ARTICLE

OBC
www.rsc.org/obc

Spin trapping of superoxide by diester-nitrones

Ahmad Allouch, Valérie Roubaud, Robert Lauricella, Jean-Claude Bouteiller and Béatrice Tuccio*

Laboratoire TRACES, case 541, Universités d'Aix-Marseille 1 et 3, Faculté de St. Jérôme, 13397 Marseille cedex 20, France. E-mail: tuccio@up.univ-mrs.fr; Tel: (33 or 0) 491-288-743

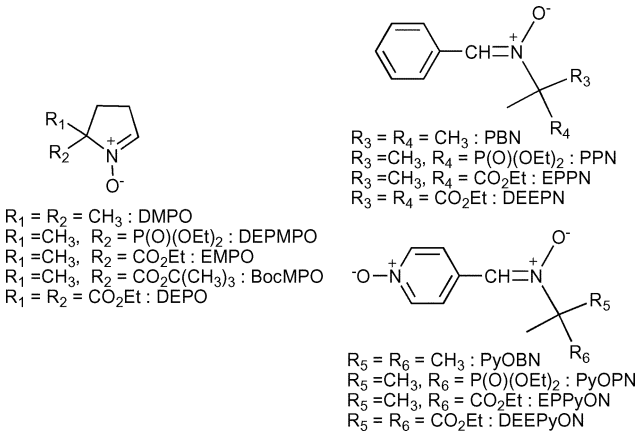
Received 24th February 2005, Accepted 5th May 2005
First published as an Advance Article on the web 24th May 2005

The nitrone *N*-[(1-oxidopyridin-1-ium-4-yl)-methylidene]-1,1-bis(ethoxycarbonyl)ethylamine *N*-oxide (DEEPyON) was synthesized and used as a spin trapping agent. The kinetic aspects of the superoxide detection by this new spin trap and by two other diester-nitrones, *i.e.* 2,2-diethoxycarbonyl-3,4-dihydro-2*H*-pyrrole-1-oxide (DEPO) and *N*-benzylidene-1,1-bis(ethoxycarbonyl)ethylamine *N*-oxide (DEEPN), were examined by determining the rate constants for the trapping reaction and for the spin adduct decay at pH 7.2. Comparing the results obtained to those given by analogous monoester-nitrones showed that both the spin trapping and the adduct decay reactions were faster in the presence of a second ester group in the cyclic nitrone series, while the superoxide trapping capacities of linear diester-nitrones were found to be dramatically weak. It follows from this study that DEPO and 2-ethoxycarbonyl-2-methyl-3,4-dihydro-2*H*-pyrrole-1-oxide (EMPO) are superior when it comes to superoxide detection. Below 0.005 mol dm^{-3}, DEPO is to date the only nitrone capable of clearly detecting superoxide, while EMPO should be preferred at higher spin trap concentration.

Introduction

The EPR/spin trapping technique has become a valuable tool in the detection of free radicals occurring in chemical or biochemical processes.[1] In this field, two kinds of nitrone spin traps have been essentially developed: five-membered cyclic nitrones such as 5,5-dimethyl-3,4-dihydro-2*H*-pyrrole *N*-oxide (DMPO) and linear nitrones, derived from either *N*-*tert*-butylbenzylideneamine *N*-oxide (PBN) or *N*-[(1-oxidopyridin-1-ium-4-yl)-methylidene]-1,1-isopropylamine (PyOBN) (Scheme 1). In the cyclic nitrone series, the first uses of DMPO to detect superoxide in aqueous media were described about thirty years ago.[2] Despite its well-known drawbacks, such as the short lifetime of its superoxide adduct at neutral pH, DMPO remained the most popular nitrone for superoxide detection for more than twenty years.[3] In 1994, the synthesis of 5-(diethoxyphosphoryl)-5-methyl-3,4-dihydro-2*H*-pyrrole *N*-oxide (DEPMPO), a much more efficient superoxide spin trap, was a major step forward.[4] The presence of an electron withdrawing dialkoxy-phosphoryl group in the β-position to the nitrone function was thus found to greatly enhance the superoxide adduct persistence.[5] More recent studies performed with 2-ethoxycarbonyl-2-methyl-3,4-dihydro-2*H*-pyrrole-1-oxide (EMPO), with 2-*tert*-butoxycarbonyl-2-methyl-3,4-dihydro-2*H*-pyrrole-1-oxide (BocMPO), or with other ester-nitrones, have shown that the presence of an ester group in the same position on the pyrrolidinic ring could also stabilise superoxide adducts.[6] In the linear nitrone series, work devoted to the elaboration of progressively better spin traps for superoxide detection was developed in the same way. PBN and PyOBN were first used and found to be poorly efficient at trapping superoxide.[3c,7] Then, more efficacious phosphorylated analogues, such as *N*-benzylidene-1-diethoxyphosphoryl-1-methylethylamine *N*-oxide (PPN) and *N*-[(1-oxidopyridin-1-ium-4-yl)methylidene]-1-diethoxyphosphoryl-1-methylethylamine *N*-oxide (PyOPN) were elaborated.[8] Finally, recent work has shown that linear ester-nitrones also yielded long-lived superoxide adducts.[9] Ester-nitrones could present certain advantages when compared to their β-phosphorylated analogues: they are often more easily prepared at high purity, and yield adducts with simpler EPR spectra. In addition, considering the benefit gained by replacing a methyl group by an ester in DMPO, PBN or PyOBN, one should wonder about the spin trapping capacities of a nitrone bearing two carboxycarbonyl groups. In the pyrrolidinic nitrone series, Karoui *et al.* recently described the synthesis of 2,2-diethoxycarbonyl-3,4-dihydro-2*H*-pyrrole-1-oxide (DEPO), a DMPO-type diester-nitrone, and its application to superoxide trapping.[10] They notably noticed that DEPO was faster than DEPMPO at trapping superoxide, though they did not measure the kinetics of this reaction. In the linear nitrone series, we previously reported on the preparation and use as a spin trapping agent of *N*-benzylidene-1,1-bis(ethoxycarbonyl)ethylamine *N*-oxide (DEEPN).[11]

