# THE SUPEROXIDE DISMUTASE ACTIVITY OF IRON COMPLEXES

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## 1. Introduction

Superoxide dismutases are enzymes which catalyse the breakdown of the superoxide radical, $O_2^{\cdot -}$, to $H_2O_2$ and triplet $O_2$. They are present in all aerobic organisms and play an important role in protection against $O_2^{\cdot -}$ generated in vivo [1–3]. Superoxide dismutases are metalloproteins, containing either copper and zinc, manganese or iron as the prosthetic group. However, free $Cu^{2+}$ and $Mn^{2+}$ ions can themselves bring about the breakdown of $O_2^{\cdot -}$ [4–6]. These observations are important to an understanding of the mechanism of action of superoxide dismutases, but they also raise the possibility that assays of tissue extracts for dismutase activity could give false results because of the presence of traces of $Mn^{2+}$ and/or $Cu^{2+}$. For example, 0.12 $\mu$M $Cu^{2+}$ or 0.82 $\mu$M $Mn^{2+}$ were sufficient to cause a 50% inhibition of the reduction of nitro-blue tetrazolium to formazan by $O_2^{\cdot -}$ generated by an illuminated methionine–riboflavin [6].

The most commonly used assays for superoxide dismutases rely on the ability of these enzymes to inhibit the reduction of either cytochrome c or of nitro-blue tetrazolium by $O_2^{\cdot -}$ generated during the oxidation of xanthine by xanthine oxidase [7,8]. These reaction mixtures usually contain EDTA. Preliminary experiments using these assays in the absence of EDTA have confirmed the inhibitory effects of $Mn^{2+}$ and $Cu^{2+}$, but have shown that the presence of EDTA is able to prevent inhibition by these metal ions (Halliwell, unpublished). Also, the inability of a $Cu^{2+}$–EDTA complex to react with $O_2^{\cdot -}$ may be deduced from previous reports [8,9].

In the present paper, the effects of $Fe^{2+}$, $Fe^{3+}$ and haematin in the presence and absence of EDTA are described.

## Materials and methods

### 2.1. *Materials*

Erythrocuprein (a superoxide dismutase containing copper and zinc), haematin, cytochrome *c*, 4-hydroxycinnamic acid and nitro-blue tetrazolium were purchased from the Sigma Chemical Corporation, Kinston-upon-Thames, Surrey, UK. Xanthine oxidase was obtained from the Boehringer Corporation, London W.5., UK. Dihydroxyfumaric acid was purchased from Koch-Light Ltd., Colnbrook, Bucks., UK. Other reagents were of the highest purity available from BDH Chemicals Ltd., Poole, Dorset, UK.

1 unit of superoxide dismutase is defined as that amount causing a 50% decrease in the rate of cytochrome *c* reduction under the assay conditions of McCord and Fridovich [8].

### 2.2. *Assays for superoxide dismutase activity*

#### 2.2.1. Using xanthine oxidase

Reduction of nitro-blue tetrazolium by a xanthine–xanthine oxidase system at pH 10.2 was followed by the increase in absorbance at 560 nm. The reaction mixture was that of Beauchamp and Fridovich [7] except that EDTA was omitted unless otherwise stated.

When the $Na_2CO_3$ buffer, pH 10.2, in this assay was replaced by 50 mM potassium phosphate buffer, pH 7.8, a slower rate of formazam production was observed, but it was still inhibited almost completely by 1 unit of superoxide dismutase.


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Reduction of cytochrome c by a xanthine–xanthine oxidase system at pH 7.8 was assayed at 550 nm as described by McCord and Fridovich [8], except that EDTA was omitted unless otherwise stated.

2.2.2. Using NADH and phenazine methosulphate

The reaction mixtures contained, in a total vol of 3.00 ml, 150 μmol $KH_2PO_4$ adjusted to pH 7.8 with KOH, 0.08 μmol nitro-blue tetrazolium and 0.1 μmol phenazine methosulphate. The reaction was initiated by adding 0.25 μmol of NADH and production of formazan was followed at 560 nm at 20°C. The iron salts added were $FeSO_4$ or $FeCl_3$.

