

Spin trapping of nitrogen dioxide and of radicals generated from nitrous acid

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Abstract

Spin trapping of nitrogen dioxide radical by several nitrones has been studied. The reaction results in the formation of persistent acyl nitroxides, after the oxidation of the intermediate spin adducts having an —ONO group on C-2 atom. The intermediate is effectively detected when DEPMPO is used as the spin trap. The reaction between PBN or 5,7-di-*tert*-butyl-3,3-dimethyl indoline *N*-oxide with nitrous acid gives the corresponding acyl nitroxide only when oxygen is present in the reaction milieu.

On the other hand, nitroso spin traps do not trap NO_2 confirming that the unpaired electron of nitrogen dioxide is localized on the oxygen atom.

Keywords: *Nitrogen dioxide, spin traps, acyl nitroxides, nitrous acid, nitrogen monoxide*

Introduction

The spin trapping technique [1,2] was developed in the late 1960s to facilitate the detection of reactive free radicals by electron paramagnetic resonance (EPR) spectroscopy. This method involves the addition of a diamagnetic molecule (the spin trap), typically an organic nitron or nitroso compound, to a system containing or producing a reactive radical. The trap reacts with the free radical to give a stable paramagnetic spin adduct which accumulates until it becomes observable by EPR.

Spin trapping is considered one of the best techniques available for characterizing the presence and the nature of reactive radicals and, in some cases, it is particularly useful for the study of a reaction mechanism involving radical intermediates [3]. But, its use in the detection of radicals such as

nitrogen monoxide and nitrogen dioxide (and eventually, in the monitoring of NO_x production in *in vitro* and *in vivo* systems) is not easy and requires special traps. In fact, attempts to detect nitric oxide in solution using the conventional spin traps (nitrones and nitroso compounds) have been unsuccessful: only some nitroso spin traps (MNP, DBNBS) were shown to be able to trap nitrogen monoxide, but the results obtained were subject to many artifacts. [4] Better results have been obtained with more complex traps such as biradicals [5], chelotropic traps [6,7], nitronyl nitroxides [8] or iron dithiocarbamates [9,10].

Very few reports on nitrogen dioxide detection by spin trapping technique can be found in the literature and these describe the trapping by nitromethane (at very alkaline pH values) or by dimethylsulfoxide of nitrogen dioxide obtained from the photochemical

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decomposition of nitramines [11,12]. Difficulties are encountered when trying to detect NO_2 in biological systems because other radicals may be trapped. In fact, in most experiments it derives from the hemolytic decomposition of peroxyntrous acid ($\text{HOONO} \rightarrow \text{NO}_2 + \text{OH}$) and the spin adduct usually observed is the one with the OH radical [13,14].

Indeed, the results reported in the present paper show that nitrogen dioxide can be trapped by various spin traps and, in all cases, stable acyl nitroxides are formed. The experiments described here also give useful information on the mechanism by which nitrogen dioxide reacts.

Materials and methods

Gaseous nitrogen dioxide was purchased, in the dimeric form N_2O_4 , from Fluka. Solutions were prepared volumetrically by condensing the gas and dissolving 50 μl of the liquid thus obtained in the previously degassed chosen solvent to a final volume of 5 ml (2.85×10^{-3} moles, 0.57 M).

PBN, DMPO, TMPO, nitrosobenzene, MNP and sodium nitrite were Sigma–Aldrich products. DEPMPO was kindly prepared and donated by Professor P. Tordo from the University of Marseille (France) according to the method described in the literature [15]. Nitrones INDO1 and INDO2 and 5,7-di-*tert*-butyl-1-hydroxy-3,3-dimethyl-1,3-dihydroindol-2-one (INDO1-H) were kindly prepared and donated by Professor D. Döpp from the University of Duisburg (Germany) according to the literature reports [16,17]. Acetic acid, benzene and dichloromethane were Sigma–Aldrich RP-ACS grade.

EPR spectra were recorded on a Bruker EMX X-band EPR spectrometer equipped with an XL Microwave frequency counter, Model 3120 for the determination of g -factors. The spectra were recorded with the following instrumental settings: modulation frequency 100 kHz, modulation amplitude 0.5, sweep width 100 or 40 G according to the particular spin adduct detected, microwave power 5 mW, time constant 0.6 s, receiver gain 5×10^3 . Computer simulation of EPR spectra were calculated using the WinSim program in the NIEHS public ESR software tools package (www.epr.niehs.nih.gov/) [18].

