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Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts

What is the physiological iron chelator?

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In the presence of physiological concentrations of iron(III) salts, superoxide radical and hydrogen peroxide can interact to form the highly-damaging hydroxyl radical. No specific added 'chelator' of iron salts is necessary for this reaction to occur.

Superoxide *Hydroxyl radical* *Iron* *Haber–Weiss reaction* *Chelating agent* *Fenton reaction*

1. INTRODUCTION

Oxygen is essential for the survival of aerobic cells but it has long been known to be toxic to them when supplied at concentrations greater than those in normal air [1–3]. The biochemical mechanisms responsible for oxygen toxicity are many and varied [3] but they include the generation of hydrogen peroxide (H_2O_2) and the superoxide radical, O_2^- [1–3]. Much of the damage done by O_2^- and H_2O_2 to living cells is due to their interaction to form a highly reactive species that can attack and destroy almost all known biomolecules. This species is the hydroxyl radical, $OH^\cdot$, and its formation requires traces of transition metal ions, of which iron is likely to be the most important in vivo [4–12]:

$$O_2^- + H_2O_2 \xrightarrow[\text{catalyst}]{\text{Fe-salt}} OH^\cdot + OH^- + H_2O$$

Traces of non-protein-bound iron salts capable of catalysing the above reaction are present in bacteria [10,13], in human extracellular fluids [7,8,14] and intracellularly in mammalian tissues [11]. The molecules to which these iron salts are attached have not been identified but presumably include organic acids, phosphate ion and phosphate esters such as ATP and ADP [11].

The original in vitro systems in which iron-dependent formation of hydroxyl radicals from O_2^- and H_2O_2 was demonstrated all contained the chelating agents EDTA, added deliberately [4,6] or present in the reagents used [15]. Iron–EDTA chelates react rapidly with O_2^- and H_2O_2 [4,16]. The concentration of chelated iron salt present was usually high (around 100 μM) to maximise the production of $OH^\cdot$ radicals and facilitate experimentation [6]. Omission of EDTA markedly decreased the number of $OH^\cdot$ radicals produced, although it did not prevent the reaction completely [6,17,18]. A number of laboratories have therefore sought for 'physiological' chelators of iron that would replace EDTA in stimulating the action of iron salts in promoting $OH^\cdot$ generation. Although one such chelator, picolinic acid, has been identified [19], a survey of a wide range of other compounds present in vivo (including amino acids, phosphate esters and organic acids) has found no other effective compounds (unpublished).

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In these experiments high concentrations of iron salts (50–100 μM) [4–6,18,19] were used in the presence of phosphate buffer, usually at pH 7.4. Phosphate is commonly employed because it is present at high concentrations in vivo and because other buffers, such as Tris and Hepes, react rapidly with OH$^{\cdot}$ radicals [20,21]. However, the physiological concentrations of non-protein-bound iron salts are usually <5 μM [7,8,14]. Here, we report the results of investigations on the rate of OH$^{\cdot}$ production in O_2^--generating systems at such physiological iron salt concentrations.

2. MATERIALS AND METHODS

2.1. *Reagents*

Desferrioxamine (Desferal®) was purchased from CIBA. Superoxide dismutase, catalase (bovine liver, thymol free), xanthine oxidase, hypoxanthine and other reagents were from Sigma.

2.2. *Assay methods*

Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts was measured by the modified salicylate hydroxylation method [22]. Solutions of $FeCl_3$ were made up fresh before use. Reaction mixtures contained a final concentration of 150 mM potassium phosphate buffer at pH 7.4.

3. RESULTS

A mixture of hypoxanthine and xanthine oxidase generates O_2^- and H_2O_2 [1,2]. These can, in the presence of a metal salt catalyst, interact to form OH$^{\cdot}$ radicals [4,6] that may be specifically and quantitatively detected by their ability to hydroxylate aromatic compounds. Hydroxylation in this system can be prevented by addition of catalase or superoxide dismutase or by omission of iron salt [6,22].

Fig.1 shows the rate of OH$^{\cdot}$ generation when an Fe^{3+}–EDTA chelate was added to a hypoxanthine-xanthine oxidase mixture in buffer at pH 7.4. The rate reached a maximum at about 50 μM Fe^{3+}–EDTA. The very low rate of OH$^{\cdot}$ production in the absence of added iron chelate is due to traces of iron contamination in the reagents [5,9,23] since it can be inhibited by the iron

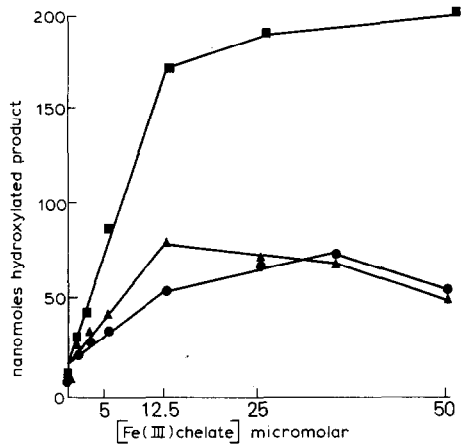

Fig.1. Rate of hydroxyl radical formation in the presence of added iron(III) chloride or iron(III) chelate: (●) $FeCl_3$ added; (▲) $FeCl_3$ pre-mixed with equimolar ATP added; (■) $FeCl_3$ pre-mixed with equimolar EDTA added. The concentrations stated were the final concentrations in the reaction mixture.

chelator desferrioxamine, which prevents iron salts form participating in OH$^{\cdot}$ generation [24,25].

In the absence of EDTA, 50 μM $FeCl_3$ alone produced much less detectable OH$^{\cdot}$ production (fig.1). EDTA could not be replaced by a wide range of biological chelating agents such as ATP, ADP, organic acids or amino acids (a typical result is shown, for ATP). However, fig.1 shows that in the biological range of iron salt concentrations (1–5 μM) $FeCl_3$ alone is almost as effective as Fe^{3+}–EDTA in allowing generation whether or not other chelators such as ATP and ADP are present.

4. DISCUSSION

When Fe(III) salts are added to water at pH 7.4 they form hydrated iron complexes that eventually precipitate as $Fe(OH)_3$ [26]. The presence of phosphate ion can retain iron in solution until the solubility product of iron(III) phosphate or iron(III) phosphate–hydroxide is exceeded. Fig.1 shows that Fe^{3+} at physiological concentrations at pH 7.4 in the presence of phosphate ion can exist in a form that allows significant OH$^{\cdot}$ formation. Addition of ADP or ATP does not markedly increase their effectiveness and the stimulation by EDTA is only slight.

Raising the added Fe^{3+} to >5 μM does not in-

crease the rate of $OH^{\cdot}$ generation, presumably because the extra iron salt then participates in formation of unreactive insoluble complexes. Adding EDTA, which prevents this precipitation but does not decrease the reactivity of the iron salt with O_2^- or H_2O_2 [16], allows a greater rate of $OH^{\cdot}$ production to be observed.

Hence the action of EDTA in greatly stimulating iron-salt-dependent $OH^{\cdot}$ generation is largely an artefact of the use of unphysiologically-high iron salt concentrations (fig.1). Sufficient iron(III) can exist in solution to allow $OH^{\cdot}$ generation in the presence of phosphate ion and phosphate esters alone at physiological pH.

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