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One-electron oxidation of "photo-Fenton" reagents and inhibition of lipid peroxidation

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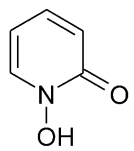
Abstract

The "photo-Fenton" reagent, 2-mercaptopyridine *N*-oxide (MPO), which releases a hydroxyl radical on ultraviolet irradiation, has been found to act as an antioxidant. In the peroxidation of linoleate initiated by a water-soluble azo-initiator, MPO has about one-third the activity of the water-soluble vitamin E analogue Trolox C. In contrast, the oxygen-containing analogue, 2-hydroxypyridine *N*-oxide (HPO), does not have measurable antioxidant activity in this system. Both reagents react with hydroxyl radical with second order rate constants very close to the diffusion-controlled limit. With the less oxidising dithiocyanate radical anion, MPO reacts approximately 50 times more rapidly than HPO at pH > 7. The more reducing properties of MPO result in its activity as an antioxidant and make it less suitable than HPO as a source of hydroxyl radicals for investigation of oxidative stress in biological systems.

Keywords: Hydroxyl radical; Peroxidation; Pulse radiolysis; Oxidative stress; Fenton reagent; Photochemical

Compounds that release a hydroxyl radical on photolysis, sometimes referred to as "photo-Fenton reagents," are of considerable interest as sources of oxidative stress in biology [1–3]. These reagents promise to be more versatile and controllable than the classical Fenton reagents which produce hydroxyl radical on reaction of transition metal ion (typically Cu^{2+} or Fe^{3+}) with H_2O_2 [4]. The most widely investigated photochemical sources of hydroxyl radical are 2-hydroxypyridine *N*-oxide (HPO, I) [5] and 2-mercaptopyridine *N*-oxide (MPO, II) [6].



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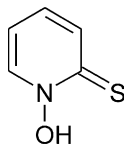

II

In aqueous solutions such reagents have been shown to produce hydroxyl radicals with quantum yields of up to ~0.3 [5,6] and are capable of yielding photo-initiated strand breaks in DNA [7,8]. These reagents therefore offer promise in investigations of oxidative stress in cellular systems where the production of hydroxyl radicals may be controlled temporally and spatially by suitable UV illumination.

Lipid peroxidation is one of the main consequences of oxidative stress in biological systems [9,10]. The oxidation of lipid molecules may lead to loss of membrane integrity and the release of toxic, reactive, and bioactive products such as 9-hydroxynonenal [11,12]. The chain reaction of lipid peroxidation may be initiated by oxidising radicals, including hydroxyl and peroxyl radicals, and is propagated by the relatively reactive lipid peroxyl radical which abstracts a hydrogen atom from a further polyunsaturated lipid molecule. It is well known that phenolic compounds, including α-tocopherol and analogous molecules ('vitamin E'), are able to reduce the lipid peroxyl radical to the hydroperoxide. The resulting tocopheroxyl radical is relatively stable and much less reactive towards a further lipid molecule than the lipid peroxyl radical. Tocopherols and similar phenols are therefore referred to as 'chain-breaking' antioxidants [13–15].

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The use of photo-Fenton reagents to investigate reactions of hydroxyl radical in dilute in vitro systems is hindered by the high reactivity of the reagent itself with the hydroxyl radical. For example, it has been reported [5] that MPO reacts with ·OH with a second order rate constant of $9 \times 10^9\,dm^3\,mol^{-1}\,s^{-1}$. We now report a further investigation of the reaction of HPO and MPO with oxidising radicals. It is shown that MPO is much more reactive than HPO. MPO, but not HPO, is able to inhibit the oxidation of linoleate by an azo-initiator. It is concluded that HPO is more suitable than MPO for future studies of light-induced oxidative stress in biological systems.

