Free Rad. Res. Comms., Vol. 4, No. 2, pp. 115–123
Photocopying permitted by license only

Printed in Great Britain

STUDIES ON THE ORIGIN OF THE HYDROXYL SPIN ADDUCT OF DMPO PRODUCED FROM THE STIMULATION OF NEUTROPHILS BY PHORBOL-12-MYRISTATE-13-ACETATE

EDWARD G. JANZEN§, LINDA T. JANDRISITS and DWAYNE L. BARBER‡

Department of Chemistry and Biochemistry, Guelph-Waterloo Centre for Graduate Work in Chemistry, University of Guelph, Guelph, Ontario N1G 2W1, Canada

(*Received February 5, 1987*)

The spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), is commonly used for the detection of short-lived free radicals produced by neutrophils during their respiratory burst. The spin adducts of both the superoxide ion ($O_2^{\cdot-}$) and the hydroxyl radical ($\cdot OH$) are detectable during this process. Since myeloperoxidase (MPO), which is also active during the respiratory burst, produces hypochlorous acid (HOCl) in the presence of chloride ions (Cl^-) and hydrogen peroxide (H_2O_2), this species has been investigated as a possible source of the DMPO-OH adduct. At concentrations of hypochlorous acid between 0.1 and 0.7 μmol/ml the DMPO-OH spin adduct is detected using electron spin resonance (ESR) techniques. Two possible mechanisms for the formation of this adduct are proposed. These findings suggest that the product of MPO, namely hypochlorous acid, is a possible source of the hydroxyl spin adduct detected during the respiratory burst.

KEY WORDS: Electron Spin Resonance, Myeloperoxidase, Phorbol-12-Myristate-13-Acetate, Neutrophils and DMPO (5,5-dimethyl-1-pyrroline-N-oxide).

INTRODUCTION

Neutrophils are part of our immune defense system in the blood. When stimulated they show an increased rate of oxygen consumption which is believed to be coupled with the production of a number of reactive microbicidal agents, including superoxide,[1] hydrogen peroxide[2] and the hydroxyl radical.[3] The series of metabolic events which lead to the production of these partially reduced species of oxygen is called the "respiratory burst". The dismutases, superoxidase dismutase[4] (SOD) and catalase, convert these damaging agents to less harmful species.

$$O_2^{\cdot-} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \quad (1)$$

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2 \quad (2)$$

The hexose monophosphate shunt, which produces NADPH from $NADP^+$, is also active during the respiratory burst.[5] It is believed that NADPH causes the reduction of oxygen to superoxide:

§Correspondence to Dr. E.G. Janzen, Department of Chemistry and Biochemistry, University of Guelph, Guelph, Ontario, Canada, N1G 2W1
‡Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada

$$NADPH + 2O_2 \longrightarrow NADP^+ + H^+ + 2O_2^- \quad (3)$$

In a model system of NADPH and NADPH oxidase the superoxide adduct has been spin trapped.[6]

The hydroxyl radical is believed to be derived from a modified Haber-Weiss reaction, where iron acts as a catalyst:

$$O_2^- + Fe^{+3} \longrightarrow O_2 + Fe^{2+} \quad (4)$$

$$Fe^{2+} + H_2O_2 \longrightarrow HO^{\cdot} + HO^- + Fe^{+3} \quad (5)$$

$$O_2^- + H_2O_2 \longrightarrow O_2 + HO^{\cdot} + HO^- \quad (6)$$

Without some form of catalysis the Haber–Weiss reaction alone is believed to be too slow to produce a significant amount of hydroxyl radicals.[7]

Both the superoxide and the hydroxyl radical spin adducts have been detected during the respiratory burst when DMPO was used as a spin trap.[8] The amount of each spin adduct detected varies depending on the type and amount of stimulant used and the amount of iron present.

