalyse the Haber-Weiss cycle is certainly supported by the observations reported here. For example, in order to reduce hydrogen peroxide to the hydroxyl radical, an iron-complex would be expected to have a redox-potential below that of the $H_2O_2/\cdot OH$ couple (0.46 V at pH 7[19]). However, the lower the redox-potential of an iron-complex, the less thermodynamically favourable will be its reduction by superoxide in the Haber-Weiss cycle. Iron-EDTA has a redox-potential which is believed to be optimal for participation in the Haber-Weiss cycle [the redox-potential for Fe(III)/Fe(II)-EDTA at pH 7 under conditions of standard concentration is 0.12 V];[19] thus it reacts rapidly in the Fenton reaction (with a second-order rate constant[5] of $1.0 \times 10^4 M^{-1}s^{-1}$), but is easily reduced back from the iron (III) to iron(II) state by superoxide. In contrast to EDTA, the redox-potential of the DTPA-complex (0.03 V[19]) may be *too* low to permit Fe(III) reduction by superoxide in the Haber-Weiss cycle (even though the Fenton reaction is fast for this complex;[5] k $1.35 \times 10^4 M^{-1}s^{-1}$).

Our findings for the reactions with succinate and citrate are particularly important, since these chelators may be responsible for the catalysis of hydroxyl-radical generation *in vivo*. Although the iron-complex of succinate evidently stimulates only a very low level of hydroxyl-radical generation, iron(III)-citrate (E^0 *ca.* 0.6 V[41]) supports a low, but significant, level of generation. Hence, iron-citrate is considerably more likely than iron-succinate to be a biological catalyst of the Haber-Weiss cycle. The behaviour of the other biological iron chelator studied, phytate, is also important. In apparent contradiction to a previous report suggesting that the claimed antioxidant behaviour of phytate is attributed to its prevention of the Fenton reaction [rather than the reduction of iron(III) by superoxide],[17,18] it is concluded here that the Fenton reaction *is* catalysed by phytate (though slowly), but the Haber-Weiss cycle as a *whole* is blocked by this agent via its effect on iron(III) reduction.

It appears initially that the failure of iron(II)-DFO to catalyse at least the Fenton reaction cannot be explained in terms of its redox potential, since the redox-potential of the complex is believed to be low enough (*ca.* −0.45 V[42]) to render the reaction thermodynamically favourable. The apparently poor ability of iron(II)-DFO to decompose hydrogen peroxide in this system may well be because the complex is such a powerful reductant that it simply reduces molecular oxygen before it can react with hydrogen peroxide (oxygen is expected to be present in incubations at higher concentration than hydrogen peroxide). In support of this conclusion we have performed oxygen-uptake studies to establish that iron(II)-DFO reduces oxygen more rapidly than a variety of other iron(II) complexes studied [e.g., iron(II)-EDTA, iron(II)-DTPA and iron(II)-citrate].[43]

The generation of the hydroxyl radical in the ascorbate-driven Fenton reaction

Reactions were performed in exactly the same manner as those described above, but with ascorbate (0.1 mM) added to the incubations: thus both superoxide and ascor-

bate are present to function as reductants of iron(III) [following its formation in the Fenton reaction] (superoxide could not be omitted from incubations because it also provides, via dismutation, the hydrogen peroxide necessary for hydroxyl-radical generation). The results presented in Table II show the levels of 2,3-DHB formed following the incubation of various iron-complexes with salicylate in the presence of superoxide and ascorbate.

Figure 1 allows a direct comparison to be made between results obtained (Tables I and II) for incubations performed in the absence and presence of ascorbate for a given iron(II)/(III)-complex. This clearly illustrates the marked effects of ascorbate on the levels of hydroxyl-radical generation. For example, whereas the generation of the hydroxyl radical in the Haber-Weiss cycle in the presence of iron-succinate and iron-citrate was found to be very poor, the addition of ascorbate to these systems was found to result in a marked stimulation in generation of the radical, suggesting that, in the presence of a good reducant for iron, succinate and citrate are very effective catalysts of hydroxyl-radical generation.