Spin trapping of nitrogen dioxide

The spin trapping experiments were carried out in an inverted U cell as described in the literature [19]. A 10^{-2} M benzene solution of the trap was prepared and 1 ml of this solution (10^{-5} moles) was placed in one leg of the U cell while 982 μl of benzene were placed in the other leg and the system was accurately degassed with argon. Benzene solution, 18 μl , of nitrogen dioxide (10^{-5} moles) were injected using a syringe into the leg containing benzene only. The two

solutions were mixed and transferred into the aqueous cell and placed in the EPR cavity. The EPR signal was immediately recorded and its evolution in time was followed. Different PBN/ NO_2 ratios were used following the same procedure, as above. Spin trapping experiments were repeated using dichloromethane instead of benzene. Small differences in the hyperfine coupling constants and in the g -factors of the acyl nitroxides were observed, as shown in the table, due to the different polarity of the two solvents. No EPR signal was obtained when nitroso spin traps were used.

Spin trapping of RNOS derived from the decomposition of nitrous acid

One milliliter of a 10^{-2} M benzene solution of the trap (PBN or INDO1) together with some drops of acetic acid were placed in one leg of the U cell; 4 mg of sodium nitrite in 1 ml of benzene were placed in the other leg. The two solutions were accurately degassed with argon, mixed together, transferred in the aqueous cell and placed in the EPR cavity. No EPR signal was observed. The same solution was exposed to air and in a few minutes, intense and well resolved signals were detected

Efficiency of NO_2 trapping by INDO1

A 0.7 mM benzene solution of INDO1-H was prepared. Different amounts of this solution were routinely taken, degassed with argon, oxidized with lead dioxide and spectra recorded in a final volume of 2 ml. In this way known concentrations of INDO1 ranging from 8.7×10^{-6} to 3.5×10^{-4} M were obtained. Intensities of the central nitrogen line of the spectra were plotted against the concentration of acyl nitroxide thus obtaining a calibration curve for nitroxide 5. The value of the slope of the straight line was the mean of three different experiments. INDO1 was reacted with nitrogen dioxide according to the procedure described above and, using the calibration curve, the concentration of 5 formed in the reaction could be deduced from the intensity of the central line of the recorded spectrum.

Macroscale reaction between INDO1 and nitrogen dioxide

INDO1 (2.5×10^{-5} moles, 7 mg) was dissolved in 2 ml of benzene in a round flask and the solution was degassed. The proper volume of a 0.57 M NO_2 benzene solution (prepared as described above) was added in order to have a 1:1 or 1:0.5 INDO1/ NO_2 ratio. Aliquots of 50 μl of both the reaction mixtures were taken, diluted to a final volume of 1 ml with benzene and degassed with argon. EPR spectra were recorded on both samples which showed the presence of acyl nitroxide 5; the intensity of the signal was proportional to the quantity of NO_2 added.

After 30 min the starting nitron was completely consumed in both mixtures as monitored by thin layer chromatography (TLC). Preparative TLC eluting with Cyclohexane/Ethyl Acetate (8:2) allowed the separation of two different products, namely 5,7-di-*tert*-butyl-3,3-dimethyl-1,3-dihydro-indol-2-one **8** and 5,7-di-*tert*-butyl-3,3-dimethyl-3*H*-indol **9** identified by comparison with authentic samples [16].

Results and discussion

The spin trapping technique was used in an attempt to detect the free radical nitrogen dioxide (NO_2) in solution. Six different nitron spin traps were examined (Figure 1), *N-tert*-butyl- α -phenylnitron (PBN), 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), 3,3,5,5-tetramethyl-1-pyrroline *N*-oxide (TMPO), 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO), 5,7-di-*tert*-butyl-3,3-dimethyl-3*H*-indole *N*-oxide (INDO1) and 6-*tert*-butyl-3,3-dimethyl-3*H*-indole *N*-oxide (INDO2). In all cases, when the spin trap solution was mixed with nitrogen dioxide dissolved in the same solvent, the formation of an acyl nitroxide radical **1–6** was observed (Figure 2). Hyperfine coupling constants and *g*-factors for nitroxides **1–6** are reported in the table and are in agreement with data found in the literature for the same compounds. In Figure 3 EPR spectra of acyl nitroxides **4** and **6**, obtained from DEPMPO and INDO2 respectively, are shown. The first step of the reaction is likely represented by the nucleophilic attack of the oxygen atom of an NO_2 molecule to the C-2 atom of the trap (Scheme 1), with formation of a C–O bond. The spin adduct thus formed is unstable and rapidly rearranges to acyl nitroxide with loss of HNO which decomposes to N_2O and water. The proposed mechanism is very similar to the one reported in the literature for the reaction between ClO_2 radical and nitron spin traps [24].