Stimulants of the respiratory burst include IgG coated latex particles, a zymosan/serum system, preopsonized zymosan, phorbol-12-myristate-13-acetate (PMA) and arachidonic acid.[9–12] Particulate stimulants, latex particles and zymosan, initiate the respiratory burst after they have been engulfed by the neutrophil in the process known as phagocytosis. Usually the hydroxyl spin adduct is detected,[9,10,12] however when an excess of stimulant is used both the hydroxyl and superoxide adducts are detected.[13] PMA and arachidonic acid are chemical membrane perturbing agents which initiate the respiratory burst without the process of phagocytosis occurring. When PMA was used as a stimulant both the hydroxyl and superoxide spin adducts were produced.[11,12] However, when DETAPAC (diethylenetriaminepentaacetic acid) was used to chelate divalent metals which cause dismutation, only the superoxide adduct was detected.[13]

It should be noted that DETAPAC lacks specificity for iron alone and also chelates other divalent ions.[14a,14b] This in turn could disrupt enzyme systems which require divalent metal ions for their activity. One of these systems is MPO, a green heme containing enzyme found in the primary granules of neutrophils. During the respiratory burst it is either transported to the phagocytic vacuoles or released extracellularly by the process of degranulation.[15–17] MPO catalyzes the conversion of hydrogen peroxide and a halide ion to water and the corresponding hypohalite ion. Since chloride is the most abundant halide found in biological systems, hypochlorite (OCl^-) is believed to be the hypohalite produced:

$$H_2O_2 + Cl^- \xrightarrow[MPO]{} OCl^- + H_2O \quad (7)$$

The iodination reaction is used to measure the ability of MPO to convert radioactive iodide to a trichloroacetic acid (TCA) protein bound precipitate.[15,17] Using this technique it was found that both Ca^{2+} and Mg^{2+} were required for enzymatic activity and that divalent ion chelators, such as EDTA (ethylenediaminetriacetic acid), effectively block MPO activity.[17]

The products of active MPO, hypochlorous acid and chlorine, have been detected using spectrophotometric techniques.[18] It has also been shown that hypochlorous acid has the same effect on various substrates as a chloride/hydrogen peroxide/MPO system.[19] Because stimulated neutrophils have increased MPO activity, it has been

postulated that hypochlorous acid may be a key microbicidal agent in addition to the superoxide ion and the hydroxyl radical.[1,17]

It has also been proposed that MPO could act as the catalyst in the Haber–Weiss reaction.[20]

$$H_2O_2 + Cl \longrightarrow HOCl + HO^- \quad (8)$$

$$HOCl + O_2^- \longrightarrow HO\cdot + O_2 + Cl^- \quad (9)$$

$$H_2O_2 + O_2^- \longrightarrow HO^- + HO\cdot + O_2 \quad (10)$$

If MPO plays a role in the respiratory burst the effect of hypochlorous acid on spin trapping experiments needs to be examined to see what effect this compound has on the spin trapping chemistry of the system.

EXPERIMENTAL

Solutions of 5% sodium hypochlorite (NaOCl) are available from Aldrich. DMPO (5,5-dimethyl-1-pyrroline-N-oxide) was available in these laboratories.[21] Samples of 100 μl of 0.2 M DMPO and various concentrations of NaOCl (final concentrations were between 0.071 μmol/ml and 0.714 μmol/ml) were added to a mixing cell[22] and brought to a final volume of 1.4 ml using 0.1 M phosphate buffer solution (pH = 7.4). For experiments partially under nitrogen, the solutions were purged with nitrogen gas for 5 to 7 minutes before they were mixed and shaken down into a flat cell. Electron spin resonance (ESR) spectra were recorded over time using a Varian E-104 X-band EPR spectrometer. When signals were very weak the spectra were accumulated and computer averaged on a Bruker ER 200D X-band spectrometer. Typically the modulation amplitude was 1.0 G and the microwave power was 20 mW.

