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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

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The ability of various iron(II)-complexes of biological, clinical and chemical interest to reduce molecular oxygen to reactive oxy-radicals has been investigated using complementary oxygen-uptake studies and e.s.r. techniques. It is demonstrated that although the rate of oxygen reduction by a given iron complex is directly related to its redox potential [thus complexes with low values of E^0 for the Fe(III)/Fe(II) couple are the most effective reductants of oxygen], the overall ability of an iron(II) complex to induce oxidative biomolecular damage is also determined by its ability to undergo redox-cycling reactions with reducing radicals formed following the reaction of hydroxyl radicals with organic substrates present in the system (e.g. metal–ion chelators and organic buffers). Evidence is presented to suggest that the "Good" buffer MOPS forms a reducing radical following attack by ·OH, and hence encourages the autoxidation of iron with the generation of oxy-radicals (as also observed for some of the chelates studied); this may have important implications for the use of such buffers in free-radical studies.

KEY WORDS: Iron autoxidation, oxygen-centred radicals, iron-chelator, electron-spin resonance, MOPS buffer.

ABBREVIATIONS: BSA, bovine serum albumin; DFO, desferrioxamine mesylate; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid; HEPES, N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulphonic acid); MES, 4-(morpholino)ethanesulphonic acid; MOPS, 4-(morpholino)propanesulphonic acid; NTA, nitrilotriacetic acid; PBN, N-t-butyl-α-phenylnitrone.

INTRODUCTION

In addition to the enzymatic sources of oxygen-centred radicals which are believed to occur *in vivo* (for example, NADPH oxidase,[1] xanthine oxidase[2] and NADH dehydrogenase[3] are believed to generate the superoxide ion $O_2^{\cdot-}$), the direct, non-enzymatic reduction of ground-state oxygen by redox-active metal ions (notably, copper[4] and iron[5]) may represent an important source of cytotoxic reactive-oxygen species (e.g.

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$O_2^{\cdot-}$,H_2O_2, ·OH) particularly under conditions in which the normal capacity of the body to sequester metal ions within storage or transport proteins is exceeded (e.g. in chronic iron overload[6] and acute iron poisoning[7,8]). Since the enzymes xanthine oxidase, NADPH oxidase and NADH dehydrogenase are capable of reducing oxygen only to superoxide ($O_2^{\cdot-}$) or H_2O_2 metal ions are also believed to be responsible for the further reduction of these species to the more reactive and biologically destructive hydroxyl radical (·OH) [reactions (1)–(3), in which M refers to a redox-active metal ion].[9,10]

$$M^{(n+1)+} + O_2^{\cdot-} \longrightarrow M^{n+} + O_2 \quad (1)$$

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

$$M^{n+} + H_2O_2 \longrightarrow M^{(n+1)+} + \cdot OH + OH^- \quad (3)$$

Metal ions are also believed to be involved in the toxicity of redox-cycling xenobiotics, evidently by bringing about the further reduction of superoxide (generated by the xenobiotic) to the hydroxyl radical [reactions (2)–(3)].[11] It is therefore believed that metal ions may play a key role in the generation of cytotoxic oxygen-derived radicals during the metabolism of oxygen and certain xenobiotics.

The chemistry involved in the reduction of oxygen by metal ions is complex, and various mechanisms have been suggested, particularly for iron (the metal implicated most frequently in the pathology of oxygen[12–16]) the autoxidation of which is often described according to reactions (4)–(7).

$$Fe^{2+} + O_2 \longrightarrow Fe^{3+} + O_2^{\cdot-} \quad (4)$$

$$Fe^{2+} + O_2^{\cdot-} + 2H^+ \longrightarrow Fe^{3+} + H_2O_2 \quad (5)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + \cdot OH + OH^- \quad (6)$$

$$Fe^{2+} + \cdot OH \longrightarrow Fe^{3+} + OH^- \quad (7)$$

The activation of oxygen by iron is subject to both kinetic and thermodynamic restraints (see, for example, ref. 17), and reactions (4)–(7) may well be an oversimplification of the process. For example, it has been suggested that oxidants other than, or in addition to, the hydroxyl radical are generated (e.g. high valent metal–oxygen complexes).[18–21]

As a result of the low redox potential for the $O_2/O_2^{\cdot-}$ couple (-0.33 V[17,22]), the initial reduction of oxygen to superoxide by iron [reaction (4)] is expected to be (thermodynamically) the most difficult reaction in the autoxidation of iron [the standard redox potential for $Fe^{3+}{}_{aq}/Fe^{2+}{}_{aq}$ is 0.77 V,[23] giving a standard Gibb's free energy change for reaction (4) of $+106.1$ kJ mol^{-1}]. Several low-molecular-weight chelating agents (e.g. EDTA) form complexes with iron which are soluble at physiological pH and which have redox potentials (Fe^{3+}/Fe^{2+}) below that of the hexaquo complex, thereby favouring reaction (4).[17] However, most of these iron-complexes have redox potentials which are still more positive than the value for $O_2/O_2^{\cdot-}$ (see, for example, ref. 17), and it is possible that, during the autoxidation of certain iron complexes, oxygen is reduced via a two-electron process to H_2O_2 without the release of free superoxide (the redox potential for the O_2/H_2O_2 couple is 0.3 V[17]). This proposal is supported by kinetic results which indicate that, in the presence of, for example, perchlorate or sulphate, the rate-determining reaction in iron autoxidation involves the direct reduction of oxygen to peroxide by two Fe^{2+} ions (as reviewed in ref. 24).

