EPR SPECTRA OF DMPO SPIN ADDUCTS OF SUPEROXIDE AND HYDROXYL RADICALS IN PYRIDINE

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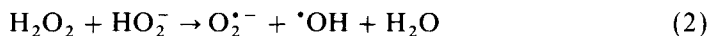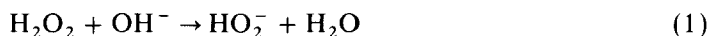
Electron spin resonance spectroscopy and the spin trapping technique were used to study the formation of the superoxide radical in pyridine. 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was employed as a trapping agent. Superoxide radical was generated using chemical (potassium superoxide) and photochemical methods with anthralin, benzanthrone, rose bengal, 1,8-dihydroxyanthraquinone and zinc tetraphenylporphyrine as photoactive pigments. Hyperfine coupling (hf) constants for $DMPO/O_2^{\cdot -}$ were determined to be $a_N = 12.36$ G, $a_H^\beta = 9.85$ G, $a_H^\gamma = 1.34$ G. The a_N and a_H^β hf constants are in good agreement with values calculated from a previously determined relationship between hf constants and solvent acceptor number (Reszka *et al.*, (1992) *Free Radical Res. Commun.*, in press). When concentrated hydrogen peroxide was added to DMPO in pyridine a similar EPR spectrum was observed. It is suggested that in this case the $DMPO/^\cdot O_2H$ adduct is formed by nucleophilic addition of H_2O_2 to DMPO to give a hydroxylamine, followed by oxidation to the respective nitroxide. The EPR spectrum observed when tetrapropylammonium hydroxide and H_2O_2 were added to DMPO in pyridine had hf couplings $a_N = 13.53$ G, $a_H^\beta = 11.38$ G, $a_H^\gamma = 0.79$ G and it was assigned to a $DMPO/^\cdot OH$ adduct. This assignment was based on similarity of this spectrum to the one produced by UV photolysis of hydrogen peroxide and DMPO in aqueous solution and subsequent transfer to pyridine.

KEY WORDS: EPR, DMPO, superoxide, hydroxyl radical, pyridine.

INTRODUCTION

Roberts and coworkers have reported that the reaction of tetrapropylammonium hydroxide (TPAH) with hydrogen peroxide (H_2O_2) in pyridine generates the superoxide radical, $O_2^{\cdot -}$, *via* reactions (1) and (2).[1]

$$H_2O_2 + OH^- \rightarrow HO_2^- + H_2O \qquad (1)$$

$$H_2O_2 + HO_2^- \rightarrow O_2^{\cdot -} + {}^\cdot OH + H_2O \qquad (2)$$

The presence of superoxide was confirmed by cyclic voltamperometry which showed that $O_2^{\cdot -}$ production was directly proportional to the concentration of TPAH. However, when PBN was used as a spin trapping agent to detect $O_2^{\cdot -}$ generated by $TPAH/H_2O_2$ in pyridine, the results were inconclusive because only the EPR spectrum of the $PBN/\cdot OH$ adduct was observed.[1] Although the hydroxyl radical is

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produced concomitantly with $O_2^{\cdot-}$ (Eq. 2) it should be scavenged primarily by solvent molecules and not by PBN.* The hyperfine couplings reported by Roberts and coworkers[1] cannot be assigned unambiguously to either PBN/$O_2^{\cdot-}$ or PBN/·OH adducts in pyridine. However, in water the EPR spectra of these adducts are quite similar[4] suggesting that their identification on the basis of EPR spectra alone, may be difficult.

DMPO is another spin trap frequently used to detect ·OH and $O_2^{\cdot-}$/$HO_2^{\cdot-}$ radicals. It has an important advantage over PBN because its adducts with $O_2^{\cdot-}$ and ·OH (I and II respectively) possess unique spectroscopic features which permit easy differentiation between these two species, both in aqueous as well as in organic media.[4-7] For that reason DMPO was later used by Damerau[8] in an attempt to detect superoxide radicals produced during the base-catalyzed decomposition of H_2O_2 in pyridine. He reported that the EPR spectrum of I consists of a triplet of doublets with $a_N = 13.8$ G and $a_H^\beta \doteq 11.9$ G. He also stated that in pyridine hf couplings of I are different from those of II, although no values for the latter adduct were given.[8] Subsequently Haseloff and coworkers,[9] using a similar approach, obtained the EPR spectrum of a DMPO adduct in pyridine with hyperfine couplings of $a_N = 14.0$ G and $a_H^\beta = 12.0$ G and assigned it to I. These authors concluded that "in solutions with a high content of pyridine the decay of the superoxide spin adduct was observed without any indication of the formation of DMPO/OH". Again no values of hf couplings of the adduct II in pyridine were given.