## 3. Results

### 3.1. *Inhibition of $O_2^{\cdot-}$-dependent reaction by $Fe^{2+}$ or $Fe^{3+}$ and EDTA*

Fig.1 shows the effects of $Fe^{2+}$, $Fe^{3+}$ and EDTA on the reduction of nitro-blue tetrazolium by $O_2^{\cdot-}$ generated by a xanthine–xanthine oxidase system at pH 10.2 [7]. Addition of 33 μM $Fe^{2+}$ or $Fe^{3+}$ had no effect. 100 μM EDTA increased the rate of formazan production, perhaps due to the removal of traces of $Cu^{2+}$ and/or $Mn^{2+}$ present in the reagents. However, addition of 33 μM $Fe^{2+}$ or $Fe^{3+}$ together with 100 μM EDTA produced a severe inhibition. It may be seen from fig.1 that 33 μM $Fe^{2+}$–EDTA or $Fe^{3+}$–EDTA complex produced an inhibition approximately equal to that obtained by addition of 1 unit of superoxide dismutase. These amounts of iron salt and EDTA produced only a slight inhibition (less than 20%) of urate formation in this reaction mixture and so the decrease in formazan production could not be explained by an inhibition of xanthine oxidase.

A xanthine–xanthine oxidase system also reduced nitro-blue tetrazolium at pH 7.8 (see Materials and methods). Again, 33 μM $Fe^{2+}$ or $Fe^{3+}$ in the presence of 100 μM EDTA almost completely inhibited formazan production, although $Fe^{2+}$, $Fe^{3+}$ or EDTA alone had little effect. Urate formation at pH 7.8 was completely unaffected by addition of these concentration of $Fe^{2+}$ or $Fe^{3+}$ and EDTA.

A mixture of $Fe^{2+}$ or $Fe^{3+}$ and EDTA also inhibited the reduction of nitro-blue tetrazolium by $O_2^{\cdot-}$ generated by a completely different system: a mixture of NADH and phenazine methosulphate at pH 7.8

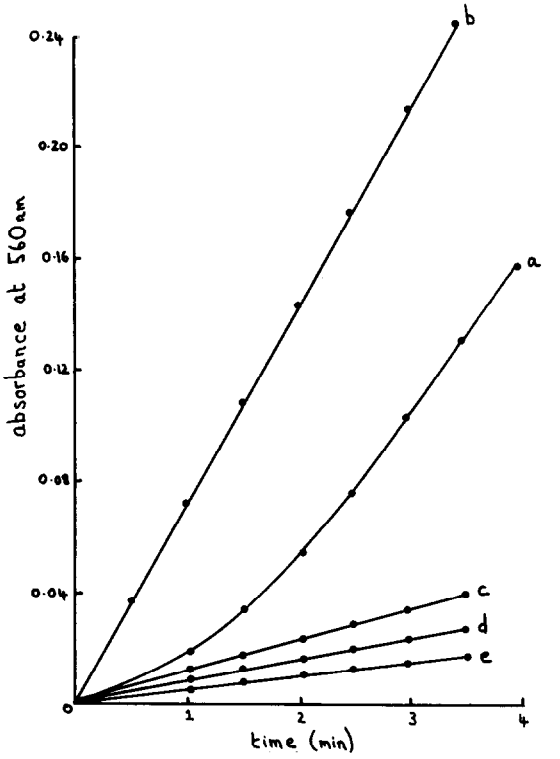


Fig.1. Effect of metal ions and EDTA on reduction of nitro-blue tetrazolium by $O_2^{\cdot-}$ at pH 10.2. Assays were carried out as described under Materials and methods. Iron was added as $FeCl_3$ or $FeSO_4$. (a) No addition. (b) Plus 100 μM. (c) Plus 33 μM $Fe^{3+}$ and 100 μM EDTA. (d) Plus 33 μM $Fe^{2+}$ and 100 μM EDTA. (e) Plus 1 unit of erythrocuprein. 33 μM $Fe^{2+}$ or $Fe^{3+}$ did not affect formazan production in the absence of EDTA. Formation of uric acid was followed by measuring the increase in absorbance at 290 nm of reaction mixtures from which nitro-blue tetrazolium had been omitted.

