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# THE EFFECT OF OXYGEN AT PHYSIOLOGICAL LEVELS ON THE DETECTION OF FREE RADICAL INTERMEDIATES BY ELECTRON PARAMAGNETIC RESONANCE

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It is well known that oxygen enhances the relaxation of free radical EPR probes through spin lattice and Heisenberg spin-spin interactions with consequent effect on the line height and width. The two relaxation processes have opposing effects on the signal heights and depend on the concentration of oxygen, the incident microwave power, and the presence of other paramagnetic species. During EPR studies of chemical, biochemical, and cellular processes involving free radicals, molecular oxygen has significant magnetic influence on the EPR signal intensity of the free radical species under investigation in addition to affecting the rates of production of the primary species and the stability of the spin adduct nitroxides. These effects are often overlooked and can cause artifacts and lead to erroneous interpretation. In the present study, the effects of oxygen and ferricyanide on the EPR signal height of stable and persistent spin adduct nitroxides at commonly employed microwave powers were examined. The results show that under commonly adopted EPR spectrometer instrumental conditions, artifactual changes in the EPR signal of spin adducts occur and the best way to avoid them is by keeping the oxygen level constant using a gas-permeable cell.

ABBREVIATIONS: DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; EPR, electron paramagnetic resonance; TEMPO, 2,2,6,6-tetramethyl-piperidinoxyl; TEMPONE, 4-oxi-2,2,6,6-tetramethyl-piperidinoxy; TEMPOL, 4-hydroxy-2,2,6,6-tetramethyl-piperidinoxyl.

KEYWORDS: DMPO, nitroxide spin-labels, spin-trapping.

## INTRODUCTION

Stable nitroxide free radicals are widely used as probes in EPR spectroscopic studies of various physiological parameters both *in vitro* and *in vivo*. Nitroxides are most frequently employed in biological applications to examine the structure and function of cell membrane, cellular redox properties, and to detect unstable free radical intermediates by spin trapping[1-4].

Collisions of dissolved paramagnetic oxygen molecules with the paramagnetic probe affect both its spin-lattice ($T_1$) and spin-spin ($T_2$) relaxation processes[2,5]. The effects of oxygen on these relaxation processes have been studied extensively and used

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for oximetric measurements of physiological and near-physiological oxygen levels[2,6,7]. At low microwave powers, molecular oxygen can broaden the signal and decrease the line height of the measured EPR probe as a consequence of enhanced $T_2$ relaxation. Therefore, monitoring of the EPR signal height has been used to quantitate oxygen concentration in the sample under investigation. However at high microwave powers, an increase in line height is correlated to oxygen evolved, since oxygen facilitates $T_1$ relaxation and increases the signal height[7]. In spin-trapping experiments coupled to EPR detection, the concentrations of the persistent nitroxide spin-adducts studied are low and, therefore, higher and partially saturating microwave power of 5–20 mW is generally employed to optimize signal to noise ratio[8]. It has been previously shown, when using non-saturating microwave power, that only the Heisenberg spin exchange effects the signal height by increasing the spectral line width of the EPR probe[2]. Conversely, higher microwave power is used as in spin-trapping studies, whereby both $T_1$ and $T_2$ relaxations are affected. As a result, the signal height depends on both microwave power, and oxygen level. The latter situation is particularly complicated in cases when over time oxygen level changes because of depletion or evolution, as frequently happens in chemical, photochemical, radiochemical, enzymatic and cellular studies. Since the EPR signal height should reflect free radical concentration, the influence of $[O_2]$ on the EPR line height of stable and persistent nitroxides at commonly used spectrometer conditions was studied and also because ignoring these effects may yield artifacts and errors in interpretation.

## MATERIALS AND METHODS

### *Chemicals*

DMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL) were from Aldrich, and 4-oxo-2,2,6,6-tetramethyl-piperidine-1-oxyl (TEMPONE) was purchased from Molecular Probes. Gases of desired $[O_2]$ were obtained from Matheson. The DMPO solution was spectrophotometrically determined using 8.0 $mM^{-1} \cdot cm^{-1}$ at 227 nm. DMPO-OH was produced by sonication[9]. Briefly: doubly distilled water containing 2 mM DMPO was sonolyzed for 10 min, and DMPO-OH was immediately used. All experiments were conducted at room temperature.

