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Detection of Superoxide Ion in Aqueous Media by N-Halopiperidines

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The reaction between N-chloro-or N-bromo-2,2,6,6-tetramethylpiperidines and superoxide radical has been studied. 2,2,6,6-tetramethylpiperidines are the main reaction products, while the corresponding piperidinoxyl spin label, has been formed as a secondary reaction product with a yield which is independent of the concentration of reagents. In this way N-halopiperidines can be used as specific spin-trappers of superoxide ion. A kinetic scheme which takes into account the consecutive reaction between piperidinoxyl and superoxide is proposed and the relative kinetic constants are given.

Introduction

There is considerable interest in the detection of superoxide anion, $O_2^{\dot{-}}$, since this species, which is generated as intermediate in the reduction of molecular oxygen both in chemical and biochemical processes, appears in the formation of very reactive and oxidizing species such as $^1\Delta O_2$ and $OH^{\cdot}$ radicals.

The free radical character of $O_2^{\dot{-}}$ permits its detection by EPR spectroscopy, but the direct measurement of $O_2^{\dot{-}}$ concentration in aqueous solution is a difficult task because of the short lifetime of this radical. In fact $O_2^{\dot{-}}$ in aqueous solution dismutes rapidly to H_2O_2 and O_2, $k \cong 10^8$ M^{-1} sec^{-1} at pH 5.

The spin trapping technique, *i.e.* the transformation of short lived radicals into long-lived radicals by reaction with particular compounds, spin trappers, may be used to overcome this difficulty.

Derivatives of pyrrolyne-1-oxide have been proposed, but they suffer from various disadvantages [1]. These compounds are unstable and cannot be used to integrate the $O_2^{\dot{-}}$ production over sufficient long periods of time because the spin adducts formed decay rapidly and show rather complicated EPR spectra.

We found that 4-hydroxy-2,2,6,6-tetramethyl-N-chloropiperidine (NCl), a quite stable compound, reacts specifically with $O_2^{\dot{-}}$ producing 4-hydroxy-2,2,6,6-tetramethylpiperidinoxyl (NO), which is a free radical, stable for months at room temperature [2]. In this way $O_2^{\dot{-}}$ steady-state concentrations as low as 10^{-9} M were measured. Pursuing this type of study we have tried to synthesize more efficient $O_2^{\dot{-}}$ spin trappers and to gain some insight into the trapping mechanism.

Experimental

Chemicals

Chemicals used throughout this work were as previously reported [2]. 4-hydroxy-2,2,6,6-tetramethyl-N-bromo-piperidine, (NBr), was prepared by the slow addition of 2 g of 4-hydroxy-2,2,6,6-tetramethylpiperidine (NH) into a hypobromite solution (1.2 g of Br_2 and 1.6 g of NaOH in 10 ml of H_2O) to which 14 ml of ethyl ether were added. The solution was stirred energically at 0 °C. After 20 minutes a concentrated NaOH solution was added until the reacting mass turned to pale yellow. The ether layer, after washing with H_2O and drying, was concentrated until complete precipitation of NBr took place. The reaction product was purified three times by precipitation from an alcoholic solution to which water was added in a ratio water/alcohol of 1. The prepared N-bromopiperidine, pale yellow crystals, m.p. 98–100 °C, shows only one spot after thin-layer chromatography on Merck silicagel 60 F254 plates. The UV–visible spectrum presented a well characterized maximum at 308 nm, $\epsilon = 310$ M^{-1} cm^{-1}. The NBr is easily reduced at the dropped mercury electrode, $E_{1/2} \cong 0$ V *vs.* standard calomel electrode (SCE).

Procedure

The kinetic measurements were performed under conditions approaching that of the continuous stirred tank reactor (C.S.T.R.) [3]. A solution of $O_2^{\dot{-}}$ in anhydrous dimethylsulfoxide (DMSO), obtained by dissolving KO_2 in the presence of 18-crown-6, $[O_2^{\dot{-}}] \cong 0.6$ M, was fed continuously at a constant rate, ranging from 2 to 6 μl/min, in 4 ml of the reacting solution. At the same time an equal volume of an H_3PO_4 solution was added to keep the pH constant. Efficient stirring in the reactor assured a uniform and homogeneous composition of the reacting mass