Materials and methods

The water-soluble azo-initiator, 2′,2′-azobis(2-methylproprionamide) dihydrochloride (AMPA), was purchased from Acros Organics Ltd. MPO, HPO, sodium linoleate, Triton X-100, and all other reagents were obtained from Sigma. Oxygen uptake by linoleate dispersions was measured in a Clarke type oxygen electrode (Rank Brothers Ltd., Cambridge, UK) thermostated to 40 °C. Pulse radiolysis experiments [16] were undertaken using the linear accelerator (10–12 MeV, 500 ns pulse) at the Free Radical Research Facility, Daresbury Laboratory, with a dose of approximately 6 Gy/pulse. Solutions were saturated with N_2O by purging and were irradiated in a capillary cell with 2.5 cm optical pathlength.

Results and discussion

Oxidation of linoleate and antioxidant effects of photo-Fenton reagents

Oxidation of sodium linoleate (10 mmol dm^{-3}) in Triton X-100 micelles (100 mmol dm^{-3}) was determined by measurement of oxygen uptake at 40 °C in a Clarke oxygen electrode in a similar manner to that described previously by Niki [17]. Oxidation was initiated by addition of AMPA to a final concentration of 16 mmol dm^{-3} in the electrode chamber. In these solutions peroxyl radicals, generated by decomposition of AMPA at a constant rate in the air-saturated solutions, initiated peroxidation of the linoleate and the consequent oxygen consumption. In the absence of any further additive, addition of an aliquot of AMPA to the linoleate micelles resulted in immediate oxygen uptake as shown in Fig. 1. In the presence of MPO (Fig. 1A) it was found that after the addition of AMPA there was a time interval (the delay period) of reduced rate of oxygen consumption before oxygen uptake resumed at the uninhibited rate. The length of this delay period increased with MPO concentration. This is the result typically found [17,18] in the presence of a compound which is capable of scavenging either the initiating azo-initiator-derived peroxyl radical or the chain propagating linoleate-peroxyl radical. In the present case, since azo-initiator (AMPA) and inhibitor (MPO) are both water soluble, the former is more likely. Fig. 1B shows the results of analogous experiments with the well-known water-soluble vitamin E analogue, Trolox C [19]. It is clear that the qualitative effect of MPO is similar to that of Trolox C.

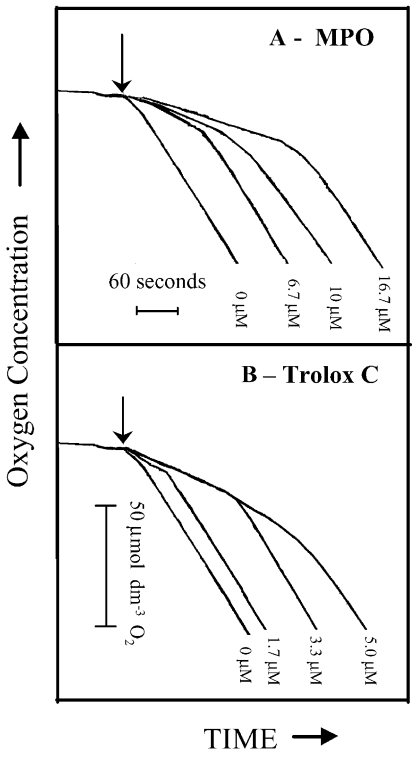


Fig. 1. Oxygen electrode traces showing oxygen consumption by solutions containing sodium linoleate (10 mmol dm^{-3}) and Triton X-100 (100 mmol dm^{-3}) at 40 °C. Oxidation was initiated by addition of AMPA (16 mmol dm^{-3}) at the point indicated by the arrow. The effects of MPO and Trolox C as inhibitors at the final concentrations indicated are shown in A and B, respectively. The same scales apply to both graphs.