### *Electron Paramagnetic Resonance (EPR) Measurements*

The EPR spectra were recorded on a Varian E9 X-band spectrometer, operating at 9.45 GHz resonant frequency, using a rectangular cavity in the $TE_{102}$ mode, and modulation frequency of 100 kHz. Samples of nitroxides were drawn into a gas-permeable, 0.8 mm inner diameter, teflon capillary. Each capillary was folded twice and inserted into a 3 mm internal diameter quartz tube and then placed within the EPR cavity. During the experiment, either air, argon, or oxygen was directed into the cavity of the spectrometer without disturbing the sample. To study changes in signal height or decay kinetics of the spin-adduct, the magnetic field was kept constant while the intensity of the EPR signal was followed by positioning the recorder pen on the peak of one of the lines of the nitroxide. No frequency drift occurred during the measurement.

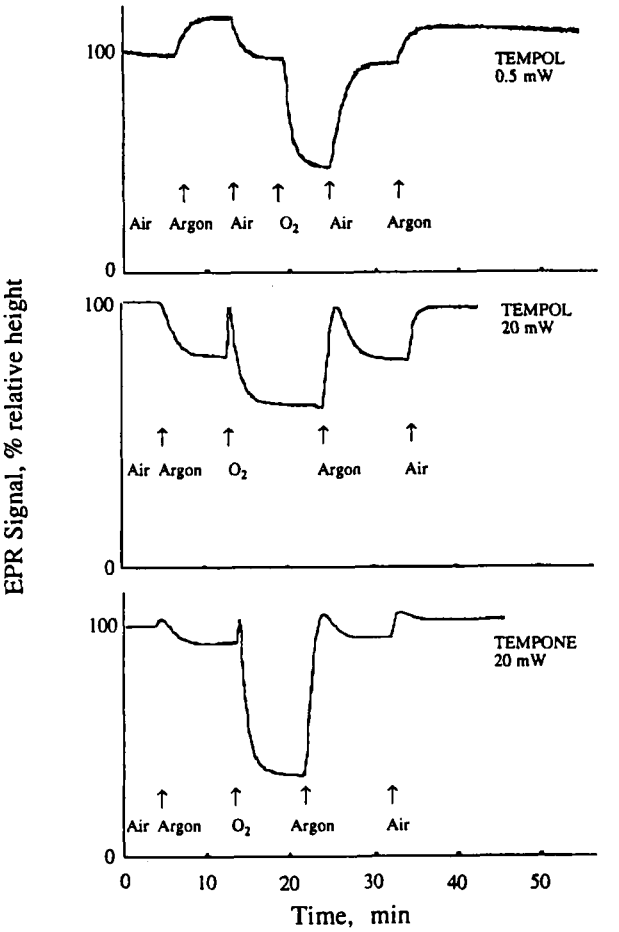


FIGURE 1 Oxygen effect on the EPR line height of nitroxides. Phosphate buffer saline at pH7.4, containing 100 $\mu$M TEMPOL was placed in a gas-permeable teflon capillary. The magnetic field was kept constant while the intensity of the nitroxide EPR spectrum was followed by positioning the recorder pen on the top of the middle line ($M_I = 0$). The equilibrating gas was changed repeatedly between air, argon, and oxygen without disturbing the alignment of the nitroxide sample in the cavity. The arrows denote the time points when gases were exchanged. Equilibration with each new gas took about 6 min. Modulation amplitude was kept at 1 gauss. a) TEMPOL, 0.5 mW, Gain 8000; b) TEMPOL, 20 mW, Gain 2000; c) TEMPONE, 20 mW, Gain 400.

## RESULTS

### *Effect of Oxygen on the EPR Signal of Stable Cyclic Nitroxides*

Solutions of 100 $\mu$M of stable nitroxides in phosphate buffered saline (PBS) were monitored for changes in EPR signal by following the height of the middle line ($M_I = 0$) under different equilibrating gases. Figure 1a shows such an experiment on TEMPOL with 0.5 mW incident microwave power maintained. When the equilibrating gas was changed from air to argon, the signal height increased by 15%. When air was re-introduced, the signal height returned to the original value. Equilibration

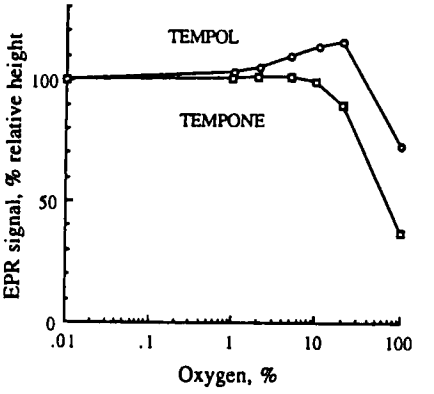


FIGURE 2 The effect of oxygen concentration on the height of the EPR line of nitroxide. Phosphate buffer saline at pH7.4, containing 100 $\mu$M TEMPOL (circles) or TEMPONE (squares) was placed in a gas-permeable teflon capillary. Modulation amplitude was kept at 1 gauss while power and gain were varied. The sample was equilibrated with gas mixtures of various oxygen concentrations at room temperature for at least 10 min before recording the EPR spectrum to monitor signal height at 1G modulation gain and 20 mW microwave power. The signal height is displayed as % of its value measured under argon.