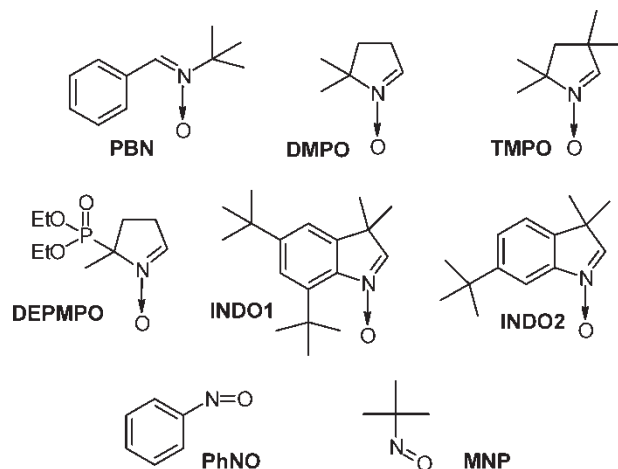


Figure 1. Chemical structures of nitron and nitroso spin traps used.

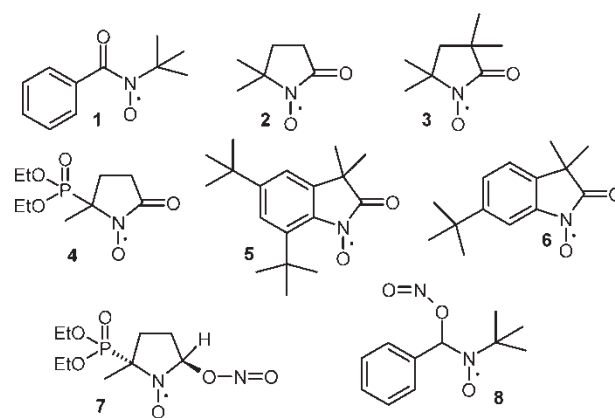


Figure 2. Chemical structure of acyl nitroxides and of the other spin adducts detected.

Different ratios PBN/ NO_2 were used and, as shown in Figure 4, an increase in the intensity of acyl nitroxide **1** proportional to the amount of nitrogen dioxide was found when these ratios varied from 1:0.25 to 1:1. The efficiency of the spin trapping was also evaluated using INDO1 as trap by comparing the peak height of the observed signal with that of known concentrations of acyl nitroxide **5**. The choice of this acyl nitroxide was determined by the availability of its reduced form which easily undergoes oxidation by lead dioxide. In this way, starting from different concentrations of INDO1-H, a calibration curve for **5** was plotted and from the slope of the straight line obtained the concentration of **5** derived from the reaction between INDO1 and NO_2 was determined. From this experiment, it was found that acyl nitroxide concentration was ca. 10% of the expected value if all INDO1 used was converted into the corresponding acylnitroxide. Although **5**, as the other acyl nitroxides, is a stable compound it may not be excluded that it further reacts giving different products which explains the low concentration of **5** detected in the EPR experiment. In fact, when the same reaction was performed on a macroscale level the starting nitron was completely consumed in a very short time. The EPR spectrum recorded on the reaction mixture immediately after NO_2 addition showed the presence of **5** whereas two products were isolated at the end of the reaction. In fact, preparative TLC of the reaction mixture afforded compounds **8** and **9** (Scheme 2). The formation of **8** could be due to deoxygenation of acyl nitroxide **5** following the reaction with another nitrogen dioxide molecule according to a mechanism similar to that reported in the literature for the reaction of aromatic nitroxides with thiyl radicals [25] or with nitrogen monoxide [26]. The formation of compound **9** is more difficult to explain, since it could likely derive from the intermediate spin adduct having the C–O–N bond after further reaction with NO_2 , but this is beyond the scope of the present discussion.