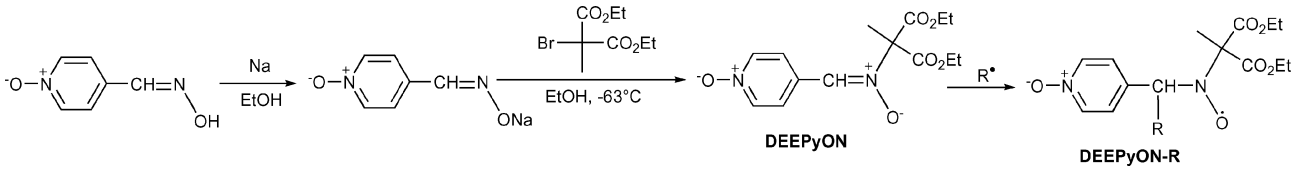
The purpose of this work was to study the kinetics of the superoxide trapping and of the decay reaction of the superoxide adducts obtained with diester-nitrones in pH 7.2 buffer, and to compare these results with those obtained with analogues bearing at the most one carboxycarbonyl group. Therefore, we first present a synthesis of *N*-[(1-oxidopyridin-1-ium-4-yl)-methylidene]-1,1-bis(ethoxycarbonyl)ethylamine *N*-oxide (DEEPyON), a new PyOBN-type diester-nitrone, and a study of its spin trapping capacities. Then, a kinetic approach recently elaborated[12] was applied to evaluate the rate constants for the superoxide trapping and for the adduct decay reactions when various mono- or diester-nitrones were employed.



Scheme 1 Formulae of various nitrone spin traps.

DOI: 10.1039/b502836a

 This journal is © The Royal Society of Chemistry 2005



Scheme 2 Synthesis of the nitrone DEEPyON and formation of its spin adduct DEEPyON–R by spin trapping of the radical R•.

Results and discussion

Synthesis and use of DEEPyON as a spin trapping agent

The simple synthetic pathway followed to prepare the nitrone DEEPyON is given in Scheme 2. The *Z*-(1-oxidopyridin-1-ium-4-yl)-4-carbaldoxime was converted into the corresponding aldoximate anion, which was reacted with diethyl bromomethyl malonate. The synthesis was performed in ethanol at −63 °C in order to avoid *O*-alkylation reaction. After recrystallisation, DEEPyON was obtained in 60% yield and in high purity.

A series of free radicals was trapped by DEEPyON in aqueous media with the aim of evaluating its potential in the detection of these transient species. Throughout this text, the aminoxyl formed by trapping a radical R• by a nitrone N will be noted N-R, in order to simplify the notation (see Scheme 2). All the DEEPyON spin adducts gave six line EPR spectra, because of hyperfine splittings with nitrogen and β-hydrogen nuclei. Their EPR parameters, determined by computer simulation of the spectra, have been reported in Table 1. Just like other linear nitrones, DEEPyON was found to trap efficiently carbon-centred radicals. Less intense EPR spectra were also recorded when superoxide, $CH_3O^{\bullet}$ and $HSO_3^{\bullet}$ free radicals were produced in the presence of DEEPyON. As an example, the spectrum of DEEPyON–O_2H obtained in pH 7.2 buffer is shown in Fig. 1. It should be mentioned here that this spectrum was obtained after noise reduction using the SVD procedure, according to a method described previously.[12] Note that a more intense EPR spectrum was obtained when EPPyON, the corresponding monoester-nitrone, was used to trap superoxide in the same medium.[11] From a qualitative point of view, it seems then that the presence of a second ethoxycarbonyl group does not enhance nitrone performance in the superoxide detection.