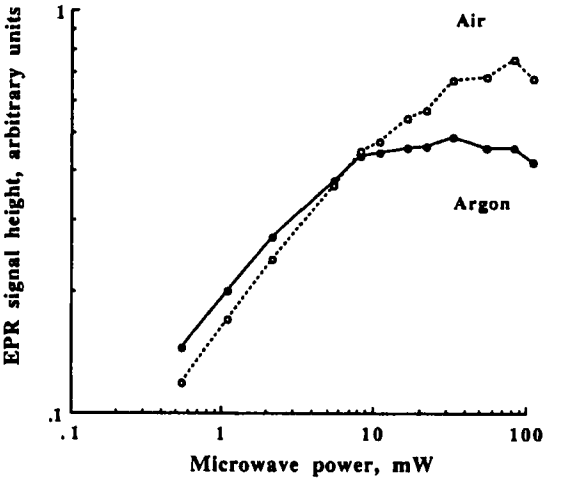


FIGURE 3 The effect of microwave power on the EPR line height of TEMPOL in air and argon. Phosphate buffer saline at pH7.4, containing 100 $\mu$M TEMPOL was placed in a gas-permeable teflon capillary. Modulation amplitude was kept at 1 gauss while power and gain were varied. The sample was equilibrated with either air (dashed line, open symbols) or argon (solid line, closed circles) for at least 10 minutes before recording the EPR spectrum to monitor signal height.

with pure $O_2$ decreased the signal height by 50% with a concomitant increase in line width. Figure 1b demonstrates TEMPOL signal dependence on the same levels of oxygen as in Figure 1a but at 20 mW microwave power. Upon changing from air to argon, the signal height decreased by 20%. The change of gas from argon to pure oxygen resulted in a temporary increase of the signal height to a value observed under air, followed by a 40% decrease. Reintroduction of argon reversed the process; the

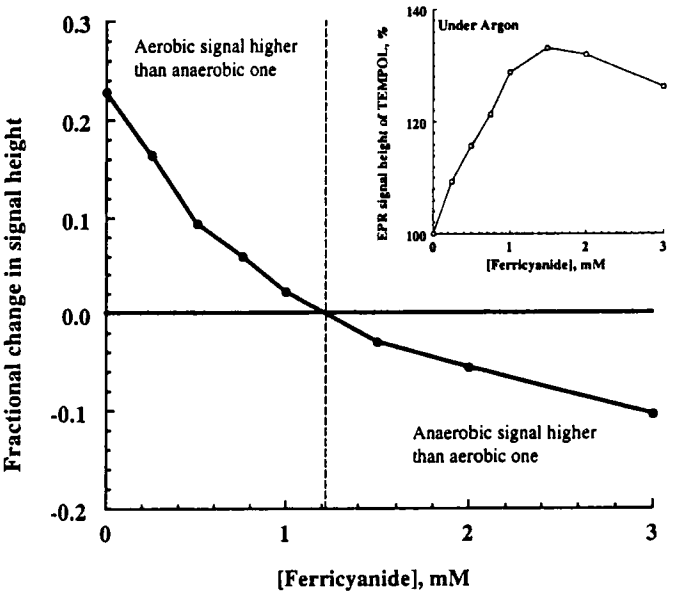


FIGURE 4 The effect of ferricyanide on the EPR line height of TEMPOL in air and argon. Phosphate buffer saline at pH7.4, containing 100 $\mu$M TEMPOL and various concentrations of ferricyanide, was placed in a gas-permeable teflon capillary. The sample was equilibrated with either air or argon for at least 10 minutes before recording the spectrum. The instrument settings were: Gain = 2000, power = 20 mW, and modulation amplitude = 1 gauss. The signal intensities were recorded under argon ($I_{argon}$) and under air ($I_{air}$) and the relative spectral change between air and argon $(I_{air} - I_{argon})/I_{air}$ was plotted vs [ferricyanide].

