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AN ATTEMPT TO DEMONSTRATE A REACTION BETWEEN SUPEROXIDE AND HYDROGEN PEROXIDE

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1. Introduction

The superoxide radical, $O_2^{\cdot-}$, is formed in living organisms and has many deleterious effects [1–3]. There is considerable evidence that systems generating $O_2^{\cdot-}$ can form the hydroxyl radical, .OH. This evidence comes both from studies employing scavengers of .OH [1,2] and also from a direct demonstration of hydroxylation of aromatic compounds by .OH in a $O_2^{\cdot-}$ generating system [4]. Production of .OH seems to be inhibited by catalase [1,2], suggesting that H_2O_2 is required [5].

In 1934, Haber and Weiss [6] proposed that H_2O_2 and $O_2^{\cdot-}$ can react together, as shown below:

$$H_2O_2 + O_2^{\cdot-} \rightarrow O_2 + .OH + OH^-.$$

The Haber-Weiss reaction has naturally been proposed as the source of .OH in systems producing $O_2^{\cdot-}$ [1–3]: it has also been suggested that the O_2 produced by this reaction is in the singlet state [7]. Despite the circumstantial evidence suggesting that the reaction does, in fact, occur [1–3,5,7], McClune and Fee [8] showed that H_2O_2 had little effect on the rate of loss of $O_2^{\cdot-}$ from aqueous solution at pH values from 8.2–10.6. Because of the importance of .OH generation as a mechanism for the cytotoxicity of $O_2^{\cdot-}$ [1–3], I have attempted to demonstrate the Haber-Weiss reaction at more physiological pH values (6.0–8.5). It is known that $O_2^{\cdot-}$ in aqueous solution reduces nitro-blue tetrazolium to formazan [9]. Since the rate constant for this reaction is low ($k = 5 \times 10^4\ M^{-1}\ s^{-1}$ at pH 7.8; $1 \times 10^4\ M^{-1}\ s^{-1}$ at pH 10.0), any enzyme [9] or compound [10] which reacts with $O_2^{\cdot-}$ strongly inhibits formazan production.

2. Materials and methods

Reagents were obtained from sources described previously [11]. Reaction mixtures contained, in a final volume of 3.00 ml, buffer, nitro-blue tetrazolium (100 μM) and any H_2O_2 required. Then 200 μl (experiments at pH 7.0–8.5) or 500 μl (experiments at pH 6.0–7.0) of a saturated solution of $K^+O_2^{\cdot-}$ in dimethylsulphoxide [11] were added. A blue colour developed almost instantaneously as formazan was produced. The absorbance of each solution was measured at 560 nm against a control in which pure dimethylsulphoxide had been added to the above reaction mixtures.

3. Results

Addition of $K^+O_2^{\cdot-}$, dissolved in dimethylsulphoxide, to a solution containing nitro-blue tetrazolium results in an immediate production of formazan, which is inhibited by reagents reacting with $O_2^{\cdot-}$ [11]. If H_2O_2 reacts with $O_2^{\cdot-}$, one would expect it to compete with nitro-blue tetrazolium in the above reaction mixtures and inhibit formazan production.

Table 1 summarises the experimental conditions used in attempts to demonstrate such an inhibition, but no inhibition was found using concentrations of H_2O_2 up to 1.0 M (compared with a concentration

Table 1
Attempts to demonstrate the Haber-Weiss reaction

Buffer	pH	Concentration of H_2O_2 tested (M)	Inhibition of formazan production by H_2O_2
Citric acid–trisodium citrate	6.0	10^{-3}–1.0	None
KH_2PO_4–Na_2HPO_4	6.5	10^{-3}–0.5	None
	7.0	10^{-3}–0.5	None
	7.5	10^{-1}–1.0	None
	8.0	10^{-3}–0.3	None
	8.5	10^{-1}–0.3	None
Tricine–KOH	7.5	10^{-3}–0.5	None
Hepes–KOH	7.8	10^{-3}–1.0	None
Triethanolamine–HCl	8.04	10^{-1}–0.5	None
Glycylglycine–KOH	8.5	10^{-1}–0.5	None