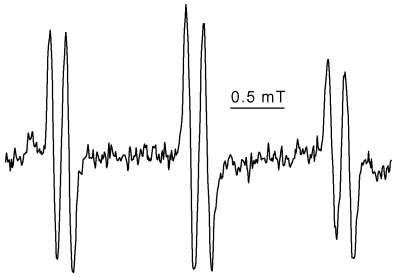


Fig. 1 EPR signal of the spin adduct DEEPyON–O_2H, obtained in a pH 7.2 buffer by generating superoxide with a xanthine–xanthine oxidase system in the presence of 0.02 mol dm^{-3} DEEPyON. The instrument settings were as follows: non-saturating microwave power, 20 mW; scan time, 5.24 s; time constant, 10.24 ms; receiver gain, 1.42 10^6; modulation amplitude, 0.1 mT.

Table 1 EPR hyperfine coupling constants for spin adducts of DEEPyON in aqueous media (tridistilled water or 0.1 mol dm^{-3} phosphate buffer, pH 7.2)

Spin adduct	a_N /mT	a_H/mT
DEEPyON–H[a]	1.56	1.08 *(2H)*
DEEPyON–CH_3[b]	1.44	0.24
DEEPyON–CH_2OH[b]	1.42	0.24
DEEPyON–CO_2H[b]	1.43	0.26
DEEPyON–C[b]	1.43	0.24
DEEPyON–OH[a]	1.40	0.19
DEEPyON–OSO_2H[b]	1.34	0.13
DEEPyON–O_2H[b]	1.29	0.15
DEEPyON–OCH_3[b]	1.34	0.13

[a] Tridistilled water. [b] pH 7.2.

Attempts to trap hydroxyl radicals with DEEPyON always failed. In accordance with previous results given by EPPN, EPPyON and DEEPN, only the signal of a carbon-centred adduct, denoted DEEPyON–C, was thus observed. However, the aminoxyl DEEPyON–OH was obtained by nucleophilic addition of water in the presence of ferric ions, which permitted the determination of its EPR parameters. Lastly, the aminoxyl DEEPyON–H was formed by incubation of DEEPyON in a $NaBH_4$ aqueous solution followed by an autoxidation, and its EPR parameters have also been reported in Table 1.

Kinetics of superoxide spin adduct formation and decay

Does the presence of a second alkoxy-carbonyl group in the β-position modify the nitrone performances in the detection of superoxide? With the aim of answering this question, kinetic studies were undertaken using the diester-nitrones DEEPyON, DEEPN and DEPO (for structures, see Scheme 1) in order to evaluate the rate constants for superoxide trapping, k_t, and superoxide adduct decay, k_d. A survey of the literature revealed major disagreements regarding the kinetics of superoxide trapping by nitrones.[3b,5a,12–14] Clearly, two categories of studies can be distinguished in this field. In the first one, the method used involves a competition towards superoxide between the nitrone of interest and a scavenger.[3b,5a,14] The second one is made up of studies based on a competition between superoxide trapping by the nitrone and spontaneous dismutation of this radical.[12,13] The model used in the first method implies that the superoxide spontaneous dismutation, the spin adduct decay and the consumption of the competitor during the course of the experiment are negligible events. In a recent paper, we brought evidence of the importance of these unduly neglected reactions, and proved that their omission generated significant overestimation of rate constants for superoxide trapping by nitrones.[15]

The kinetic method used in the present work, based on a competition between the superoxide trapping and its spontaneous dismutation at pH 7.2, has been extensively explained elsewhere and will not be reiterated here.[12] Worth mentioning is that it only refers to the rate constants for superoxide spontaneous dismutation, k_{dis}, and does not necessitate the calibration of the superoxide source. It permits the consideration of the whole kinetic curve of superoxide adduct formation and decay. EPR spectra of the nitrone–superoxide adduct, N–O_2H, were recorded as a function of time at various nitrone concentrations, in the presence of an internal reference. Using both singular value decomposition and pseudo-inverse deconvolution methods, kinetic curves indicating the time-dependent changes in the N–O_2H concentration were achieved. Their modelling with the help of a home-made computer program permitted the evaluation of k_t and k_d, the rate constants for superoxide trapping by the nitrone N and for the decay of the spin adduct N–O_2H formed, respectively. This kinetic approach was applied to the three diester-nitrones DEEPyON, DEEPN and DEPO. The results obtained have been reported in Table 2, along with data previously determined with the monoester-nitrones EMPO,

Table 2 Rate constants for the spin trapping of superoxide by nitrones (k_t) and for the decay of nitrone–superoxide spin adducts (k_d), at pH 7.2 and at various nitrone concentrations ([N]).