In contrast, free superoxide has been detected during the autoxidation of iron in MOPS and HEPES buffers (via its reduction of nitroblue tetrazolium),[25] and the rate law for the autoxidation of iron in the presence of inorganic phosphate (rate = $k[Fe^{2+}]pO_2[\text{phosphate}]^2$) is believed to indicate the formation of free superoxide in the rate limiting reaction.[24,26] Therefore, under certain circumstances [e.g. in which the redox potential of Fe^{3+}/Fe^{2+} is lowered via chelation of the metal ion, and under physiological conditions (for which the redox potential for $O_2/O_2^{\cdot-}$ is calculated[17,27] as *ca.* 0.2 V)] the autoxidation of iron may well involve the formation of free superoxide.

In view of the critical role apparently played by chelating agents in determining the reactivity of iron-chelate complexes towards oxygen, considerable attention has been directed towards the identification of chelators which *prevent* the activation of oxygen by iron.[28–31] For example, phytic acid is believed to prevent oxidative deterioration in plant materials via its chelation of iron,[32] and desferrioxamine (DFO), a powerful iron chelator isolated from *Streptomyces pilsus*, is used clinically to treat iron-overload conditions (although its use for treatment is associated with neurological damage).[33–35] In contrast, the *toxicity* of some iron chelators has been attributed to their formation of complexes with iron which react readily with oxygen to form reactive oxygen species, notably the nephrotoxin and carcinogen NTA.[36–38]

In this study we intended to compare directly the ability of various iron complexes to promote (or inhibit) the generation of reactive oxygen species capable of inducing biomolecular damage. The chelators chosen for study included examples of those which may form complexes with iron *in vivo* (e.g. inorganic phosphate, citrate, succinate[39] and, in plants, phytate[32]), and those having therapeutic value (i.e. desferrioxamine[33–35]), or which form complexes with iron (notably EDTA[9,10,40] and NTA[38]) which are believed readily to undergo redox reactions with oxygen and its reduced intermediates. Attention has been given to any possible modifying effects of the presence of organic buffers on autoxidation (see e.g. ref.[25]).

Various techniques are available for the study of iron autoxidation, including, for example, the method in which 1,10-phenanthroline is added to an aliquot of the reaction mixture at a specified time and the concentration of iron(II) remaining calculated from the absorbance at 515 nm (see, for example, ref.[25]). Alternatively, the autoxidation process can be followed by monitoring the consumption of oxygen [according to reactions (4)–(7)].[38] In this study we employed the latter method, since the phenanthroline method may be subject to interference resulting from any competition for the binding of iron(II) between the chelator under investigation and 1,10-phenanthroline itself. In an attempt to facilitate the direct observation of any radical species formed, the technique of e.s.r. spin trapping (reviewed in ref.[41]) was also employed.

MATERIALS AND METHODS

All chemicals purchased were of analytical quality, and reagent solutions were prepared using doubly-distilled water. Solutions of DMPO were purified prior to use via filtration through activated charcoal. DMPO, EDTA (disodium salt), MOPS, NTA (disodium salt), and PBN were purchased from Sigma Chemical Company, and desferrioxamine mesylate was obtained from Ciba Giegy. SOD (S-2515, 31,500 units mg^{-1}), catalase (C-40, 14,100 units mg^{-1}) and BSA (98–99%, fatty-acid free) were

obtained from Sigma Chemical Company. All other chemicals were purchased from Fisons plc.

Oxygen-electrode studies

Changes in oxygen concentration were monitored upon the addition of a small aliquot (typically 0.1 ml) of $FeCl_2$ (from a concentrated stock solution prepared freshly in N_2-purged water) to the cavity of a Clark-type oxygen electrode (maintained at 37°C) containing a solution of NaCl (50 mM), MOPS (10 mM) and chelator (2 mM) (adjusted to pH 7.4 using HCl or NaOH), to give a final Fe^{2+} concentration of either 0.24 or 0.48 mM and a total volume of 5 ml. Where indicated, incubations were also performed at different chelator concentrations, or with the omission of either MOPS or the chelator. In order to estimate the molar ratio of oxygen uptake to Fe^{2+}-addition, it was assumed that the initial oxygen concentration in incubations, before Fe^{2+} addition, was *ca.* 210 μM.

E.s.r. spin-trapping investigations

Reactions were initiated via the addition of a small aliquot of $FeCl_2$ (from a concentrated stock solution prepared freshly in N_2-purged water) to a glass test-tube containing either PBN (82 mM) or DMPO (54 mM), NaCl (50 mM), MOPS(10 mM), and the chelator (2 mM) (adjusted to pH 7.4 using HCl or NaOH), to give a final Fe^{2+} concentration of 1 mM and a total volume of 5 ml. Where indicated, reactions were performed with the omission of MOPS and the addition of either catalase (500 $\mu g\,ml^{-1}$), superoxide dismutase (400 $\mu g\,ml^{-1}$) or bovine serum albumin (500 $\mu g\,ml^{-1}$). Reactions were left at room temperature (open to the atmosphere) for 10 min., before being transfered to a flattened aqueous e.s.r. cell. E.s.r. spectra were recorded using a Bruker ESP 300 spectrometer, employing 100 kHz modulation and an X-band klystron.

Continuous-flow e.s.r. experiments

The Ti^{3+}/H_2O_2 reaction was employed as a source of the hydroxyl radical in three-way continuous flow experiments[9] in order to observe directly both the reaction of the hydroxyl radical with MOPS buffer and the reaction of any organic radicals formed with H_2O_2 and Fe^{3+}. Reactant concentrations before mixing in the spectrometer cavity were: $TiCl_3$, 5 mM (stream 1); H_2O_2 2.5 mM (stream 2); MOPS, 40 mM (stream 3). Where indicated, the concentration of H_2O_2 was increased from 2.5 to 7.2 mM, and $FeCl_3$ was added to stream 3 (to a pre-mixing concentration of 9.9 mM). In all experiments reagent solutions were deoxygenated before and during use by purging with N_2 and the post-mixing pH was adjusted to 2.2 via the addition of H_2SO_4 to the $TiCl_3$, H_2O_2 and MOPS streams. Spectra were recorded on a Bruker ESP 300 spectrometer.

