# PRODUCTION OF HYDROXYL RADICALS FROM PARAQUAT RADICALS AND $H_2O_2$

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Received 21 April 1981

## 1. Introduction

The herbicidal action and toxicity of paraquat ($PQ^{2+}$) involve its metabolic reduction to the paraquat radical ($PQ^{+}$) and subsequent reactions of the radical [1–4]. $PQ^{+}$ reacts very rapidly with $O_2$ to give $O_2^-$ ($k_2 = 7.7 \times 10^8\ M^{-1} . s^{-1}$) [1], and it has been postulated that this reaction, and subsequent reactions of $O_2^-$ to give more reactive products such as singlet oxygen ($^1O_2$) and hydroxyl ($OH^{\cdot}$) radicals, are the basis of its toxicity [2–5]. However, whether $^1O_2$ can be produced from $O_2^-$ in biological systems, and whether catalysts required for $OH^{\cdot}$ production are available are still matters of speculation [6–10].

This paper describes a reaction between $PQ^{+}$ and $H_2O_2$ to give $OH^{\cdot}$:

$$PQ^{+} + H_2O_2 \rightarrow PQ^{2+} + OH^{-} + OH^{\cdot} \quad (1)$$

It is shown that the reaction does not require a metal catalyst, and is very fast and able to compete with the reaction of the radical with $O_2$. The requirements for $OH^{\cdot}$ production via this mechanism should be met in biological systems, and the reaction could play an important role in paraquat toxicity.

## 2. Methods

Paraquat radicals were generated by the reaction of xanthine oxidase ($4 \times 10^{-3}$ U/ml) with xanthine (0.2 mM) and paraquat (1 mM). The reactions were carried out at ~28°C in closed 12 ml tubes, in 0.05 M phosphate buffer (pH 7.4) containing 2 mM methional, and 0.3 mM $H_2O_2$ where indicated (total vol. 2.5 ml). At intervals, 0.4 ml gas samples were removed and the ethylene concentration measured as in [11]. All biochemicals were obtained from Sigma (St Louis MO) except for superoxide dismutase from Diagnostic Reagents (Oxon).

## 3. Results

Xanthine oxidase was found to catalyse electron transfer from xanthine to paraquat. Anaerobically a blue solution with the absorption spectrum of $PQ^{+}$ [2] was produced, but on introduction of air the colour rapidly disappeared due to the reaction of $PQ^{+}$ with $O_2$. When the enzyme reaction was carried out anaerobically in the presence of $H_2O_2$ no $PQ^{+}$ formation was detected, and $H_2O_2$ introduced anaerobically into a solution of $PQ^{+}$ caused the rapid disappearance of the radical.

The reaction between $PQ^{+}$, generated anaerobically by the xanthine oxidase reaction, and $H_2O_2$ caused the production of ethylene from methional (fig.1). Ethylene production from methional, provided it can be inhibited by $OH^{\cdot}$ scavengers, is usually indicative of $OH^{\cdot}$ production [12]. Benzoate (15 mM and 30 mM) gave 50% and 72% inhibition of the reaction and 30 mM ethanol gave 30% inhibition. With no obvious mechanism for forming other sufficiently reactive radicals in this system, $OH^{\cdot}$ radicals are therefore strongly implicated. In the absence of $PQ^{2+}$ or xanthine oxidase, very little ethylene was produced (fig.1). Omission of $H_2O_2$ decreased the rate to ~15%, and this reaction was a further 95% inhibited by catalase (1000 U/ml). Recrystallization of the $PQ^{2+}$ from aqueous ethanol did not alter its behaviour. The rate of ethylene production did not vary over 1–4 mM methional, indicating efficient trapping of $OH^{\cdot}$ radicals. The rate of ethylene production was ~80% of the rate of $PQ^{+}$ generation (measured as cytochrome *c* reduction) under comparable conditions suggesting that most of the $PQ^{+}$ formed gave rise to $OH^{\cdot}$.

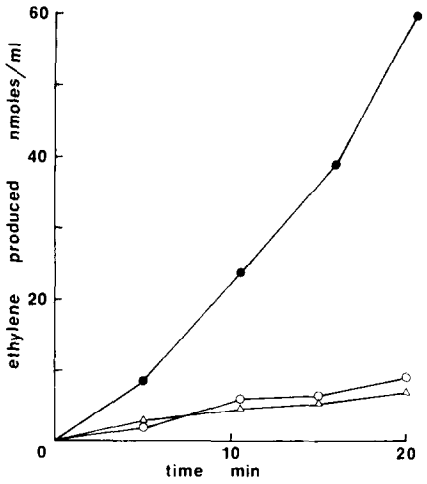


Fig.1. Ethylene production from $H_2O_2$ and $PQ^+$. For experimental conditions see section 2. The rate of enzymic $PQ^+$ formation was 4.2 nmol . $ml^{-1}$ . $min^{-1}$, determined in a separate experiment measuring cytochrome *c* reduction under similar conditions: (•) all reactants present; (○) no xanthine oxidase; (△) no paraquat.