Whereas the addition of ascorbate to incubations initiated with the iron complexes of EDTA resulted in the stimulation of hydroxyl-radical generation, ascorbate ad-

TABLE II

The generation of the hydroxyl radical (determined via the hydroxylation of salicylate to 2,3-DHB) by various Fe(II)/(III)-complexes in the presence of superoxide and ascorbate.

	nmol 2,3-DHB formed[b]	
Chelator[a]	Fe(II)	Fe(III)
Succinate	57.6 ± 2.3	26.6 ± 5.5
Citrate	55.4 ± 7.6	28.6 ± 0.3
DFO	6.4 ± 3.8	3.5 ± 0.3
DTPA	47.6 ± 3.4	15.6 ± 0.0
EDTA	95.5 ± 26.8	64.8 ± 5.5

[a]Incubations contained 50 mM NaCl, 10 mM K_2HPO_4/KH_2PO_4, 2.5 mM salicylate, 0.2 mM xanthine, 0.01 unit ml^{-1} xanthine oxidase, 0.4 mM chelator, 0.1 mM ascorbate, plus 0.1 mM $FeCl_2$ or $FeCl_3$.

[b]2,3-DHB was determined following 90 min. incubation at 37°C. Values are corrected against control incubations (in which iron was omitted). Errors represent ± 1 S.D. (n = 2).

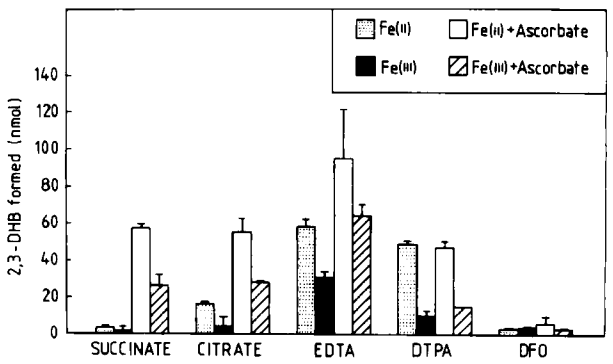

FIGURE 1 Comparison of the ability of ascorbate to stimulate hydroxyl-radical generation (determined via the hydroxylation of salicylate to 2,3-DHB) by different iron-complexes in the Haber-Weiss cycle. The results are a summary of those presented in Tables I and II.

dition to reactions initiated with the DTPA-complexes had no significant effect on the generation of the radical (see Figure 1). This observation suggests that ascorbate is more effective than superoxide in the reduction of iron(III)-EDTA (under the incubation conditions employed here), and that both superoxide and ascorbate are very poor reductants of iron(III)-DTPA. As might be expected on the basis of the very low redox-potential for the DFO-iron complex (*ca.* $-0.45\,V$[42]), no significant increase in the rate of hydroxyl-radical generation was observed following the addition of ascorbate to incubations with this chelate.

A stopped-flow e.s.r. study of the reduction of iron(III)-EDTA by ascorbate

The reduction of iron(III) complexes by ascorbate has previously been studied largely via spectrophotometric techniques. Evidence was sought here, via the direct observation by e.s.r. spectroscopy of the ascorbyl radical anion, for the assertion that the ability of ascorbate to promote hydroxyl-radical generation in the Fenton reaction is determined by its ability to reduce iron(III). In an initial experiment, the reaction between iron(III)-EDTA (0.5 mM $FeCl_3$ and 4 mM EDTA) and ascorbate (100 mM) at pH 7.4 was investigated using a two-stream stopped-flow apparatus as a function of time with the e.s.r. spectrometer's magnetic field locked onto the first peak of the spectrum of the ascorbyl radical-anion (see Figure 2).

The time-dependence of the concentration of the radical shows that the maximum concentration of the radical is achieved within *ca.* 10–20 ms of mixing, followed by rapid initial decay and then an apparently slower decay process. Ascorbyl radicals are