RESULTS

Oxygen-uptake studies

The extent of uptake of oxygen recorded during the autoxidation of various iron-complexes at pH 7.4 in MOPS buffer is shown in Figure 1: the nature of the iron

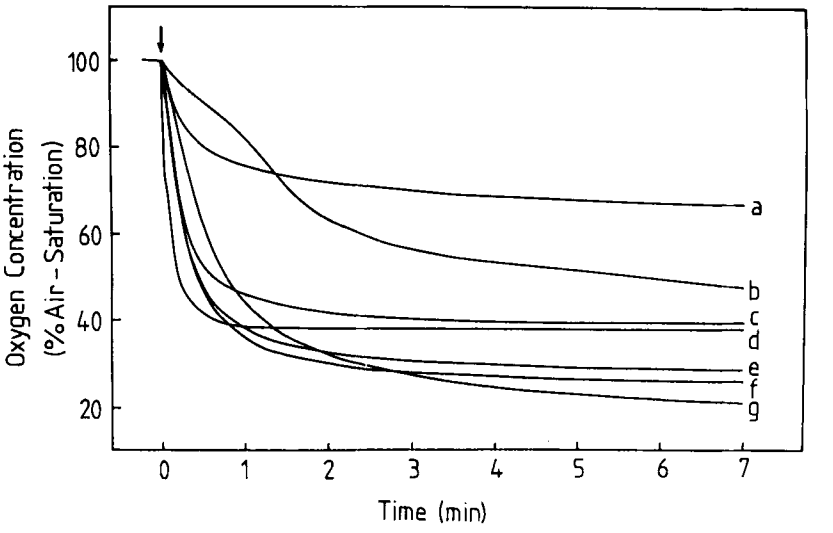


FIGURE 1 Oxygen-uptake occurring during iron(II)-autoxidation in the presence of various iron-chelators. $FeCl_2$ was added (to a final concentration of 0.48 mM), to a solution containing NaCl (50 mM), MOPS (10 mM) and the chelator indicated (2 mM) [adjusted to pH 7.4 and maintained at 37°C] in the cavity of an oxygen-electrode and changes in oxygen concentration were monitored with time. Key: a, inorganic phosphate; b, succinate; c, phytate; d, DFO; e, NTA; f, EDTA; g, citrate.

chelator is found to affect both the initial rate and overall extent of oxygen-uptake. For example, DFO stimulates the greatest *initial* rate of uptake [presumably a reflection of the very low redox potential of the Fe(III)DFO/Fe(II)DFO couple (*ca.* -0.45 V[42,43]], whereas the NTA, EDTA and citrate complexes exhibit the greatest *overall* levels of oxygen uptake after *ca.* 5 minutes incubation.

Oxygen-electrode techniques have been employed previously to determine the stoicheiometry of the reaction between Fe^{2+} and oxygen, and hence to derive kinetic and mechanistic information.[31,45] An $Fe^{2+}:O_2$ ratio of 4:1 is expected on the basis of

TABLE I

Fe^{2+}/O_2 ratios measured for the autoxidation of various iron(II)-chelate complexes determined using oxygen-uptake measurements

Iron-chelator[a]	Fe^{2+}/O_2 ratio[b]
Inorganic phosphate	7.1/1
Succinate	4.5/1
Phytate	3.9/1
Desferrioxamine	3.7/1
NTA	3.3/1
EDTA	3.3/1
Citrate	3.0/1

[a]The oxygen uptake resulting from iron(II)-complex autoxidation was measured directly from the oxygen-electrode recordings (after seven minutes' incubation) presented in Figure 1, in which full experimental details are given.

[b]The concentration of oxygen was assumed to be 210 μM for the determination of Fe^{2+}/O_2 ratios. Typical values, showing less than 4% variation.

reactions (4)–(7) (see earlier), and this value has been reported for the reactions of both iron(II)tris(4,7-dihydroxy-1,10-phenanthroline)[44] and iron(II)phytate[32] with oxygen. As indicated in Table 1, the stoicheiometry observed here is dependent markedly upon the nature of the iron complex under investigation. Since oxygen-uptake is still occurring at the end of the seven-minute incubation period in the presence of phosphate and succinate, the high Fe^{2+}/O_2 ratios observed with these chelators evidently reflects the relatively low reactivity of the phosphate and succinate iron-complexes with oxygen, and therefore probably the failure of the reaction to reach completion (rather than the stoicheiometry of the completed reaction). In contrast, the reaction of the phytate and DFO complexes with oxygen appears to reach completion within the incubation time, and the $Fe^{2+}:O_2$ ratios for these complexes are, within reasonable error, 4:1. Most interestingly, the low $Fe^{2+}:O_2$ ratios observed with NTA, EDTA, and citrate, which are still decreasing at the end of the incubation period, indicate strongly the operation of additional chain reactions,