The reaction did not appear to depend on trace metal contaminants. Diethylenetriamine-pentaacetic acid (DTPA), the iron complex of which is a poor catalyst of $OH^\cdot$ production from $O_2^-$ and $H_2O_2$ [13], did not inhibit ethylene production from $PQ^+$ and $H_2O_2$, and addition of up to 10 $\mu$M $Fe^{2+}$ in the presence of DTPA caused a $\leqslant$20% increase in rate. This contrasts with a much greater effect of $Fe^{2+}$(DTPA) on $OH^\cdot$ production from $O_2^-$ and $H_2O_2$ (table 1). Likewise, although 10 $\mu$M $Fe^{2+}$ gave a 7-fold increase in rate of ethylene production from $O_2^-$ and $H_2O_2$ in the presence of EDTA, it only doubled the rate with $PQ^+$ and $H_2O_2$ (table 1). However, both DTPA and EDTA approximately doubled the rate of ethylene production from $PQ^+$ and $H_2O_2$ (table 1). EDTA also increased the rate in the absence of $PQ^{2+}$ but DTPA had an effect only if $PQ^{2+}$ and xanthine oxidase were both present. Since ethylene production in the presence and absence of DTPA was dependent on the same factors, and because DTPA suppresses contaminant metal ion-catalysed $OH^\cdot$ production from $H_2O_2$ and $O_2^-$ [13], experiments examining the effect of $O_2$ on the paraquat reaction were carried out in the presence of 0.2 mM DTPA.

Table 1
Effects of chelators and iron on the rate of ethylene production from $H_2O_2$ and either $PQ^+$ or $O_2^-$

| Chelator | $Fe^{2+}$ added ($\mu$M) | Rate of ethylene production (nmol . $ml^{-1}$ . 10 $min^{-1}$) | |
|---|---|---|---|
| | | $PQ^+ + H_2O_2$[a] | $O_2^- + H_2O_2$[b] |
| – | 0 | 34 | |
| DTPA (0.2 mM) | 0 | 63 | 5 |
| | 5 | 70 | 11 |
| | 10 | 75 | 19 |
| EDTA (0.2 mM) | 0 | 75 | 9 |
| | 5 | 105 | 38 |
| | 10 | 150 | 63 |

[a,b] For reaction conditions see section 2. Reactions were carried out: [a] in $N_2$; [b] in air in the absence of $PQ^{2+}$

In the presence of $O_2$ the rate of $OH^\cdot$ production from $PQ^+$ and $H_2O_2$ was substantially decreased. It decreased gradually as the $O_2$ concentration increased, but even in air, the rate was ~3-times higher than in the absence of $PQ^{2+}$ (table 2). The slightly higher rate of radical production by xanthine oxidase in the presence of $PQ^{2+}$ is not enough to account for this difference. Superoxide dismutase inhibited the reaction in the presence, but not in the absence of $O_2$ (table 2). Inhibition by 10 $\mu$g/ml SOD was almost complete in air, but was somewhat less at lower $O_2$ concentrations.

$OH^\cdot$ production in the absence of $O_2$ was decreased to ~15% if no $H_2O_2$ was added. Introduction of $O_2$, which should react with either xanthine oxidase or $PQ^+$ and give rise to $H_2O_2$ resulted in a sharp increase in $OH^\cdot$ production (fig.2). This rose to a maximum with increasing $O_2$ concentration and declined gradually as normal air concentration was approached. Superoxide dismutase was inhibitory in much the same way as with added $H_2O_2$ (fig.2).

Table 2
Effects of $O_2$ and superoxide dismutase on $OH^\cdot$ production from $PQ^+$ and $H_2O_2$

| % $O_2$ | Relative reaction rate | | % Inhibition by 10 $\mu$g/ml superoxide dismutase |
|---|---|---|---|
| | 1 mM $PQ^{2+}$ | No $PQ^{2+}$ | |
| 0 ($N_2$) | 100 | | 0 ± 10 |
| 2 | 70 | 4 | 70 |
| 6 | 32 | 3 | 80 |
| 20 (air) | 9 | 3 | 86 |

Reaction conditions are described in section 2, except that 0.2 mM DTPA was also present. Air/$N_2$ mixtures were made by filling the tubes with $N_2$, and using a syringe, replacing a known volume with air. Rates of radical generation (cytochrome *c* reduction): 1 mM $PQ^{2+}$, $N_2$ 50 nmol. $ml^{-1}$ . $min^{-1}$; 1 mM $PQ^{2+}$, air 3.6 nmol . $ml^{-1}$ . $min^{-1}$; no $PQ^{2+}$, air 2.3 nmol . $ml^{-1}$ . $min^{-1}$