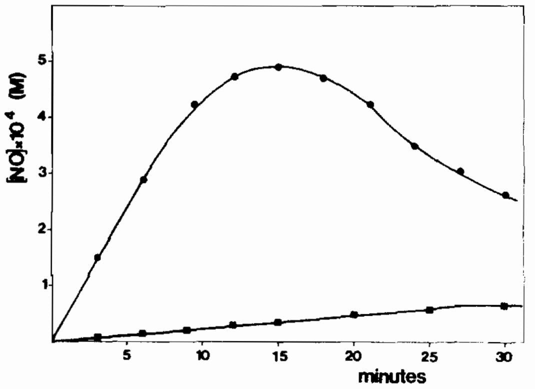


Fig. 1. Concentration of 4-hydroxy-2,2,6,6-tetramethylpiperidinoxyl *vs.* time when a solution of KO_2-18-crown-6 in DMSO, $[O_2^{\cdot-}] = 0.61$ *M*, was fed continuously at a rate of 4 µl/min into 1 ml of an aqueous solution of the corresponding N-halopiperidines: (■) NCl 10^{-2} *M*; (●) NBr 10^{-2} *M*. The solution also contains sodium borate 0.1 *M*, EDTA 2 × 10^{-4} *M* and catalase 10^{-7} *M*. The catalase was added to prevent the accumulation of H_2O_2. To keep the pH at 9.2 an H_3PO_4 solution was perfused into the stirred solution at the same rate as the $O_2^{\cdot-}$ solution.

throughout its whole volume. Since the volumes of the added H_3PO_4 and $O_2^{\cdot-}$ solutions were kept negligible with respect to the total volume, the C.S.T.R. simplifications can be applied to the kinetic equations [3]. 20 µl samples of reacting solution were used to measure the NO and N-halopiperidines concentrations by EPR and by DC voltametry with synchronized dropping mercury electrode ($E_{1/2} \cong$ 0 V *vs.* SCE) in borate buffer 0.1 *M*, pH 10, respectively.

The $O_2^{\cdot-}$ steady-state concentrations were evaluated from the spectroscopic measurements of the reduction degree of ferricytochrome *c* in experiments in which the N-halopiperidines were substituted by ferricytochrome *c*. EPR and NMR spectra were taken with a Varian mod. V-4500 and with a Varian mod. EM-390 spectrometers respectively. The electronic spectra were recorded with a Varian Techtron mod. 635 spectrometer. Polarographic analyses were performed by an Amel mod. 472 polarograph.

Results

Addition of $O_2^{\cdot-}$ to an aqueous solution of NBr leads to the production of the corresponding nitroxide. As in the case of NCl the formation of NO is a specific reaction of the superoxide radical. In fact, among the reactive species which could be formed in the reduction of molecular oxygen, such as $O_2^{\cdot-}$, singlet oxygen, ˙OH radicals, H_2O_2, only $O_2^{\cdot-}$ can bring about the formation of nitroxide from N-bromopiperidine.

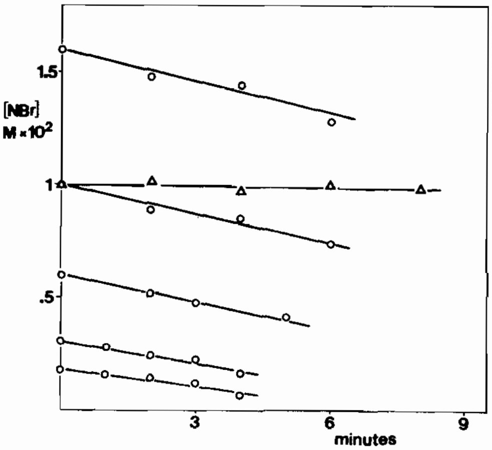


Fig. 2. Initial decay of N-halopiperidine concentrations *vs.* time in the presence of superoxide radical at different initial concentrations of NBr (○) and NCl (△). The corresponding NO formation is reported in Fig. 3. Rate of $O_2^{\cdot-}$ feeding: 0.83 × 10^{-5} *M* sec^{-1}. The other experimental conditions are those reported in Fig. 1.

In Fig. 1 the results obtained when NCl and NBr are used as $O_2^{\cdot-}$ spin trappers in experiments carried out in analogous conditions are reported. The most important features of these experiments are:

– NBr appears a more efficient scavenger of $O_2^{\cdot-}$ radicals than NCl.

– The maximum concentration of nitroxide is much lower than the initial N-halopiperidine concentration.

– The disappearance of NO in the final part of the experiment, which is particularly evident in the case of NBr. This indicates a consecutive reaction of the nitroxide with reagents or products present in the reaction system. To investigate this result, the reactivity of NO was checked with NBr, H_2O_2 and $O_2^{\cdot-}$. It results from these experiments that only the superoxide radical reacts with the nitroxide. In fact the nitroxide in the presence of a constant concentration of $O_2^{\cdot-}$, decays following a first order kinetic whose rate is directly proportional to the $O_2^{\cdot-}$ concentration. A second order rate constant of 730 M^{-1} sec^{-1} can be calculated for the reaction between $O_2^{\cdot-}$ and NO carried out in conditions similar to those of Fig. 1. Since these radical reactions are very sensitive to impurities the initial rates of the reactions have been considered in the kinetic analysis of the nitroxide formation from NCl and NBr. In fact in our experimental conditions, owing to the impurities present in the reaction system the $O_2^{\cdot-}$ decay follows a first-order kinetics, k_0 = 23 sec^{-1}, as we have observed in experiments of the reduction of ferricytochrome *c* by $O_2^{\cdot-}$, instead of the expected second-order decay [4].