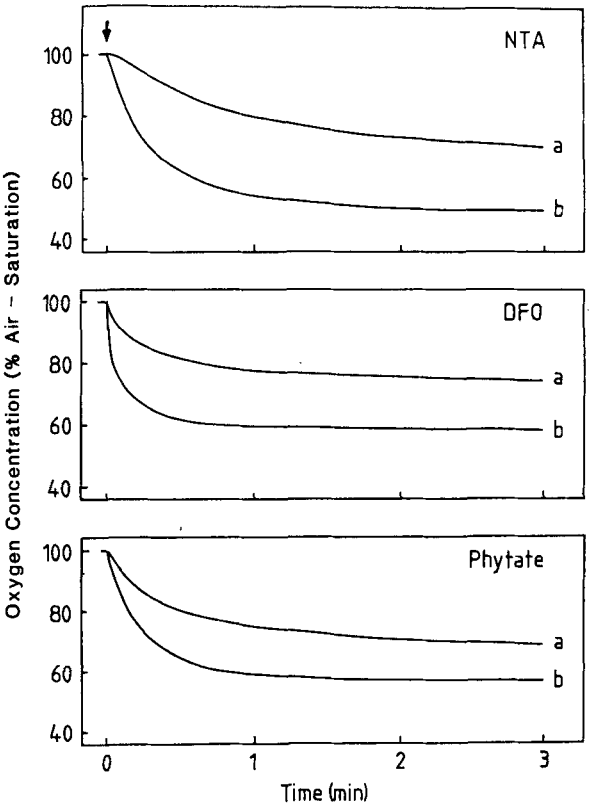


FIGURE 2 The effect of MOPS buffer on the extent of oxygen-uptake occurring as a result of the autoxidation of iron(II) in the presence of different iron-chelators. $FeCl_2$ was added (to a final concentration of 0.24 mM) to a solution containing NaCl (50 mM) and the iron-chelator indicated (2 mM) [adjusted to pH 7.4 and maintained at 37°C] in the cavity of an oxygen-electrode and changes in oxygen concentration were monitored with time. Reactions were performed in the absence (a) and presence (b) of MOPS (10 mM).

possibly involving the regeneration of iron (II), leading to a higher level of oxygen uptake than might be expected from the direct interaction of 4 moles of Fe^{2+} with 1 mole of O_2.

Experiments with NTA, DFO, and phytate were performed in both the presence and absence of buffer: as indicated in Figure 2 the presence of the buffer (in excess of the chelate) results in a considerable increase in the level of oxygen-uptake recorded. In order to investigate the possibility that MOPS buffer itself increases the reactivity of iron(II) towards oxygen (despite its apparent lack of metal-binding capability)[45] the autoxidation of iron was monitored in MOPS alone (i.e. in the absence of the chelating agent). The results shown in Figure 3 demonstrate clearly that the presence of MOPS (in saline) allows iron(II) to react with oxygen whereas in non-buffered saline no oxygen uptake is detectable. Likewise as shown in Figure 4, MOPS stimulates oxygen uptake in the presence of EDTA at a low [EDTA]:[iron] ratio (0.52:1) but not when the chelator EDTA is present in excess (EDTA forms a tightly bound iron complex with 1:1 stoicheiometry).[46]

It is possible that MOPS itself is able to form a (weakly bound) complex with iron (in the absence of other chelators) which causes a sufficient decrease in the redox potential (Fe^{3+}/Fe^{2+}) to permit reactions with oxygen to occur (in the same manner as the other chelators studied). On the other hand it has also been suggested that under such conditions the metal ion is not chelated to the buffer[25,47] and that the effect of MOPS is simply to maintain the pH of the system at a high enough value that the relatively stable complex $Fe(H_2O)_6^{2+}$ (which predominates at slightly lower pH values and for which autoxidation is considerably retarded)[47] is hydrolysed via deprotonation of the water molecules, to give species with reduced E^{θ} and greater susceptibility to autoxidation[48,49] [E^{θ} for aqueous Fe(III)/Fe(II) at pH 1 is 0.77 V, whereas at pH 7 the value is 0.11 V].[48] Since MOPS buffer would certainly not be expected to complex

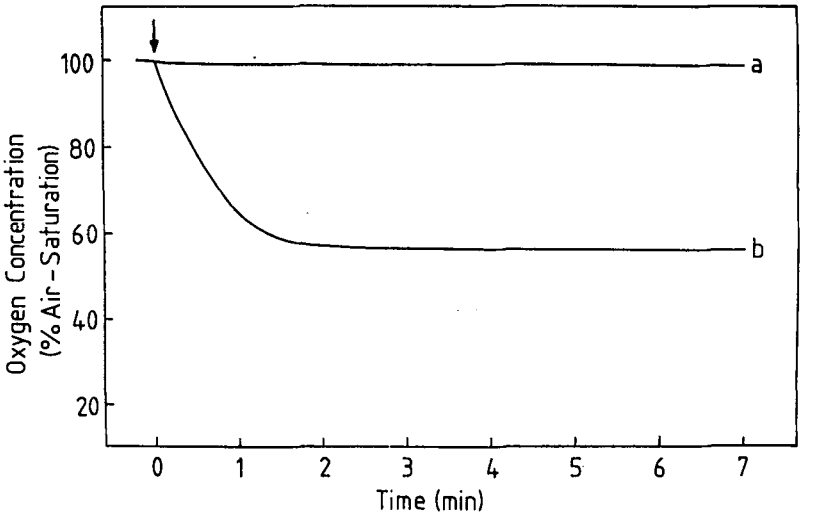


FIGURE 3 Determination of the level of iron(II)-autoxidation (assessed via oxygen-uptake as a function of time) stimulated by NaCl (a) and NaCl plus MOPS (b). $FeCl_2$ was added (to a final concentration of 0.48 mM) to NaCl (50 mM) in the absence of (a) and presence (b) of MOPS (pH 7.4, 37°C) in the cavity of an oxygen-electrode.

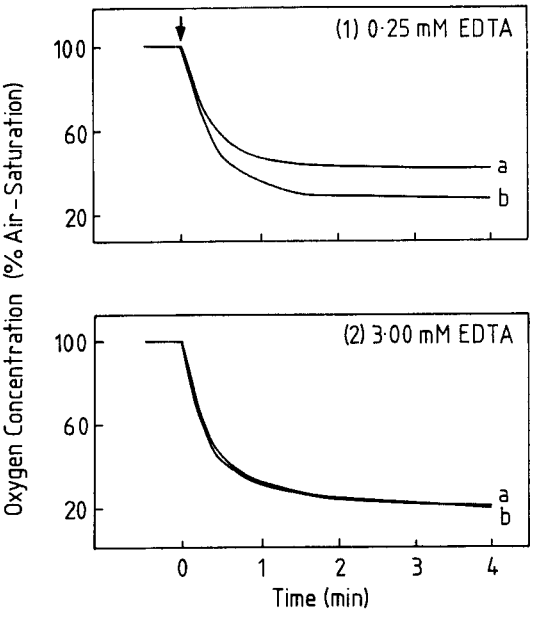


FIGURE 4 The effect of the chelator-to-iron ratio on the stimulation of oxygen-uptake by MOPS during iron(II)-EDTA autoxidation. $FeCl_2$ was added (to a final concentration of 0.48 mM) to a solution containing NaCl (50 mM) and EDTA [either 0.25 mM (1), to give an [EDTA]/[Fe] ratio of 0.521, or 3.00 mM (2), to give an [EDTA]/[Fe] ratio of 6.2/1], in the absence (a) presence (b) of MOPS (adjusted to pH 7.4 and maintained at 37°C) in the cavity of an oxygen-electrode and changes in oxygen concentration monitored with time.

