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THE EFFECT OF IRON ON THE DISTRIBUTION OF SUPEROXIDE AND HYDROXYL RADICALS AS SEEN BY SPIN TRAPPING AND ON THE SUPEROXIDE DISMUTASE ASSAY

GARRY R. BUETTNER, LARRY W. OBERLEY and SUSAN W. H. CHAN LEUTHAUSER
The Radiation Research Laboratory, The University of Iowa, Iowa City, IA 52242, U.S.A.

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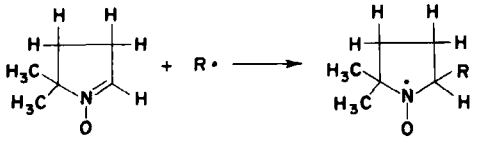
Abstract—Using the spin trap 5,5-dimethyl-1-pyrroline-1-oxide we have demonstrated the presence of ·OH in the xanthine–xanthine oxidase system when iron and/or iron–EDTA (ethylenediamine-tetraacetic acid) is present. With increasing iron (or iron–EDTA) concentration the intensity of the $O_2^{\cdot-}$ spin adduct decreased while that of ·OH increased. However, use of diethylenetriaminepentaacetic acid (DETAPAC) as a metal chelator in the reaction mixture suppressed the ·OH spin adduct signal while maintaining the intensity of the signal from the $O_2^{\cdot-}$ spin adduct.

Use of EDTA to eliminate the interfering effects of metal ions in the superoxide dismutase assay employing xanthine oxidase and nitroblue tetrazolium introduces an artifact from the iron present. The interference in the assay from metal ions, including iron, can be eliminated with use of DETAPAC as a metal chelator. Thus, it is possible to make comparisons of measured superoxide dismutase activities even when there are variations in the amount of iron present in the samples.

INTRODUCTION

The superoxide radical, $O_2^{\cdot-}$, is formed in many living organisms and it or subsequent reaction products can have many deleterious effects (Fridovich, 1974; Bors *et al.*, 1974; Halliwell, 1974). Some of these effects are thought to be the result of hydroxyl radical, ·OH, as there are numerous reports of ·OH reaction products in $O_2^{\cdot-}$ generating systems (Weiss *et al.*, 1977; Tauber and Babior, 1977; Halliwell and Ahluwalia, 1976; Fong *et al.*, 1973; Beauchamp and Fridovich, 1970 are but a few examples).

We have used the technique of spin trapping (see Janzen, 1971) in an attempt to observe the $O_2^{\cdot-}$ and ·OH that can be formed from the enzymatic reaction of xanthine oxidase acting on xanthine. The spin trapping technique involves the addition reaction of a short lived free radical to a compound, spin trap, to produce a relatively long-lived free radical product, spin adduct, which is easily studied by electron spin resonance, EPR. For the spin trap used in this study, 5,5-dimethyl-1-pyrroline-1-oxide (DMPO), this reaction may be represented as:



where $R^{\cdot}$ may be $O_2^{\cdot-}$, ·OH, $\cdot CH_3$, phenyl radical, etc. As both the β-proton and the nitrogen hyperfine splitting of the nitroxide spin adduct are sensitive to the character of $R^{\cdot}$, it is possible in some cases to identify $R^{\cdot}$ or, if no unique assignment is feasible, it is still possible to learn something about the nature of $R^{\cdot}$ (Janzen and Liu, 1973; Harbour *et al.*, 1974).

We were able to observe both the $O_2^{\cdot-}$ and ·OH spin adducts of DMPO in the xanthine–xanthine oxidase system. However, it was only possible to observe the $O_2^{\cdot-}$ spin adduct of DMPO in solutions having low iron content or by using a judicious choice of metal chelator. Use of ethylenediaminetetraacetic acid, EDTA, as a chelating agent resulted in little or no signal from the $O_2^{\cdot-}$ spin adduct, depending on the amount of iron present, while use of diethylenetriaminepentaacetic acid, DETAPAC, as a metal chelating agent allows the observation of the signal from the $O_2^{\cdot-}$ spin adduct of DMPO over a wide range of iron concentrations in the xanthine–xanthine oxidase system.

MATERIALS AND METHODS

The spin trap 5,5-dimethyl-1-pyrroline-1-oxide was purchased from Aldrich Chemical Co. Inc., Milwaukee, Wisconsin. Xanthine, xanthine oxidase, superoxide dismutase, catalase and diethylenetriaminepentaacetic acid were obtained from Sigma Chemical Co., St. Louis, Missouri.

