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Free hydroxyl radicals are formed on reaction between the neutrophil-derived species superoxide anion and hypochlorous acid

Luis P. Candeias*, Kantilal B. Patel, Michael R.L. Stratford, Peter Wardman

Cancer Research Campaign Gray Laboratory, PO Box 100, Mount Vernon Hospital, Northwood, Middlesex HA6 2JR, UK

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Superoxide anion reacts with hypochlorous acid to yield free hydroxyl radicals, as shown by the hydroxylation of benzoate. This reaction is analogous to the Haber–Weiss reaction but in the absence of metal ions is at least six orders of magnitude faster.

Hypochlorous acid; Superoxide anion; Hydroxyl radical; Radiolysis

1. INTRODUCTION

There is currently much interest in the chemistry of oxidative damage induced by activated neutrophils [1]. These cells release hypochlorous acid (HOCl) and the superoxide radical ($O_2^{\bullet -}$), and their cytotoxic effects may be related to the formation of hydroxyl radicals ($^{\bullet}OH$):

$$O_2^{\bullet -} + HOCl \rightarrow {}^{\bullet}OH + Cl^- + O_2 \quad (1)$$

in a reaction analogous to the Haber–Weiss reaction where H_2O_2 replaces HOCl:

$$O_2^{\bullet -} + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- + O_2 \quad (2)$$

The latter reaction has attracted enormous interest because of its possible involvement in ischaemia-reperfusion injury, inflammation and other responses to cellular oxidative stress [2,3]. However, reaction **(2)** occurs significantly only in the presence of metal ions such as iron and copper [3,4]. In contrast, reaction **(1)** is rapid [5], although direct evidence for the formation of *free* $^{\bullet}OH$ is lacking. In view of the role of activated neutrophils in diverse diseases, it is important to identify the damaging chemical species involved in the respiratory burst. We have used radiolysis to generate radicals and prove free $^{\bullet}OH$ is produced in reaction **(1)**.

2. MATERIALS AND METHODS

Stock solutions of hypochlorous acid (ca. 0.05 $mol \cdot dm^{-3}$) were prepared by diluting solutions of NaOCl (Aldrich) and adjusting the pH to 5.5 with concentrated perchloric acid (Merck). The concentration and purity of these solutions were checked by spectrophotometry, using the extinction coefficients of HOCl, $\varepsilon_{235\,nm} = 99.9\ dm^3 \cdot mol^{-1} \cdot cm^{-1}$ and $\varepsilon_{290\,nm} = 27.1\ dm^3 \cdot mol^{-1} \cdot cm^{-1}$ [6]. No changes of the absorption spectrum of the stock solutions (protected from light) were observed until 2 h after preparation. Other chemicals were from Aldrich, Merck or Sigma and of analytical grade. Solutions were prepared using water purified with a 'Milli-Q' system (Millipore) and saturated with oxygen, nitrous oxide (< 10 ppm O_2) or with a mixture of N_2O + O_2 20% (v/v), all purchased from BOC. All experiments were performed at room temperature (20 ± 2°C).

Steady-state γ-irradiations were performed using a ^{60}Co source with a nominal activity of 2,000 Ci. The samples were contained in 15 ml gas-tight vials and degassed for ≥ 30 min before irradiation. The dose rate was 32.9 $Gy \cdot min^{-1}$, determined by Fricke dosimetry [7]. The fluorescence of the samples was measured < 15 min after irradiation using a Perkin Elmer LS 50B luminescence spectrometer.

Irradiated samples were analyzed by HPLC using a Novapak C_{18} cartridge column (105 × 4 mm, Millipore) with detection by UV absorption (254 nm) and fluorescence (excitation 295 nm, emission 400 nm). Elution was achieved with a linear gradient (20% to 50% in 5 min.) of 75% acetonitrile in water against 0.02 $mol \cdot dm^{-3}$ H_3PO_4 and 0.02 $mol \cdot dm^{-3}$ KH_2PO_4.