iron in the presence of such good chelating agents as NTA, DFO or EDTA we believe that the buffer action referred to above provides a convincing explanation of the results shown in Figure 3 and that the enhancement of oxygen uptake shown in Figure 2 must be due to the effect of the buffer in reacting with *radicals* formed from the Fe^{2+}/O_2 reaction, thereby presumably initiating a chain reaction, a suggestion which was subsequently tested (see below).

The observations that, as the concentration of EDTA is increased in the presence of MOPS the enhancement induced by the buffer is eliminated (see Figure 4), and that the level of oxygen uptake increases as [EDTA] is increased from 2 to 3 mM (compare Figure 1 and 4) strongly suggests likewise that excess of the chelate (EDTA in this case) can act effectively as a stimulant for chain reactions to enhance autoxidation.

E.s.r. spin-trapping studies

In an attempt to provide *direct* evidence for the generation of free radicals during iron autoxidation, the technique of e.s.r. spin-trapping was employed. Autoxidation experiments were performed initially in MOPS-buffered solutions of various chelators in the presence of the spin-trap PBN. Although not all incubations yield a detectable e.s.r. signal, those which did so provide what appears to be the same spectrum [$a(N) = 15.98$ G, $a(H) = 2.34$ G)], although its intensity is influenced by the nature

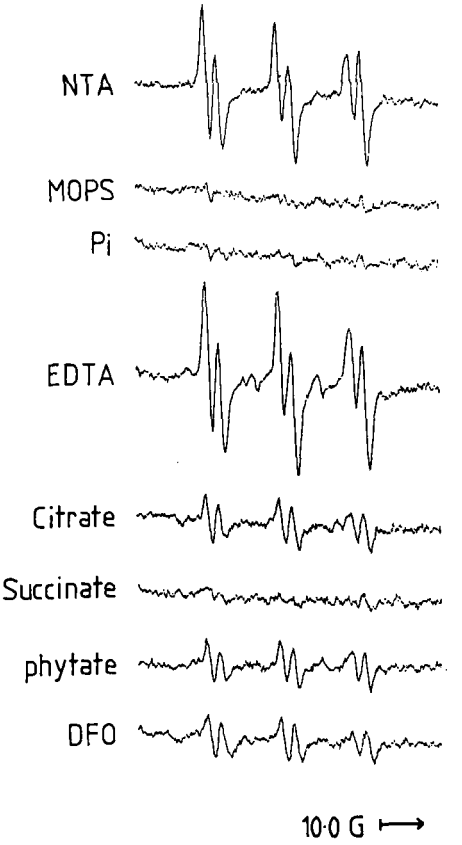


FIGURE 5 E.s.r. signals detected following the autoxidation of various iron(II)-complexes in the presence of PBN. $FeCl_2$ was added (to a final concentration of 1 mM) to a solution containing PBN (82 mM), NaCl (50 mM), MOPS (10 mM) and the chelator indicated (2 mM) [adjusted to pH 7.4]. Following incubation at room-temperature for 10 minutes the reaction was transferred to an e.s.r. cell for analysis.

of the chelator (Figure 5). It is of interest to note that the five iron-complexes which give clear e.s.r. signals (NTA, EDTA, citrate, phytate and DFO) also exhibit the greatest overall levels of oxygen uptake in the studies described above (see Figure 1). Control incubations performed with EDTA in the absence of MOPS resulted in the loss of the predominant signal, to leave a different, weaker signal [$a(N)$ = 15.16 G, $a(H)$ = 2.80 G] (Figure 6). This finding strongly suggests that the buffer itself is attacked by the hydroxyl radical [generated via reactions (4)–(6)] to form the major radical being trapped. (The rate constant for the reaction between ·OH and MOPS is believed[50] to be 2–2.6 × $10^9 dm^3 mol^{-1} s^{-1}$).

When hydrogen peroxide was added to incubations (to 40 mM) with iron(II)EDTA [in order to stimulate ·OH generation via reaction (6)] additional lines, corresponding to the PBN/·OH adduct [$a(N)$ = 15.49 G, $a(H)$ = 2.61 G[51]], were present in the spectra (Figure 6c). This finding eliminates the possibility that the major adduct observed in the experiment described above it that of the hydroxyl radical, although