In the reaction between $O_2^{\cdot-}$ and NCl a linear increase of the initial rate of NO formation, V_{NO}^{0},

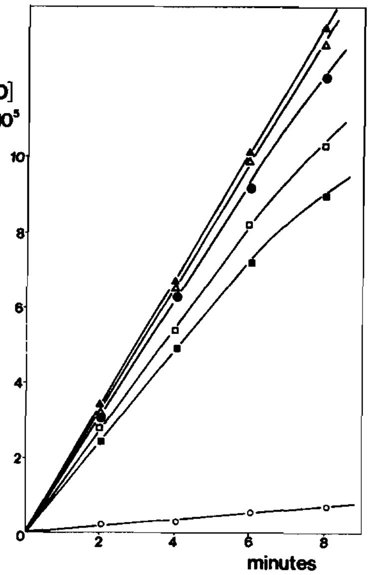


Fig. 3. Initial NO concentrations *vs.* time obtained in the experiments of Fig. 2. The initial N-halopiperidine concentrations are: (▲) $[NBr]_0$ 1.8 × 10^{-2} *M*; (△) $[NBr]_0$ 1.0 × 10^{-2} *M*; (●) $[NBr]_0$ 0.6 × 10^{-2} *M*; (□) $[NBr]_0$ 0.3 × 10^{-2} *M*; (■) $[NBr]_0$ 0.18 × 10^{-2} *M*; (○) $[NCl]_0$ 1.0 × 10^{-2} *M*.

with the initial NCl concentration, $[NCl]_0$, was found for $[NCl]_0$ ranging between 10^{-4} and 5 × 10^{-3} *M*. Furthermore from the slope of the plot of $\log[NO]_0$ *vs.* $\log[NCl]_0$ a reaction order of one with respect to the halopiperidine was deduced.

In analogous experiments carried out by substituting NBr for NCl a non-linearity between NBr concentration and NO formation rates was observed. The initial parts of the plots regarding the NBr decay and NO formation at different initial concentrations of N-bromopiperidine, $[NBr]_0$, are reported in Figs. 2 and 3 respectively. From these plots the initial rates $d[NBr]/dt = V^{\circ}_{NBr}$ and $d[NO]/dt = V^{\circ}_{NO}$ were calculated.

For the sake of comparison the plots relative to an experiment carried out with $[NCl]_0 = 10^{-2}$ *M* are reported in Figs. 2 and 3. As can be easily seen, NBr reacts with $O_2^{\dot{-}}$ with a higher rate than NCl. The NO yields, calculated as $V^{\circ}_{NO}/V^{\circ}_{NBr}$ and $V^{\circ}_{NO}/V^{\circ}_{NCl}$, for an initial N-halopiperidine concentration of 10^{-2} *M* are 0.04 and 0.05 respectively. As regards the other products of the reaction between N-halopiperidines and $O_2^{\dot{-}}$, a NMR analysis has shown that 4-hydroxy-2,2,4,4-tetramethylpiperidine is formed with an almost quantitative yield.

The possibility that NO could be formed in the reaction between $^1\Delta O_2$, which according to Kearns [5] is one of the products of $O_2^{\dot{-}}$ dismutation, and the NH produced in the reaction [6] has been ruled-out experimentally. In fact no NO was detected after the addition of KO_2–DMSO to an aqueous solution of 4-hydroxy-2,2,6,6-tetramethylpiperidine. Furthermore the participation of molecular oxygen to the NO formation has been excluded, the NO yields being independent of O_2 partial pressure in the range 0–1 atm. Finally from a survey of the data of Figs. 2 and 3 it appears that on increasing $[NBr]_0$, the values of V°_{NO} and V°_{NBr} tend parallely towards a saturation limit. This phenomenon shows the characteristic of a 'substrate saturation effect', which is confirmed by the linearity of the plots $1/V^{\circ}_{NO}$ and $1/V^{\circ}_{NBr}$ *vs.* $1/[NBr]_0$, see Fig. 4.

