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A New Method of Detecting O_2^- Production

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There is increasing interest in the superoxide anion (O_2^-) chemistry since O_2^- appears to be generated in a variety of chemical and biochemical processes involving oxygen [1, 2]. However, in aqueous solutions, its concentration appears to be quite low owing to its very fast dismutation into H_2O_2 and O_2 [3] which is increased by the presence of trace amounts of metal ions such as Cu^{++}, Mn^{++} *etc.*, free [4] or complexed in superoxide dismutase [2], a class of metalloenzymes widely distributed among living organisms. Therefore the direct measurement of such low O_2^- concentrations by spectroscopic methods has not yet been possible. The trapping of O_2^- by suitable scavengers, which integrate the O_2^- production over a certain time, circumvents this difficulty partially. These scavengers take advantage of the redox properties of the superoxide ion which is a mild reducing and oxidizing reagent [5]. Among chromophoric scavengers, ferricytichrome c (cyt c(III)), $k = 9 \times 10^5$ M^{-1} sec^{-1} at pH 8 [6], tetranitromethane, $k = 1.9 \times 10^9$ at pH 8.5 [7] and nitroblue-tetrazolium [8] have been used as oxidizing scavengers while epinephrine [9] resulted to be a rather reducing scavenger. However these trappers lack specificity as they react to a different extent with many reducing or oxidizing compounds and in particular with other oxygen derivatives like $^1O_2$, H_2O_2 and $^{\bullet}OH$ which can be formed in the O_2^- dismutation [2].

Bolton and coworkers [10, 11] used unsaturated compounds, *i.e.* pyrroline oxide, able to add free radicals such as $HO_2^{\bullet}$, $HO^{\bullet}$ *etc.* with the production of spin adducts which can be detected by electron spin resonance. However these spin adducts decay too rapidly to permit an efficient integration of the O_2^- production.

We report here a new method for detecting the generation of O_2^- by the formation of stable nitroxides, spin labels, from sterically hindered piperidine derivatives. These radicals are very stable at room temperature and can be easily detected by EPR spectroscopy at very low concentration and therefore appear to be very suitable to study the O_2^- production in chemical and biological systems.

Experimental

Cytochrome c and KO_2 were Fluka and K & K respectively. Superoxide dismutase was prepared from bovine red blood cell according to McCord and Fridovich [8]. Bovine liver catalase was from Sigma and was purified from superoxide dismutase by passage through a Sephadex G-75 column, 40 X 1 cm, equilibrated with 0.5 *M* phosphate buffer, pH 7.3. Other chemicals were reagent grade and were used without further purification. 4-Hydroxy-2,2,6,6-tetramethyl-4-piperidine (I) was obtained by reduction of 2,2,6,6-tetramethyl-4-piperidine with $LiAlH_4$ in tetrahydrofuran; 4-hydroxy-2,2,6,6-tetramethylpiperidinoxyl (II) was prepared from (I) according to Rozantsev [12]. The reduction of II with $LiAlH_4$ in tetrahydrofuran gave the corresponding hydroxylamine (III). 4-Hydroxy-2,2,6,6-tetramethyl-N-chloropiperidine (IV) was prepared by slow addition of a 2 *M* aqueous solution of I to 1 *M* NaClO kept at 0 °C. IV was extracted twice from n-hexane; m.p. 86–87 °C, yield about 50%. The UV spectrum of IV together with that of the corresponding nitroxide is reported in Fig. 1. 1,4,7,10,13,16-hexaoxacyclooctadecane (18-crown-6) was synthesised according to Pederson [13].

Optical spectra were recorded with a Varian Techtron mod. 635 spectrophotometer and room temperature EPR spectra with a Varian spectrometer.

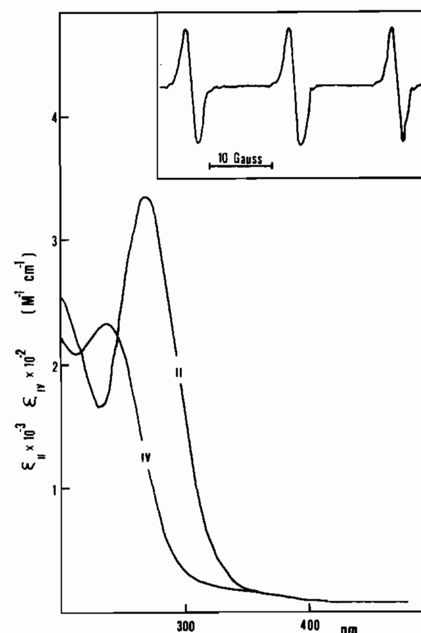

Figure 1. 4-Hydroxy-2,2,6,6-tetramethylpiperidinoxyl (II) and 4-hydroxy-2,2,6,6-tetramethyl-N-chloropiperidine(IV) spectra. Borate buffer 0.02 N pH 9.2. Inset: ESR spectrum of 10^{-4} *M* II in water; temperature 25 °C.

TABLE I. Production of Nitroxide from N-substituted 2,2,-6,6-Tetramethylpiperidines. The experiments were performed at pH 9.2 in 0.1 borate buffer at T = 25 °C. Singlet oxygen, $^1O_2$, was produced by addition of NaClO 10^{-2} *M* to H_2O_2 10^{-2} *M*; $^{\bullet}OH$ radicals were obtained from photolysis of H_2O_2 10^{-2} *M* at wavelength > 300 nm. O_2^- was added as KO_2–crown ether complex in DMSO, see text. The symbols +++, ++, + and – correspond to high, medium, low and zero yields respectively.