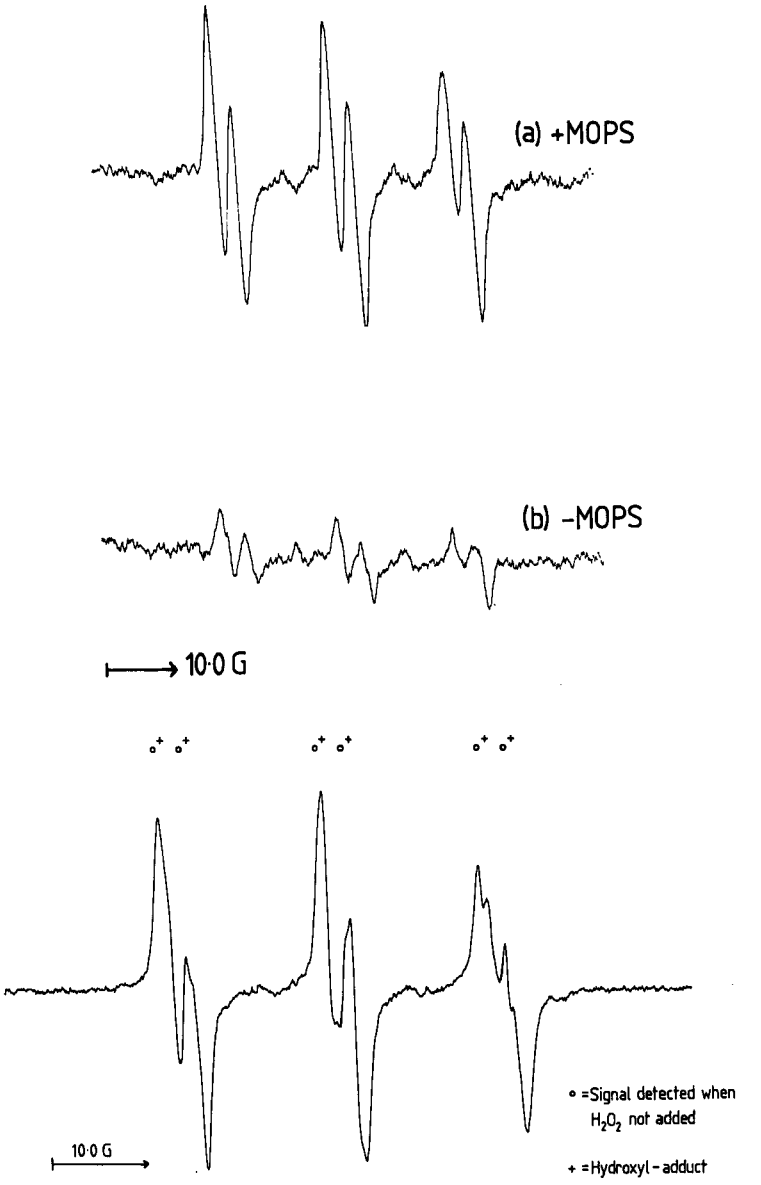


FIGURE 6 The effect of MOPS on the e.s.r. signal detected following the autoxidation of iron(II)-EDTA in the presence of PBN. $FeCl_2$ was added (to a final concentration of 1 mM) to a solution containing PBN (82 mM), NaCl (50 mM) and EDTA (2 mM) [adjusted to pH 7.4] in the presence (a) and absence (b) of MOPS. Following incubation for 10 minutes at room temperature the reaction was transferred to an e.s.r. cell for analysis. Figure 6c shows the e.s.r signal detected following the autoxidation of iron(II)-EDTA in the presence of added H_2O_2. $FeCl_2$ was added (to a final concentration of 1 mM) to a solution containing PBN (82 mM), NaCl(50 mM), H_2O_2 (40 mM), MOPS (10 mM) and EDTA (2 mM) [adjusted to pH 7.4]. Following incubation at room temperature for 10 minutes the reaction was transferred to an e.s.r. cell for analysis.

weak signals from this adduct may well be present in spectra (obscured by other more intense signals). When catalase, but not superoxide dismutase, was added to incubations with iron(II)EDTA (without the addition of H_2O_2) a marked decrease in signal intensity was observed (results not shown), thus indicating the hydrogen–peroxide dependence of adduct formation (bovine serum albumin failed to offer such protection, indicating an *enzymatic* function for catalase rather than the non-specific scavenging of radicals). These findings are consistent with those of a recent study[52] in which it is reported that formation of the DMPO/·OH adduct during the autoxidation of iron(II)DFO is inhibited by catalase but not superoxide dismutase.

The demonstration of the hydrogen-peroxide dependence, and hence the likely hydroxyl-radical dependence, of the formation of the major adduct detected, and the invariance found in the values of its hyperfine-coupling constants in the presence of different chelating agents provides further evidence for the trapping of a MOPS-derived radical, resulting from the reaction of ·OH with the buffer. The weaker signal trapped in the absence of the buffer is believed to be from the iron chelator (i.e. EDTA in Figure 6b).

Findings from similar experiments employing the spin-trap DMPO support this conclusion. The spectra obtained following the autoxidation of iron(II) EDTA in MOPS with DMPO indicate clearly the trapping of two species [one having $a(N)$ 15.81 G and $a(N)$ 22.64 G and the other values of 15.59 G and 22.92 G] (Figure 7). The

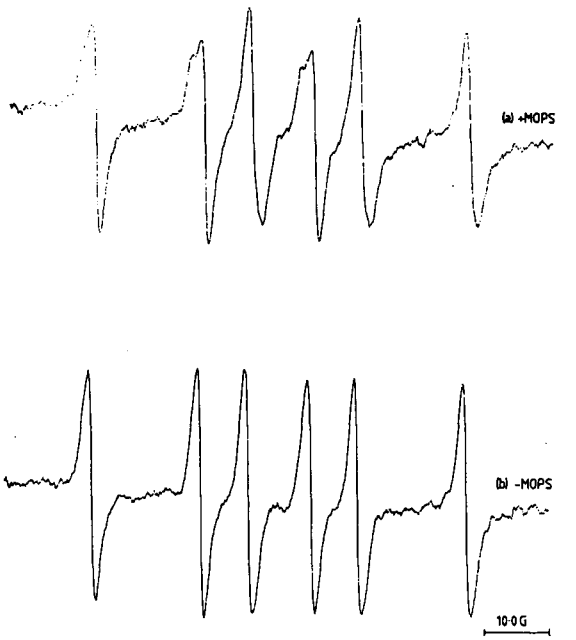


FIGURE 7 E.s.r. signals detected following the autoxidation of iron(II)-EDTA in the presence (a) and absence (b) of MOPS using the spin-trap DMPO. $FeCl_2$ was added (to a final concentration of 1 mM) to a solution containing DMPO (54 mM), (50 mM), EDTA (2 mM) in the presence (a) and absence (b) of MOPS (10 mM) (adjusted to pH 7.4). Following 5 minutes incubation at room temperature reactions were transferred to an e.s.r. cell for analysis.