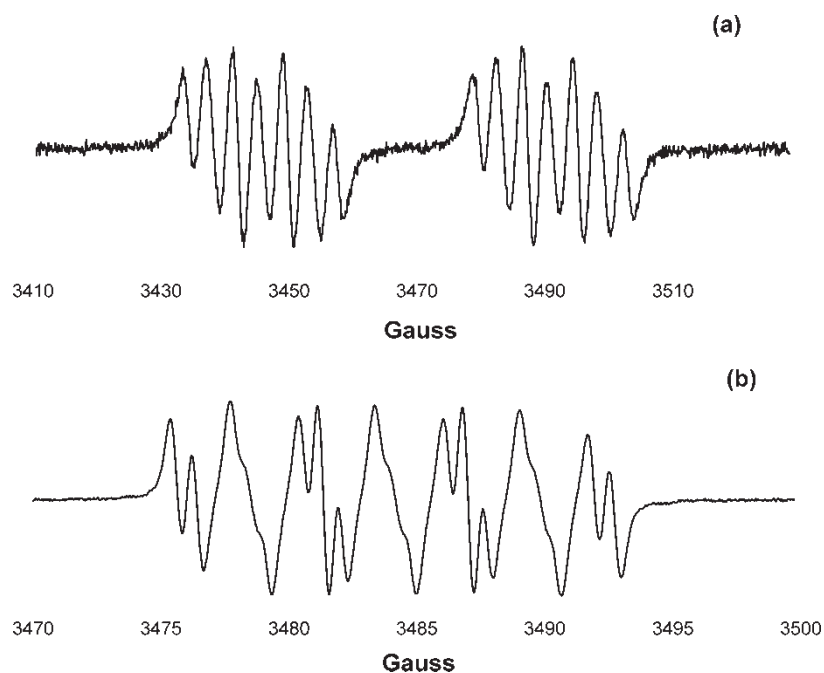
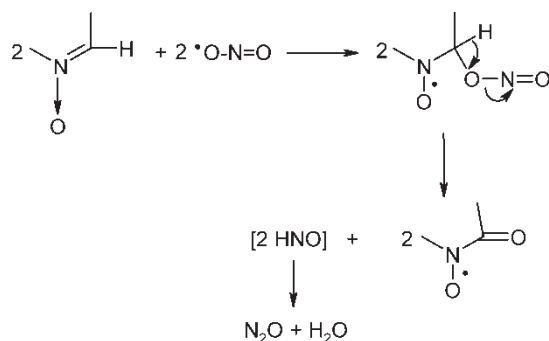


Figure 3. EPR spectra of acyl nitroxides 4 (a) and 6 (b) generated by the reaction of the spin traps DEPMPPO and INDO2 with nitrogen dioxide, respectively. The spectra were recorded after mixing benzene solutions of NO_2 (10^{-5} moles) and of the trap (10^{-5} moles) with the following instrumental settings: modulation frequency 100 kHz, modulation amplitude 0.5, sweep width 100 G for 4 and 40 G for 6, microwave power 5 mW, time constant 0.6 s, receiver gain 5×10^3 . Spectrum (a) was recorded after the disappearance of the intermediate spin adduct 7 (Figure 4).

In the case of DEPMPPO, the intermediate spin adduct 7 (Figure 2) was effectively detected and was stable enough to allow recording of the spectrum (Figure 5) before rearrangement to acyl nitroxide 4. Hyperfine coupling constants were measured and were confirmed by computer simulation. The EPR spectrum consists of two doublets ($a_{\text{P}} = 46.797$ G) of triplets ($a_{\text{N}} = 12.216$ and $a_{\text{H}} = 9.587$ G) with additional splitting on the γ -protons ($a_{\text{H}} = 0.466$ (2H); $a_{\text{H}} = 0.196$ (2H) G) and a g -factor of 2.0095₀. These values are consistent with the values found in the literature for spin adducts between DEPMPPO and oxygen-centered radicals [27]. The presence of two chiral centers in spin adduct 7 results in two different diastereomeric forms [27] with distinctive

