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Comparison of Superoxide with Other Reducing Agents in the Biological Production of Hydroxyl Radicals

By Christine C. WINTERBOURN
Department of Clinical Biochemistry, Christchurch Hospital, Christchurch, New Zealand

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Iron–EDTA was shown to catalyse OH· production from H_2O_2 and ascorbate by a mechanism largely independent of superoxide. When ascorbate and superoxide were both present, the ascorbate mechanism was more important than superoxide as a source of OH·, and would appear to be more significant biologically.

The one-electron reduction of O_2 produces $O_2^{-\cdot}$, and occurs in a wide range of both chemical and metabolic processes. Superoxide appears to be toxic to living systems, as the enzyme superoxide dismutase, which breaks it down, is an essential constituent of all organisms that utilize O_2 (McCord *et al.*, 1971; Fridovich, 1975). Although superoxide dismutase has been shown to inhibit a number of reactions that would be deleterious to the cell, it has seldom been possible to define direct reactions of superoxide that could be responsible for the damage (Fridovich, 1975; Goldberg & Stern, 1977; Michelson & Durosay, 1977). In many cases, however, there is evidence that $O_2^{-\cdot}$ acts as a percursor of the more reactive OH· (Cohen, 1977). The mechanism for production was originally postulated to be the Haber–Weiss reaction (Fridovich, 1975):

$$O_2^{-} + H_2O_2 \rightarrow OH^{\cdot} + O_2 + OH^{-} \quad (1)$$

but kinetic data have subsequently shown that this is too slow to be of significance (Halliwell, 1976; McClune & Fee, 1976; Czapski & Ilan, 1978). However, this reaction occurs more rapidly when catalysed by iron–EDTA, and a mechanism of this type has been suggested as an alternative (Cohen, 1977; McCord & Day, 1978). EDTA-complexed Fe^{2+} reacts with H_2O_2 by a Fenton-type reaction (2), and the role of the $O_2^{-\cdot}$ is to reduce the ferric complex thus formed (3), the net result being reaction (1).

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

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

However, the requirement for $O_2^{-\cdot}$ is only to reduce the ferric complex, a role that other reducing agents may be able to perform equally well. The most common reducing agents in biological systems are ascorbate, GSH, NADH and NADPH. In the present paper, the ability of each of these to produce ethylene from methional [an indication of OH· production (Beauchamp & Fridovich, 1970)] in the presence of iron–EDTA and H_2O_2 has been investigated, and the significance of these reactions in relation to the $O_2^{-\cdot}$-dependent process considered.

Abbreviation used: GSH, reduced glutathione.

Experimental

$FeSO_4$ and H_2O_2 were AnalaR grade from BDH, Poole, Dorset, U.K. Other chemicals were obtained from Sigma Chemical Co., St Louis, MO, U.S.A. No superoxide dismutase activity was detectable in the catalase. Reactions were carried out in 32ml vials fitted with rubber septa in the lids. The vials, containing 5ml of reaction mixture, were placed on a rotating mixer. The temperature for each experiment was maintained within ±1°C, in the range 25–30°C. Reactants were introduced by syringe, and gas samples for analysis of ethylene removed at intervals with a 1ml syringe. Gas chromatography was performed at 70°C on a column packed with Chromosorb 102. Under the conditions used, a peak height of 100 corresponded to approx. 60nmol of ethylene/ml of reaction mixture. Rates of $O_2^{-\cdot}$ generation from xanthine and xanthine oxidase were determined by measuring rates of cytochrome *c* reduction under conditions where increasing the cytochrome *c*/xanthine oxidase ratio did not increase the amount of reduction.

Results

Ascorbate

In accordance with previous results (Beauchamp & Fridovich, 1970), equimolar amounts of H_2O_2 and $FeSO_4$ in EDTA/sodium phosphate buffer, pH7.4, in the presence of methional produced ethylene. As expected for a reaction involving OH· radicals,

ethylene production was inhibited by ethanol and benzoate (Table 1). There was slight inhibition of the reaction by superoxide dismutase. With catalytic Fe^{2+} concentrations (5 μM or 1 μM) and 50 μM-H_2O_2, ethylene was produced very much more slowly and the reaction was almost completely inhibited by superoxide dismutase. In this and other experiments in which superoxide dismutase was inhibitory, bovine serum albumin at twice the superoxide dismutase concentration had no effect.

