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SINGLET OXYGEN-TRAPPING REACTION AS A METHOD OF $^1O_2$ DETECTION: ROLE OF SOME REDUCING AGENTS

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The production of singlet oxygen by H_2O_2 disproportionation and via the oxidation of H_2O_2 by NaOCl in a neutral medium was monitored by spin trapping with 2,2,6,6 tetramethyl-4-piperidone (TMPone). The singlet oxygen formed in both reactions oxidized 2,2,6,6 tetramethyl-4-piperidone to give nitroxide radicals. However the production of nitroxide radicals was relatively small considering the concentrations of H_2O_2 and NaOCl used in the reaction systems. Addition of electron donating agents: ascorbate, Fe^{2+} and desferrioxamine leads to an increase in the production of nitroxide radicals. We assumed that a very slow step of the reaction sequence, the homolytic breaking of the O-O bond of N-hydroperoxide (formed as an intermediate product during the reaction of $^1O_2$ with TMPone) could be responsible for the relatively small production of nitroxide radicals. Electron donating agents added to the reaction system probably raise the rate of the hydroperoxide decomposition by allowing a more rapid heterolytic cleavage of the O-O bond leading to a greater production of nitroxide radicals. The largest effect was observed in the presence of desferrioxamine. Its participation in this process is proved by the concomitant appearance of desferrioxamine nitroxide radicals. The results obtained demonstrate that the method proposed by several authors and tested in this study to detect singlet oxygen is not convenient for precise quantitative studies. The reactivity of TMPone towards $O_2^{\cdot-}/HO_2^{\cdot}$ and $^{\cdot}OH$ has been also investigated. It has been found that both $O_2^{\cdot-}/HO_2^{\cdot}$ and $^{\cdot}OH$ radicals formed in a phosphate buffer solution (pH 7.4, 37°C), respectively by a xanthine-oxidase/hypoxanthine system and via H_2O_2 UV irradiation, do not oxidize 2,2,6,6 tetramethyl-4-piperidone to nitroxide radicals.

KEY WORDS: EPR, Spin trapping, Singlet oxygen, Desferrioxamine, Nitroxide free radical

Abbreviations: $^1O_2$, Singlet oxygen; EPR, Electron Paramagnetic Resonance; TMPone, 2,2,6,6 tetramethyl-4-piperidone; TMPone nitroxide, 2,2,6,6 tetramethyl-4-piperidone-N-oxide; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; SP, Strong Peach; PMN, Polymorphonuclear Leukocytes

INTRODUCTION

Among the reactive oxygen species (ROS),[1,2] the singlet oxygen $^1O_2$ plays the role of a powerful oxidant for various biological molecules.[3–6] Its formation, from the two-electron oxidation of H_2O_2 by compounds containing chlorine in its +1 formal oxidation state, in activated PMN, has been suggested by Klebanoff.[7] Thus it is necessary to detect its possible formation in various media used to mimic the conditions which allow for the appearance of an oxidative stress. Up till now various methods have been used to detect singlet oxygen. Most of the methods used in the gas

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swept out of solution into the gas. Because of the difficulties associated with spectroscopic methods, it has been necessary to resort to chemical methods for detecting singlet oxygen in solution.

In 1976 Lion[8] *et al.* proposed a new chemical method to detect singlet oxygen, involving the generation of stable nitroxide radicals when excited oxygen reacts with sterically hindered amines. Other studies[9–14] have been devoted to this type of detection but sometimes with contradictory results on its specificity and poor agreement with the expected results for singlet oxygen formation. For example Rosenthal *et al.*[14] have suggested that conversion of TMPone to nitroxide could be carried out with hydroxyl radicals generated by photolysis of H_2O_2. However, Rigo *et al.*[11] find that only singlet oxygen is able to oxidize TMPone to TMPone radicals, but species like $^{\bullet}OH$ or $O_2^{\bullet-}$ are not effective. Among the sterically hindered amines, we have chosen TMPone because its pKa (7.6) makes it possible to work with a pH nearest to that of the physiological medium, and permits the detection of $^1O_2$ in neutral solutions.[13] Indeed it is the only non-protonated form of these amines that has the lone electron pair of the nitrogen which is necessary for the electrophilic attack of the amine by $^1O_2$. It leads to the formation of an hydroperoxide, which then produces a nitroxide radical that is very stable and easily detectable by EPR spectroscopy at concentrations as low as 100 nM.[8]