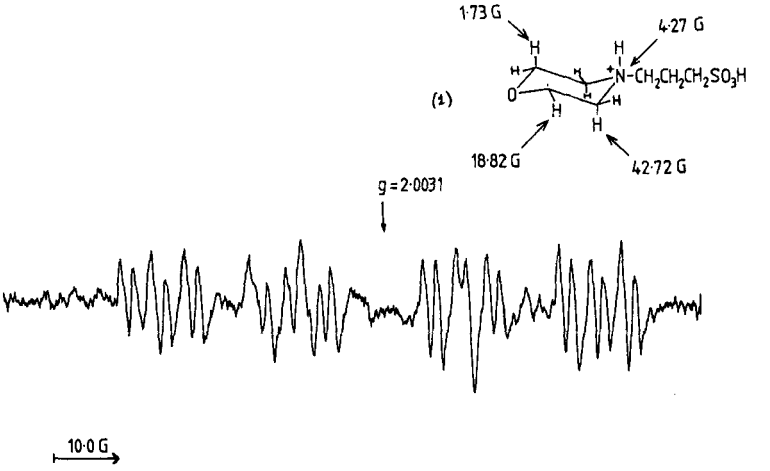


FIGURE 8. E.s.r. spectrum of the radical detected following the reaction of ·OH with MOPS (at pH 2.2). The radical was generated via the continuous rapid mixing of $TiCl_3$ (5 mM), H_2O_2 (2.5 mM) and MOPS (40 mM) in the e.s.r. spectrometer cavity (the final pH was adjusted to 2.2 via the addition of H_2SO_4).

coupling constants for neither of these species correspond to the values reported for the superoxide- or hydroxyl-radical adducts of DMPO[41] and the signal from the latter adduct was not observed when MOPS was omitted from incubations; it is therefore suggested that the latter species is a MOPS-radical adduct to DMPO and that the former species is the EDTA-derived radical adduct.

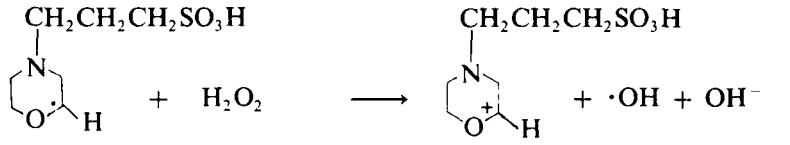
Continuous-flow e.s.r. studies

Continuous-flow techniques were employed in order to permit the direct observation of any radicals formed in the reaction of the hydroxyl radical (generated using the Ti^{3+}/H_2O_2 couple) with MOPS buffer.

The reaction of ·OH with MOPS at pH 2.2 (under optimal conditions for ·OH generation) gives the spectrum showed in Figure 8 which is attributed to the oxygen-centred radical derived from the buffer. The hyperfine-coupling constants assigned to the MOPS radical (1) (shown in Figure 8) are consistent with those of similar radicals which result from the reaction of ·OH with the related substrates morpholine and N-ethylmorpholine (and analysed in terms of a preferred ring conformation in which the substituent adopts the equatorial position).[53] It is also of interest to note here that other workers have reported the detection of (relatively stable) nitrogen-centred radicals (observed by direct e.s.r.) following the reaction of ·OH with piperazine-based "Good" buffers (e.g. HEPES), but signals were not detected from the morpholine-derived buffer MES (a structural analogue of MOPS).[54]

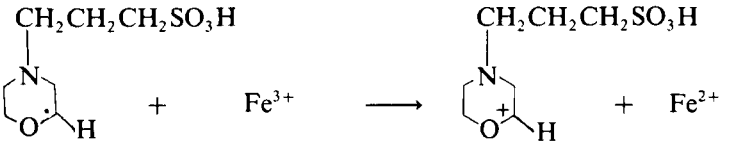
The signal intensity of the MOPS radical was found to depend markedly upon the concentration of H_2O_2. Although it might be expected that increasing the concentration of H_2O_2 would lead to an increase in formation of the MOPS radical (1) (via the stimulation of ·OH generation), the opposite effect was seen: increasing the final concentration (i.e. after mixing) of H_2O_2 from 0.83 mM to 2.4 mM resulted in a marked decrease in the intensity of the radical signal. This is believed to result from

the destruction of the radical by one-electron transfer to H_2O_2 [reaction (8)] a reaction previously established[9] for α-oxygen substituted radicals such as ·CHMeOH and ·CMe_2OH.



(8)

In order to investigate the possibility that the MOPS radical might also interact with iron (which would be expected to complicate further the mechanism of iron autoxidation in MOPS buffer), experiments were also performed in which Fe^{III} was added to the substrate stream in continuous-flow experiments. Addition of $FeCl_3$ (to give a post-mixing concentration of 3.3 mM) resulted in complete loss of the signal from the MOPS radical (1), indicating that the radical had indeed undergone rapid oxidation [reaction (9)], a type of reaction which has also been demonstrated for α-oxygen substituted radicals from alcohols.[9] Using steady-state approaches (coupled with kinetic simulation), as described previously,[9] it is possible to estimate the rate constant for reactions (8) and (9) as *ca.* 10^5 and 10^9 $dm^3mol^{-1}s^{-1}$, respectively.



(9)

The very effective reduction of iron by electron-rich organic radicals of this type [e.g. via reaction (9)] and its subsequent oxidation by oxygen, superoxide and H_2O_2 [reaction (4)–(6)] would thus be expected to result in the continuous redox-cycling of the metal ion, with the concomitant generation of the hydroxyl radical.