3. RESULTS AND DISCUSSION

Radiolysis of water by ^{60}Co γ-rays generates radical, ionic and molecular species [8,9]:

$$H_2O \rightarrow {}^{\bullet}OH\ (0.28),\ e_{aq}^-\ (0.27),\ H^-\ (0.34),\ H^{\bullet}\ (0.06),\ H_2\ (0.05),\ H_2O_2\ (0.07) \quad (3)$$

where the numbers in brackets are the approximate yields in $\mu mol \cdot J^{-1}$. In the presence of N_2O the hydrated electrons are converted into $^{\bullet}OH$ (0.6 $\mu mol \cdot J^{-1}$):

$$N_2O + e_{aq}^- + H_2O \rightarrow {}^{\bullet}OH + N_2 + OH^- \quad (4)$$

As a probe for hydroxyl radicals [10] we used the hydroxylation of benzoate in aqueous solution to yield a mixture of 2-, 3- and 4-hydroxybenzoate [11], of which the 2- isomer (salicylate) is easily detected by fluorescence spectroscopy (λ_{exc} = 305 nm, λ_{em} = 410 nm).

*Corresponding author. Fax: (44) (923) 83 52 10.

Table I

Radiation chemical yields (nmol·J^{-1}) for formation of salicylate on irradiation of aqueous solutions of benzoate (5 mmol·dm^{-3}) at pH 5.0.

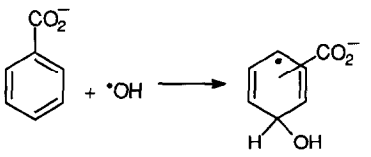
	Initial yield of •OH	No HOCl	HOCl 10^{-4} mol·dm^{-3}
N_2O	≈ 600	11.1 ± 0.2	21 ± 1
O_2	≈ 300	59.5 ± 0.2	388 ± 12
$N_2O + O_2$ (20% v/v)	≈ 600	93 ± 2	
Air + formate (0.1 mol·dm^{-3})	0	5.5 ± 0.2	

Upon irradiation of solutions containing benzoate, these became fluorescent due to the formation of salicylate in concentration proportional to the radiation dose (Fig. 1). In N_2O-saturated solution the yield of salicylate was small (Table I) but it increased ca. 5-fold in O_2-saturated solution. However, in the latter case the yield of •OH is half that in N_2O-saturated solution, because e^-_{aq} reacts with O_2 rather than with N_2O:

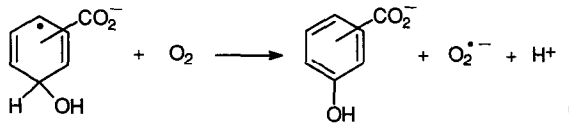
$$e^-_{aq} + O_2 \rightarrow O_2^{\bullet -} \quad \textbf{(5)}$$

Superoxide does not cause the conversion of benzoate to salicylate; in aerated solution of benzoate containing 0.1 mol·dm^{-3} sodium formate (yield of $O_2^{\bullet -}$ 0.6 μmol·J^{-1}) there was negligible formation of salicylate. The increase of the yield of salicylate in oxygen-saturated solution arises because •OH adds to the aromatic ring of benzoate to give the hydroxycyclohexadienyl radical, followed by *oxidation* and deprotonation (Eq. **(6)**) [12]. Therefore, the presence of oxidants increases the yield of hydroxylation by increasing the efficiency of the oxidation step [11].

A similar mechanism can account in part for the increased yields of salicylate observed in the presence of HOCl, as shown by the results with the N_2O-saturated



(6a)



(6b)

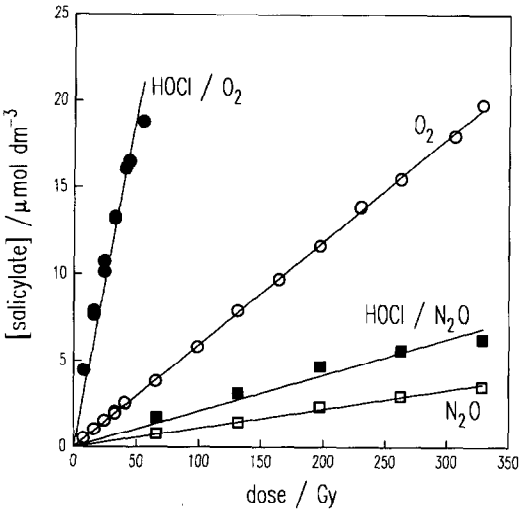


Fig. 1. Increase of the concentration of salicylate on γ-irradiation of aqueous solutions of benzoate (5 mmol·dm^{-3}) saturated with N_2O (squares) or O_2 (circles) in the presence of 1.0×10^{-4} mol·dm^{-3} HOCl (solid symbols) or in its absence (open symbols).

solutions (Table I), although HOCl is a less efficient oxidant than oxygen.