Nitrone	k_t/dm^3 mol^{-1} s^{-1}	[N]/mmol dm^{-3}	k_d/10^{-3} s^{-1}
DEEPyON[a]	0.16	90	9.5
		60	5.6
		30	3.3
EPPyON[b]	0.33	80	6.9
		50	4.8
DEEPN [ac]	—	10	0.73
EPPN[b]	0.02	50	2.4
		20	2.1
DEPO[a]	31.1	15	2.1
		10	1.3
		5	1.0
EMPO[b]	10.9	200	1.25
		30	0.65
		10	0.6
BocMPO[a]	3.45	175	0.86
		50	0.81
		20	0.68

[a] This work. [b] From ref. 12. [c] From ref. 11.

EPPN and EPPyON using the same procedure.[12] Since the disparity in the results given in literature for the superoxide trapping kinetics using BocMPO is noteworthy,[13,14] the rate constants k_t and k_d were also determined in the present work for this nitrone. As an example, experimental and calculated kinetic curves obtained by trapping superoxide using DEPO at pH 7.2 have been represented in Fig. 2. It is important to specify here that these k_t values are pH dependent apparent rate constants, which includes the contribution of both $O_2^{-\bullet}$ and $HO_2^{\bullet}$ trapping.

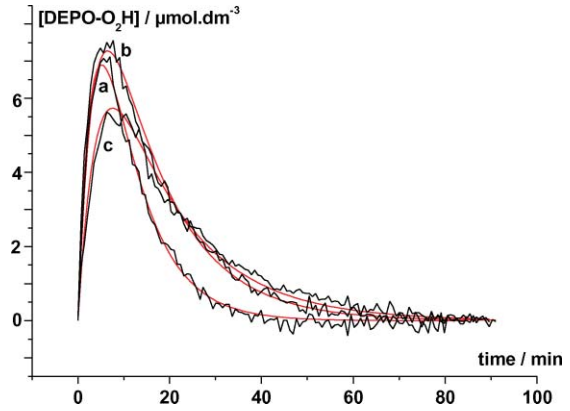


Fig. 2 Experimental (black) and calculated (red) kinetic curves indicating the time-dependent changes in the spin adduct DEPO–O_2H concentration. The spin adduct was produced at pH 7.2 by generating superoxide in the presence of (a) 0.015 mol dm^{-3} DEPO, (b) 0.01 mol dm^{-3} DEPO and (c) 0.005 mol dm^{-3} DEPO. Calculated curves, obtained from computer simulation using the kinetic model given in the experimental section, led to the following kinetic parameters: second-order rate constant for the trapping reaction, $k_t = 31.1$ dm^3 mol^{-1} s^{-1}; first-order rate constant for the adduct decay reaction, (a) $k_d = 2.1\ 10^{-3}$ s^{-1}, (b) $k_d = 1.3\ 10^{-3}$ s^{-1}, (c) $k_d = 10^{-3}$ s^{-1}.

As mentioned in a previous paper,[12] these results confirm that the superoxide spin adduct decay increases with the nitrone concentration, whatever the nitrone, and that cyclic nitrones are incomparably better at trapping superoxide than linear spin traps. This general trend has already been observed previously,[3c,12,16] and this work seems to confirm that efforts to elaborate a more efficient PBN–nitrone for superoxide detection in aqueous media are doomed to failure. Note however that this kind of spin trap also presents important advantages for *in vivo* applications, such as a lipophilicity and a biodistribution easily modulated. But all the results obtained with any of the various linear nitrones represented in Scheme 1 indicate that these traps are essentially useful to detect carbon-centred radicals. Attempts to determine k_t for DEEPN were never successful. Since the adduct DEEPN–O_2H does not decay particularly rapidly (see k_d in Table 2), this failure is probably due to a very slow superoxide trapping reaction, as well as poor water solubility. Comparing results given by DEEPyON and EPPyON confirms that the presence of a second withdrawing ethoxycarbonyl group in the β-position to the nitrone function is harmful to superoxide trapping in this series: it resulted in significantly increasing the adduct decay rate, while the superoxide trapping was found to be twice as slow.