Inset: The TEMPOL signal height recorded under argon plotted vs ferricyanide concentration.

signal height, temporarily increased to a value observed under air, and subsequently decreased again by 20%. Several commonly used nitroxides such as TEMPO, TEMPONE, 3-carboxy PROXYL, 3-carbamoyl PROXYL, and 3-carbamoyl-3-pyrroline were studied and similarly responded to oxygen under 20 mW incident microwave power. In the case of TEMPONE, however, a maximal signal height was observed at $0\% < O_2 < 20\%$ (Figure 1c).

The dependence of the EPR signal height on $[O_2]$, under commonly used microwave power of 20 mW, differed for different nitroxides. 100 $\mu$M of TEMPOL and TEMPONE were equilibrated with various oxygen concentrations by flowing argon-oxygen gas mixtures of varying compositions around the teflon capillary. The EPR signal heights were measured without moving the sample and the variation of signal height as a function of oxygen concentration was monitored and displayed in Figure 2 (the residual oxygen level under argon was < 10 ppm.)

### *Effect of Microwave Power*

The experiment was repeated using various microwave power values and repeatedly changing the equilibrating gas between air and argon. Figure 3 displays the signal height dependence on microwave power for the two equilibrating gases. Below 7.5 mW the signal height obtained under argon exceeded that observed under air.

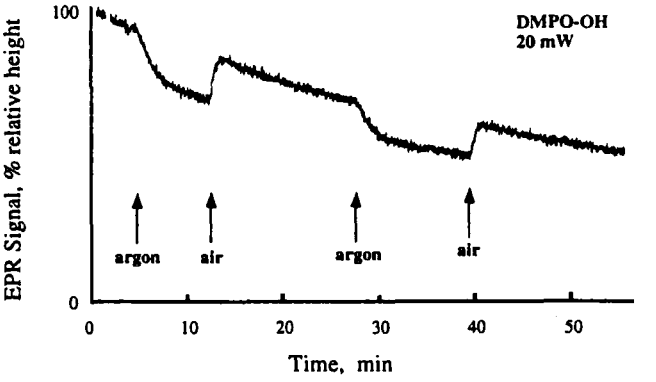


FIGURE 5 Oxygen effect on the EPR line height of DMPO-OH. DMPO-OH prepared by sonolysis in phosphate buffer saline pH7.4 was placed in a gas-permeable teflon capillary under a constant gas flow of desired oxygen level. The magnetic field was kept constant while the intensity of the nitroxide EPR spectrum was followed by positioning the recorder pen on the top of the 2nd line of the 1:2:2:1 quartet. The equilibrating gas was changed repeatedly between air and argon without disturbing the alignment of the spin-adduct sample in the cavity. The arrows denote the time points when gases were exchanged. Equilibrium with each new gas took about 6 min. The instrument settings were: Gain = 50000, power = 20 mW, and modulation amplitude = 1 gauss. It was always ensured that there was no frequency drift.

Above 7.5 mW the presence of air increased the signal height of TEMPOL when compared to that found under argon (Figure 3).

### *Effect of Ferricyanide*

Since oxygen also affects the spin-lattice relaxation, its influence on signal height of TEMPOL in the presence of another relaxing agent was examined. The nitroxide EPR signal was monitored at 20 mW incident microwave power with either air or argon, in the presence of various concentrations of $K_3Fe(CN)_6$. The effect of $K_3Fe(CN)_6$ on TEMPOL signal under argon is presented in the inset to Figure 4. The signal height of TEMPOL increased with the increase of $K_3Fe(CN)_6$ concentration up to 1.5 mM. Figure 4 demonstrates the dependence of the relative change of TEMPOL signal on $[K_3Fe(CN)_6]$ upon switching from air to argon. Below 1.2 mM $K_3Fe(CN)_6$, argon decreased TEMPOL signal; whereas, above this concentration, argon increased the signal height. The results indicate that both paramagnetic agents, $O_2$ and $K_3Fe(CN)_6$, similarly affect the relaxation process and consequently the EPR signal.