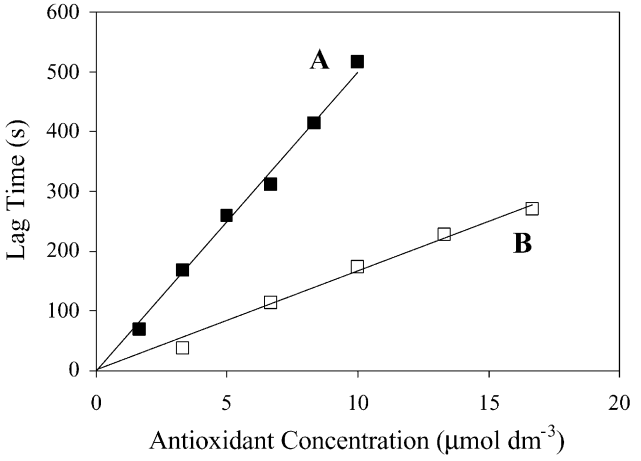


Fig. 2. The effects of Trolox C (A) and MPO (B) on the duration of the delay period in the oxidation of sodium linoleate in Triton X-100 micelles at 40 °C initiated by AMPA.

Fig. 2 shows that there is a linear relationship between lag time and concentration of inhibitor for both MPO and Trolox C. The slopes of 50.1 and 16.6 s dm^3 μmol^{-1} for Trolox C and MPO, respectively, indicate that MPO has an efficiency of about 30% of that of Trolox C in inhibiting linoleate oxidation. In contrast there was no observable lag period during AMPA-initiated oxidation of linoleate at pH 7.0 on addition of HPO at concentrations of up to 500 μmol dm^{-3}. It therefore appears that HPO has negligible reactivity with peroxyl radicals in this system.

One-electron oxidation of HPO and MPO studied by pulse radiolysis

The absolute reactivity of HPO and MPO with oxidising radicals was investigated by pulse radiolysis. The second order rate constant for reaction of the hydroxyl radical with HPO was determined by competition [20] with the thiocyanate anion. The absorbance values, A_0 and A, of the dithiocyanate radical anion [$(SCN)_2^{-\cdot}$] at 500 nm were measured immediately after the 500 ns radiation pulse in the absence and presence of HPO, respectively. The rate constant for reaction of ·OH with HPO (k_{HPO}) was evaluated by the equation:

$$\frac{A_0}{A} - 1 = \frac{k_{HPO}[HPO]}{k_{SCN}[SCN^-]}, \quad (1)$$

where the second order rate constant for reaction of ·OH with SCN^- (k_{SCN}) is 1.1×10^{10} dm^3 mol^{-1} s^{-1} [20]. Since HPO undergoes deprotonation with a pK_a of 6.0 [5,21], the rate constant was measured below and above the pK_a at pH values of 4.6 and 8.3, respectively. The slopes of the plots in Fig. 3, according to Eq. (1), give values of k_{HPO} reported in Table 1. At both pH values, k_{HPO} is effectively almost at the diffusion-controlled limit for reaction of a small solute with ·OH ($\sim 2 \times 10^{10}$ dm^3 mol^{-1} s^{-1}) and it is

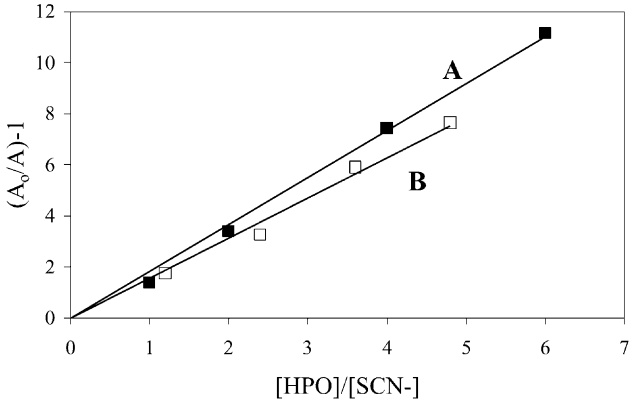


Fig. 3. Competition plots for $(SCN)_2^{-\cdot}$ radical absorption at 500 nm measured immediately (<1 μs) after pulse radiolysis of solutions of NaSCN (1.0 mmol dm^3) and HPO (0–6 mmol dm^{-3}) at pH 8.3 (A) and pH 4.6 (B).