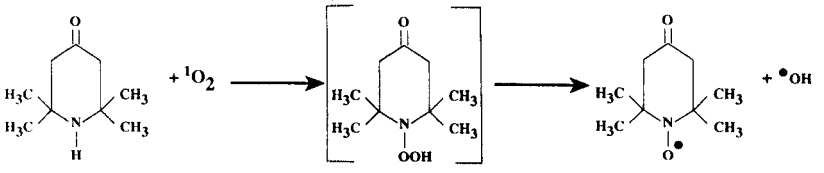
In the present study, we have used two procedures for singlet oxygen production, the H_2O_2 disproportionation[15] and the two-electron oxidation of H_2O_2 by OCl^- ion in a neutral medium.[16] The reactions (1) and (2) describe these processes:

$$HO_2^- + H_2O_2 \rightarrow (^1O_2, {}^3O_2) + H_2O + OH^- \quad (1)$$

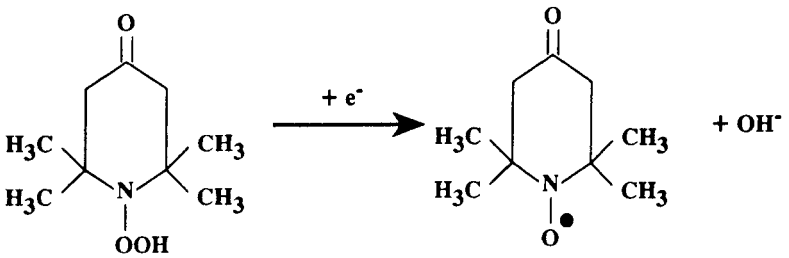
$$H_2O_2 + OCl^- \rightarrow {}^1O_2 + Cl^- + H_2O \quad (2)$$

Lion *et al.*[12] have proposed a sequence of reactions for nitroxide radical formation by reaction of the singlet oxygen with TMPone (Scheme 1), with an intermediate state (a charge transfer complex) as the first step. But these authors have not specified the nature of the OH ($^{\bullet}OH$ or OH^-). Taking into account the number of electrons, we note that for Scheme 1 it is necessary to envisage in the last reaction the formation of an $^{\bullet}OH$ radical by the homolytical breaking of the O-O bond in the hydroperoxide. It is probable that this reaction is slow, and a more rapid reaction could be possible if an electron donor were added to the medium (see Scheme 2).

In our studies we have used various electron donating agents to check this hypothesis and we have found that the addition of these agents leads effectively to a greater production of nitroxide radicals.



SCHEME 1. Sequence of reactions for nitroxide radical formation by reaction of singlet oxygen with TMPone (An intermediate species between square brackets is probably relatively stable.)



SCHEME 2. Heterolytic cleavage of O-O bond in N-hydroperoxide appearing in the presence of electron donating agents.

MATERIALS AND METHODS

2,2,6,6 tetramethyl-4-piperidone hydrochloride 98% was obtained from Aldrich-Chemie (Germany), 5,5′ dimethyl-1-pyrroline-N-oxide (DMPO) was purchased from the Sigma Chemical Company, USA, and purified by filtering through activated charcoal. DMPO prepared in this manner is a colorless liquid and is devoid of EPR signals.[17] H_2O_2 (30%) INTEROX type 0010 and hypochlorite of sodium (NaOCl) with 5% active chlorine were used. Catalase from bovine liver (19 900 units per mg of solid) was obtained from Sigma Chemical Company, USA. Desferrrioxamine (Desferal) was obtained from CIBA Laboratory. The classical couple hypoxanthine (Sigma, 4 mM)-xanthine oxidase (Sigma, 48 mU/ml) in a Na phosphate buffer (20 mM, pH 7.4) was used to furnish $O_2^{\bullet-}$.

Dissolution of the reagents used in these studies was carried out in distilled water or in the sodium phosphate buffer. In order to remove traces of transition metal impurities the phosphate buffer solution and the distilled water used to prepare the other solutions were purified with the aid of CHELEX-100 resin (Bio-Rad Laboratories).

The typical experimental procedure was as follows. Reactants were introduced into the reactor as described in tables 1–5. The glass reactor was thermostated at 37°C and protected from exposure to light. The reaction solution was mixed using an oscillating table. In all experiments the final volume of the mixture was 2 mL. Times indicated in Tables and Figures were counted from the moment of addition of the last compound. Aliquots were withdrawn, filtered through a 0.65 μm porosity filter, then placed in a flat quartz cell. EPR spectra were immediately recorded at room temperature on a Varian CSE spectrometer operating in the X band mode (9.4 GHz). The intensities of the signals were calculated from EPR measurements (peak-to-trough method). The g factors were calculated by calibration against SP (Strong Peach) ($g = 2.0029$).

TMPone (50 mM) was used as a spin trapping agent. The reaction of singlet oxygen with this amine leads to the formation of a TMPone nitroxide free radical.[12] Detection of this radical was performed by EPR spectroscopy after different times of reaction. For each test several measurements were made. The intensity of the TMPone nitroxide radical signal is given in arbitrary units (a.u.), where an intensity of 1000 a.u. corresponds approximately to $3.4 \cdot 10^{18}$ spins L^{-1} (see Nejjari[18]). DMPO (50 mM) was used for trapping hydroxyl radicals formed from H_2O_2 in the reaction systems during UV-irradiation. This irradiation was performed using a high-pressure mercury lamp with wavelenghs ranging from 250 to 600 nm.