EPR spectra. In this case, however, only one of the two diastereomers was detected, most likely the isomer with the phosphoryl and $-\text{ONO}$ groups in *trans* relationship. The attack of the oxygen atom on the trap and the formation of spin adduct 7 in the reaction with DEPMPPO and of similar intermediates, not detected with the other traps, is fully justified if the different resonance forms possible for nitrogen dioxide are considered. In fact, since the unpaired electron in NO_2 may be delocalized throughout the molecule, nitrogen dioxide may exist in four different mesomeric forms (Figure 6). The two structures which mainly contribute to stabilization of the resonance hybrid are those neutral ones having the unpaired electron on the oxygen atom (structures A and B) therefore they are most likely responsible for the formation of 7 and hence of acyl nitroxides. The existence of different resonance forms for nitrogen dioxide molecule suggests that it can react not only through the oxygen, but also through the nitrogen atom. This multiple reactivity was observed in the reaction between nitrogen dioxide and substituted phenols [28]. In fact, different products, i.e. nitrophenols or quinones, were obtained according to the particular mesomeric form involved in the reaction. In the present work, the spin adduct deriving from the reaction of NO_2 through its nitrogen atom was never observed. This could be justified by the higher nucleophilicity of the oxygen with respect to



Scheme 1. The proposed mechanism for acyl nitroxides formation in the spin trapping of nitrogen dioxide by nitrones.

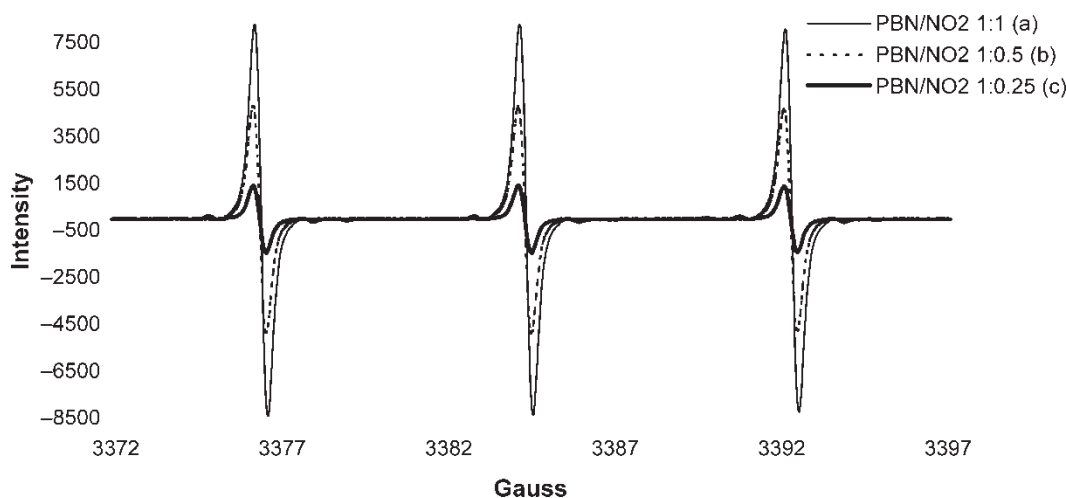


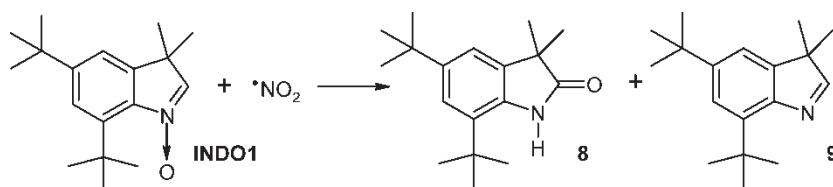
Figure 4. EPR spectra, in benzene, of acyl nitroxide **1** obtained at different NO_2 concentrations: 10^{-5} moles of PBN were used to trap 10^{-5} (a), 5×10^{-6} (b) and 2.5×10^{-6} (c) moles of nitrogen dioxide in the same volume of 2 ml.

the nitrogen atom and hence the attack of the oxygen with the formation of a C–O bond in the spin adduct is favored.

Nitroso spin traps were also used to trap nitrogen dioxide, without any success. In fact either with nitrosobenzene PhNO or MNP (Figure 1) no EPR signal was recorded. This fact confirms that when the NO_2 radical is trapped the unpaired electron is localized on the oxygen atom, because the nitroso spin trap cannot form a stable spin-adduct with an oxygen-centered radical whose the oxygen is bound to another electronegative atom, such as nitrogen.

