# The First ESR Spin-trapping Evidence for the Formation of Hydroxyl Radical from the Reaction of Copper(II) Complex with Hydrogen Peroxide in Aqueous Solution

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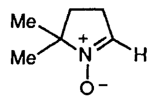
The formation of hydroxyl radical (OH·) during the reaction of copper(II) ion with hydrogen peroxide ($H_2O_2$) is determined by ESR spectroscopy using water-soluble spin-traps, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO, **1**), α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN, **2**) and sodium 3,5-dibromo-4-nitrosobenzenesulphonate (DBNBS, **3**).

It has been reported that hydroxyl radical plays a major role in the indirect action of radiation on cells. Hydroxyl radicals are produced when water is exposed to high-energy ionizing radiation. It is a highly active radical and will react with virtually any chosen molecule.

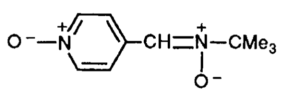
Most of the hydroxyl radical generated *in vivo* is derived from the metal ion-dependent breakdown of hydrogen peroxide ($H_2O_2$) according to the general eqn. (1), where $M^{n+}$ is an unidentified endogenous metal ion, such as $Fe^{II}$, $Cu^{I}$, $Ti^{III}$ or $V^{IV}$. Realistically only $Fe^{II}$ and $Cu^{I}$ occur *in vivo*, and then only under abnormal physiological conditions.[1]

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + OH\cdot + OH^- \qquad (1)$$

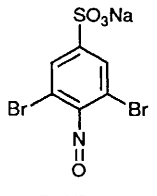
Great attention has been given to the iron decomposition of $H_2O_2$. It has been suggested, however, that the rate constant for the reaction of $Cu^+$ with $H_2O_2$ is several orders of magnitude greater than that for $Fe^{2+}$.[2] Simpson *et al.* have detected hydroxyl radical generated from the $Cu^{2+}$–$H_2O_2$ system, by lucigenin-amplified chemiluminescence, deoxyribose degradation, and benzoate hydroxylation.[3] Further, it has been shown that $Cu^{2+}$ ion can reduce $H_2O_2$ to generate hydroxyl radical capable of degrading deoxyribose with the formation of thiobarbituric acid (TBA)-reactive products.[4] We have previously shown that the copper(II) complex of ethylenediamine (en), $Cu^{II}(en)_2$, has the highest activity towards $H_2O_2$ that yields hydroxyl radical by use of the TBA methods.[5] This is the first ESR study, however, on the formation of hydroxyl radical from the reaction of $Cu^{2+}$ ion with $H_2O_2$. In order to detect the radicals formed by the reaction of $Cu^{2+}$ ion with $H_2O_2$, the spin-trapping experiments using water-soluble spin traps, DMPO **1**, POBN **2** and DBNBS **3**, were undertaken.



DMPO, **1**

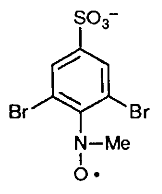


POBN, **2**



DBNBS, **3**

The ESR measurements were carried out on a JEOL-RE-1X spectrometer (X-band) with 100 kHz field modulation. The hyperfine coupling constants and *g*-factors were calib-



**4**

**Fig. 1** ESR spectrum of DMPO–OH adduct generated from $Cu^{II}(en)_2$–$H_2O_2$ system in the presence of DMPO at pH 7.4. *Reaction conditions*: $Cu^{II}$ $(en)_2$, 0.001 mol $dm^{-3}$; $H_2O_2$, 0.1 mol $dm^{-3}$; DMPO, 0.05 mol $dm^{-3}$.