TABLE 1
Comparison of intensity of nitroxide radical signals obtained during the reaction of TMPone with singlet oxygen in different reaction systems.

Reaction systems	Intensity of the TMPone nitroxide radicals signal (a.u.) after:		
	10 min	30 min	60 min
TMPone + H_2O_2	40 ± 5	120 ± 10	200 ± 10
TMPone + H_2O_2 + Cat	N.D.	N.D.	N.D.
TMPone + H_2O_2 + NaOCl	220 ± 10	490 ± 15	800 ± 15
TMPone + H_2O_2 + NaOCl + Cat	N.D.	N.D.	N.D.
TMPone + NaOCl	N.D.	N.D.	N.D.

Concentrations: TMPone (50 mM), NaOCl (25 mM), H_2O_2 (50 mM), Cat (catalase) (5000 units mL^{-1}) sodium phosphate (20 mM, pH 7.4)
N.D. – Not detectable. a.u. – arbitrary units
Assays were carried out as described in Materials and Methods.
Each result represents the average ± SD of five experiments.
EPR spectrometer settings: microwave power 10 mW, central field 3380 G, sweep width 50 G, frequency power 9.464 GHs, modulation amplitude 2 G, time constant 0.5 s

RESULTS

We have studied the formation of nitroxide radicals in three situations: (i) in the presence of $O_2^{\cdot-}$ (ii) in the presence of H_2O_2 with and without of UV irradiation, (iii) in presence of H_2O_2 and OCl^-. In the second case we can compare the effect of H_2O_2 disproportionation and the possible effect of a complementary contribution of $^{\cdot}OH$ obtained by UV homolytic breaking of H_2O_2. In the third case, in agreement with different authors[16,19] an excess of H_2O_2 with respect to OCl^- was used.

The formation of superoxide anion by the couple hypoxanthine-xanthine oxidase in our test was checked by EPR spectroscopy after trapping by DMPO (results not presented).[20,21] When $O_2^{\cdot-}$ was formed under similar conditions but in the presence of TMPone, the nitroxide radical signal did not appear in the EPR spectrum. The addition of desferrioxamine (1 mM) to this reaction medium did not change the result.

The results of the tests with H_2O_2 and H_2O_2 + OCl^- are presented in Table 1. About four times more $^1O_2$ appears in the H_2O_2 (50 mM) oxidation by OCl^- (25 mM) than in the H_2O_2 (50 mM) disproportionation. Production of nitroxide radicals increases as a function of time for both reaction systems probably due to the existence of a limiting step in the sequence of reactions. In the presence of catalase or in the absence of H_2O_2, nitroxide radicals were not observed in the reaction systems.

The effect of H_2O_2 concentration on nitroxide radical formation in the tests with H_2O_2 and H_2O_2 + OCl^- is shown in Figure 1. With increasing concentrations of H_2O_2, the intensity of the nitroxide radical signal increases in the both cases, with and without NaOCl, but rises more rapidly in the presence of OCl^-, particularly for small concentrations of H_2O_2.

The nitroxide radical obtained in these reactions by oxidation of TMPone amine is characterized by three lines with a 15.7 splitting between components and a g-factor of 2.0062 (see Table 2). This nitroxide radical is very stable. As seen from Table 2, our results are identical within error limits to those obtained by Rozantzev *et al.*[10]

Since Rosenthal *et al.*[14] have considered that $^{\cdot}OH$ is able to transform TMPone into nitroxide radicals (contrary to the results of Rigo[11]), we have tried to verify this hypothesis by forming $^{\cdot}OH$ in our system. $^{\cdot}OH$ was formed by UV irradiation of H_2O_2.