In the spin trapping experiments, typical reaction mixtures consisted of 0.1 m*M* xanthine, EDTA or DETAPAC, 80 m*M* DMPO, and iron as ferrous ammonium sulfate. Xanthine oxidase was present at approximately 60 n*M*. The reaction mixture was composed of various combinations of the above in 0.05 *M* phosphate buffer at pH 7.8. The EDTA and DETAPAC concentrations used were at least one order of magnitude greater than the iron concentration in the sample.

The standard superoxide dismutase assay of Beauchamp and Fridovich (1971) was used (xanthine oxidase/nitroblue tetrazolium [NBT]) with the addition of one unit per m/ of catalase. In the various cases tested, the xanthine oxidase concentration was adjusted to give approximately equal initial rates of blue formazan formation. The concentration of EDTA or DETAPAC used in each assay mixture was at least ten fold greater than that of the iron.

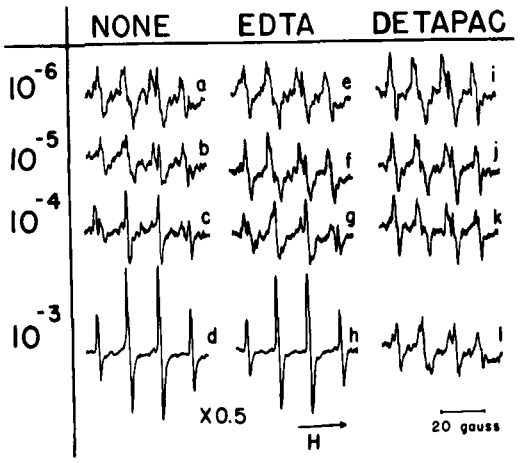


Figure 1. EPR spectra of the spin adducts of DMPO in the xanthine–xanthine oxidase system. Each row represents the spectra observed at different iron concentrations, ranging from 10^{-6} *M* to 10^{-3} *M*, with the different metal chelators present. Spectra i, j and k are examples of the O_2^- spin adduct of DMPO while spectra d and h are examples of the ·OH spin adduct. (In recording spectra d and h the receiver gain was reduced by a factor of two). Spectra c and g show a mixture of ·OH and O_2^-· spin adducts. This figure shows that in the presence or absence of EDTA, as the iron concentration is increased in the reaction mixture, the intensity of the ·OH spin adduct signal of DMPO increases while that of the O_2^-· spin adduct decreases. However, with DETAPAC as a metal chelator, the ·OH spin adduct formation is suppressed while little change is observed in the O_2^- spin adduct signal intensity.

RESULTS

In the xanthine–xanthine oxidase system, the particular DMPO spin adduct observed was dependent upon the concentration of iron in the system as well as the particular metal chelating agent used. As shown in Fig. 1, at low iron concentrations the EPR spectrum observed is that which has been assigned to the O_2^- spin adduct of DMPO (see Harbour *et al.*, 1974 for the assignment of the ·OH and O_2^- spin adduct spectra of DMPO). As the concentration of iron in the reaction mixture is increased the relative intensity of the ·OH spin adduct increases while the intensity of the O_2^- spin adduct decreases even in the presence of EDTA. However, if DETAPAC is used as a chelating agent in the reaction mixture, there is no significant change in the EPR spectrum observed as the iron concentration is varied from 10^{-6} through 10^{-3} mol/*l*.

Traces of iron are present in most biological systems. Fong *et al.* (1976) have found that the concentration of inorganic iron in liver cytosol can range from 10–130 μM with most values around 55 μM. Our observations as well as those of Halliwell (1975) show that iron–EDTA reacting with O_2^- can interfere with the routine assays for superoxide dismutase (Beauchamp and Fridovich, 1971; McCord and Fridovich, 1969). Using the superoxide dismutase assay of Beauchamp and Fridovich (1971), we found that the presence of iron can significantly affect the sensitivity of the assay. As shown in Table 1, there is a significant variation in the sensitivity of the assay when using EDTA or DETAPAC as a metal chelating agent even at 1.0 μM iron (0.1–1.0 μM iron is the approximate concentration of iron expected with the use of an 0.05 *M* phosphate buffer prepared from the usual commercial reagents). When the concentration of iron is raised to 0.1 m*M* the sensitivity of the assay is maintained when using DETAPAC as a metal chelator. However, if EDTA is used, essentially all the sensitivity of the assay is lost.