Neutrophils were isolated according the procedure of Babior and coworkers.[23] A 50 μl sample (4×10^7 cells/ml) of neutrophils from mature dogs suspended in 0.1 M PBS was treated with 50 μl of 10 μg/ml PMA in a solution containing 25 μl 0.1 M DMPO and 50 μl Krebs Ringer phosphate buffer with 2×10^{-3} M glucose. Signals were recorded on a Bruker ER 200D X-band spectrometer.

RESULTS

A solution partially degassed with nitrogen and containing sodium hypochlorite at 0.714 μmol/ml and 14.3 mM DMPO gave the ESR spectrum shown in Figure 1(a). The pattern obtained is characteristic of the DMPO-OH adduct. At lower concentrations, the ESR signal is less intense (Figure 1(b)) and at concentrations as low as 0.071 μmol/ml NaOCl no spectrum is observed (Figure 1(c)).

When dog neutrophils are stimulated with PMA in solutions containing 14.3 mM DMPO, a similar spectrum is obtained (Figure 2(a)) which increases in intensity with time (Figure 2(b)). A maximum is reached at 15 to 20 minutes and eventually the signal decreases to undetectable levels.

With sodium hypochlorite solutions exposed to air, weak ESR signals are obtained at concentrations of 0.714 μmol/ml (Figure 3(a)). Accumulated spectra show the signal to be that of the DMPO-OH adduct (Figure 3(b)).