Buffers were tested at final concentrations of 50–100 mM. Presence of mannitol (0.1 or 0.2 M) in the reaction mixtures did not affect the results. The concentrations of H_2O_2 specified were the final concentrations in the 3 ml reaction mixtures.

of nitro-blue tetrazolium of 10^{-4} M). Even when the reaction mixtures also contained 0.2 M mannitol to scavenge any .OH that might be formed and prevent it from undergoing further reactions, no inhibition by H_2O_2 was found. (Mannitol itself did not affect formazan production under the various reaction conditions listed in table 1.) In any case, dimethylsulphoxide itself is probably a powerful scavenger of .OH [12].

When buffers were used at concentrations lower than 50 mM, they were unable to maintain the pH of the reaction mixture when $K^+O_2^{\cdot-}$ was added. However, the rise in pH (due to consumption of H^+ when $O_2^{\cdot-}$ dismutates to H_2O_2 and O_2) was the same whether or not H_2O_2 was present. Under these conditions an apparent inhibition of formazan production by H_2O_2 at high concentrations was seen: table 2 shows a typical experiment, carried out at pH 7.5 in 10 mM phosphate buffer. Since both KCl and glucose also inhibit, it seems that this effect is not caused by a reaction of H_2O_2 with $O_2^{\cdot-}$, but is simply due to the presence of

Table 2
Inhibition of formazan production at low buffer concentration

Compound present	Concentration (M)	% Inhibition of formazan production
H_2O_2	0.007	0
	0.033	4
	0.1	19
	0.2	27
	1.0	32
Glucose	0.2	14
	1.0	25
KCl	0.2	35
	1.0	36

The experiments were carried out in the presence of 10 mM KH_2PO_4–Na_2HPO_4 buffer, pH 7.5. The buffer was unable to maintain the pH of the reaction mixture, which shifted to 8.0 when $K^+O_2^{\cdot-}$ was added. However, the ΔpH was the same in all reaction mixtures. None of the above compounds inhibited when the buffer concentration was raised to 100 mM.

polar molecules, which probably facilitate the nonenzymic dismutation of $O_2^{\cdot -}$ ($O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$), a reaction which competes with reduction of nitro-blue tetrazolium.

4. Discussion

The results presented in this paper are essentially negative: the Haber-Weiss reaction cannot be demonstrated. This does not, of course, prove that the reaction does not occur, but direct demonstration of it will be required before it can be accepted a mechanism for generation of .OH. Nevertheless, that .OH is generated by some mechanism in systems producing $O_2^{\cdot -}$ [1–4] and that H_2O_2 is involved [1,2] seems almost certain.

References

[1] Fridovich, I. (1975) Ann. Rev. Biochem. 44, 147–159.
[2] Bors, W., Saran, M., Lengfelder, E., Spottl, R. and Michel, C. (1974) Curr. Top. Radiat. Res. Q. 9, 247–309.
[3] Halliwell, B. (1974) New Phytol. 73, 1075–1086.
[4] Halliwell, B. and Ahluwalia, S. (1976) Biochem. J. 153, 513–518.
[5] Hodgson, E. K. and Fridovich, I. (1976) Biochim. Biophys. Acta 430, 182–188.
[6] Haber, F. and Weiss, J. (1934) Proc. R. Soc. London Ser. A 147, 332–351.
[7] Kellogg, E. W. and Fridovich, I. (1975) J. Biol. Chem. 250, 8812–8817.
[8] McClune, G. J. and Fee, J. A. (1976) FEBS Lett. 67, 294–298.
[9] Beauchamp, C. and Fridovich, I. (1971) Anal. Biochem. 44, 276–287.
[10] Halliwell, B. (1975) FEBS Lett. 56, 34–38.
[11] Henry, L. E. A., Halliwell, B. and Hall, D. O. (1976) FEBS Lett. 66, 303–306.
[12] McCord, J. M. and Fridovich, I. (1969) J. Biol. Chem. 244, 6056–6063.