Table 1. The effect of iron and chelating agent on the assay for superoxide dismutase. The table entries represent the amount of pure bovine superoxide dismutase giving one unit of activity using the xanthine oxidase/NBT assay method of Beauchamp and Fridovich (1971). (Note that 1×10^{-9} kg/*l* is 1 ng/m*l*)

Iron Concentration	DETAPAC	EDTA
10^{-6} *M*	13×10^{-9} kg/*l*	50×10^{-9} kg/*l*
10^{-5} *M*	12×10^{-9} kg/*l*	very large
10^{-4} *M*	14×10^{-9} kg/*l*	very large

DISCUSSION

The reaction of superoxide radical with iron complexes of EDTA have recently been studied by Ilan and Czapski (1977), McClune *et al.* (1977) and Halliwell (1975). Their observations can be rationalized in terms of the reaction sequence:

$$Fe^{III}\text{-EDTA} + O_2^- \longrightarrow Fe^{II}\text{-EDTA} + O_2$$

$$Fe^{II}\text{-EDTA} + O_2^- \longrightarrow [Fe^{III}\text{-EDTA-}O_2]^{3-}$$

$$[Fe^{III}\text{-EDTA-}O_2]^{3-} \overset{H^+}{\rightleftharpoons} Fe^{III}\text{-EDTA} + H_2O_2$$

The net result is the dismutation of O_2^- to form O_2 and H_2O_2. With the introduction of H_2O_2 into the system, it is then possible to form ·OH via a Fenton-type reaction (see Walling, 1975; Bors *et al.*, 1974; and references therein), i.e.

$$Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + OH^- + \cdot OH.$$

Fong *et al.* (1976) have also observed the iron mediated formation of ·OH in a O_2^- generating system. Our observations are consistent with this general reaction scheme.

In the superoxide dismutase assay procedure, such as that of Beauchamp and Fridovich (1971), EDTA is added to remove the interfering effects of trace metal ions such as Cu^{2+} and Mn^{2+} Halliwell (1975) has shown that 33 μM iron does not interfere with the conversion of NBT to formazan in the absence of EDTA. However, in the presence of EDTA, iron can significantly inhibit formazan production. We have also made this observation.

Marklund and Marklund (1974) have developed an assay for superoxide dismutase activity by following

the inhibition of pyrogallol autoxidation. They found that the use of DETAPAC as the metal chelator in the assay medium prevented interference from Fe^{2+} as well as Cu^{2+} and Mn^{2+}. We have found that DETAPAC will also prevent interference from iron in the xanthine oxidase/NBT superoxide dismutase assay as well as suppress the ·OH formation from the Fenton-type reaction of iron and H_2O_2.

The ability to remove the interfering effects of iron from the superoxide dismutase assay allows for a more meaningful comparison of superoxide dismutase levels in various tissues that may have a wide range of iron concentrations.

REFERENCES

Beauchamp, C. and I. Fridovich (1971) *Anal. Biochem.* **44,** 276–287.
Beauchamp, C. and I. Fridovich (1970) *J. Biol. Chem.* **245,** 4641–4646.
Bors, W., M. Saran, E. Lengfeller, R. Spöttl and C. Michel (1974) *Curr. Top. Radiat. Res. Quart.* **9,** 247–309.
Fong, K., P. B. McKay, J. L. Poyer, B. B. Keele and H. Misra (1973) *J. Biol. Chem.* **248,** 7792–7797.
Fong, K., P. B. McKay, J. L. Poyer, H. P. Misra and B. B. Keele (1976) *Chem.-Biol. Interac.* **15,** 77–89.
Fridovich, I. (1974) *Adv. Enzymol.* **41,** 35–97.
Halliwell, B. (1974) *New Phytol.* **73,** 1075–1086.
Halliwell, B. (1975) *FEBS Lett.* **56,** 34–38.
Halliwell, B. and S. Ahluwalia (1976) *Biochem. J.* **153,** 513–518.
Ilan, Y. and G. Czapski (1977) *Biochim. Biophys. Acta* **498,** 386–394.
Harbour, J., V. Chow and J. R. Bolton (1974) *Can. J. Chem.* **52,** 3549–3553.
Janzen, E. G. (1971) *Acc. Chem. Res.* **4,** 31–40.
Janzen, E. G. and J. Liu (1973) *J. Mag. Res.* **9,** 510–512.
Marklund, S. and G. Marklund (1974) *Eur. J. Biochem.* **47,** 469–474.
McClune, G., J. Fee, G. McCluskey and J. Groves (1977) *J. Am. Chem. Soc.* **99,** 5220–5222.
McCord, J. M. and I. Fridovich (1969) *J. Biol. Chem.* **244,** 6049–6055.
Tauber, A. I. and B. M. Babior (1977) *J. Clin. Invest.* **60,** 374–379.
Walling, C. (1975) *Acc. Chem. Res.* **8,** 125–131.
Weiss, S. J., G. W. King, and A. F. Lobuglio (1977) *J. Clin. Invest.* **60,** 370–373.