Things are very different for pyrrolidinic nitrones. As can be seen in Table 2, EMPO was found to be superior to BocMPO for detecting superoxide: its rate constant for the trapping reaction, k_t, was more than three times higher, while both superoxide adducts showed more or less the same stability at pH 7.2. Note however that the efficiency of BocMPO at trapping superoxide is very similar to that of DEPMPO,[12] while the former is more easily prepared at high purity and gives adducts showing EPR spectra with half the lines. In the cyclic nitrone series, the second ester group clearly induced an acceleration of the trapping reaction. Thus, DEPO trapped superoxide *ca.* three times faster than EMPO, its monoester analogue. In a recent study, Karoui *et al.*[10] have shown that one ethoxycarbonyl group of DEPO was equatorial, thereby facilitating the approach of the attacking radical. Note also that DEPO shows two enantiotopic faces, while the faces of EMPO, BocMPO or DEPMPO are diastereotopic. Thus, the faster superoxide trapping by DEPO could be explained by these steric effects. On the other hand, DEPO–O_2H decayed much faster than EMPO–O_2H. According to Olive *et al.*,[17] replacing the methyl group of DEPMPO by a second diethoxyphosphoryl group also resulted in increasing the superoxide adduct decay. All these results clearly point to the fact that the presence of two identical electron withdrawing groups in the β-position to the nitrone function accelerate the adduct decay, though introducing one group only enhances the superoxide adduct stability. When the nitrone concentration was set to 0.01 mol dm^{-3}, DEPO–O_2H decayed *ca.* twice as fast as EMPO-O_2H. This effect is even more marked at higher nitrone concentration. For example, the half-life ($t_{1/2}$) was estimated to be *ca.* 5.5 min for DEPO–O_2H when the nitrone concentration was only 0.015 mol dm^{-3}, while EMPO–O_2H showed a half-life of 9.5 min when a much higher nitrone concentration of 0.2 mol dm^{-3} was used. When experiments were conducted using DEPO concentrations over 0.05 mol dm^{-3}, the spectra recorded showed mainly the presence of the hydroxyl radical adduct DEPO–OH, which, according to Karoui *et al.*,[10] could be formed after the decomposition of DEPO–O_2H. It follows from these observations that the nitrone DEPO is a more efficient superoxide detector at very low concentration.

Conclusion

The different results presented herein clearly show that the use of PBN-type nitrones in superoxide detection should be avoided, though these compounds remain efficient at trapping carbon-centred radicals. In this series, the presence of a second ester group yielded a decrease in the superoxide trapping rate, while an opposite effect was observed with cyclic nitrones: DEPO trapped superoxide *ca.* three and nine times faster than EMPO and BocMPO, respectively. On the other hand, DEPO–O_2H decayed much faster than EMPO–O_2H, particularly at high nitrone concentration. Consequently, it is not easy to determine which is the best trap for superoxide detection. At a concentration lower than 0.005 mol dm^{-3}, DEPO is obviously the only nitrone described to date capable of clearly detecting superoxide. Above, EMPO should be preferred, since DEPO–O_2H decay was found to increase greatly with the nitrone concentration.

Experimental

All chemicals were purchased from ACROS or Sigma-Aldrich Chemical Companies. The enzymes were obtained from the Boerhinger Mannheim Biochemica Company. Aqueous media were prepared from tridistilled water and buffers were stirred for 6 h in the presence of a chelating iminodiacetic acid resin (40 g dm^{-3}) to remove trace metal impurities. ^{1}H and ^{13}C NMR spectra were recorded on Bruker instruments. The chemical shifts (δ) in ppm are reported with respect to internal TMS and J values are given in Hz. The nitrones BocMPO,[6c] DEPO,[10] and DEEPN,[11] were synthesised, purified and identified in our laboratory according to procedures described previously.

Synthesis of DEEPyON

Z-(1-oxidopyridin-1-ium-4-yl)-4-carbaldoxime was prepared beforehand from 4-methylpyridine *N*-oxide, following the method of Schnekenburger.[18] Then, a synthetic route analogous to that used to prepare DEEPN was followed.[11] A solution containing Na (20 mmol) and *Z*-(1-oxidopyridin-1-ium-4-yl)-4-carbaldoxime (20 mmol) was prepared in absolute ethanol (90 cm^3) and was cooled down in a liquid air–chloroform bath. An ethanolic solution of diethyl bromomethylmalonate (20 mmol in 90 cm^3 of absolute ethanol) was added to this mixture, the medium was kept for 3 h at −63 °C, and then slowly brought back to room temperature. After evaporation of the solvent under reduced pressure, the nitrone was extracted with benzene from the solid obtained with the help of a Soxhlet apparatus. Evaporation of the solvent and recrystallisation from benzene–hexane (1 : 1; vol : vol) yielded crystals (3.9 g, 60%); mp 105 °C. Elemental analysis calculated for $C_{14}H_{18}N_2O_6 \cdot 0.25\ H_2O$ (314.5): C, 54.19; H, 5.85; N, 9.04; found: C, 53.73; H, 5.87; N, 9.45%. δ_H ($CDCl_3$, 200.13 MHz) 1.33 (6H, t, J 7.2, OCH_2–CH_3), 2.05 (3 H, s, CH_3), 4.34 (4H, q, J 7.2, OCH_2), 7.66 (1H, s, N=C*H*), 8.16 (4H, s, *aromatic H*); δ_c ($CDCl_3$, 75.47 MHz) 14.25 (2C, OCH_2–CH_3), 21.35 (1C, CH_3), 63.77 (2C, OCH_2), 83.99 (1C, C–CH_3), 125.39 (2C, *aromatic C*), 127.52 (1C, *aromatic C*), 131.79 (1C, HC=N), 139.49 (2C, *aromatic C*), 166.20 (2C, C=O).