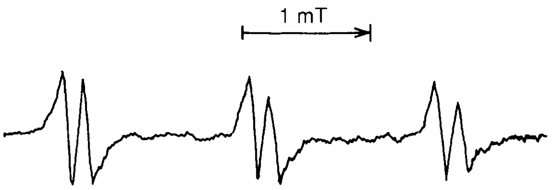


**Fig. 2** ESR spectrum of POBN–OH adduct generated from $Cu^{II}$-$(en)_2$–$H_2O_2$ system in the presence of POBN at pH 7.4. *Reaction conditions*: $Cu^{II}$ $(en)_2$, 0.001 mol $dm^{-3}$; $H_2O_2$, 0.1 mol $dm^{-3}$; POBN, 0.05 mol $dm^{-3}$.

rated by comparison with DPPH ($g = 2.0036$) and a standard sample of $Mn^{2+}/MgO$. $Cu^{II}(en)_2 \cdot ClO_4$ was synthesized as described previously.[5] Since the stability constant of $Cu^{II}(en)_2$ is 10.76 and 9.37 at 25 °C,[6] main structure of copper complex with en may be $Cu^{II}(en)_2$ in aqueous solutions. Spin-traps, DMPO and POBN, were purchased from Sigma Chemical Co. DBNBS was prepared from 3,5-dibromosulphanilic acid by oxidation with $H_2O_2$ in glacial acetic acid, as described previously.[7,8] $H_2O_2$ (30%) was commercially available. Dimethyl sulphoxide (DMSO) was distilled at reduced pressure from $CaH_2$ and stored over freshly activated 4 Å molecular sieves under dry argon. Deionized and triply distilled water was used in these experiments. Reactions were performed at pH 7.4 in 0.1 mol $dm^{-3}$ phosphate buffer. ESR spin-trapping experiments carried out under air-saturated conditions were done by mixing spin-traps, $H_2O_2$, DMSO (when required) immediately prior to addition of $Cu^{II}(en)_2$ solution. After addition of $Cu^{II}(en)_2$ solution, the samples were mixed and transferred to an ESR quartz flat cell and their ESR spectra recorded 2 min after mixing.

Aqueous solutions of $Cu^{II}(en)_2$ showed the ESR spectrum to consist of four broad lines ($a^{Cu} = 8.773$ mT, $g = 2.097$), which is typical of copper(II) complexes.[9] When aqueous solutions of $Cu^{II}(en)_2$ were mixed with those of $H_2O_2$, the ESR spectrum due to $Cu^{II}(en)_2$ was not observed, nor was a new ESR signal observed. Further, visible spectrum of $Cu^{II}(en)_2$ ($\lambda_{max}$ 555 nm) completely disappeared within 1 min of being mixed with $H_2O_2$ solution, but no absorption was observed in the visible region. These results indicate that paramagnetic $Cu^{2+}$ ion is reduced to diamagnetic $Cu^+$ ion.

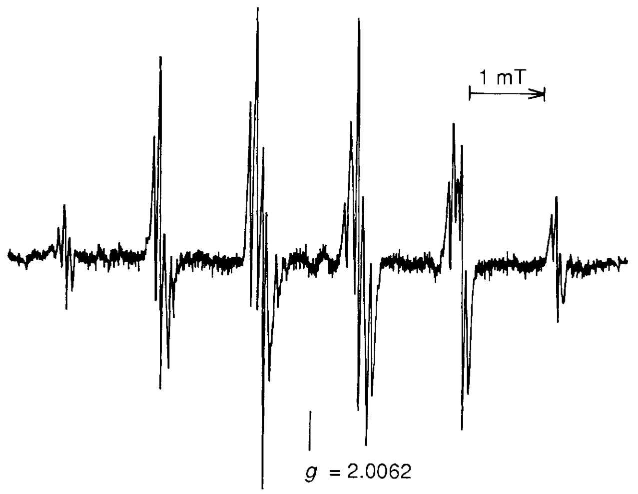


**Fig. 3** ESR spectrum observed by the oxidation of DMSO with $Cu^{II}$-$(en)_2$–$H_2O_2$ system in the presence of DBNBS at pH 7.4. *Reaction conditions*: $Cu^{II}$ $(en)_2$, 0.001 mol $dm^{-3}$; $H_2O_2$, 0.1 mol $dm^{-3}$; DMSO, 0.05 mol $dm^{-3}$; DBNBS, 0.05 mol $dm^{-3}$.