Ascorbate markedly increased the rate of ethylene production from H_2O_2 and catalytic amounts of iron–EDTA (Fig. 1, lines *a* and *b*). The reaction was predominantly independent of $O_2^{-\cdot}$ as there was only about 20% inhibition by superoxide dismutase (Fig. 1, line *c*). Stimulation of ethylene production from H_2O_2, ascorbate and methional was evident with as low as 1 μM-Fe^{2+} ions. The rate of production increased with increasing ascorbate concentration in the range 10–100 μM, but there was little difference between 25 μM- and 50 μM-H_2O_2. Ethylene production was inhibited by ethanol and benzoate (Table 1).

Ascorbate and iron–EDTA alone also produced ethylene from methional, although the rate of production was less than with H_2O_2 present (Fig. 1, line *d*). However, the reaction was H_2O_2-dependent, as it was eliminated by catalase (Fig. 1, line *e*). It was also inhibited only slightly by superoxide dismutase (Fig. 1, line *f*). When H_2O_2 was present, there was no

Table 1. *Inhibition of ethylene production by ethanol and benzoate*

Reaction conditions were the same as described for Fig. 1.

Reaction mixture	Percentage inhibition of ethylene production: 5mM-Ethanol	5mM-Sodium benzoate
5 μM-Fe^{2+}+50 μM-H_2O_2	64	82
5 μM-Fe^{2+}+50 μM-H_2O_2+50 μM-ascorbate	48	70
5 μM-Fe^{2+}+50 μM-H_2O_2+50 μM-NADH	50	71

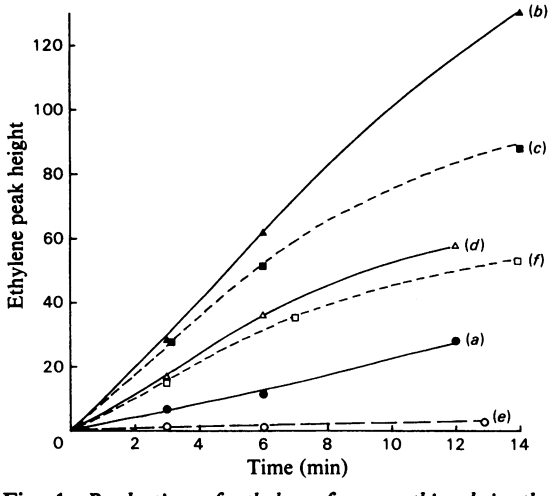

Fig. 1. *Production of ethylene from methional in the presence of iron–EDTA, H_2O_2 and ascorbate*

Reactions were carried out in 10mM-sodium phosphate buffer, pH 7.4, containing 1mM-methional, 20 μM-EDTA and 5 μM-$FeSO_4$. Additional reagents were: (*a*, ●) 50 μM-H_2O_2; (*b*, ▲) 50 μM-H_2O_2+50 μM-sodium ascorbate; (*c*, ■) 50 μM-H_2O_2, 50 μM-ascorbate+4.1 μg of superoxide dismutase/ml; (*d*, △) 50 μM-ascorbate; (*e*, ○) 50 μM-ascorbate+20 μg of catalase/ml; (*f*, □) 50 μM-ascorbate+superoxide dismutase.

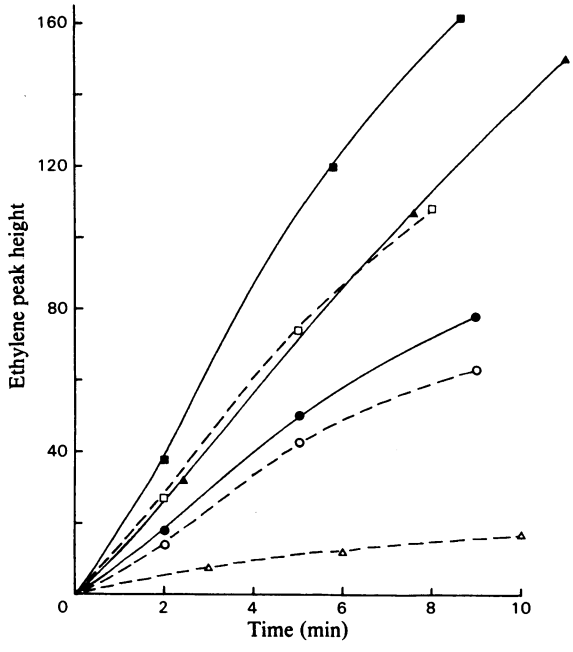

Fig. 2. *Comparison of ethylene production from $O_2^{-\cdot}$, produced from xanthine and xanthine oxidase, and ascorbate, in the presence of iron–EDTA and H_2O_2*