Reduction of DEEPyON by $NaBH_4$

Reduction of DEEPyON (30 mmol dm^{-3}) by $NaBH_4$ (30 mmol dm^{-3}) was performed in tridistilled water. Autoxidation of the hydroxylamine thus formed gave the corresponding aminoxyl radical DEEPyON–H.

Obtention of the aminoxyl DEEPyON–OH

The aminoxyl radical DEEPyON–OH was generated by nucleophilic addition of water in the presence of $FeCl_3$ (1 mmol dm^{-3}) and DEEPyON (10 mmol dm^{-3}).

Spin trapping of free radicals by DEEPyON

All the experiments were conducted in 0.1 mol dm^{-3} phosphate buffer, pH 7.2, in the presence of 10 mmol dm^{-3} DEEPyON, unless otherwise stated. Hydroxyl radical $HO^{\bullet}$ was produced by a standard Fenton system (0.2% H_2O_2, 2 mmol dm^{-3} ethylenediaminetetracetic acid, and 1 mmol dm^{-3} $FeSO_4$). The radicals $^{\bullet}CH_3$, $^{\bullet}CH_2OH$, $^{\bullet}CO_2H$, and $HSO_3^{\bullet}$ were produced by performing a Fenton reaction (see conditions above) in the presence of dimethyl sulfoxide (30%), methanol (20%), sodium formate (0.2 mol dm^{-3}), or Na_2SO_3 (30 mmol dm^{-3}), respectively. The methoxyl radical was produced by heating a 10 mmol dm^{-3} $K_2S_2O_8$ solution in phosphate buffer–methanol (80 : 20, vol : vol). The superoxide was produced by a xanthine–xanthine oxidase system (X–XO system: 0.8 mmol dm^{-3} xanthine, 1 mmol dm^{-3} diethylenetriaminepentacetic acid, 0.04 unit per cm^{-3} xanthine oxidase) in the presence of 20 mmol dm^{-3} DEEPyON. EPR assays were carried out at 20 °C in capillary tubes by using a Bruker EMX spectrometer operating at X-band with 100 kHz modulation frequency. In the case of the EPR spectra of the superoxide adduct, the singular value decomposition method described in a previous paper was applied to reduce the noise.[12] For the various adducts, hyperfine coupling constants were evaluated by computer simulation of experimental EPR spectra.

Achievement of experimental kinetic curves

All measures were made at pH 7.2 in 0.1 mol dm^{-3} phosphate buffer and using a X–XO superoxide generator. In a standard experiment, the medium contained a nitrone N (concentration ranging from 5 to 175 mmol dm^{-3}), 0.8 mmol dm^{-3} xanthine, 1 mmol dm^{-3} diethylenetriaminepentacetic acid, 3-carboxy-2,2,5,5-tetramethylpyrrolidin-1-oxyl (3CP, 0.5–1.1 μmol dm^{-3}) used as internal standard, and 0.04 unit per cm^{-3} xanthine oxidase. Air was bubbled into the medium for one min before addition of xanthine oxidase. A part of the EPR signal showing at least one line of the superoxide adduct N–O_2H and one line of 3CP spectra was recorded every 42 s for at least 90 min. Noise was then reduced using the SVD procedure, and the kinetic curves giving the adduct concentration [N–O_2H] *vs*. time were obtained after deconvolution of the signal using the pseudo-inverse method.[12] Three experimental kinetic curves were thus obtained for each nitrone. Considering that DEPO–O_2H decayed too rapidly at high nitrone concentration, it was not possible to perform an experiment with an amount of DEPO high enough to trap out all the superoxide produced. In this case, the procedure was modified as described previously.[12] The recording of the curve at the highest nitrone concentration, whose shape does not depend on the rate constants k_t and k_{dis}, was achieved using 200 mmol dm^{-3} DEPMPO instead of DEPO. The same procedure modification was employed in the case of DEEPyON because of the very low rate of superoxide trapping by this nitrone.