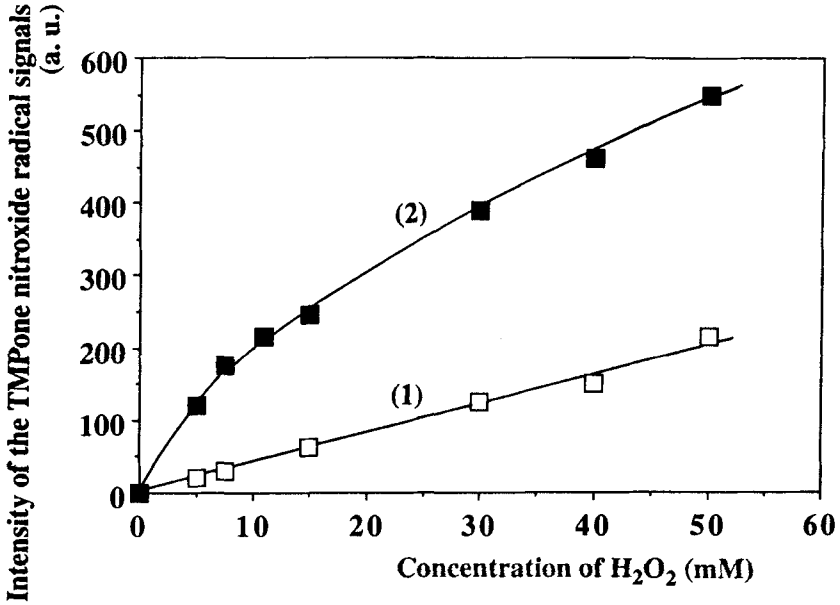


FIGURE 1. Effect of the concentration of hydrogen peroxide on formation of TMPone nitroxide radical in the absence (1) and presence (2) of NaOCl.

In all measurements TMPone (50 mM) was used as a singlet oxygen scavenger. Final concentration of NaOCl was 25 mM. H_2O_2 and NaOCl were diluted in sodium phosphate buffer 20 mM, pH 7.4 Each point represents the average of five experiments obtained after stirring (30 min) of the reaction medium at 37°C. EPR spectrometer settings were the same as for table 1.

Its formation was checked by spin trapping with DMPO.[22,23] A 1:2:2:1 quartet due to a DMPO spin adduct of $^{\bullet}$OH (DMPO-OH)$^{\bullet}$: g = 2.0073, $a_N = a_{\beta H} = 14.95$ G, appeared indicating the generation of $^{\bullet}$OH radicals (spectrum not presented). The results presented in Table 3 show a significant production of this adduct during UV irradiation, suggesting the generation of a great amount of $^{\bullet}$OH radicals in the system. In spite of this, the amount of nitroxide radicals increases only slightly. The small increase in nitroxide radical production (compare line 3 and 4 in the Table 3) may be due to an

TABLE 2
EPR parameters for nitroxide radicals from 2,2,6,6 tetramethyl-4-piperidone and desferrioxamine

	g-factor
Nitroxide radicals	
– our results	2.0062
– Rozantzev[10]	2.0064
Desferrioxamine radicals	
– our results	2.0065
– Morehouse[31]	2.0065

TABLE 3
Effect of UV irradiation on formation of nitroxide and (DMPO,OH)$^{\bullet}$ radicals.

Reaction systems	Intensity of the nitroxide and (DMPO,OH)$^{\bullet}$ radicals signals (a.u.) after:			
	30 min		60 min	
	TMPone radicals	(DMPO,OH)$^{\bullet}$	TMPone radicals	(DMPO,OH)$^{\bullet}$
TMPone + H_2O_2	120 ± 10	–	200 ± 10	–
TMPone + H_2O_2 + UV (1 min)	150 ± 10	–	270 ± 15	–
TMPone + H_2O_2 + DMPO	70 ± 5	80 ± 5	90 ± 5	90 ± 5
TMPone + H_2O_2 + DMPO + UV (1 min)	120 ± 10	2000 ± 40	140 ± 10	1200 ± 40

Concentrations in sodium phosphate buffer: TMPone (50 mM), H_2O_2 (50 mM), DMPO (50 mM)
Each result represents the average + SD of five measurements.
The UV irradiation (1 min) takes place either after 30 min or 60 min of stirring of reaction system and the EPR spectra are obtained about 3 min after the end of the UV irradiation.

increase in homolytical breaking by UV irradiation of the N-hydroperoxide formed as an intermediate in the reaction sequence leading to the nitroxide radicals (Scheme 1).

A complementary result concerning the influence of $^{\bullet}OH$ trapping by DMPO in the mechanisms of H_2O_2 disproportionation is given in Table 3. This trapping leads to a decrease in the intensity of the nitroxide radical signals (compare line 1 and 2 in Table 3). This phenomenon may be due to an inhibition of reaction (3) proposed by Halliwell and Gutteridge[24] and reaction (4) proposed by Arneson[25] and Koppenol[26] which could lead to the formation of singlet oxygen.

$$^{\bullet}OH + H_2O_2 \rightarrow O_2^{\bullet-} + H^+ + H_2O \quad (3)$$

$$^{\bullet}OH + O_2^{\bullet-} \rightarrow OH^- + {}^1O_2 \quad (4)$$

The effects of reducing agents on the formation of TMPone nitroxide radicals for the system containing H_2O_2 with NaOCl are shown in Tables 4 and 5. A comparison of the intensities of the TMPone nitroxide radical signals obtained when reducing agents were introduced into the reactor at the same time as all the other reaction compounds is found in Table 4. Whereas Table 5 compares the results obtained when

TABLE 4
Effect of reducing agents on the formation of TMPone nitroxide radicals.