[13]. Details of the assay are given under Materials and methods. Neither 33 μM $Fe^{2+}$ or $Fe^{2+}$ nor 100 μM EDTA had any effect on formazan production in this system, but, in the presence of 100 μM EDTA, these concentrations of iron salt inhibited it almost completely.

Fig.2 shows the effects of $Fe^{2+}$ and $Fe^{3+}$ on the reduction of cytochrome *c* by $O_2^{\cdot-}$ generated by a xanthine–xanthine oxidase system at pH 7.8 [8]. Addition of 100 μM EDTA or of 67 μM $Fe^{2+}$ had no effect, but addition of both $Fe^{2+}$ and EDTA decreased cytochrome *c* reduction: it may be seen from fig.2 that 33 μM $Fe^{2+}$–EDTA complex produced an inhibi-

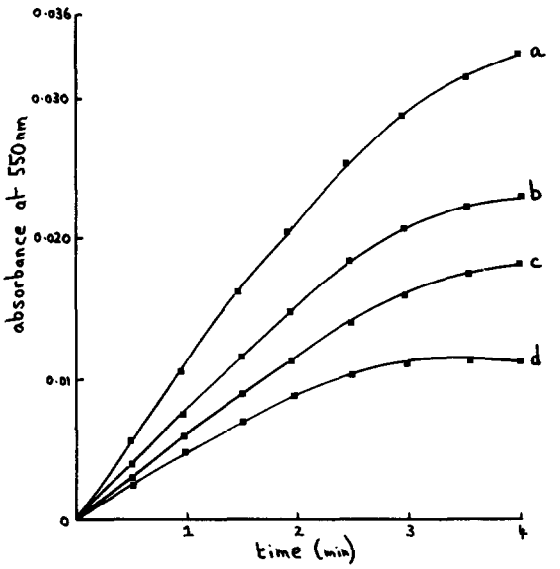


Fig.2. Effect of metal ions and EDTA on reduction of cytochrome *c* by $O_2^{\cdot -}$ at pH 7.8. Assays were carried out as described under Materials and methods. Iron was added as $FeCl_3$ or $FeSO_4$. (a) No addition. (b) Plus 16 μM $Fe^{2+}$ and 100 μM EDTA. (c) Plus 33 μM $Fe^{2+}$ and 100 μM EDTA. (d) Plus 67 μM $Fe^{2+}$ and 100 μM EDTA. . Neither 67 μM EDTA alone affected cytochrome *c* reduction. 1 unit of superoxide dismutase inhibited reduction by 50%. Formation of uric acid was followed at 290 nm in reaction mixtures from which cytochrome *c* had been omitted.

tion approximately equal to that observed on adding 1 unit of superoxide dismutase (50%). Hoewever, there was no inhibition of cytochrome *c* reduction by 33 μM $Fe^{3+}$–EDTA; it was necessary to add 167 μM $Fe^{3+}$ and 200 μM EDTA before 50% inhibition was seen. (Both EDTA and $Fe^{3+}$ were required). The above amounts of $Fe^{2+}$ or $Fe^{3+}$ and EDTA dit not affect urate production in this assay.

To confirm these results, a completely-different system was used. The conversion of 4-hydroxycinnamic acid to 3,4-dihydroxycinnamic acid by a mixture of horseradish peroxidase and dihydroxyfumarate at pH 6 is dependent upon $O_2^{\cdot -}$ [14]. Table 1 shows that addition of 10 μM $Fe^{2+}$ or of 100 μM EDTA had little effect on the rate of hydroxylation, but addition of both $Fe^{2+}$ and EDTA caused an inhibition approximately equal to that obtained by addition of 1 unit of superoxide dismutase. $Fe^{3+}$ also inhibited in the presence of EDTA, but it cannot be ruled out that this was due to its reduction to $Fe^{2+}$ by dihydroxyfumarate [15].

### 3.2. *Inhibition of $O_2^{\cdot -}$ dependent reaction by haematin*

Kovacs and Matkovics [16] reported that several haemoproteins showed a 'non-specific superoxide dismutase activity'. This prompted an examination of the effect of free haematin, on $Fe^{3+}$–porphyrin.