	$^3O_2$	$^1O_2$	O_2^-	H_2O_2	$^{\bullet}OH$
I N–H	–	++	–	–	–
III N–OH	+	+++	+++	++	–
IV N–Cl	–	–	+++	–	–

Results and Discussion

The reactivity of N-substituted 2,2,4,4-tetramethylpiperidines was tested toward O_2^- and other oxygen reactive derivatives such as H_2O_2, $^{\bullet}OH$ and singlet oxygen. The results are reported in Table 1. It appears that IV and III react with O_2^- giving the nitroxide II. However the formation of II from III is not specific for O_2^-, since oxygen, either singlet or triplet, and H_2O_2 produce the nitroxide though to a lower extent. Further experiments showed that the formation of II is enhanced on increasing the pH from 7 to 12. On the contrary IV, a chloroamine, appears to be a specific O_2^- spin trap. In fact this chloroamine is stable for days at room temperature in the pH range 2–12 and reacts specifically with O_2^- to form the corresponding nitroxide which can be stored for months at 5 °C and can be easily detected by its room temperature EPR spectrum (see the inset of Fig. 1). In our experiments steady-state O_2^- concentrations were achieved by continuous mechanical infusion of a DMSO solution of KO_2 containing 18-crown-6 (molar ratio KO_2/crown $\simeq 2$) into an efficiently stirred buffered aqueous solution. Small amounts of superoxide dismutase were added to control the O_2^- steady-state concentration while catalase was added to eliminate H_2O_2. When these experiments are performed in the presence of the chloroamine, $\rangle N{-}O^{\bullet}$ production occurs. The rate of the spin adduct formation is reported in Fig. 2 for experiments performed at different pH values and different superoxide dismutase concentrations.

The O_2^- steady-state concentration, $[O_2^-]_{ss}$, was measured from the initial rate of cyt c(III) reduction at 550 nm, whilst in parallel experiments cyt c(III) was substituted for IV. The $[O_2^-]_{ss}$ was calculated according to the eqn. $-[(d\text{cyt c(III)})/dt] = k\text{cyt c(III)}[O_2^-]_{ss}$; the k values used were those reported by Koppenol *et al.* [6]. It appears (see Fig. 2) that O_2^- steady-state concentrations in the range of 10^{-9}–10^{-8} *M* can be easily detected with the use of IV.

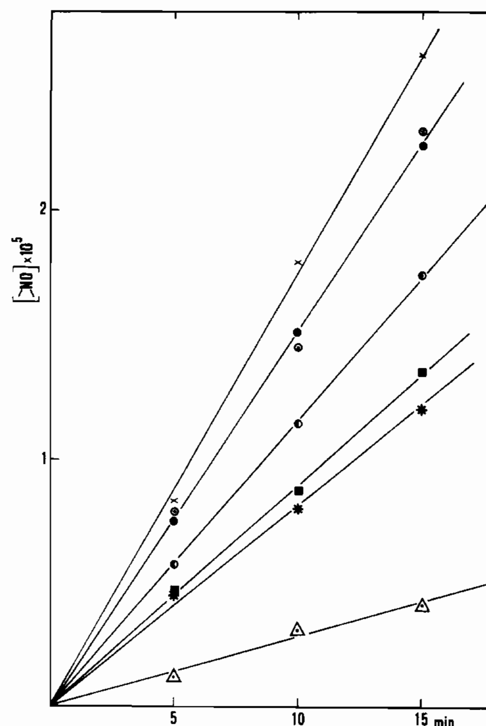

Figure 2. Nitroxide concentration against time when 0.8 *M* O_2^- in DMSO, as KO_2–crown complex, was pumped continuously, under efficient stirring, into a 0.1 N borate solution, 10^{-4} *M* EDTA, containing 10^{-2} *M* chloroamine. Rate of O_2^- introduction 1.7×10^{-5} *M*/sec. The experimental conditions were:

	pH	Sod	Catalase	$[O_2^-]_{ss}$ [a]
×	9.7	3.0×10^{-9}	9×10^{-8}	4.4×10^{-7}
△	9.7	9.3×10^{-8}	9×10^{-8}	3.2×10^{-8}
*	8.8	3.0×10^{-9}	9×10^{-8}	6.0×10^{-8}
■	7.8	3.0×10^{-9}	9×10^{-8}	5.6×10^{-9}
⊙	7.8	–	9×10^{-8}	1.1×10^{-8}
●	7.8	–	–	not measured
◐ [b]	7.8	–	9×10^{-8}	not measured

[a] $[O_2^-]_{ss}$ has been calculated from competition measurements with cyt c(III), see text. [b] N_3^- 10^{-2} *M* was present.

Further experiments carried out in presence of H_2O_2 or azide, a very efficient singlet oxygen quencher [14], showed that H_2O_2 or singlet oxygen have no effect on the $\rangle N{-}O^{\bullet}$ production from chloroamine and O_2^-, see Fig. 2.

In conclusion a new simple method of detecting O_2^- at steady-state concentrations as low as 10^{-9} *M* can be derived from experiments reported here. Work on possible applications of this method to chemical and biological systems is in progress.

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