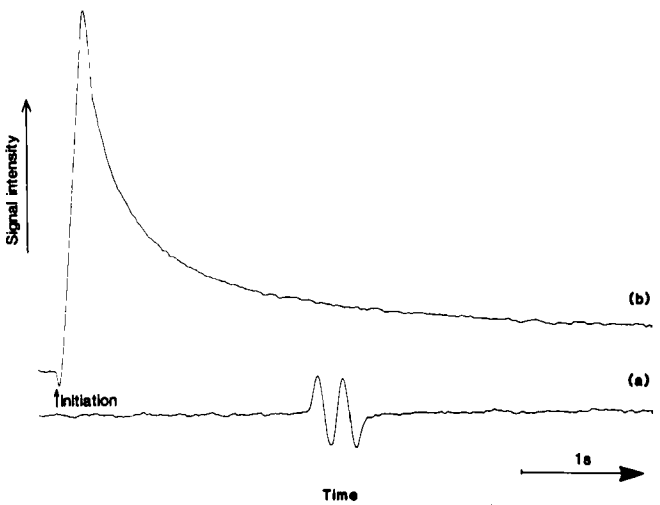

FIGURE 2 Variation with time of the ascorbyl radical anion concentration during the oxidation of ascorbate by Fe(III)-EDTA at pH 7.4 (in phosphate buffer). (a) The base-line shown is the e.s.r. spectrum of the radical anion recorded using a static sample, *ca.* 5 minutes after mixing. (b) Stopped-flow experiments: the magnetic field of the spectrometer was locked onto the first peak of the spectrum (see (a)). The spectrometer was then set to scan in the time-mode and changes in radical concentration monitored following the rapid mixing of the reagents using a twin-syringe stopped-flow apparatus. Reagent concentrations before mixing were 100 mM ascorbate, 4 mM EDTA and 0.5 mM $FeCl_3$ (arrow indicates reaction-initiation).

believed to undergo disproportionation [reaction (6)] with a rate constant of the order 10^5–$10^6\,M^{-1}s^{-1}$ at pH 7 (the exact value being dependent upon ionic strength, temperature and the presence of phosphate buffers).[27] It is also possible that ascorbyl radical anions can decay via their oxidation to dehydroascorbate by iron(III) [reaction (9)].

$$\text{Fe(III)-EDTA} + \text{AscH}^{\cdot} \rightarrow \text{Fe(II)-EDTA} + \text{Asc} \qquad (9)$$

Ascorbate was always employed in excess initial concentration over that of iron(III), so we can assume that all the iron(III) initially present is reduced to iron(II) by ascorbate, and therefore that the oxidation of ascorbyl radicals by iron(III) [reaction (9)] will be negligible during the initial phase of decay. As expected on this basis, the decay of $Asc^{\cdot-}$ (as determined via measure of the absolute radical concentration) was found to be second-order, at least for the initial stages of reaction (and as judged by the linearity of the variation of $[Asc^{\cdot-}]^{-1}$ with time): a value of the rate constant for decay of *ca.* $5 \times 10^5\,M^{-1}s^{-1}$ is estimated.

To determine the rate constant for the initial reaction between iron(III) and ascorbate, it was assumed that at the time of the reaction at which the ascorbyl-radical signal is at a maximum (i.e. at the point of inflection), a steady-state exists in which the rate of radical generation via reaction (4) equals its rate of loss via disproportionation [see equation (10)].

$$k_{obs}[\text{AscH}^-]_t[\text{Fe(III)}]_t = 2k_t[\text{AscH}\cdot]_t^2 \qquad (10)$$

where k_{obs} = observed rate constant for the initial reaction between ascorbate and Fe(III)

$[\text{AscH}^-]_t$ = concentration of ascorbate at time the specified time, t

$[\text{Fe(III)}]_t$ = concentration of iron(III) at time t

$2k_t$ = rate constant for the disproportionation (termination) of ascorbyl radical anion

$[\text{AscH}\cdot]_t$ = concentration of ascorbyl radical anion at time t

An attempt was first made to simulate the experimentally observed time-course for the ascorbyl radical anion. It was found possible to match both the initial (*ca.* 20 ms) growth and initial decay of the radical (see Figure 3), using values for k_{obs} and $2k_t$ of $4.0 \times 10^2\,M^{-1}s^{-1}$ and $5.0 \times 10^5\,M^{-1}s^{-1}$ respectively (the finding that the simulated time-course for the initial growth of radicals does not fit the experimental observation exactly is believed to reflect both the time required for the syringe-drive to mix the reactants and the response-time of the spectrometer). Since the values obtained here are in good agreement with those reported elsewhere,[27] it is believed that equation (10) is probably valid for the initial stage of reaction under the conditions employed here.