Table 1
Second order rate constants[a] for reaction of ·OH and $(SCN)_2^{-\cdot}$ with HPO and MPO

		·OH	$(SCN)_2^{-\cdot}$
HPO	pH 4.6	$(1.57 \pm 0.05) \times 10^{10}$	$(1.5 \pm 0.6) \times 10^6$
	pH 8.3	$(2.02 \pm 0.04) \times 10^{10}$	$(1.9 \pm 0.1) \times 10^7$
MPO	pH 7	9×10^{9}[b]	$(1.1 \pm 0.1) \times 10^9$

[a] Units dm^3 mol^{-1} s^{-1}.
[b] From [5].

clear that deprotonation of HPO results in only a small measured increase in reactivity. The second order rate constant for reaction of ·OH with MPO has been reported by Aveline et al. [6] to be slightly lower at 9×10^9 dm^3 mol^{-1} s^{-1}, but in this case it appears to have been measured from the rate of formation of product absorbance. Since product radical absorbance might arise from transformations following the initial hydroxyl radical reaction, such measurements may underestimate the rate constant for hydroxyl radical reaction.

Such high reactivity must be considered when employing photo-Fenton reagents in vitro to produce hydroxyl radical-induced damage to a target molecule at low concentration, since scavenging of a substantial fraction of the hydroxyl radicals by the reagent itself is possible. For example, attempts have been made to demonstrate photo-induced initiation of linoleate oxidation by HPO but have been unsuccessful. The lack of success is attributed to scavenging of ·OH by HPO itself and also to interfering background oxidation of linoleate in micelles of both Triton X-100 and SDS induced by illumination at wavelengths between 300 and 330 nm. The use of such relatively short wavelength is required to match the absorption maximum of HPO at ~310 nm (pH 7). Further attempts to initiate photooxidation of polyunsaturated lipids by such reagents appear to require photo-Fenton reagents which have absorption spectra shifted to longer wavelength, and which are more hydrophobic so as to partition into the lipid phase and make reaction of hydroxyl radicals with lipid more effective. Reagents activated by illumination at longer wavelengths have been investigated [22], but tend to involve type I and II photochemical oxidations in addition to hydroxyl radical generation.

In the present case, measurement of peroxyl radical reactivity is difficult due to the weak absorbance of aliphatic peroxyl radicals in the near-UV and visible spectrum. In order to compare the rates of one-electron oxidation of HPO and MPO with a less powerful oxidising radical species than ·OH (one-electron reduction potential at pH 7, E'_0, 2.73 V vs. NHE [23]), the rate constants for reaction of the $(SCN)_2^{-\cdot}$ radical anion (E'_0, 1.33 mV vs. NHE [23]) were determined from the decay of its absorption at 480–500 nm. The inset to Fig. 4 shows good pseudo-first order kinetics for the reaction between

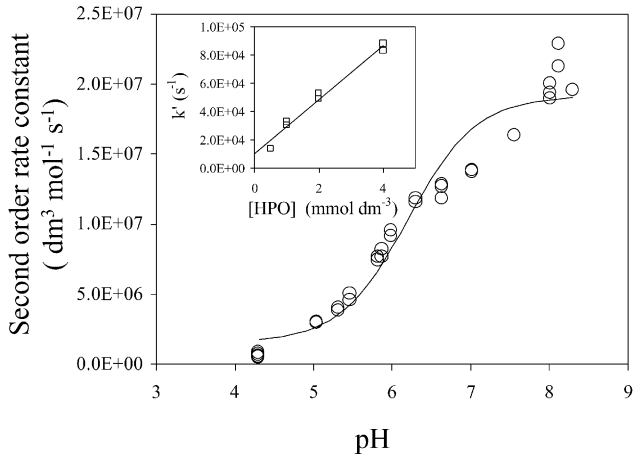
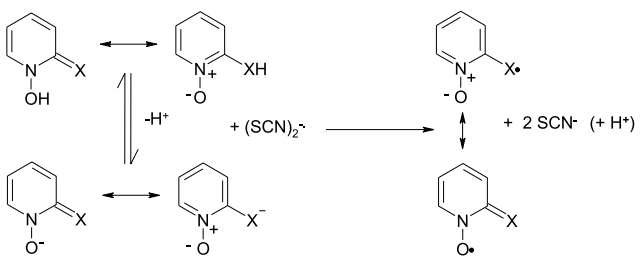