Determination of kinetic parameters

The kinetic model considered can be described by eqns. (1)–(4), in which k_x, k_{dis}, k_t and k_d are the rate constants for the apparent first-order production of superoxide, for the second-order spontaneous dismutation of superoxide, for the second-order trapping of superoxide by the nitrone N, and for the first-order decay of the spin adduct N–O_2H, respectively, Y representing EPR silent products, and X an intermediate derived from xanthine.[12]

$$X \xrightarrow{k_x} O_2^{\bullet -} \quad (1)$$

$$2\,O_2^{\bullet -} + 2H^+ \xrightarrow{k_{dis}} O_2 + H_2O_2 \quad (2)$$

$$O_2^{\bullet -} + N + H^+ \xrightarrow{k_t} N\text{-}O_2H \quad (3)$$

$$N\text{-}O_2H \xrightarrow{k_d} Y \quad (4)$$

The rate eqns. (5)–(8) can be written from these reactions.

$$d[X]/dt = -k_x\,[X] \quad (5)$$

$$d[O_2^{-\bullet}]/dt = k_x\,[X] - k_t\,[N][O_2^{-\bullet}] - 2\,k_{dis}\,[O_2^{-\bullet}]^2 \quad (6)$$

$$d[N\text{–}O_2H]/dt = k_t\,[N][O_2^{-\bullet}] - k_d\,[N\text{–}O_2H] \quad (7)$$

$$d[N]/dt = -k_t\,[N][O_2^{-\bullet}] \quad (8)$$

Computer modelling of the kinetic curves obtained was achieved using the computer program Kalidaphnis and according to eqns. (5)–(8). At pH 7.2, the value of the apparent rate

constant k_{dis} is 4.03 10^5 dm^3 mol^{-1} s^{-1},[19] the nitrone concentration is an experimental parameter, and the initial concentrations of superoxide and of N–O_2H are equal to zero. The standard least-square method was applied to fit the experimental curves, yielding the other parameters, *i.e.* initial concentration $[X]_0$, k_x, k_t and k_d. As mentioned in our previous paper, the values obtained for the concentration $[X]_0$ and for the rate constant k_x only came up as an empirical model and had no real meaning.[12] Since they might vary with the solutions of either xanthine or of xanthine oxidase used, experiments at various nitrone concentrations were performed with exactly the same superoxide generator.

References

1 (*a*) C. A. Evans, *Aldrichimica Acta*, 1979, **12**, 23–29; (*b*) M. J. Perkins, *Adv. Phys. Org. Chem.*, 1980, **17**, 1–64; (*c*) G. R. Buettner, *Free Radical Biol. Med.*, 1987, **3**, 259–303; (*d*) C. Mottley and R. P. Mason, *Biol. Magn. Reson.*, 1989, **8**, 489–546; (*e*) S. Pou, H. J. Halpern and G. M. Rosen, *Acc. Chem. Res.*, 1999, **32**, 155–161.

2 (*a*) J. R. Harbour, V. Chow and J. R. Bolton, *Can. J. Chem.*, 1974, **52**, 3549–3553; (*b*) J. R. Harbour and J. R. Bolton, *Biochem. Biophys. Res. Commun.*, 1975, **64**, 803–807.

3 (*a*) G. R. Buettner and L. W. Oberley, *Biochem. Biophys. Res. Commun.*, 1978, **83**, 69–74; (*b*) E. Finkelstein, G. M. Rosen and E. J. Rauckman, *J. Am. Chem. Soc.*, 1980, **102,**, 4994–4999; (*c*) E. Finkelstein, G. M. Rosen, E. J. Rauckman and J. Paxton, *Mol. Pharmacol.*, 1979, **16**, 676–685; (*d*) S. Pou and G. M. Rosen, *Anal. Biochem.*, 1990, **190**, 321–325; (*e*) R. Konaka, M. Kawai, H. Noda, M. Kohno and R. Niwa, *Free Radical Res.*, 1995, **23**, 15–25.

4 C. Fréjaville, H. Karoui, B. Tuccio, F. Le Moigne, M. Culcasi, S. Pietri, R. Lauricella and P. Tordo, *J. Chem. Soc., Chem. Commun.*, 1994, 1793–1794.

5 (*a*) C. Fréjaville, H. Karoui, B. Tuccio, F. Le Moigne, M. Culcasi, S. Pietri, R. Lauricella and P. Tordo, *J. Med. Chem.*, 1995, **38**, 258–265; (*b*) B. Tuccio, R. Lauricella, C. Fréjaville, J. C. Bouteiller and P. Tordo, *J. Chem. Soc., Perkin Trans. 2*, 1995, 295–298; (*c*) S. Barbati, J. L. Clément, G. Olive, V. Roubaud, B. Tuccio and P. Tordo, in *Free Radicals in Biology and Environment, NATO ASI Series, Series A: Life Sciences*, ed. F. Minisci, Kluwer Academic Publishers, Dordrecht, 1997, vol. 27, p. 39.