Reaction conditions were the same as for Fig. 1, except that all solutions contained 50 μM-H_2O_2 and 0.5mM-xanthine. Additional reagents were: ●, 50 μM-ascorbate; ○, 50 μM-ascorbate+4.1 μg of superoxide dismutase/ml; ▲, 0.017unit of xanthine oxidase/ml (rate of $O_2^{-\cdot}$ generation 12nmol/min per ml); △, xanthine oxidase+superoxide dismutase; ■, 50 μM-ascorbate+0.017 unit of xanthine oxidase/ml; □, ascorbate, xanthine oxidase+superoxide dismutase.

requirement for O_2, but in the absence of H_2O_2 no ethylene was produced under N_2.

To compare the abilities of $O_2^{-\cdot}$ and ascorbate to produce $OH^\cdot$ radicals, $O_2^{-\cdot}$ was generated from xanthine and xanthine oxidase at rates of 6 and 12nmol/min per ml. In the presence of methional, H_2O_2 and iron–EDTA this produced ethylene at approx. 0.8 and 1.4 times the rate observed with 50μM-ascorbate. Both catalase and superoxide dismutase prevented ethylene production. When ascorbate and xanthine oxidase were both present, ethylene was produced more rapidly than with each individually, but the rate of production was less than the sum of the individual rates (Fig. 2). Most of the ethylene was produced by a mechanism dependent on ascorbate but not on $O_2^{-\cdot}$, as superoxide dismutase only slightly inhibited the reaction. The proportion of the reaction that was $O_2^{-\cdot}$-dependent was comparable with that observed with ascorbate and no xanthine oxidase. It increased slightly with increasing rate of $O_2^{-\cdot}$ generation, but was always a minor fraction.

Reduced glutathione

GSH did not enhance $OH^\cdot$ radical formation from iron–EDTA and H_2O_2. The low rate of production of ethylene from methional in the presence of H_2O_2 and catalytic amounts of iron–EDTA (as shown in Fig. 1, line *a*) was unchanged by up to 150μM-GSH. With higher GSH concentrations (1mM) there was slight inhibition.

NADH and NADPH

NADH and NADPH both enhanced the rate of ethylene production from H_2O_2, iron–EDTA and methional, but to a much lesser extent than did ascorbate. Under the conditions of Fig. 1, 100μM-NADH or -NADPH approximately doubled the rate, and 50μM had a lesser effect. H_2O_2 was required, but the mechanism differed from that with ascorbate in that $O_2^{-\cdot}$ was involved and the reaction was almost completely inhibited by superoxide dismutase.

Ethylene production in the presence of xanthine, xanthine oxidase and NADH (or NADPH) was approximately equal to the sum of the individual rates, as would be expected if the role of the NADH were as an additional source of $O_2^{-\cdot}$.

Discussion

It is apparent from these results that ascorbate and H_2O_2, in the presence of catalytic amounts of iron–EDTA, react to form $OH^\cdot$ radicals. Although it has been shown that ethylene production from methional is a complex reaction and is not necessarily specific for $OH^\cdot$ (Pryor & Tang, 1978), in this instance it is highly likely that $OH^\cdot$ was responsible. The reaction between Fe^{2+} and H_2O_2 is a well-established producer of $OH^\cdot$. (It also produces OH^-, but the solutions were sufficiently buffered for it to have had no effect.) With ascorbate present, $OH^\cdot$ should be produced by a similar mechanism, and it is much more likely to have been the source of ethylene than is any other reaction product. In addition, ethylene production in each case was inhibited by the $OH^\cdot$ scavengers, benzoate and ethanol.

