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# Reaction of Dithionite with Nitroxides. A New Possible Spin Trapping Agent of Superoxide Ion

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Although the formation of superoxide ion, $O_2^{\cdot-}$, has been proposed in autoxidations and in oxygen metabolizing organisms, its detection is still a debated argument because of the low sensitivity and specificity of $O_2^{\cdot-}$ trapping agents [1]. These detection methods require the use of Cu, Zn superoxide dismutase (SOD), an enzyme which reacts with $O_2^{\cdot-}$ at a rate close to the diffusion limit, and therefore $O_2^{\cdot-}$ production is calculated from the fraction of the trapping reaction inhibited by SOD.

In the search for species able to react with $O_2^{\cdot-}$, producing relatively stable free radicals such as nitroxides which can be detected by EPR spectroscopy at room temperature and at concentrations of the order of $10^{-7}$ *M*, new chemical species (which appear to meet these requirements) have been obtained from the reaction between nitroxides themselves and free radicals. This study describes the preparation of such a type of spin trap (NOS) from 4-amino-2,2,6,6-tetramethylpiperidinoxyl, (I), and $SO_2^{\cdot-}$ radical and its used in the detection of short lived $O_2^{\cdot-}$ radicals.

## Experimental

### *Preparation of the $O_2^{\cdot-}$ Spin Trapping Agent (NOS)*

The addition of small aliquots of $Na_2S_2O_4$ (which in aqueous solutions give rise to the equilibrium $S_2O_4^{=} \rightleftarrows 2SO_2^{\cdot-}$ [2]) into a nitrogen-bubbled $10^{-2}$ *M* I solution buffered at pH 8.0 with 0.1 *M* sodium phosphate determined the decrease of EPR spectrum of I until its complete disappearance when the molar ratio [dithionite]/[I] $\simeq$ 0.65 was reached. In these conditions the EPR spectrum of the reaction mixture was completely flat, indicating that the species $SO_2^{\cdot-}$ was still absent. Voltammetric measurements showed the same behaviour. In fact the polarographic reduction waves of I and of $S_2O_4^{=}$, which occur at –330 and –420 mV vs saturated calomel electrode respectively, were completely absent at a ratio [dithionite]/$[I]_0 \simeq 0.65$, while no polarographic wave due to the reaction product NOS could be observed.

The reaction mixture so obtained, containing the reaction product between I and $SO_2^{\cdot-}$, can be stored, frozen under nitrogen, for several days.

The addition of 1 *M* $H_2O_2$ to the NOS solution regenerates the starting nitroxide I, with a yield of 90%, as it results from EPR spectra taken after a hour from the $H_2O_2$ addition.

### *$SO_2$ Determination*

Small portions of the analysing solutions were brought to pH 1.0 by concentrated HCl and then nitrogen was bubbled into solutions to withdraw the $SO_2$ eventually formed. The stripped $SO_2$ was measured by differential pulse voltammetry [3]. In the case of NOS solutions a ratio $[SO_2]/[I]_0 \simeq 0.1$ has been calculated, where $[I]_0$ is the starting nitroxide concentration.

## Results and Discussion

Steady-state concentrations of superoxide radicals have been generated by slow perfusion of a DMSO solution containing 0.5 *M* 18-crown-6 and 0.4 *M* $KO_2$ into buffered aqueous solutions efficiently stirred. The addition of $10^{-3}$ *M* NOS to this system results in an EPR spectrum identical to that of I, which suggests that the reaction between NOS and $O_2^{\cdot-}$ leads to the formation of the nitroxide I again. In these conditions the $SO_2$ analysis in the reaction system gives a ratio $[SO_2]/[I]_{formed} \simeq 1$ independently of the $O_2^{\cdot-}$ added. The addition of $10^{-6}$ *M* SOD strongly inhibits the formation of nitroxide I (see Fig. 1) while the presence of $10^{-3}$ *M* $NaN_3$ has practically no effect on the rate of appearance of the EPR signal. Since $NaN_3$ scavenges efficiently ${}^1\Delta O_2$ [4], which according to some authors is generated in the spontaneous dismutation of $O_2^{\cdot-}$ [5], it appears that ${}^1\Delta O_2$ is not implicated in the formation of nitroxide I from NOS.

A sensitive autoxidation of NOS to nitroxide by molecular oxygen has been observed only at a pH higher than 9, while the addition of $10^{-4}$ *M* $H_2O_2$ to $10^{-3}$ *M* NOS regenerates nitroxide I at a rate of 2.1 $\mu$ *M* min$^{-1}$ which is completely independent of pH. Since, by addition of catalase at micromolar concentrations, the $H_2O_2$ can be easily brought to concentrations which are orders of magnitude lower than $10^{-4}$ *M*, the increase of EPR signal due to $H_2O_2$ can be completely abolished. Efforts to elucidate directly the chemical structure of NOS has been unsuccessful because of the impossibility of its separation from aqueous solutions. However, the formation of $SO_2$

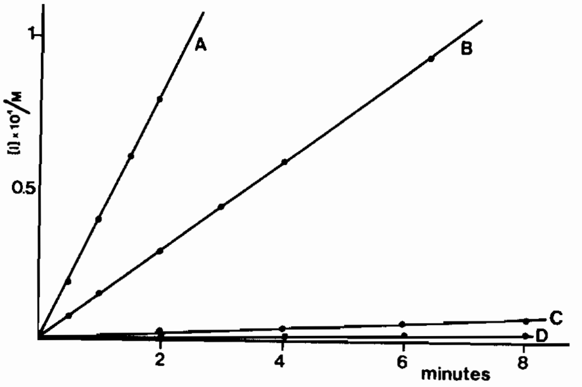
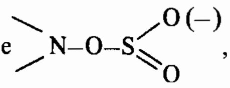
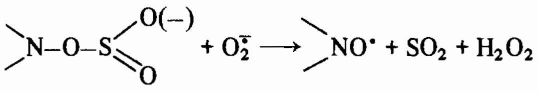


Fig. 1. Rates of formation of 4-amino-2,2,6,6-tetramethylpiperidinoxyl when 0.4 *M* $O_2^{\cdot-}$ in DMSO as $KO_2$-18-crown-6 complex was added continuously with efficient stirring into buffered solutions containing $10^{-3}$ *M* NOS and 5 × $10^{-7}$ *M* catalase. Rate of $O_2^{\cdot-}$ introduction 4 × $10^{-4}$ *M* $min^{-1}$. Curve A, 0.1 *M* sodium borate pH 8.9. Curve B, 0.1 *M* potassium phosphate pH 8.9. Curve C, as curve A plus $10^{-6}$ *M* SOD. Curve D, as B plus $10^{-6}$ *M* SOD.

and I in equimolecular amounts in the reaction between NOS and $O_2^{\cdot-}$, together with the practical absence of $SO_2$ in the reaction between I and dithionite, suggests that dithionite does not act as reducing agent of >NO· group of nitroxide and that therefore NOS should be formed in a coupling reaction between >NO· group and $SO_2^{\cdot-}$ radicals leading to a structure of the type  , which in turn is oxidized by $O_2^{\cdot-}$ according to the following reaction scheme:



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