Thus, when DMPO was included in the $Cu^{II}(en)_2$–$H_2O_2$ system, a typical ESR spectrum [$a^N(1) = a^H(1) = 1.49$ mT] due to DMPO–OH adduct[10] was observed as shown in Fig. 1. In the absence of either $Cu^{II}(en)_2$ or $H_2O_2$, no ESR spectrum was observed. This result indicates that hydroxyl radical can be generated from the reaction of $Cu^{II}(en)_2$ with $H_2O_2$. The ESR spectrum due to DMPO–OH adduct gradually decreased its signal intensity, but it could still be observed 1 h after mixing. The formation of hydroxyl radical was also determined by use of POBN as a spin-trap. Fig. 2 shows the ESR spectrum [$a^N(1) = 1.49$ mT, $a^H(1) = 0.17$ mT] due to POBN–OH adduct.[11]

The ESR spectrum due to POBN–OH adduct could be observed at 30 min after mixing, although its signal intensity decreased. However, when DBNBS was included in the $Cu^{II}(en)_2$–$H_2O_2$ system, no ESR signal was observed. Since DBNBS does not form the stable adduct with hydroxyl radical,[7,8] in this reaction system the formation of hydroxyl radical is not clear. However, when DMSO was added to $H_2O_2$ solutions containing DBNBS before being mixed with the solutions of $Cu^{II}(en)_2$, an intensive ESR spectrum was observed as shown in Fig. 3. In the absence of either $Cu^{II}(en)_2$ or $H_2O_2$, no ESR spectrum was observed. ESR parameters can be determined as follows: $a^N(1) = 1.493$ mT, $a^H(3) = 1.279$ mT, $a^H(2) = 0.082$ mT and $g = 2.0062$. ESR spectrum shown in Fig. 3 is almost identical with that of methyl radical adduct of DBNBS generated either by the photolysis of aqueous solutions of $H_2O_2$ containing DMSO[7] or by the oxidation of DMSO with Fenton-type reaction systems such as $Ti^{3+}$–$H_2O_2$ and $Fe^{2+}$–$H_2O_2$.[12] Therefore, the radical species observed by the reaction of $Cu^{II}(en)_2$ with $H_2O_2$ in the presence of both DMSO and DBNBS can be assigned to the methyl radical adduct **4**. This radical species was very stable and its ESR spectrum could still be observed at 30 min after mixing.

In $Cu^{2+}$–$H_2O_2$ system[4] containing DMSO, methyl radical may be formed as in eqns. (2)–(6).

$$Cu^{2+} + H_2O_2 \rightarrow Cu^+ + 2H^+ + O_2^- \qquad (2)$$

$$Cu^{2+} + O_2^- \rightarrow Cu^+ + O_2 \qquad (3)$$

$$Cu^+ + H_2O_2 \rightarrow Cu^{2+} + OH^\cdot + OH^- \qquad (4)$$

$$OH\cdot + Me_2SO \rightarrow Me\cdot + MeSOOH \qquad (5)$$

$$Me\cdot + \text{DBNBS} \rightarrow \text{methyl radical adduct } \mathbf{4} \qquad (6)$$

Superoxide ion, $O_2^-$, which may be formed by the first reaction step between $Cu^{2+}$ and $H_2O_2$ [eqn. (2)], was not trapped by the spin-traps used here. It is known that the reaction of $O_2^-$ with spin-traps is low. For example, the second-rate constant for the reaction of $O_2^-$ with DMPO is only 10 $dm^3 mol^{-1} s^{-1}$.[13] On the other hand, some copper complexes can catalyse $O_2^-$ dismutation more rapidly at low pH than equimolar amounts of Cu, Zn–SOD (the rate constant for $O_2^-$ dismutation is *ca.* $1.6 \times 10^9$ $dm^3 mol^{-1} s^{-1}$), although at physiological pH it is almost the same or less.[14] From these facts it may be suggested that the rate of oxidation of $O_2^-$ by $Cu^{2+}$ ion is faster than that of trapping of $O_2^-$ by spin-traps.

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