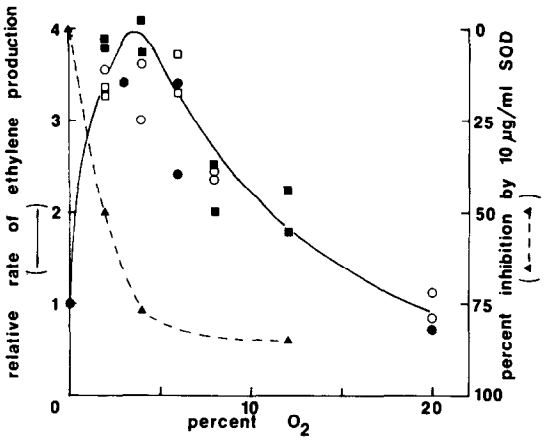


Fig.2. Effects of $O_2$ and superoxide dismutase on $OH^{\cdot}$ production from $PQ^+$ generated by xanthine oxidase in the absence of added $H_2O_2$. Reaction conditions were the same as for table 2 except no $H_2O_2$ was added. (○,●,□,■) represent results obtained on 4 occasions.

## 4. Discussion

These results provide evidence for a reaction between $H_2O_2$ and $PQ^+$ that produces $OH^{\cdot}$ radicals (reaction (1)). No requirement for a metal catalyst was found, contrasting with the analogous reaction involving $O_2^-$ [13]. In biological terms, therefore, this reaction provides a mechanism for producing $OH^{\cdot}$ radicals from $PQ^{2+}$ without the need to invoke the existence of a suitable metal catalyst. The reaction of $PQ^+$ with $H_2O_2$ appears to be very fast and able to compete with its reaction with $O_2$. Competition between these reactions reduced the yield of $OH^{\cdot}$, but even in air the reaction between $H_2O_2$ and $PQ^+$ was detectable. Because $PQ^+$ reacts with $O_2$ very rapidly, it is often assumed that other reactions of $PQ^+$ in air can be disregarded [1–5]. The reaction between $PQ^+$ and $H_2O_2$ appears to invalidate this assumption, and especially in partially deoxygenated conditions direct production of $OH^{\cdot}$ from $PQ^+$ is likely to occur.

In the absence of added $H_2O_2$, $OH^{\cdot}$ production from $PQ^+$ was very low unless $O_2$, which could act as a source of $H_2O_2$, was present. As biological $H_2O_2$ production depends on $O_2$ metabolism, biological systems should also require $O_2$ for this reaction. At higher $O_2$ concentrations the function of $O_2$ as a source of $H_2O_2$ was overshadowed by its removal of $PQ^+$ via reaction (2), and the stimulation of $OH^{\cdot}$ production was replaced by inhibition:

$$PQ^+ + O_2 \rightleftharpoons PQ^{2+} + O_2^- \quad (2)$$

Although $OH^{\cdot}$ production from $PQ^+$ and $H_2O_2$ was not due to $O_2^-$, the reaction in air was inhibited by superoxide dismutase. This is analogous to the effect of superoxide dismutase on reactions of a number of semiquinone radicals with cytochrome *c* or methaemoglobin, and can be explained if reaction (2) is reversible [14,15]. Superoxide dismutase, by removing $O_2^-$, can displace this equilibrium to the right and indirectly inhibit reactions of $PQ^+$. This finding has the important implication that inhibition of $OH^{\cdot}$-dependent reactions by superoxide dismutase does not necessarily imply participation of $O_2^-$.

Paraquat toxicity is dependent on a variety of factors. Reduction to $PQ^+$ is an essential step, $O_2$ is important, $H_2O_2$ appears to be involved, and there is some evidence that superoxide dismutase is inhibitory [2–5,16]. Lipid peroxidation, and other evidence suggesting free radical involvement, has also been observed [3,5,17]. From the data presented here, it is apparent that all these features can be accommodated if the toxic mechanism involves $OH^{\cdot}$ production from $PQ^+$ and $H_2O_2$. Firstly, production of $OH^{\cdot}$ radicals should initiate tissue-damaging reactions [6,7]. $O_2$ would be essential not as a source of $O_2^-$ but of $H_2O_2$, and superoxide dismutase would be protective by removing $PQ^+$ rather than $O_2^-$.

Production of $OH^{\cdot}$ from $PQ^+$ and $H_2O_2$ may therefore be of major significance in paraquat toxicity. A further implication of these results, however, is that the effects of the $PQ^+$ radical cannot be assumed to be the same as the effects of $O_2^-$, and paraquat-induced tissue damage cannot therefore be considered as analogous to oxygen toxicity.

## Acknowledgement

This work was supported by a grant from the Medical Research Council of New Zealand.

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