The effect of HOCl is much more pronounced in O_2-saturated solution: the yield of salicylate increases >6-fold compared to the O_2-saturated solution without HOCl. Under these conditions the concentration of oxygen is sufficient to convert all the e^-_{aq} to superoxide anion (Eq. **(5)**). The large increase of the yield of salicylate implies that superoxide reacts with HOCl to give a species that causes the hydroxylation of benzoate which we show to be the hydroxyl radical (Eq. **(1)**). However, this reaction alone would cause the yield of salicylate to double. We attribute the ca. 6-fold increase to the reaction of oxygen with the hydroxycyclohexadienyl radicals generating $O_2^{\bullet -}$ (Eq. **(6b)**) [13], which in turn reacts with HOCl, inducing a small chain reaction (chain length ca. 3).

In order to confirm that the reactive species generated by reaction of $O_2^{\bullet -}$ with HOCl is the free hydroxyl radical, we analyzed the HPLC the irradiated benzoate solution saturated with oxygen and containing HOCl. The resulting chromatogram (Fig. 2) shows the peaks of the three isomeric hydroxylated derivatives of benzoate (identified using appropriate standards). For comparison, Fig. 2 also shows the isomer distribution resulting from the action of *free* •OH radicals obtained by irradiation of a benzoate solution saturated with N_2O (and 20% v/v O_2 in order to ensure complete conversion of the hydroxycyclohexadienyl radicals to hydroxylated products). The relative areas of the peaks show that the same distribution of isomers is obtained in both cases. This is conclusive evidence for the increased formation of •OH in the presence of oxygen and HOCl, i.e. for the formation of •OH on reaction of $O_2^{\bullet -}$ with HOCl.

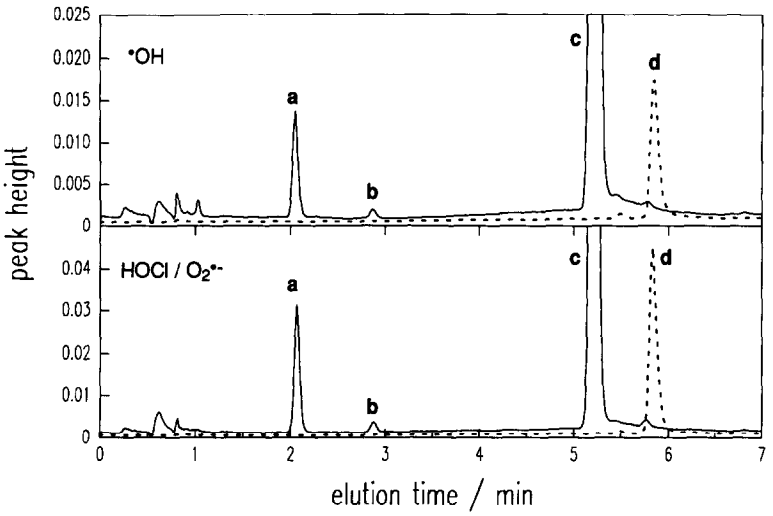


Fig. 2. HPLC elution pattern showing that hydroxyl radicals (•OH) or superoxide in the presence of hypochlorous acid ($O_2^{\bullet-}$/HOCl) induce the formation of the same derivatives of benzoate and in the same relative yields. Peak identification: (a) 4-hydroxybenzoate; (b) 3-hydroxybenzoate; (c) benzoate; (d) 2-hydroxybenzoate (salicylate). The solid and dotted lines were obtained by absorption (254 nm) or fluorescence (λ_{exc} = 295 nm, λ_{em} = 400 nm) detection, respectively.