To the best of our knowledge, no evidence for acyl nitroxide formation in the spin trapping of nitrogen dioxide has been reported in the literature. Only Shi et al. describe the formation of an acyl nitroxide during the decomposition of peroxyxynitrite in the presence of DMPO [29]. But as reported in that paper, DMPO was directly oxidized to acyl nitroxide by peroxyxynitrite with no participation of nitrogen dioxide or of other radicals. In our case, oxidation of the trap by nitrogen dioxide ($E = 0.99\text{ V}$ in water [30]), has to be excluded since NO_2 is not able to oxidize the various traps used [31,32]. In addition, an intermediate is formed and also detected, although only in the case of DEPMPO deriving from the nucleophilic attack of $\text{O}=\text{N}=\text{O}$ to the nitron. The intermediate nitroxide thus obtained is eventually oxidized to acyl nitroxide.

In our experiments, gaseous NO_2 dissolved in the chosen solvent was used. Other authors indicate sodium nitrite as a convenient source of nitrogen dioxide: its decomposition in the presence of air and of an acid leads to the formation of nitrogen dioxide. Nitrous acid produced in the neutralization of sodium nitrite is unstable (Scheme 3): it may exist in equilibrium with dinitrogen trioxide, [33] it may disproportionate to nitrogen monoxide and nitric acid [34] or it may decompose to nitrogen monoxide and nitrogen dioxide [33]. Spin trapping of the radical species formed after the decomposition of nitrous acid (HNO_2) was also attempted. No EPR signal was detected when sodium nitrite (NaNO_2) and acetic acid (in order to dissociate NaNO_2) were added to a degassed solution of PBN. But, on minimum exposure to air, and therefore to oxygen, a signal consisting of a triplet of doublets overlapping the three lines of the acyl nitroxide was recorded. Simulation of the spectrum allowed the determination of the hyperfine coupling constants of both acyl nitroxide **1** (see Table I) and of the other radical species: $a_{\text{N}} = 13.2$ (1N) and $a_{\text{H}} = 1.8$ (1H), most likely the intermediate spin adduct **8**, having the $-\text{ONO}$ group linked to C-2 (similar to spin adduct **7** detected with DEPMPO). These values are consistent with those published for oxygen-centered radicals trapped by PBN [35]. But as time proceeded, the acyl nitroxide signal increased while, simultaneously, the three doublets gradually



Scheme 2. Macroscale reaction between INDO1 and nitrogen dioxide.