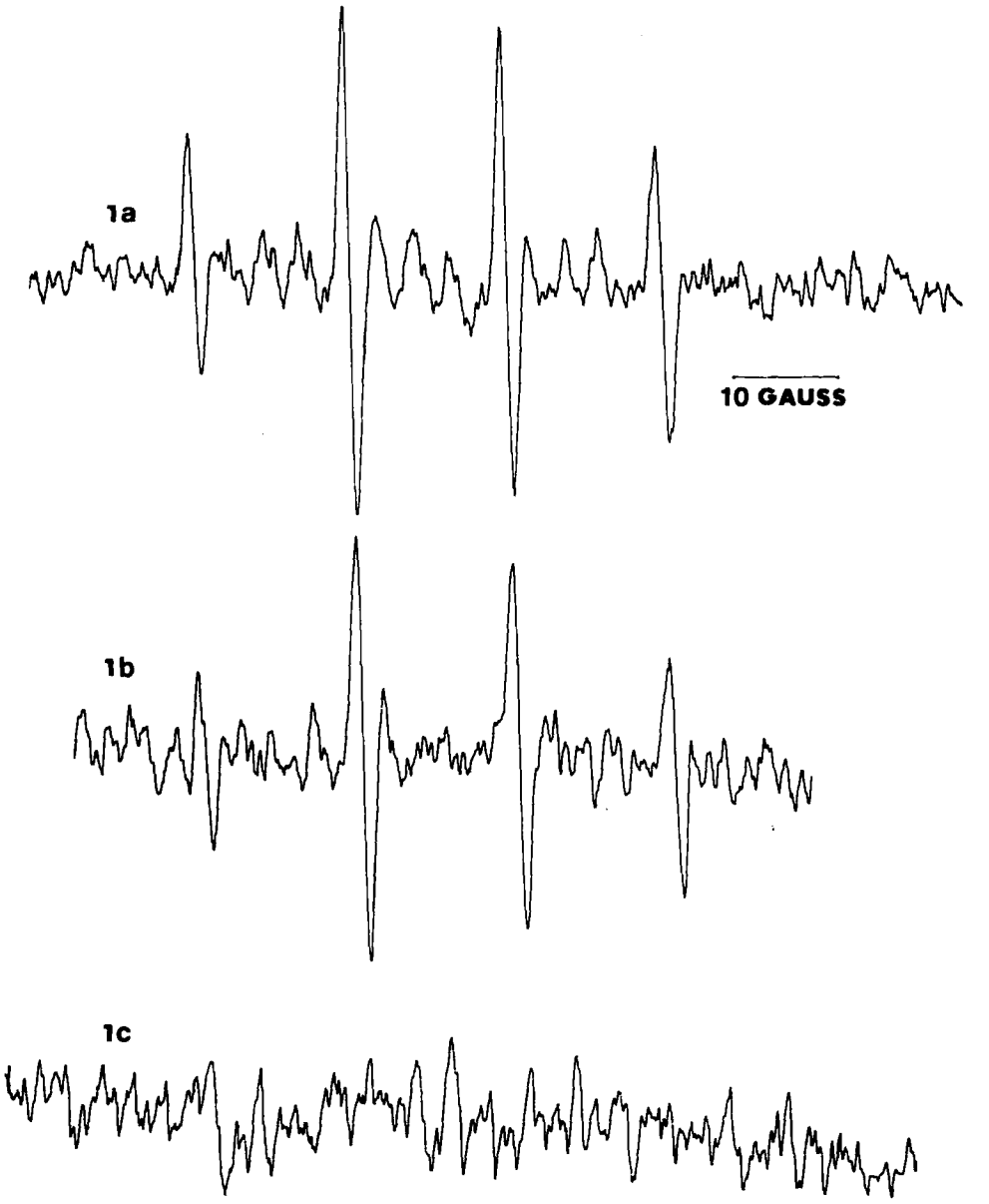


FIGURE 1 ESR spectra obtained from solutions purged for 5 minutes with nitrogen before mixing prepared from 100 μl of 0.2 M DMPO, 20 μl diluted HOCl and 1.38 mls of 0.1 M phosphate buffer (pH = 7.4). (a) Final concentration 0.714 μM HOCl 17 minutes after mixing. (b) Final concentration 0.357 μM HOCl 24 minutes after mixing. (c) Final concentration 0.071 μM HOCl 20 minutes after mixing.