6 (*a*) G. Olive, A. Mercier, F. Le Moigne, A. Rockenbauer and P. Tordo, *Free Radical Biol. Med.*, 2000, **28**, 403–408; (*b*) H. Zhang, J. Joseph, J. Vasquez-Vivar, H. Karoui, C. Nzanzumuhire, P. Martásek, P. Tordo and B. Kalyanaraman, *FEBS Lett.*, 2000, **473**, 58–62; (*c*) H. Zhao, J. Joseph, H. Zhang, H. Karoui and B. Kalyanaraman, *Free Radical Biol. Med.*, 2001, **31**, 599–606; (*d*) K. Stolze, N. Udilova and H. Nohl, *Biol. Chem.*, 2002, **383**, 813–820; (*e*) K. Stolze, N. Udilova, T. Rosenau, A. Hofinger and H. Nohl, *Biol. Chem.*, 2003, **384**, 493–500; (*f*) K. Stolze, N. Udilova, T. Rosenau, A. Hofinger and H. Nohl, *Biochem. Pharmacol.*, 2005, **69**, 297–305.

7 (*a*) E. G. Janzen, D. E. Nutter, E. R. Davis, B. J. Blackburn, J. L. Poyer and P. B. McCay, *Can. J. Chem.*, 1978, **56**, 2237–2242; (*b*) E. G. Janzen, Y. Y. Wang and R. V. Shetty, *J. Am. Chem. Soc.*, 1978, **100**, 2923–2925.

8 (*a*) A. Zeghdaoui, B. Tuccio, J. P. Finet, V. Cerri and P. Tordo, *J. Chem. Soc., Perkin Trans. 2*, 1995, 2087–2089; (*b*) V. Roubaud, R. Lauricella, B. Tuccio, J. C. Bouteiller and P. Tordo, *Res. Chem. Intermed.*, 1996, **22**, 405–416; (*c*) C. Rizzi, S. Marque, F. Belin, J. C. Bouteiller, R. Lauricella, B. Tuccio and P. Tordo, *J. Chem. Soc., Perkin Trans. 2*, 1997, 2513–2518.

9 (*a*) V. Roubaud, R. Lauricella, J. C. Bouteiller and B. Tuccio, *Arch. Biochem. Biophys.*, 2002, **397**, 51–56; (*b*) K. Stolze, N. Udilova, T. Rosenau, A. Hofinger and H. Nohl, *Biochem. Pharmacol.*, 2004, **68**, 185–194.

10 H. Karoui, J. L. Clément, A. Rockenbauer, D. Siri and P. Tordo, *Tetrahedron Lett.*, 2004, **45**, 149–152.

11 A. Allouch, V. Roubaud, R. Lauricella, J. C. Bouteiller and B. Tuccio, *Org. Biomol. Chem.*, 2003, **1**, 593–598.

12 R. Lauricella, A. Allouch, V. Roubaud, J. C. Bouteiller and B. Tuccio, *Org. Biomol. Chem.*, 2004, **2**, 1304–1309.

13 (*a*) I. Yamazaki, L. H. Piette and T. A. Grover, *J. Biol. Chem.*, 1990, **265**, 652–659; (*b*) A. Keszler, B. Kalyanaraman and N. Hogg, *Free Radical Biol. Med.*, 2003, **35**, 1149–1157.

14 (*a*) F. A. Villamena and J. Zweier, *J. Chem. Soc., Perkin Trans. 2*, 2002, 1340–1344; (*b*) P Tsai, K. Ichikawa, C. Mailer, S. Pou, H. J. Halpern, B. Robinson, R. Nielsen and G. M. Rosen, *J. Org. Chem.*, 2003, **68**, 7811–7817; (*c*) S. Goldstein, G. M. Rosen, A. Russo and A. Samuni, *J. Phys. Chem. A*, 2004, **108**, 6679–6685; (*d*) J. Weaver, P. Tsai, S. Pou and G. M. Rosen, *J. Org. Chem.*, 2004, **69**, 8423–8428.

15 R. Lauricella, J. C. Bouteiller and B. Tuccio, *Phys. Chem. Chem. Phys.*, 2005, **7**, 399–404.

16 N. Gotoh and E. Niki, *Chem. Lett.*, 1990, 1475–1478.

17 G. Olive, F. Le Moigne, A. Mercier, A. Rockenbauer and P. Tordo, *J. Org. Chem.*, 1998, **63**, 9095–9099.

18 J. Schnekenburger, *Arch. Pharm.*, 1969, **302**, 494–504.

19 D. Behar, G. Czapski, J. Rabani, L. M. Dorfman and H. A. Schwarz, *J. Phys. Chem.*, 1970, **74**, 3209–3213.