The more gradual decay of the signal observed after the initial rapid decay is believed to indicate the operation of additional reactions leading to the *generation* of the radical, resulting in a decrease in the *observed* overall decay rate. Measurement of the differences between radical concentrations for the observed and simulated time-courses provides a profile for the build-up of radicals via secondary reactions (as indicated in Figure 3). It is suggested that the secondary reactions involve redox-cycling of the metal-ion, possibly involving its reduction of oxygen to the hydroxyl radical, which may then in turn attack ascorbate to generate ascorbyl.

In further experiments designed to investigate this possibility, it was found that the time for which the ascorbyl radical anion signal can be observed is markedly dependent upon the concentration of oxygen in the system. For example, the signal was

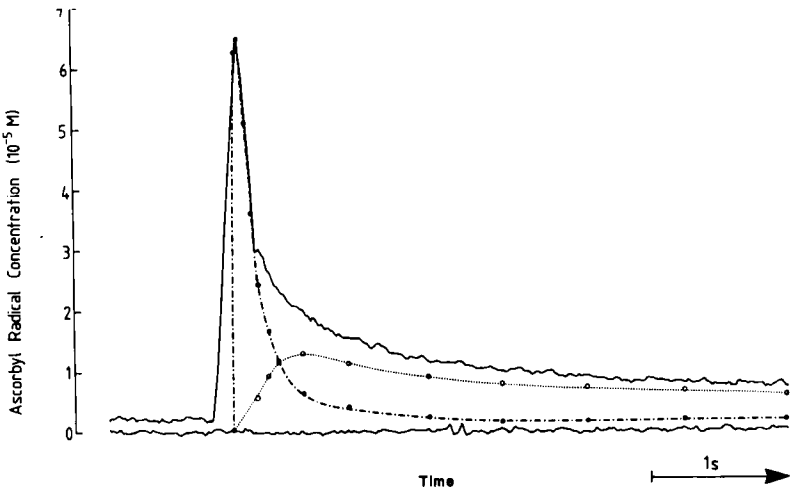

FIGURE 3 Experimental and simulated time-courses of ascorbyl radical anion concentration during the oxidation of ascorbate by Fe(III)-EDTA at pH 7.4 (in tris buffer) in a stopped-flow experiment. A calculated time-course for the generation of the radical via secondary reactions (see text) is also shown. Reagent concentrations before mixing were 100 mM ascorbate, 4 mM EDTA and 0.5 mM $FeCl_3$.

—— observed variation in [$Asc^{\cdot-}$] with time

–·–·– simulated variation in [$Asc^{\cdot-}$]

· · · · · calculated contribution to $Asc^{\cdot-}$ formation via secondary reactions.

observed to decay rapidly when the reactant solutions were deoxygenated (by purging with nitrogen) prior to use (Figure 4a), whereas the loss of signal could be retarded by using oxygen-saturated solutions (see Figure 4b).

The possibility was then investigated that oxygen is reduced to superoxide (and hence hydrogen peroxide) by the iron(II)-EDTA formed in the initial reaction. When experiments were performed using deoxygenated solutions, but with added hydrogen peroxide, the loss of the ascorbyl-radical signal was again retarded (see Figure 4c), suggesting that a process involving the reduction of oxygen through to hydrogen peroxide is indeed responsible for the prolonged observation of the radical in the experiments described above. In order to confirm the dependence of radical formation on the presence of hydrogen peroxide, an experiment was performed in which catalase was added to an oxygen-saturated system: this was found to prevent the expected prolonged observation of the radical in the presence of oxygen (Figure 4d), verifying the role of hydrogen peroxide in radical formation.

It is known that the hydroxyl radical can react with ascorbate to form the ascorbyl radical anion;[33] it is therefore proposed that, following the reduction of iron(III)-EDTA by ascorbate, iron(II)-EDTA undergoes rapid autoxidation to generate the hydroxyl radical, which then attacks further ascorbate (present in excess) to generate more ascorbyl radical anion. The iron(III)-EDTA complex generated following the oxidation of iron(II)-EDTA by oxygen, superoxide and hydrogen peroxide is also expected to generate further hydroxyl radicals via reaction with ascorbate (present in excess) [reactions (1),(6) and (11)–(15)].