Ascorbate can therefore be substituted for $O_2^{-\cdot}$ as the reducing agent in reaction (3), and a combination of reactions (2) and (4) can be proposed for $OH^\cdot$ production from ascorbate and H_2O_2:

$$2\ Fe^{3+}\text{–EDTA} + \text{ascorbate} \rightarrow 2\ Fe^{2+}\text{–EDTA} + \text{dehydroascorbate} \quad (4)$$

When both ascorbate and $O_2^{-\cdot}$ are present, it appears that the reaction involving ascorbate predominates, and there is even inhibition of the $O_2^{-\cdot}$-dependent pathway. Halliwell & Foyer (1976) have also found evidence for reaction (4) and that ascorbate can react with $O_2^{-\cdot}$ and inhibit $O_2^{-\cdot}$-dependent reactions.

Production of $OH^\cdot$ via an O_2-dependent pathway was observed with ascorbate and iron–EDTA in the absence of added H_2O_2, but involving H_2O_2 as an intermediate. It is likely that this reaction was initiated by the autoxidation of ascorbate, catalysed by iron–EDTA (Halliwell & Foyer, 1976), with the reaction of the H_2O_2 formed with the iron–EDTA accounting for the $OH^\cdot$ radicals.

GSH, NADH and NADPH, unlike ascorbate, did not reduce Fe^{3+}–EDTA sufficiently rapidly to enable it to catalyse $OH^\cdot$ formation from H_2O_2. NADH and NADPH, however, did make a small contribution to $OH^\cdot$ production, but the mechanism involved $O_2^{-\cdot}$. Autoxidation of the NADH (or NADPH) catalysed by the iron–EDTA is the most probable source of the $O_2^{-\cdot}$.

It has been suggested that the reaction of $O_2^{-\cdot}$ with chelated iron and H_2O_2 may be an important source of $OH^\cdot$ in biological systems, and that one of the prime functions of superoxide dismutase may be to prevent such reactions (Cohen, 1977; McCord & Day, 1978). This conjecture depends on the existence of complexes such as iron–EDTA. This is not too unrealistic, as, although most tissue iron stores such as ferritin would be unavailable, some iron, particularly during transport, should be present in low-molecular-weight complexes or protein complexes such as transferrin. Transferrin has been shown to have the ability to catalyse $OH^\cdot$ formation (McCord & Day, 1978).

If chelated iron is an important biological catalyst for production of $OH^\cdot$, the relative significance of ascorbate and $O_2^{-\cdot}$ in the process can be considered in terms of the results of this study. The 50μM-ascorbate concentration used is comparable with that found in human plasma and erythrocytes, and

lower than the concentration in most other cells (Henry, 1964). The ascorbate mechanism should therefore be feasible intracellularly or in plasma. $O_2^{-\bullet}$ concentrations can be less precisely estimated. Production rates for activated granulocytes have been estimated as 0.07 and 17μmol/min per 10^{10} cells (Babior *et al.*, 1973; Weening *et al.*, 1975). This would correspond to approx. 0.04 or 10nmol of $O_2^{-\bullet}$ produced/min per ml of blood. The higher estimate is comparable with the level of $O_2^{-\bullet}$ generated by xanthine oxidase in this study. It would be expected that $O_2^{-\bullet}$ concentrations in blood containing activated granulocytes would be some of the highest encountered, but the present results suggest that even under these conditions the ascorbate mechanism for generating $OH^\bullet$ is likely to predominate. White-cell ascorbate concentrations are several times higher than plasma concentrations, so this should favour the ascorbate mechanism still further.

$O_2^{-\bullet}$-dependent $OH^\bullet$ generation could be more significant in some instances, such as when activated granulocytes are concentrated round a target. Nevertheless, in combination with H_2O_2 and chelates such as iron–EDTA, ascorbate would appear to be generally more important than $O_2^{-\bullet}$ as a source of $OH^\bullet$, and, unless a more efficient $O_2^{-\bullet}$-dependent mechanism can be recognized, there must be some doubts about whether the primary role of superoxide dismutase is to prevent $OH^\bullet$ production. What appears more relevant is the involvement of H_2O_2 in both processes, and efficient removal of H_2O_2 should be a more crucial preventive measure.

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