### *Effect of Oxygen on the EPR Signal of DMPO-OH*

The detection and quantitation of persistent nitroxide spin adducts resulting from the reaction of a spin trap with unstable free radical intermediates produced in radiolytic, photochemical, enzymatic, and cellular processes is commonly carried by EPR. When using the spin trapping technique, the EPR spectrum of the spin adduct is typically monitored under 10–20 mW microwave power to ensure optimal signal/noise ratio. To examine the effect of $O_2$ on the signal height of spin adducts, in the present study, DMPO-OH was generated sonolytically as previously described[9]. The DMPO-OH solution was placed in gas-permeable teflon capillary within the EPR

cavity, and the decay of the EPR signal was monitored while repeatedly switching the equilibrating gas between air and argon. As seen in Figure 5, the DMPO-OH signal progressively decreased reflecting the finite life time of this spin adduct. However, when the saturating gas was changed from air to argon (Figure 5, arrow 1), a transient decrease by ~ 20% in signal height was observed. The rate of this transient decrease reflects the rate of oxygen removal from the sample. Subsequently, the original rate of signal decay resulting from irreversible nitroxide spin-loss was re-established. In order to verify that the deaeration-induced temporary decrease in signal height resulted from a power saturation phenomenon, rather than a chemical reaction, the sample was re-aerated (2nd arrow, Figure 5). Whereupon, the signal height increased and reached a value expected from the anticipated DMPO-OH half life. This behavior was reproducible over several cycles of aeration/deaeration (3rd & 4th arrows, Figure 5).

## DISCUSSION

EPR detection of spin adduct nitroxides has provided valuable information with respect to the identification and quantitation of various free radical intermediates. While studying such intermediates, $[O_2]$ might change and, consequently, affect their generation or decay. For instance, radical production from stimulated neutrophils as well as the relative formation rates of $O_2^{\cdot-}$ and $H_2O_2$ by hypoxanthine/xanthine oxidase depends on $[O_2]$[10]

For valid EPR measurement it is, therefore, necessary that the respective EPR signal should reflect the concentration of free spins in a sample. The results displayed in Figures 1 and 5 show that this is not always the case, since the EPR signal changes without a change in radical concentration. Although the effect of oxygen on EPR signal of nitroxide probes is well documented[11], the microwave power commonly used to improve signal/noise ratio in detecting spin-adducts is typically between 5–20 mW[8,12,13]. Under these partially saturating microwave power conditions, the two opposing effects of oxygen on the signal height operate. In aerobic solutions, the decrease in signal intensity as a result of microwave power saturation is prevented by the alternate spin-lattice relaxation pathway provided by dissolved oxygen. However, under conditions where $[O_2]$ varies, this relaxation pathway is modified, leading to an artifactual change in the EPR signal. Consequently, an erroneous estimation of the concentration of the radical under study might occur. This is particularly true in kinetics experiments[12,13]. As demonstrated in Figures 2, 3 and 4, both oxygen and $K_3Fe(CN)_6$, exert two opposing magnetic effects on the EPR signal of the radical. Consequently, changing from argon to air in the presence of 1 mM ferricyanide had little effect on the signal intensity of TEMPOL (Figure 4). When using partially saturating microwave power, physiological $[O_2]$ increased the EPR signal intensity, whereas high $[O_2]$ lowered the signal height (Figure 1b). At high microwave power, the EPR transition saturates because of the long spin-lattice relaxation times ($T_1$) of nitroxides. However, traces of molecular oxygen or any other paramagnetic species can facilitate this relaxation pathway and subsequently cause an increase in the line height. Even the common use of ferricyanide to restore nitroxides from their respective hydroxylamine can cause a non-chemical increase of the EPR signal by as much as 30%, if the solution is not properly aerated at these powers.

To avoid this artifact, a constant replenishment of air is necessary. Use of non-saturating microwave power also eliminates this artifact, but at the expense of

signal/noise. The presence of small amounts of paramagnetic metal complexes could provide a relaxation pathway that would enhance the signal height, yet such a maneuver might interfere with the free radical reactions under investigation. Of the three alternative routes for avoiding the artifact, the most straight forward would appear to be replenishment of air to the test sample, using gas-permeable teflon cell.

## *In Conclusion*

Variations in oxygen concentration can effect the EPR signal intensity of the nitroxide in two different ways. i) A chemical mechanism in which the rates of radical production and decay of the primary as well as secondary spin adducts generated in cellular and biochemical reactions depend on oxygen level. ii) Magnetic mechanisms: spin-lattice relaxation ($T_1$) and spin-spin relaxation ($T_2$) processes. While chemical mechanisms may be the subject of research, the magnetic effects artifactually obscure the observed results. In view of subtle non-chemical effects of oxygen at physiological and sub-physiological levels, the present results demonstrate the complex dependence of the EPR signal on microwave power, oxygen, and other paramagnetic species and call for proper instrumental and reaction conditions when employing EPR for free radicals kinetics studies.



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