Fig. 4. Effect of pH on the second order rate constant for reaction of the $(SCN)_2^{\cdot}$ radical with HPO in solutions containing NaSCN (100 mmol dm^{-3}). Inset: Plot of pseudo-first order rate constant (k') for decay of the $(SCN)_2^{-\cdot}$ radical absorption at 500 nm vs. HPO concentration in solutions containing NaSCN (1.0 mmol dm^{-3}) at pH 8.3.

$(SCN)_2^{-\cdot}$ and HPO measured at pH 8.3. Determination of the second order rate constant over a range of pH values resulted in the curve shown in the main part of Fig. 4. Fitting the data to a single pK_a curve shows that the rate constant increases from 1.5×10^6 dm^3 mol^1 s^1 at pH < 4 to 1.9×10^7 dm^3 mol^{-1} s^{-1} at pH > 8. The same fit gives a pK_a value of 6.2 ± 0.1, in good agreement with the published pK_a value of 6.0 for deprotonation of HPO [5,21] from potentiometric and spectrophotometric measurements. The second order rate constant for reaction of $(SCN)_2^{-\cdot}$ with MPO was found to be $(1.1 \pm 0.1) \times 10^9$ dm^3 mol^{-1} s^{-1} at pH 6.8. Since MPO has been reported to have a pK_a of 4.67 [24], this was taken to reflect the reactivity of the anion at pH $\geqslant 6$. The results therefore show that the anion of MPO is oxidised by the $(SCN)_2^{-\cdot}$ radical approximately 50 times more rapidly than the anion of HPO. This reflects the difference in one-electron reduction potential of the resulting two radicals, formed as illustrated in Scheme 1. Tautomerism between enol/thiol and keto/thione forms for HPO/MPO has been reported, but the results indicate that in aqueous solution the keto/thione forms predominate [21,24]. It is concluded that the one-electron reduction potential of the MPO radical must be considerably lower than that of HPO as a result of the difference in electronegativities of the sulfur and oxygen atoms. For comparison, it may be noted that the one-electron reduction potentials (E^o ($C_6H_5X^{\cdot}/C_6H_5\ X^-$), X = O or S) of the phenoxyl and phenylthiol radicals are 0.79 and 0.69 V vs. NHE, respectively [25].



Scheme 1.

The reactivity of aliphatic peroxyl radicals is considerably lower than that of hydroxyl radical [26]. The greater ease of oxidation of the anion of MPO relative to that of HPO demonstrated for the dithiocyanate radical anion in the pulse radiolysis experiments predicts that MPO will have higher reactivity than HPO with alkyl (lipid) peroxyl radicals. This explains the difference in the abilities of MPO and HPO to inhibit the AMPA-initiated oxidation of linoleate described above. It is concluded that HPO is likely to be a superior photo-Fenton reagent for studies of the effects of hydroxyl radical in biological systems since it is less likely than MPO to be involved in subsequent repair-type reactions with damaged targets. It has already been shown that MPO also has the disadvantage of being a less selective source of hydroxyl radicals than HPO [6,27] and that MPO may also act as a photoreductant [2].

Acknowledgments

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