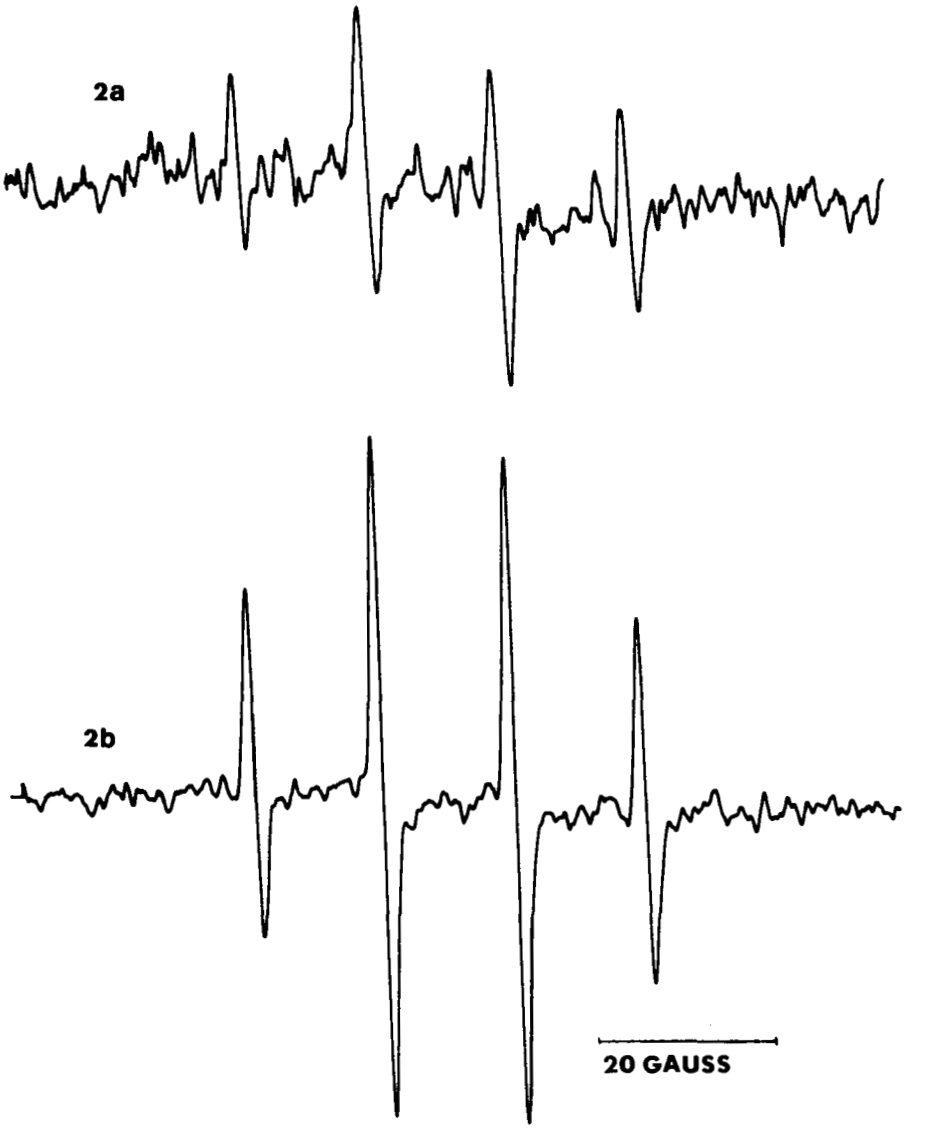


FIGURE 2 ESR spectra obtained from 50 μM of a 4 $\times$ 10^7 cells/ml solution of neutrophils stimulated with 50 μl of 10 μl/ml PMA in the presence of 25 μl 0.1 M DMPO and 50 μl KRPG. (a) Spectrum obtained 4.5 minutes after addition of PMA (gain in (a) is twice gain in (b)). (b) Spectrum obtained 25 minutes after addition of PMA.

At higher concentrations of hypochlorite, either in air or under partial degassing with nitrogen, the ESR spectra change to patterns which appear to be due to oxidation products of DMPO or DMPO spin adducts (see Figure 3(b) for some evidence of such lines). Although these spectra have not been found to date in spin