Reaction systems	Intensity of the TMPone nitroxide radicals (a.u.) after:	
	10 min	60 min
TMPone + H_2O_2 + NaOCl	215 ± 10	800 ± 15
TMPone + H_2O_2 + NaOCl + Fe^{2+}	4700 ± 30	5900 ± 30
TMPone + H_2O_2 + NaOCl + Asc.	1340 ± 25	4400 ± 30
TMPone + H_2O_2 + NaOCl + DFO	55 000 ± 400	60 000 ± 400

Concentrations: TMPone (50 mM), NaOCl (25 mM), H_2O_2 (50 mM), Asc. (Ascorbate) (5 mM), Fe^{2+} (5 mM), DFO (Desferrioxamine) (2 mM)
All the reactants were added at the beginning of the reactions. Each result represents the average ± SD of five experiments.

TABLE 5
Effect of reducing agents on the formation of TMPone nitroxide radicals[a]

Reaction systems	Intensity of the TMPone nitroxide radicals (a.u.) after: 10 min	60 min
TMPone + H_2O_2 +NaOCl + stirring[b]	380 ± 15	1000 ± 20
TMPone + H_2O_2 + NaOCl + stirring[b] + Fe^{2+}	10 000 ± 50	8400 ± 40
TMPone + H_2O_2 + NaOCl + stirring[b] + Asc.	1600 ± 30	4400 ± 30
TMPone + H_2O_2 + NaOCl + stirring[b] + DFO	62 000 ± 400	80 000 ± 400

[a] The reducing agents were added to the systems 20 min after initiation of the reaction.
[b] 20 min of stirring of reaction system
Concentrations: TMPone (50 mM), NaOCl (25 mM), H_2O_2 (50mM), Fe^{2+} (10 mM), Asc. (Ascorbate) (10 mM), DFO (Desferrioxamine) (2 mM)
EPR spectrometer settings were the same as for table 1.
The intensities were calculated from EPR signal and each result represents the average ± SD of five experiments.

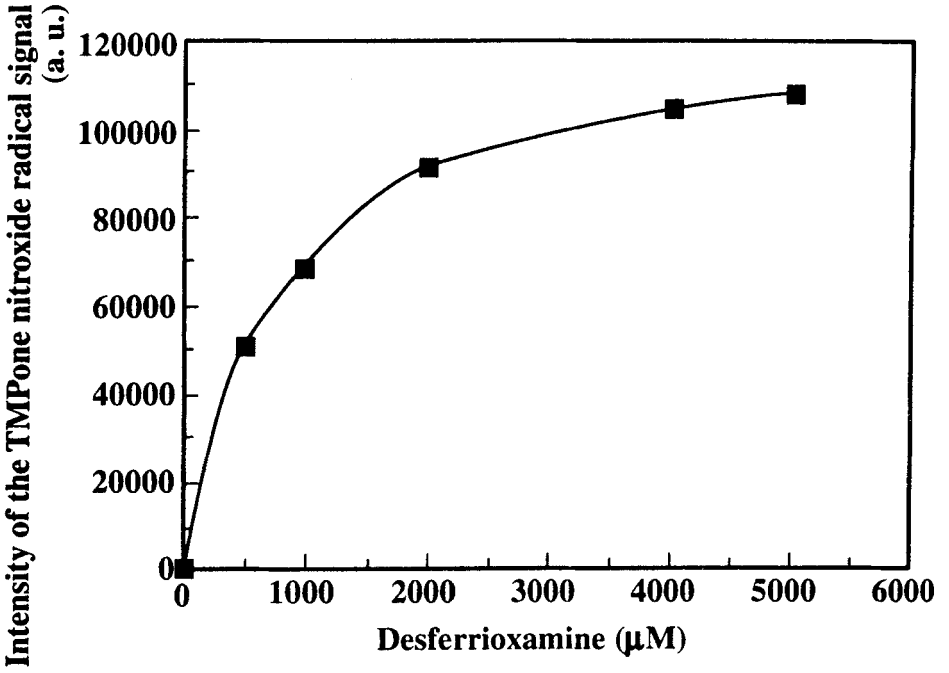


FIGURE 2. Effect of desferrioxamine concentration on the TMPone nitroxide radical formation through oxidation of TMPone by singlet oxygen.

Conditions: The oxidation of TMPone by singlet oxygen (formed by NaOCl, H_2O_2 system diluted in sodium phosphate buffer, pH 7.4, 20 mM) was carried out as described under Materials and Methods. The final concentration of TMPone was 50 mM, of NaOCl 25 mM, of H_2O_2 50 mM. Each point represents the average of three experiments obtained after stirring (30 min) of the reaction medium at 37°C. EPR spectrometer settings were the same as for table 1.