DISCUSSION AND CONCLUSIONS

Although the free-radical chemistry of iron in biological systems is often considered in terms of its reactions with enzymatically-generated superoxide and hydrogen peroxide, this study demonstrates that iron itself can initiate reactions leading to molecular damage via its direct reductive activation of molecular oxygen.

Iron-complexes with low redox potentials for the Fe^{III}/Fe^{II} couple [e.g. DFO–Fe^{III}/Fe^{II}, −0.45 V;[42,43] EDTA–Fe^{III}/Fe^{II}, 0.12 V;[17] and NTA–Fe^{III}/Fe^{II}, 0.23 V[55]] are expected to reduce oxygen more readily than those with higher redox potentials [e.g. citrate–Fe^{III}/Fe^{II}, 0.6 V[56]]. In the oxygen-electrode studies reported here it is shown that only the *initial* rates of oxygen uptake appear to correlate with the redox potential of the metal complex; thus after seven minutes' incubation, for example, the *iron-citrate* complex had stimulated the greatest level of oxygen uptake, despite its relatively high redox potential. It has also been shown that the observed overall stoicheiometry of the reactions ($Fe^{2+}:O_2$), when determined using oxygen-uptake techniques, shows considerable dependence upon the nature of the iron chelator used, and can differ markedly from the 4:1 value expected on the basis of reactions (4)–(7) (see below).

The similarity observed in the Fe^{2+}/O_2 ratio determined for Fe(II)NTA and Fe(II)EDTA complexes (and indeed in their abilities to generate the MOPS adduct to PBN) suggest that FeNTA is at least as effective as FeEDTA in the catalysis of free-radical reactions. Indeed, in a recent study involving the generation of ·OH from the reaction of iron(III) chelates with H_2O_2 it was found[57] that FeNTA is more potent than FeEDTA in the catalysis of damage to DNA bases, and, using the salicylate hydroxylation assay we have shown[27] that both complexes are good catalysts of ·OH generation via the Haber–Weiss cycle.

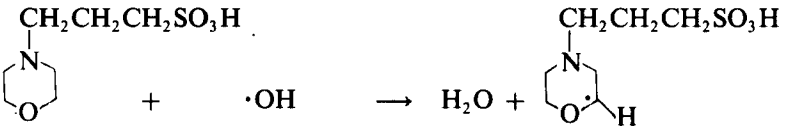
The detection of buffer-derived radicals (using e.s.r. spin-trapping techniques), and the demonstration of their ability to reduce both iron(III) and hydrogen peroxide (via continuous-flow e.s.r. experiments), suggests that additional reactions take place when iron undergoes autoxidation in the presence of certain organic substrates, which might explain why low Fe^{2+}/O_2 ratios are observed in the autoxidation of some iron complexes (such as iron-EDTA and iron-citrate). For example, following the reduction of 1 mole of oxygen to ·OH during the oxidation of 3 moles of Fe^{2+} [reactions (4)–(6)], the ·OH radical might then oxidise the organic buffer [reaction (10)], rather than a fourth Fe^{2+} [reaction (7)], and thereby initiate a series of chain reactions in which iron can reduce further molecules of oxygen as it is redox-cycled between the +2 and +3 oxidation states by the MOPS-derived radical and oxygen (and its reduced forms) [reactions (4)–(6), (9) and (10)].

$$Fe^{2+} + O_2 \longrightarrow Fe^{3+} + O_2^{\cdot-} \quad (4)$$

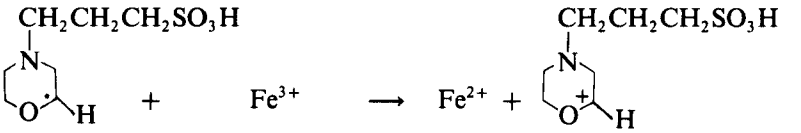
$$Fe^{2+} + O_2^{\cdot-} + 2H^+ \longrightarrow Fe^{3+} + H_2O_2 \quad (5)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {\cdot}OH + OH^- \quad (6)$$

$$Fe^{2+} + {\cdot}OH \longrightarrow Fe^{3+} + OH^- \quad (7)$$



(10)



(9)

Organic free radicals which are good one-electron donors [like (1)] would also be expected to result from the reactions of the ·OH radical with metal-ion chelators, a suggestion which is supported by the trapping here of EDTA radicals in addition to MOPS radicals and by numerous observations in pulse radiolysis and e.s.r. studies. For example, it has been demonstrated that the reaction of ·OH with the chelating agent DTPA leads to the generation of a DTPA-centred radical which can reduce the Fe^{III}-DTPA complex.[58]

The observed failure of iron-DFO to stimulate oxygen uptake after the first minute of incubation in the oxygen-electrode studies is believed to reflect the probable failure of organic radicals to promote redox cycling of iron-DFO. The similar behaviour of iron-phytate might also reflect the failure of organic radicals to reduce the iron(III)-complex. (Indeed, we have previously reported that the phytate-chelation of iron prevents its redox cycling by superoxide,[27] the reductant in the Haber–Weiss cycle.)

Therefore, although desferrioxamine and phytate are able to act as antioxidants by preventing redox cycling of iron, they do not prevent, but greatly stimulate, reactions in which the metal ion is oxidised, such as the reduction of oxygen through to the hydroxyl radical [cf. the detection of spin-trap adducts in the autoxidation of their iron(II)-complexes in MOPS buffer].

It is also clear therefore that oxygen-uptake techniques cannot be used in the determination of Fe^{2+}:O_2 ratios during the autoxidation of iron in the presence of organic buffers or organic-chelating agents, and that the 4:1 ratio measured for the oxidation of iron in the presence of phytate, reported both in this work and elsewhere,[32] may be coincidental, and cannot be taken to reflect the four-electron reduction of oxygen to water.

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