Table 1
Effect of $Fe^{2+}$ and EDTA on the hydroxylation of 4-hydroxycinnamic acid

| Compound added | nmoles 3,4-dihydroxycinnamic acid formed in 30 min |
|---|---|
| None | 54 |
| 10 μM $FeSO_4$ | 49 |
| 100 μM EDTA | 54 |
| 10 μM $FeSO_4$ + 100 μM EDTA | 17 |
| 1 unit superoxide dismutase | 20 |

A mixture of 4-hydroxycinnamic acid (2.5 μmol), dihydroxyfumaric acid (30 μmol) and $KH_2PO_4$ (8.3 μmol) was adjusted to pH 6 with KOH and incubated, in a total of vol of 1.00 ml, at 25°C for 0.5 hr in the presence of an amount of horseradish peroxidase sufficient to catalyse an absorbance change of 0.036 $min^{-1}$ at 436 nm using the guaiacol assay [10]. After 0.5 hr, 10 μl of conc. HCl was added and the products extracted into ether. A sample of the upper layer was taken, the ether removed by evaporation on a water-bath at 50°C and the residue dissolved in 1 ml of $H_2O$. 3,4-dihydroxycinnamic acid was assayed by a colorimetric method [11,12].

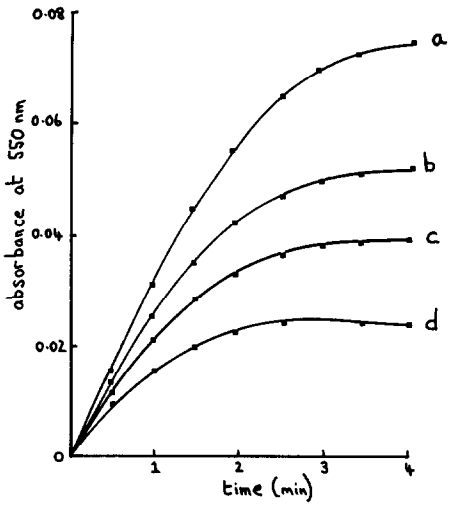


Fig.3. Effect of haematin on reduction of cytochrome *c* by $O_2^{\cdot -}$ at pH 7.8. Assays were carried out as described under Materials and methods. (a) No addition (b) Plus 30 μM haematin. (c) Plus 40 μM haematin. (d) Plus 100 μM haematin. Formation of uric acid was followed at 290 nm in reaction mixtures from which cytochrome *c* had been omitted.

Fig.3 shows that haematin inhibited reduction of cytochrome *c* by $O_2^{\cdot -}$ generated by a xanthine–xanthine oxidase system at pH 7.8: approximately 40 μM haematin was required to achieve 50% inhibition. It had no effect on urate production. Haematin also inhibited reduction of nitro-blue tetrazolium by this system at pH 7.8 (data not shown).

These results were not due to the presence of contaminating iron salts in the haematin preparation, since the degree of inhibition at various concentrations was the same whether or not EDTA was present.

## 4. Discussion

It may be concluded that at pH 6, 7.8 or 10.2, a complex of an iron salt with EDTA can react with superoxide. Both $Fe^{2+}$–EDTA and $Fe^{3+}$–EDTA are almost equally effective in assays which use nitro-blue tetrazolium to detect $O_2^{\cdot -}$, but $Fe^{2+}$–EDTA was much more effective in assays using cytochrome *c*.

Cytochrome c has a much higher affinity for $O_2^{\cdot -}$ than has nitro-blue tetrazolium [7,8]. Indeed, cytochrome *c* inhibits the reduction of nitro-blue tetrazolium by $O_2^{\cdot -}$: formazan is not produced until the cytochrome *c* has been reduced (Halliwell, unpublished).

The observation reported in the present paper strongly suggest that $Fe^{3+}$–EDTA reacts with $O_2^{\cdot -}$, becoming reduced to $Fe^{2+}$–EDTA:

$$Fe^{3+}\text{–EDTA} + O_2^{\cdot -} \rightarrow O_2 + Fe^{2+}\text{–EDTA} \qquad (1)$$

If the rate constant for this reaction is low, then cytochrome *c* could efficiently compete for the avialable $O_2^{\cdot -}$, whereas nitro-blue tetrazolium could not.