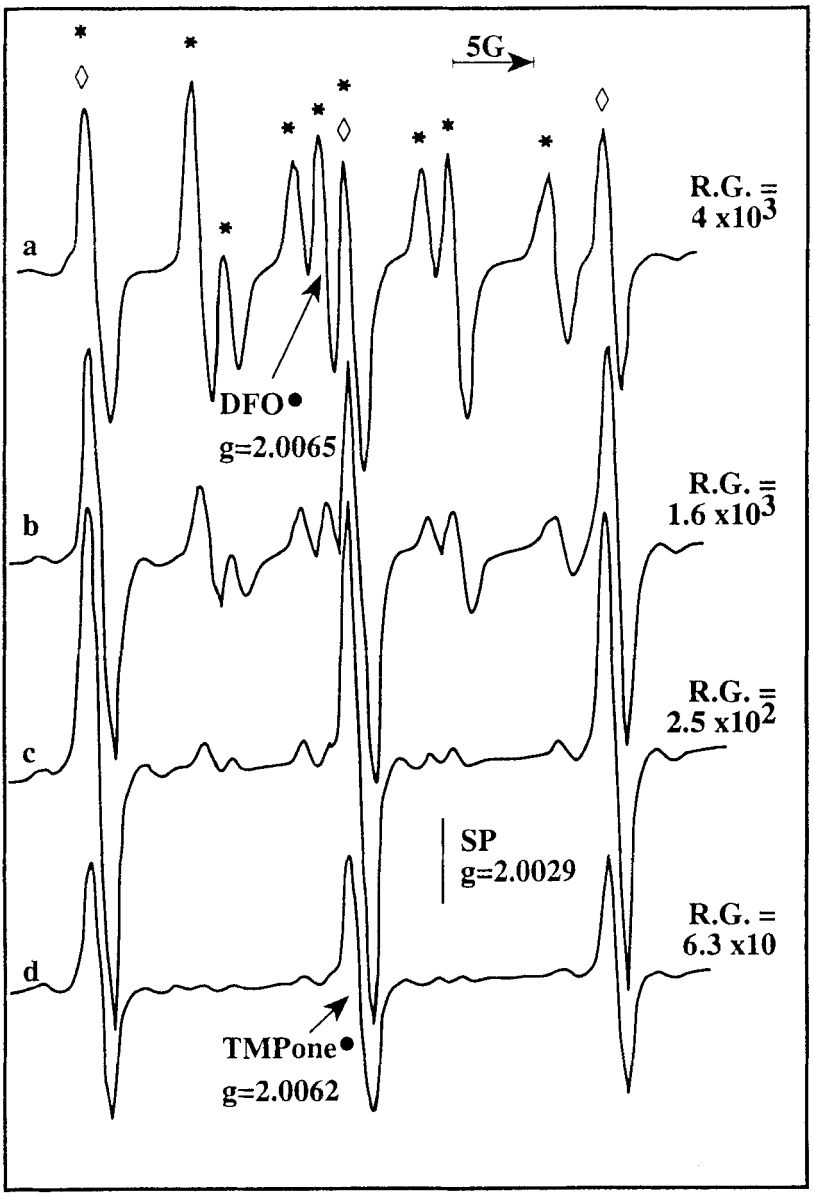


FIGURE 3. EPR spectra assigned to TMPone nitroxide radicals (a_N = 15.7 G) and desferrioxamine nitroxide radical (a_N = 7.85 G, a_{2H} = 6.35 G).

Conditions: The spectra were registered after (a) 10 sec, (b) 60 sec, (c) 5 min and (d) 10 min of stirring of the reaction mixture containing desferrioxamine (1 mM), TMPone (50 mM), H_2O_2 (50 mM) and NaOCl (25 mM) in sodium phosphate buffer (pH 7.4, 20 mM). EPR spectrometer settings: microwave power 10 mW, central field 3380 G, sweep width 50 G, frequency 9.464 G, modulation amplitude 2 G, time constant 0.064 sec R.G. – Receiver Gain. Locations of peaks corresponding to TMPone nitroxide (◊) and desferrioxamine nitroxide (*) are shown.

the same reducing agents were added to the system 20 min after initiation of the reactions leading to the formation of the N-hydroperoxide. As shown in both tables, the addition of reducing agents into reaction systems lead to a considerably larger production of these radicals. In particular, in the presence of desferrioxamine a considerable increase in the formation of nitroxide radicals was observed. Desferrioxamine is known not only as an excellent chelating agent for Fe^{3+}[27,28] or a powerful $^{\bullet}OH$ and $O_2^{\bullet-}$ scavenger,[29,30] but also as a reducing agent.[31] In the presence of ascorbate or Fe^{2+}, the increase in production of nitroxide radicals was not as considerable, but was significant nonetheless and the results were several fold higher than without the reducing agents. The most important effect of desferrioxamine on the formation of nitroxide radicals was seen when this agent was added after previous stirring of the reaction mixture containing TMPone with H_2O_2 and NaOCl during 20 min.