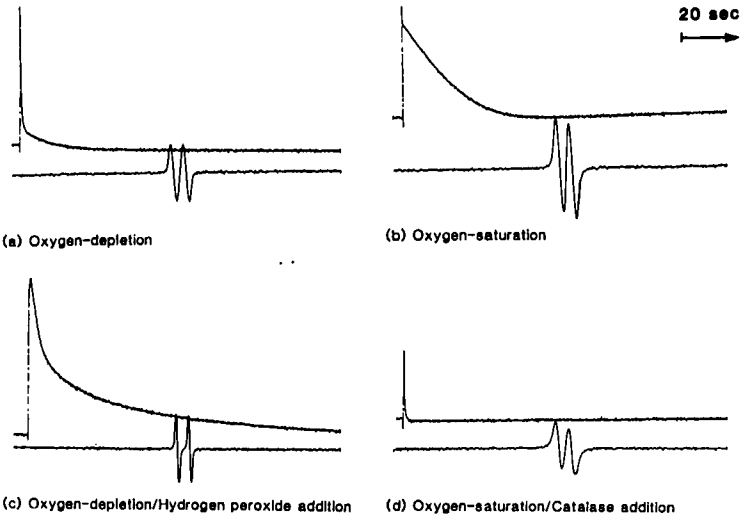

FIGURE 4 The effect of oxygen-depletion (a), oxygen-saturation (b), H_2O_2 addition (c), and catalase addition, (d), on the concentration of ascorbyl radical anion detected in the reaction between Fe(III)-EDTA and ascorbate at pH 7.4 (in phosphate buffer). Reagent concentrations before mixing were 100 mM ascorbate, 4 mM EDTA, 0.5 mM $FeCl_3$ 250 mM H_2O_2 and 4×10^4 units ml^{-1} catalase.

$$Fe(III)\text{-}EDTA + Asc^{2-}(AscH^-) \rightarrow Fe(II)\text{-}EDTA + Asc^{\cdot-} (+ H^+) \quad (11)$$
$$2Asc^{\cdot-} + H^+ \rightarrow AscH^- + Asc \quad (6)$$
$$Fe(II)\text{-}EDTA + O_2 \rightarrow Fe(III)\text{-}EDTA + O_2^{\cdot-} \quad (12)$$
$$Fe(II)\text{-}EDTA + O_2^{\cdot-} + 2H^+ \rightarrow Fe(III)\text{-}EDTA + H_2O_2 \quad (13)$$
$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (1)$$
$$Fe(II)\text{-}EDTA + H_2O_2 \rightarrow Fe(III)\text{-}EDTA + \cdot OH + OH^- \quad (14)$$
$$AscH^- + \cdot OH + H^+ \rightarrow H_2O + AscH\cdot \quad (15)$$

Despite the complexity of this system the above experiments suggest that e.s.r. spectroscopy, in conjunction with a stopped-flow system, is a valuable technique for the study of the reactions of iron with ascorbate and oxygen: although a total kinetic analysis of the system has not been attempted, a semi-quantitive study of the ability of ascorbate to reduce various iron(III)-complexes was undertaken, in order to provide an indication of their ability to catalyse the ascorbate-driven Fenton reaction.

The rôle of the metal-ion chelator in the reduction of iron(III) by ascorbate

Stopped-flow e.s.r. experiments were performed to monitor the reactions of ascorbate with the iron(III)-complexes described above. The time-courses of ascorbyl radical-anion concentration obtained for the iron complexes of EDTA, DTPA, citrate, succinate, phytate, inorganic-phosphate and DFO were found to differ both in the maximum concentration of the radical acquired in the first 10–20 ms of reaction and also in the subsequent decay rate of the radical (see Figure 5). These results suggest that the ability of the chelators to promote initial reaction between iron(III) and ascorbate [reaction (4)] is in the order EDTA > citrate > phytate > succinate $\approx$ DTPA $\gg$ DFO. Values for k_{obs} have not been calculated because the time-courses indicate that secondary reactions may significantly contribute to the initial maxim-