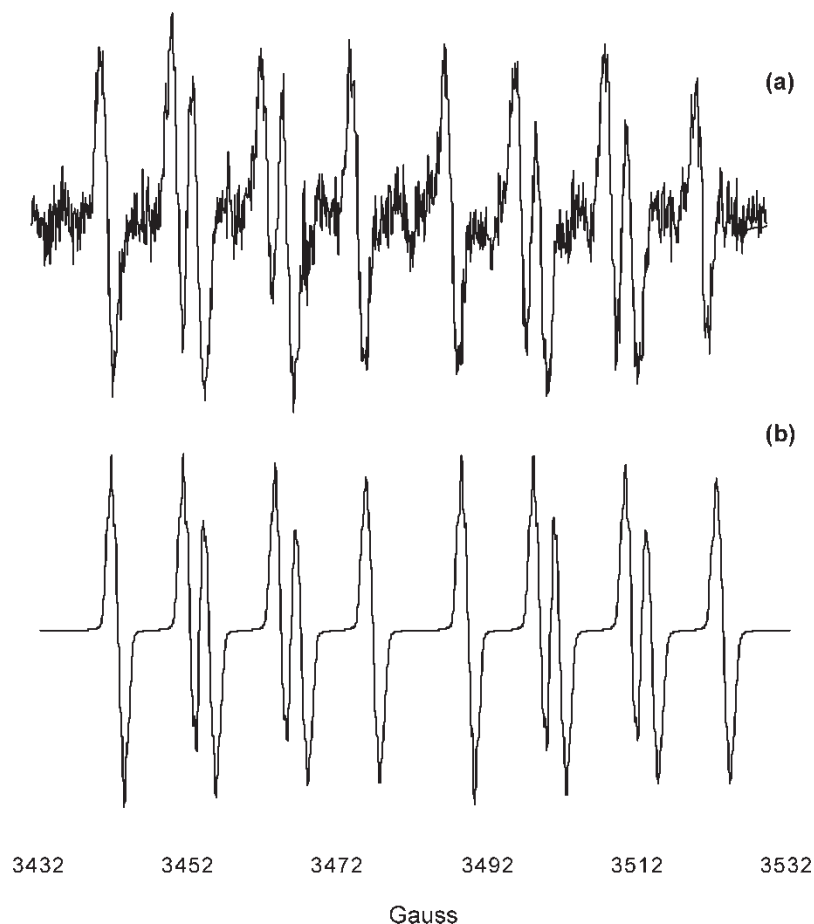


Figure 5. (a) EPR spectrum of spin adduct 7 recorded immediately after mixing benzene solutions of DEPMPO and NO_2 . Same experimental conditions and instrumental settings as in Figure 3 were used. (b) Computer simulation of spin adduct 7. The best fit was observed with the following set of coupling constants: $a_{\text{P}} = 46.797 \text{ G}$, $a_{\text{N}} = 12.216 \text{ G}$, $a_{\text{H}} = 9.587 \text{ G}$ (1H), $a_{\text{H}} = 0.466 \text{ G}$ (2H) and $a_{\text{H}} = 0.196 \text{ G}$ (2H).

disappeared (Figure 7). This fact confirms our hypothesis that acyl nitroxides are not formed in the oxidation of the nitrones by nitrogen dioxide but they derive from the nucleophilic addition of nitrogen dioxide to the trap followed by loss of HNO .

The same experiment was repeated using INDO1 as trap. No spin adduct was detected when oxygen was excluded from the reaction milieu, but as soon as the reaction mixture was aerated, the signal of acyl nitroxide 5 started to appear and after 8 min it was very intense. In this reaction acyl nitroxide was the sole radical detected and no other intermediates were observed. Both the experiments suggest that from the decomposition of nitrous acid, nitrogen dioxide is not formed. Nitrogen monoxide is the sole radical species present in the reaction milieu but it cannot be trapped by nitrones (spin trapping experiments, not reported

here, carried out using nitrones and gaseous NO were unsuccessful). According to the equilibrium in Eq. 1 (Scheme 3) dinitrogen trioxide could also be present, but it may dissociate to NO and NO_2 (Eq. 4), a process that begins to be significant above -30°C [33]. If this was our case, acyl nitroxide signal should have been recorded also in the absence of oxygen. Acyl nitroxide 1 (or 4) is formed only when the solution is aerated and in fact an EPR signal rapidly appears. The presence of oxygen, and hence of NO_2 ($2\text{NO} + \text{O}_2 \rightarrow 2\text{NO}_2$ $k = 2.4 \times 10^{-6} \text{ M}^{-2} \text{ s}^{-1}$) [36] is necessary for the formation of acyl nitroxides.

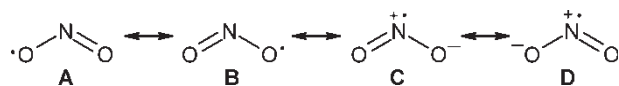
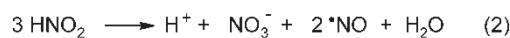
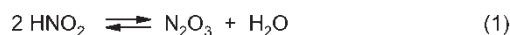


Figure 6. Resonance forms for nitrogen dioxide.



Scheme 3. Possible reactions for the decomposition of nitrous acid.

Table I. Hyperfine coupling constants (h.f.c.s.) in Gauss and *g*-factors for acyl nitroxides 1–6.