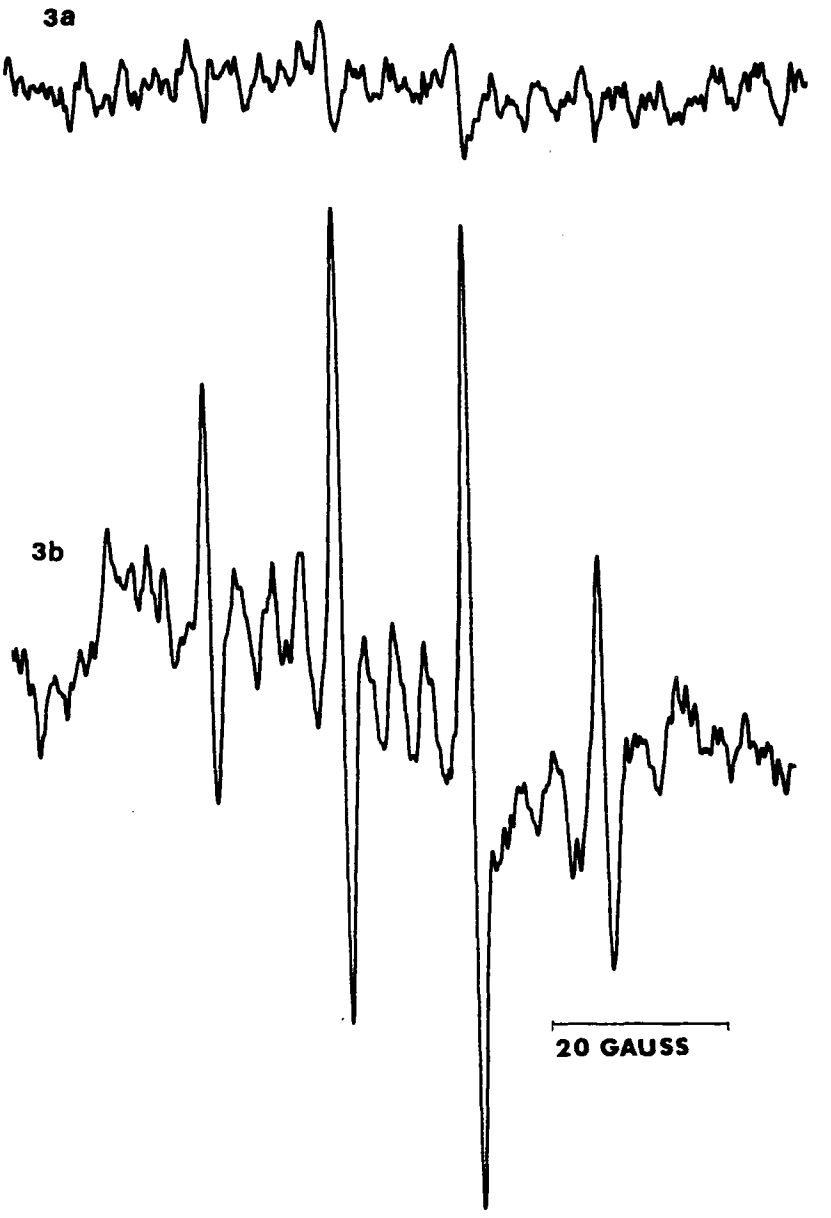


FIGURE 3 ESR spectra of a mixture of 0.714 μM HOCl and 100 μl 0.2 M DMPO in 1.38 mls 0.1 M phosphate buffer (pH = 7.4) exposed to air. (a) One scan taken 20 minutes after mixing. (b) Thirty accumulated scans.

trapping experiments with neutrophils, further work will be needed to identify these species in the chemical systems.

DISCUSSION

Since these results show that sodium hypochlorite in solution produces the hydroxyl adduct of DMPO it follows that the hypochlorous acid believed to be produced by MPO in neutrophils could also give the DMPO-OH spin adduct. Since the divalent ions Ca^{2+} and Mg^{2+} are required for MPO activity, chelating agents such as DETAPAC could block the formation of hypochlorous acid and hence prevent the formation of the DMPO-OH adduct derived from this source.[13]

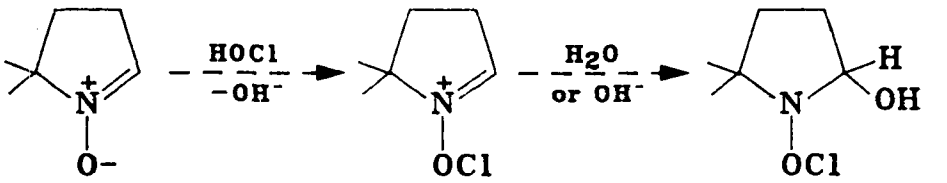
It has been proposed that hydroxyl radicals can be detected using α-keto-δ-methiol butyric acid (KMBA), since in the presence of hydroxyl radicals KMBA produces ethylene. By measuring the formation of ethylene formed from KMBA in the presence of neutrophils, it was found that the addition of myeloperoxidase *increased* the amount of ethylene produced.[24] While these results suggest that MPO produces hydroxyl radicals, spin trapping experiments with neutrophils have shown that the addition of myeloperoxidase actually *decreases* the strength of the DMPO-OH spin adduct signal.[9]

Our results provide an explanation which involves hypochlorous acid and the role it plays in spin trapping experiments. If hypochlorous acid is causing the decomposition of the spin adduct as well as its formation then its addition to spin trapping systems could lead to less intense signals being observed. Clearly this is a very complex system since the DMPO spin adduct now is becoming a transient radical itself i.e. the lifetime of the spin adduct and thus its intensity depends critically on two rates: the rate of formation of the DMPO-OH adduct, and the rate of its decay:

$$\text{DMPO} \longrightarrow \text{DMPO-OH} \longrightarrow \text{Decay Product} \qquad (11)$$