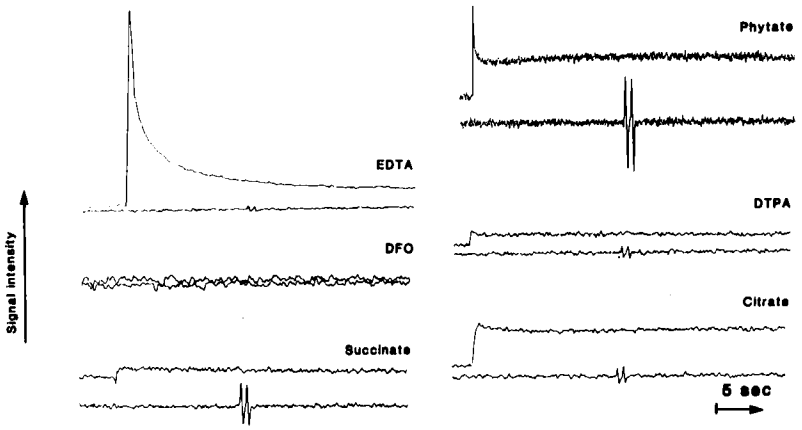

FIGURE 5 The effect of the metal-ion chelator on the oxidation of ascorbate by Fe(III) (determined via the measurement of ascorbyl radical anion concentration using stopped-flow e.s.r. spectroscopy) at pH 7.4 (in tris buffer). Reagent concentrations before mixing were 100 mM ascorbate, 4 mM chelator and 0.5 mM $FeCl_3$.
Receiver gain settings used: EDTA, 1×10^4; phytate, 2.5×10^5; DFO, 2×10^5; DTPA, 2.5×10^5; succinate, 8×10^4; citrate, 5×10^4.

um radical concentration detected. Secondary reactions appear to be particularly significant with the phytate and citrate iron(III)-complexes, since a considerable steady-state longer-term radical concentration is achieved (see Figure 5).

The results of the semi-quantitative e.s.r. analysis presented above are thus in good agreement with the results of the salicylate-hydroxylation experiments in the presence of ascorbate on the ability of the ascorbate to promote the generation of the hydroxyl radical by various iron-complexes (EDTA > citrate > succinate > DTPA ≫ DFO).

CONCLUSIONS

It is concluded that, for an iron-complex to catalyse the generation of the hydroxyl radical in the presence of an enzymatic source of superoxide (and hence, hydrogen peroxide), the complex must permit both the efficient reduction of iron(III) *and* the subsequent reaction of iron(II) in the Fenton reaction. Examples of iron-chelators have been identified which catalyse either one or both of these reactions: for example, DTPA and phytate are believed to catalyse the Fenton reaction, but are believed to block continuous hydroxyl-radical generation by preventing reduction of the metal-ion by superoxide (and indeed ascorbate). In contrast, EDTA and NTA are both believed to be good catalysts of the Haber-Weiss cycle, a finding which may be of relevance to the known toxicity and carcinogenicity of NTA.[44–46]

Of the biological chelators studied, only citrate was found to promote a significant level of hydroxyl-radical generation via the Haber-Weiss cycle. However, both citrate and succinate were found to be good catalysts of ·OH generation in the ascorbate-driven Fenton reaction: this finding may be of crucial relevance to the toxicity of iron within a reducing intracellular environment.

The findings presented here also indicate that desferrioxamine, which has been used in the treatment of iron-overload,[39] prevents *both* reactions of the Haber-Weiss cycle (and also the ascorbate-driven Fenton reaction): the iron(III)-reduction step [reaction (2)] via its reduction of the metal ion's redox potential below that of the $O_2/O_2^{\cdot-}$ couple, and the Fenton reaction [reaction (3)] via promotion of the rapid oxidation of the metal-ion by oxygen (hence preventing its reduction of hydrogen peroxide).

In addition to the identification of iron-chelators which selectively inhibit or promote specific reactions of the Haber-Weiss cycle (both in the presence and absence of ascorbate), this study has also provided direct e.s.r. evidence for the operation of prolonged redox-cycling reactions between iron, oxygen, and ascorbate, reactions which may even cause direct cell death. This suggests that the processes responsible for the toxicity of endogenously-generated oxygen radicals may indeed be similar to those responsible for the toxicity of xenobiotics (e.g. menadione[3]) which are known to induce oxidative stress via related mechanisms.

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