The rate constant for the reaction between superoxide and HOCl had previously been determined ($k = 7.5 \times 10^6$ $dm^3 \cdot mol^{-1} \cdot s^{-1}$) by monitoring the decay of the absorption of $O_2^{\bullet-}$ in the presence of HOCl; it was suggested that singlet molecular oxygen could be formed in this reaction [5]. Singlet oxygen is able to hydroxylate aromatic compounds, but its regioselectivity, i.e. the distribution isomeric products formed is distinct from that of •OH [14,15]. The formation of •OH in reaction **(1)** can also account for the reported hydroxylation of phenol by a combination of the enzymatic systems, xanthine/xanthine oxidase and myeloperoxidase/H_2O_2/Cl^- [16]. Furthermore, the production of •OH by neutrophils by a mechanism dependent on myeloperoxidase and not involving metal ions has been inferred from spin trapping of the $^{\bullet}CH(CH_3)OH$ formed in the presence of ethanol [17]. Hydroxylation of benzoate, generating a characteristic spectrum of products (essentially a *fingerprint*) now provides unequivocal evidence for the generation of free •OH in reaction **(1)**.

The interaction of HOCl with $O_2^{\bullet-}$ to yield hydroxyl radicals is potentially important in the biological action of neutrophils. It presents an alternative pathway for the toxicity of HOCl, in addition to the well established formation of chloramines and inactivation of antiprotease [18]. It is also a mechanism by which, through the release of HOCl, neutrophils can transform the unreactive $O_2^{\bullet-}$ into the highly reactive •OH. In the absence of metal ions, reaction **(1)** is at least six orders of magnitude faster than the Haber–Weiss reaction [5,19].

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REFERENCES

[1] Jesaitis, A.J. and Dratz, E.A., eds. (1992) The Molecular Basis of Oxidative Damage by Leukocytes, CRC Press, Boca Raton.
[2] Sies, H., Ed. (1991) Oxidative Stress, Academic Press, London.
[3] Halliwell, B. and Gutteridge, J.M.C. (1989) Free Radicals in Biology and Medicine, Clarendon Press, Oxford.
[4] Winterbourn, C.C. (1993) Free Rad. Biol. Med. 14, 85–90.
[5] Long, C.A. and Bielski, B.H.J. (1980) J. Phys. Chem. 84, 555–557.
[6] Morris, J.C. (1966) J. Phys. Chem. 70, 3798–3805.
[7] Tabata, Y., Ito, Y. and Tagawa, S. Eds. (1991) Handbook of Radiation Chemistry, CRC Press, Boca Raton.
[8] Buxton, G.V. (1987) in: Radiation Chemistry (Farhataziz and Rodgers, M.A.J. Eds.) pp. 321–349, VCH, New York.
[9] von Sonntag, C. (1987) The Chemical Basis of Radiation Biology, Taylor & Francis, London.
[10] Halliwell, B., Grootveld, M. and Gutteridge, J.M.C. (1989) Methods Biochem. Anal. 33, 59–90.
[11] Klein, G.W., Bhatia, K., Madhavan, V. and Schuler, R.H. (1975) J. Phys. Chem. 79, 1767–1774.
[12] Bhatia, K. and Schuler, R.H. (1974) J. Phys. Chem. 78, 2335–2338.
[13] Howard, J.A. and Ingold, K.U. (1967) Can. J. Chem. 45, 785.
[14] Briviba, K., Devasagayam, T.P.A., Sies, H. and Steenken, S. (1993) Chem. Res. Toxicol. 6, 548–553.
[15] Kalyanaraman, B., Ramanujam, S., Singh, R.J., Joseph, J. and Feix, J.B. (1993) J. Am. Chem. Soc. 115, 4007–4012.
[16] Subrahmanyam, V.V., Kolachana, P. and Smith, M.T. (1991) Free Rad. Res. Comm. 15, 285–296.
[17] Ramos, C.L., Pou, S., Britigan, B.E., Cohen, M.S. and Rosen, G.M. (1992) J. Biol. Chem. 267, 8307–8312.
[18] Test, S.T. and Weiss, S.J. (1986) Free Rad. Biol. Med. 2, 91–116.
[19] Ross, A.B., Mallard, W.G., Helman, W.P., Bielski, B.H.J. and Buxton, G.V. (1992) NDRL-NIST Solution Kinetics Database – ver. 1, NIST Standard Reference Data, GaitheCrsburg.