An $Fe^{2+}$–EDTA complex must also combine with $O_2^{\cdot -}$, perhaps by the reaction

$$Fe^{2+}\text{–EDTA} + 2H^+ + O_2^{\cdot -} \rightarrow Fe^{3+}\text{–EDTA} + H_2O_2 \qquad (2)$$

The results of fig.2 show that $Fe^{2+}$–EDTA inhibits the reduction of cytochrome *c* by $O_2^{\cdot -}$ at much lower concentrations than does $Fe^{3+}$–EDTA, and so it must have a greater affinity for $O_2^{\cdot -}$.

Thus a mixture of an iron salt and EDTA is potentially capable of bringing about the catalytic breakdown of $O_2^{\cdot -}$, the bound iron undergoing alternate oxidation and reduction. This cannot happen in the cytochrome *c* assay, because cytochrome *c* reacts with $O_2^{\cdot -}$ much more quickly than does $Fe^{3+}$–EDTA and so, when added $Fe^{2+}$–EDTA is used up, $Fe^{3+}$–EDTA will accumulate and cytochrome *c* should be reduced. In the assays using nitro-blue tetrazolium, catalytic breakdown may occur. For example, addition of $Fe^{2+}$–EDTA will result in its conversion to $Fe^{3+}$–EDTA. When the concentration of $Fe^{2+}$–EDTA has fallen to a low level, $Fe^{3+}$–EDTA can then begin to react with $O_2^{\cdot -}$ and so the process will continue, since, although the affinity of $Fe^{3+}$–EDTA for $O_2^{\cdot -}$ seems to be much lower than that of $Fe^{2+}$–EDTA, it is still greater than that of nitro-blue tetrazolium.

Traces of free iron are present in most biological systems and so the application of routine assays for dismutase [7,8] to such systems may produce artifacts. Fig.1 shows that as little as 100 nmol of $Fe^{2+}$ or $Fe^{3+}$ produce almost complete inhibition in the assay system of Beauchamp and Fridovich [7], which contains 100 μM EDTA.

The observations reported in the present paper may help to explain the different reports of the effects of EDTA on lipid peroxidation, a process known to involve $O_2^{\cdot -}$ under certain conditions [17–23]. For

example, Pederson and Aust [18,19] showed that EDTA stimulated lipid peroxidation, whereas other workers reported an inhibition [24–27]. EDTA should promote $O_2^{\cdot -}$-dependent lipid peroxidation by chelating any $Mn^{2+}$ and/or $Cu^{2+}$ present, so preventing them from scavenging $O_2^{\cdot -}$. On the other hand, a complex of EDTA with any iron salt present might be expected to inhibit. Addition of $Fe^{2+}$–EDTA is sometimes found to enhance the rate of lipid peroxidation during studies in vitro [19,28], but its presence may well change the mechanism of the reaction. The final effects of EDTA will depend on the amounts of the different metal ions present.

Haematin can also react with $O_2^{\cdot -}$, presumably, at least initially, by undergoing reduction to the ferrous form. This is consistent with the observations of Kovacs and Matkovics [16], who reported a 'non-specific dismutase activity' of haemoproteins such as myoglobin, catalase and haemoglobin. However, the methods used to prepare the haemoproteins for these experiments [16] might not have separated them from the superoxide dismutases present in the tissues of origin [29]. Halliwell [30] did not detect any dismutase activity in highly-purified catalase: the dismutase present in less-pure preparations could easily be removed by Sephadex gel filtration (Halliwell, unpublished). Other workers have reported similar observations [31–33]. If these haemoproteins do react with $O_2^{\cdot -}$, it seems unlikely that they have a substantially greater affinity for it then does free haematin and so the reaction is unlikely to be detected except at high protein concentration (40 $\mu$M haematin was equivalent to 1 unit of dismutase in the cytochrome *c* assay). Interestingly, however, high concentrations of haematin and haemoproteins can inhibit lipid peroxidation [34].

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