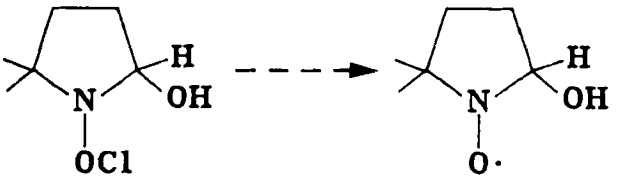
However it should be noted that not all available information fits this simple scheme since it has been shown that the addition of inhibitors to myleperoxidase such as sodium azide cause an increase in the amount of hydroxyl adduct detected.[12]

The mechanism of the reaction of hypochlorous acid with DMPO is unknown but we assume similar reactions to those involved in chlorohydrin formation from olefins.[25] Since the first step in the reaction with a carbon–carbon double bond is the addition of the chloronium ion (Cl^+) to the more electronegative carbon, the analogous addition to nitrones would involve addition at the nitronyl oxygen. This addition product should react rapidly with water:



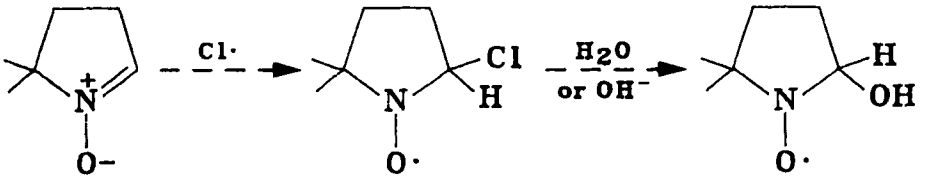
(12)

Spontaneous loss of a chlorine atom is expected to produce DMPO-OH.



(13)

The chlorine atom should also be trapped by DMPO but this adduct has never been detected.[26] Since the hydrolysis of β-chloroalkyl radicals is known to be fast,[27] rapid hydrolysis of the DMPO-Cl adduct would explain its absence and the formation of the DMPO-OH adduct in its stead.



(14)

It should be noted that hypochlorous acid and chloride ions are in equilibrium with dichlorine in aqueous solution and in the presence of light chlorine atoms could also be produced from photolysis of dichlorine.[28]

$$HOCl + Cl^- + H^+ \rightleftharpoons H_2O + Cl_2 \qquad (15)$$

In conclusion we propose that myeloperoxidase through its product, hypochlorous acid, may in part be a source of the hydroxyl spin adduct of DMPO. The implications of this statement are being further explored in these laboratories.[29]

Acknowledgements

We thankfully acknowledge support from the Natural Sciences and Engineering Research Council of Canada. Help in initiating this project is gratefully acknowledged: Prof. F.W. Kleinhans, Department of Medical Research, Methodist Hospital of Indiana and Department of Physics, Indiana University-Purdue, University at Indianapolis, for kindling our interest and Dr. S.T. Barefoot for teaching us methodology; Professors Wood and Mellors of this department for use of equipment and biochemicals; Ms. Y. Koggel, Animal Care Service, Ontario Veterinary College for providing dog blood samples.

References

1. B.M. Babior, R.S. Kipnes and J.T. Curnutte, *J. Clin. Invest.*, **52**, 741, (1973).
2. R.K. Root and J.A. Metcalf, *J. Clin. Invest.*, **60**, 1266, (1977).
3. A.I. Tauber and B.M. Babior, *J. Clin. Invest.*, **60**, 374, (1977).
4. J.M. McCord and I. Fridovich, *J. Biol. Chem.*, **244**, 6049, (1969).
5. G.Y.N. Iyer, M.F. Islam and J.H. Quastel, *Nature*, **192**, 535, (1961).
6. J.V. Bannister, P. Bellavite, M.C. Serra, P.J. Thornalley and F. Rossi, *FEBS Lett.*, **145**, 323, (1982).
7. J.M. McCord and E.D. Day, *FEBS Lett.*, **86**, 139, (1978).
8. J.V. Bannister and W.H. Bannister, *Environ. Health. Perspec.*, **64**, 37–43 (1985). Janzen, E.G., in Free Radicals in Biology, Editor: W.A. Pyror, Academic Press Inc., **4**, 115, (1980).
9. H. Rosen and S.J. Klebanoff, *J. Clin. Invest.*, **64**, 1725, (1979).
10. D.A. Hawley, F.W. Kleinhans and J.L. Biesecker, *Am. J. Clin. Path.*, **79**, 673, (1983).