The results presented in Figure 2 confirm the important role of desferrioxamine in our reaction system. A small amount of this compound is sufficient to allow a significant increase of production of TMPone nitroxide radical. The nitroxide radical formation increased considerably until the concentration of desferrioxamine reached 2 mM, where the increase was weaker.

Figure 3 shows that after a very short time of stirring of the reaction system (containing desferrioxamine, TMPone, H_2O_2 and NaOCl), TMPone nitroxide radical and desferrioxamine radical (DFO$^{\bullet}$) are formed simultaneously. The DFO$^{\bullet}$ radical containing the structural component $-CH_2-NO^{\bullet}-CO-$ gives the 9 line spectrum as a result of splitting of the nitroxide nitrogen coupling ($a_N = 7.85$ G) by two protons ($a_{2H} = 6.35$ G) from the neighbouring CH_2 group.[31] This radical with a g-factor of 2.0065 is not very stable and has a half-life of approximately 7 min at 37°C. As is shown in Figure 3 the intensity of this DFO$^{\bullet}$ signal decreased, and that of the TMPone nitroxide radical increased as a function of the time of stirring of the reaction system. After 10 min the TMPone nitroxide radical dominated in the EPR spectrum.

In Figure 4 the kinetics of the formation of TMPone nitroxide radicals for the NaOCl + H_2O_2 + TMPone system, with and without desferrioxamine are shown. The intensity of the TMPone radicals is considerably higher when desferrioxamine is present in the reaction medium. In the absence of DFO, the intensity of this radical increased slowly as a function of time (curve 1), but in the presence of DFO (curve 2) the increase is rapid during the first 15 min, and after 40 min the intensity of the EPR signal increased very slowly.

DISCUSSION

The results presented here confirm that in phosphate buffer $^1O_2$, formed by H_2O_2 disproportionation or by oxidation of H_2O_2 with NaOCl, reacts with TMPone giving a nitroxide radical. If we take into consideration the H_2O_2 disproportionation process to produce $^1O_2$, various pathways are possible. We observe easily (Table 1) that catalase addition destroys H_2O_2 and consequently inhibits $^1O_2$ formation. According to some authors[15,24,25] singlet oxygen could be formed not only by reaction (1) but also by reactions (5) + (4) or (5) + (6).

$$2H_2O_2 \rightarrow O_2^{\bullet-} + 2^{\bullet}OH + 2H^+ \quad (5)$$

$$O_2^{\bullet-} + HO_2^{\bullet} + H^+ \rightarrow {}^1O_2 + H_2O_2 \quad (6)$$

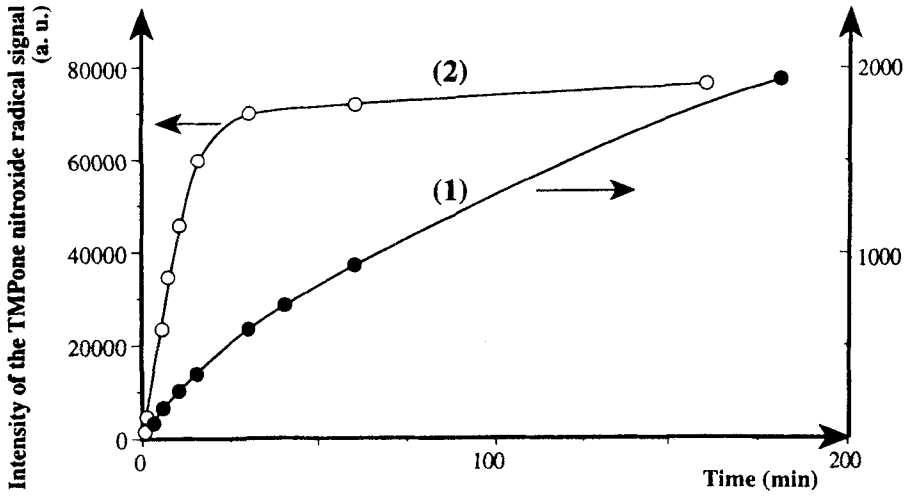


FIGURE 4. Kinetics of formation of TMPone nitroxide radicals for NaOCl + H_2O_2 + TMPone system, (2) with and (1) without desferrioxamine in sodium phosphate buffer solution at 37°C.

Conditions: TMPone (50 mM), H_2O_2 (50 mM), NaOCl (25 mM), desferrioxamine (1 mM), in sodium phosphate buffer (pH 7.4, 20 mM). The intensities were calculated from the EPR signal after a definite time and each point represents the average of five measurements. EPR spectrometer settings were the same as for table 1 except that the time constant in the case of test (2) was 0.064 sec.