Acyl nitroxide	h.f.c.s.	Solvent	<i>g</i> -Factor	Reference
1	$a_N = 8.00$ (1N)	C_6H_6	2.0068 ₆	[20]
	$a_N = 8.15$ (1N)	CH_2Cl_2	2.0068 ₃	
2	$a_N = 6.68$ (1N); $a_H = 3.51$ (1H); $a_H = 3.48$ (1H)	CH_2Cl_2	2.0070 ₈	
3	$a_N = 6.30$ (1N)	C_6H_6	2.0072 ₇	[21]
	$a_N = 6.46$ (1N)	CH_2Cl_2	2.0072 ₁	
4	$a_N = 7.02$ (1N); $a_P = 40.92$ (1P); $a_H = 3.99$ (1H); $a_H = 2.99$ (1H)	C_6H_6	2.0070 ₅	[22]
	$a_N = 7.86$ (1N); $a_P = 45.27$ (1P); $a_H = 4.33$ (1H); $a_H = 2.88$ (1H)	CH_2Cl_2	2.0070 ₇	
5	$a_N = 5.85$ (1N); $a_H = 0.80$ (1H); $a_H = 0.65$ (1H); $a_H = 0.24$ (1H)	C_6H_6	2.0061 ₇	[23]
	$a_N = 5.98$ (1N); $a_H = 0.81$ (1H); $a_H = 0.64$ (1H); $a_H = 0.24$ (1H)	CH_2Cl_2	2.0061 ₂	
6	$a_N = 5.63$ (1N); $a_H = 2.70$ (1H); $a_H = 2.34$ (1H); $a_H = 0.79$ (1H); $a_H = 0.13$ (1H)	C_6H_6	2.0063 ₂	[23]
	$a_N = 5.91$ (1N); $a_H = 2.84$ (1H); $a_H = 2.38$ (1H); $a_H = 0.83$ (1H); $a_H = 0.14$ (1H)	CH_2Cl_2	2.0062 ₂	

Conclusions

The spin trapping technique provides an useful tool to study the reactivity of radical species or to determine the nature of the radicals present in a particular system.

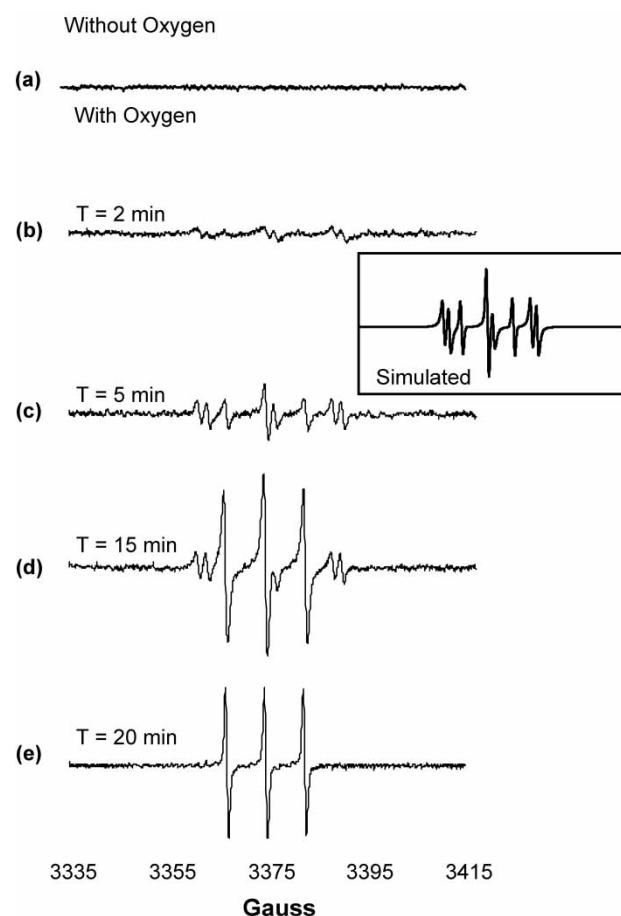


Figure 7. EPR spectra recorded during the decomposition of HNO_2 in the presence of PBN in benzene: (a) in the absence of oxygen; (b–e) in the presence of oxygen at different time intervals. Spectra b–d resulted from the overlapping of acyl nitroxide 1 and spin adduct 8 signals. The inset shows the computer simulation of the two nitroxides: hyperfine splitting constants are $a_N = 8.00$ G for 1 and $a_N = 13.2$ G and $a_H = 1.8$ G for 8.

This paper shows that nitrogen dioxide, a biologically relevant species, is trapped by electrophilic neutral compounds such as nitrones. The reactions proceed through the formation of intermediate spin adducts having the $-ONO$ group bound to C-2 of the trap indicating that nitrogen dioxide, in this case, reacts through its nucleophilic oxygen atom. These spin adducts are unstable and difficult to be detected; in fact only in two cases their EPR signals were recorded. Oxidation of these intermediates lead to acyl nitroxides, which are stable compounds detectable by EPR spectroscopy. The results obtained with sodium nitrite indicate that nitrous acid decomposes to nitrogen monoxide and nitric acid and, if oxygen is strictly excluded from the reaction milieu, it could be a useful source of nitrogen monoxide.

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