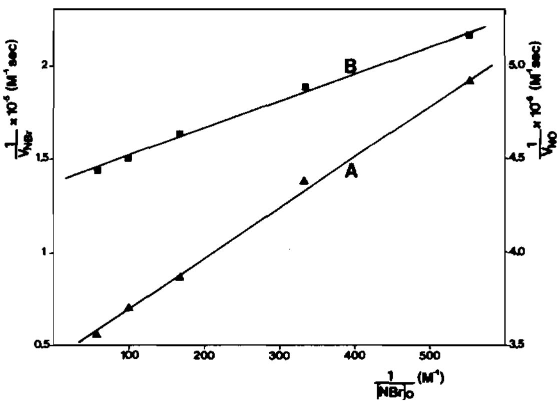
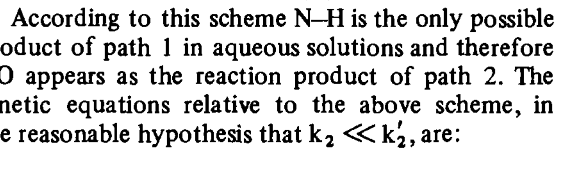


Fig. 4. Double reciprocal plot of initial rate of NO formation (▲) and NBr decay (■) *vs.* the corresponding initial NBr concentrations. The rate values were obtained from the data of Figs. 2 and 3.

Discussion

The ease of the reduction of N–X bond suggests an electron transfer from $O_2^{\dot{-}}$ to N-halopiperidine as first stage of the studied reaction followed by the cleavage of the N–X bond. On account of the similar electronegativities of N, Cl and Br atoms the reaction may proceed via the parallel paths 1 and 2 reported in the following reaction scheme where the experimental results are summarized:



According to this scheme N–H is the only possible product of path 1 in aqueous solutions and therefore NO appears as the reaction product of path 2. The kinetic equations relative to the above scheme, in the reasonable hypothesis that $k_2 \ll k_2'$, are:

$$V_{NX} = (k_1 + k_2)[NX]\,[O_2^{\dot{-}}] \qquad (2)$$

$$V_{NO} = k_2[NX]\,[O_2^{\dot{-}}] \qquad (3)$$

Furthermore from C.S.T.R. approximation it results:

$$\frac{d[O_2^{\dot{-}}]}{dt} = V_o - (k_1 + k_2)[NX]\,[O_2^{\dot{-}}] - k_0[O_2^{\dot{-}}] \cong 0 \qquad (4)$$

where V_o is the rate ($M\,sec^{-1}$) at which $O_2^{\dot{-}}$ is fed into the reaction system. From the rearrangement of equations 2–4 it can be easily obtained:

$$\frac{1}{V_{NBr}} = \frac{1}{V_o} + \frac{k_0}{(k_1 + k_2)\cdot V_o} \cdot \frac{1}{[NBr]} \qquad (5)$$

$$\frac{1}{V_{NO}} = \frac{k_1 + k_2}{k_2} \cdot \frac{1}{V_o} + \frac{k_0}{k_2 \cdot V_o} \cdot \frac{1}{[NBr]} \qquad (6)$$

According to these equations the experimental value of $k_2/(k_1 + k_2)$ obtained from the intercept of the plot of $1/V_{NO}$ *vs.* $1/[NBr]_o$, curve A of Fig. 4, is 0.04. This value, which according to the kinetic scheme presented above represents the yield of NO formation from NBr, is in good agreement with the yield of NO calculated directly from V^{o}_{NO}/V^{o}_{NBr}.

From the k_0 value of 23 sec^{-1} and from the slopes of curve A and B of Fig. 4, $k_1 = 21 \times 10^3\ M^{-1}\ sec^{-1}$ and $k_2 = 1.1 \times 10^3\ M^{-1}\ sec^{-1}$ were calculated. These data indicate that $O_2^{\dot{-}}$ reacts almost quantitatively with NBr, at NBr concentrations 10^{-2} M (see eq. 4), thus explaining the observed saturation effect.

This effect was not observed with NCl, according to its lower reactivity. In this case $k_1 = 76$ and $k_2 = 4.0\ M^{-1}\ sec^{-1}$ for the reaction between NCl and $O_2^{\dot{-}}$ were calculated according to eqns. 2 and 3, from the data reported in Figs. 2 and 3.

In conclusion it has been demonstrated that N-halopiperidines can be utilized as spin trappers to integrate the $O_2^{\dot{-}}$ production in aqueous media since in the reaction with $O_2^{\dot{-}}$ they generate the stable piperidinoxyl radical with a constant yield. From the kinetic constants of the reactions of $O_2^{-\cdot}$ with NCl and NBr it can be deduced that the NO production is proportional to the $O_2^{\dot{-}}$ concentration until the final concentration of NO is $10^{-3} \times [NCl]_o$ or $10^{-2} \times [NBr]_o$, respectively and therefore according to the kinetic equations previously reported the rate of $O_2^{\dot{-}}$ production could be quantitatively evaluated.

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