11. K. Cheung, J. Lark, M.F. Robinson, P.J. Pomery and S. Hunter, *Aust. J. Exp. Biol. Med. Sci.*, **64**, 157, (1986).
12. M.R. Green, H.A.O. Hill, M.J. Okolow-Zukowska and A.W. Segal, *FEBS Lett.*, **100**, 23, (1979).
13. B.E. Britigan, G.M. Rosen, Y. Chai and M.S. Cohen, *J. Biol. Chem.*, **261**, 4426, (1986).
14a. D.D. Perrin, *Stability Constants of Metal Ion Complexes, Part B: Organic Ligands (IUPAC Chemical data Series No. 22)* Pergamon Press Toronto, 996, (1979).
14b. J.J. Christensen and R.M. Izatt, *Handbook of Metal Ligand Heats and Related Thermodynamic Quantities*, Third Edition, Marcel Dekker Inc. New York, 393, (1983).
15. J.A. Roth and M.L. Kaeberle, *Vet. Immun. Immunopath.*, **2**, 157, (1981).
16. S.J. Klebanoff and C.B. Hamon, *J. Reticuloendothel. Soc.*, **12**, 170, (1972).
17. S.J. Klebanoff and R.A. Clark, *J. Lab. Clin. Med.*, **89**, 675, (1977).
18. J.E. Harrison and J. Schultz, *J. Biol. Chem.*, **251**, 1371, (1976).
19. K. Agner, in: *Structure and Function of Oxidation-Reduction Enzymes*, Proceedings of the Wenner-Gren symposium (1970), Editors: Å. Åkeson and A. Ehrenberg, Pergamon Press, Toronto, **18**, 329, (1972).
20. H.A.O. Hill and M.J. Okolow-Zubkowska, in: *Oxygen and Life*, Special Publication Royal Soc. of Chem., London 98, (1981).
21. D.L. Haire, J.W. Hilborn and E.G. Janzen, *J. Org. Chem.*, **51**, 4298, (1986).
22. G.A. Russell, E.G. Janzen and E.T. Strom, *J. Am. Chem. Soc*, **86**, 1807, (1964).
23. M. Markert, P.C. Andrews and B.M. Babior, *Meth. Enzym.*, **105**, 358, (1984).
24. K. Takanaka and P.J. O'Brien, *FEBS Lett.*, **110**, 283, (1980); However it has recently been shown that KMBA reacts with HOCl to give ethylene (Z. Adams, A. Borochov and S. Mayak, *Free Rad. Res. Comms.* **2**, 137, (1986).
25. L.S. Boguslavskaya, *Russ. Chem. Rev.*, **41**, 740, (1972).
26. D. Rehorek, C.M. DuBose and E.G. Janzen, *Z. Chem.*, **24**, 188, (1984).
27. G. Koltzenburg, G. Behrens and D. Schulte-Frohlinde, *J. Am. Chem. Soc.*, **104**, 7311, (1982).
28. M. Eigen and K. Kustin, *J. Am. Chem. Soc.*, **84**, 1355, (1962) and R.E. Connick and Y.T. Chia, *J. Am. Chem. Soc.*, **81**, 1280, (1959).
29. Some of these results were reported in part at the 28th Rocky Mountain Conference, Denver, Colorado, Aug. 3–7, (1986).

Accepted by Dr. J.V. Bannister