Decreasing generation of nitroxide radicals in the presence of DMPO (see Table 3) could be related to inhibition of formation of $^1O_2$ in reaction (4) as a result of trapping of $^{\bullet}OH$ radicals. So, the results presented in Table 3 seem to indicate that this pathway of $^1O_2$ formation is probable.

We have tried in our studies to check the participation of pathway (6) (suggested by Arneson[25]) in $^1O_2$ formation by monitoring the formation of nitroxide radicals during $O_2^{\bullet-}$ production. If pathway (6) takes place in the reaction system containing xanthine oxidase + hypoxanthine + TMPone, TMPone nitroxide radical should appear. However we did not observe this phenomenon. It is very probable that the small rate constant for dismutation, $k = 2 \cdot 10^5\ M^{-1}\ s^{-1}$ [26] (reaction 6) and a very short life-time for $O_2^{\bullet-}$ is responsible for the absence of singlet oxygen formation. Morever Arneson[25] suggested that reaction (4) may have a higher yield of singlet oxygen than reaction (6).

The second method of $^1O_2$ production, via the oxidation of hydrogen peroxide by NaOCl, has often been used to produce electronically excited molecular oxygen.[16,19] The rate constant for this reaction at 25°C is about $4.4 \cdot 10^7\ M^{-1}\ s^{-1}$.[16] As shown in Table 1 and in Figure 1, the oxidation of H_2O_2 by NaOCl gives a greater formation of nitroxide radical than the disproportionation of H_2O_2 at the same concentration. These results are in agreement with reactions (1) and (2), which describe both processes. As was demonstrated[15,16,19] in reaction (1), during dismutation of H_2O_2, both singlet and triplet oxygen are formed, whereas in the stoichiometric reaction (2) all oxygen appears in a $^1O_2$ state.

The slow increase in the production of nitroxide radicals as a function of time for

both reaction systems is probably related to the presence of a limiting step in the sequence of reactions (see Table 1 and curve (1) in Figure 4). The increase of nitroxide radical production after the addition of reducing agents, ascorbate, Fe^{2+}, desferrioxamine, suggests the appearance of an additional more rapid step (Table 4). The greater effect was observed when reducing agents were added to the system 20 min after initiation of the reaction (Table 5). These results indicate that reducing agents operate not at the level of $^1O_2$ formation but at the last step of the reaction chain of nitroxide radical formation (Scheme 1). The homolytic breaking of the O-O bond (appearing under the influence of light and heat) of N-hydroperoxide (formed as an intermediate product during the reaction of $^1O_2$ with TMPone) could be responsible for the relatively small production of nitroxide radicals. Electron donating agents added to the reaction system increase the rate of hydroperoxide decomposition by allowing a more rapid heterolytic cleavage of the peroxide bond (Scheme 2). The greater effect was observed in the presence of desferrioxamine, its participation in the process being proved by the concomitant appearance of desferrioxamine radicals.

The existence of a relatively slow step in the reaction sequence leading to nitroxide radicals shows that the spin trapping method is not appropriate for the quantitative detection of $^1O_2$.

In agreement with Rigo *et al.*[11] our studies also show that species like $O_2^{\cdot-}$ and $^{\cdot}OH$ do not seem to be able to oxidize TMPone to nitroxide radicals. It is probable that the production of TMPone nitroxide radicals obtained by Rosenthal *et al.*[14] with γ-irradiation of water is not only due to $^{\cdot}OH$ generation but is probably also related to the presence of $H^{\cdot}$ and/or $(e^-)_{aq}$ in the system.

The small increase in the production of nitroxide radicals after UV irradiation in our test, in spite of a significant production of hydroxyl radical (checked by using a DMPO spin trap, Table 3), tends to confirm the above suggestion. Moreover the increase in the production of nitroxide radical in the presence of $FeSO_4$ does not seem to be due to hydroxyl radical formation in the mixture of TMPone, H_2O_2 and $FeSO_4$ (as suggested by Rosenthal *et al.*[14]) but is probably due to a more rapid heterolytic cleavage of the O-O bond of N-hydroperoxide in the presence of an electron donating agent (here Fe^{2+}). In accord with Rosenthal *et al.*,[14] we have observed about 4 times more nitroxide radicals after addition of Fe^{2+} (5 mM) into the TMPone + H_2O_2 system (results not presented). Similar or greater effects were also observed in the presence of other reducing agents such as ascorbate and desferrioxamine. Logically this effect was greater when the $^1O_2$ was formed during the oxidation of H_2O_2 by NaOCl because in this reaction more $^1O_2$ was formed and consequently more hydroperoxide, which then could be decomposed by heterolytic cleavage of the O-O bond with the participation of electron donating agents.

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