I   II
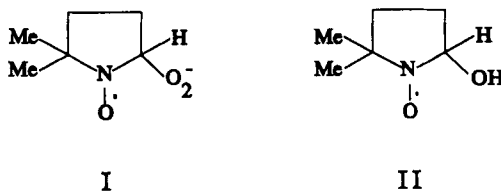
In pyridine, the reported spectra assigned to I[8,9] have only 6 hyperfine components whereas the spectra of the same adduct in other organic solvents have 12 components.[5,7,10] Additionally, the values of hyperfine couplings[8,9] are close to those observed for I in water ($a_N = 14.1$ G and $a_H^\beta = 11.3$ G in aqueous solution), whereas it is known that hyperfine couplings decrease as solvent polarity decreases.[5,7,10,11] These discrepancies suggest that the assignment of the DMPO adduct produced by TPAH/H_2O_2 in pyridine to I[8,9] might be incorrect.

The present study was undertaken to establish the EPR parameters of spin adducts I and II in pyridine and to identify the DMPO adduct produced in this solvent by the TPAH/H_2O_2 system. To generate the superoxide radical we used crown ether-solubilized KO_2 (refs. 10, 12) and photoactive pigments, such as 1,8-dihydroxy-anthraquinone (1,8-DHAQ), anthralin (An), benzanthrone (BZ), rose bengal, and Zn-tetraphenylporphyrine (ZnTTP), which are known to possess photosensitizing capabilities. EPR spectra obtained using these approaches in pyridine were compared with spectra of I in other organic solvents.

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* Rate constants for ·OH reaction with pyridine and PBN were reported to be 3×10^9 and 1×10^9 M^{-1} s^{-1} respectively.[2,3]

MATERIALS AND METHODS

Tetrapropylammonium hydroxide (TPAH; 1.0 M solution in water), 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), 1,8-dihydroxyanthraquinone (1,8-DHAQ), zinc tetraphenylporphyrin (ZnTTP), benzanthrone (BZ), rose bengal (RB), cetyl pyridinium chloride (CPC), potassium superoxide (KO_2), 18-crown-6 ether and pyridine were purchased from Aldrich Chemical Company (Milwaukee, WI). Anthralin (An, 1,8-dihydroxy-9[10]-anthracene, Dithranol) was from Sigma (St. Louis, MO). DMPO was purified by vacuum distillation and stored under nitrogen at $-20°C$ before use. RB/CPC ion pairs were prepared as described earlier.[13] 1,8-DHAQ was recrystallized from toluene.

EPR spectra were recorded using a Varian E-109 Century line EPR spectrometer operating at 9.5 GHz with 100 kHz modulation. Samples were irradiated directly inside the microwave cavity of the spectrometer using a 1 kW Xe lamp equipped with a Schoeffel grating monochromator. Photosensitizing dyes were irradiated at or near their absorption maxima (see Results and Discussion). A stock solution of KO_2 was prepared by adding ca. 4 mg of KO_2 powder to 1 mL of pyridine containing 0.1 M 18-crown-6 ether. To facilitate solubilization of KO_2 the sample was vortexed for 1 min. An aliquot of the KO_2 stock solution was added to pyridine containing 80 mM DMPO to a final volume of 500 μL. The sample was gassed with nitrogen for 1 min prior EPR recording. Hyperfine couplings (hf) were obtained by simulating and optimizing spectra using a PC computer.

RESULTS AND DISCUSSION

EPR spectrum of $DMPO/O_2^{\bullet-}$ (I) in pyridine

Chemical generation. It has been shown that crown ether-solubilized KO_2 produces $O_2^{\bullet-}$ efficiently in various organic solvents. We have previously found that under carefully controlled conditions 18-crown-6 ether-solubilized KO_2 and DMPO in benzene or toluene yield adduct I.[5,10] In this work we used the same procedure to prepare I in pyridine. Figure 1A shows the EPR spectrum observed when an aliquot of the stock solution of KO_2 was added to pyridine containing DMPO (80 mM) and the solution was deaerated by bubbling with N_2 for 1 min. The spectrum has hf couplings $a_N = 12.36$ G, $a_H^\beta = 9.85$ G and $a_H^\gamma = 1.34$ G and it was assigned to adduct I. The number of hf components and values of the coupling constants ($a_N > a_H^\beta$) are characteristic of a DMPO adduct with $HO_2^{\bullet-}$ or $O_2^{\bullet-}$ radicals. In particular the ratio of the nitrogen to β-hydrogen coupling (a_N/a_H^β) of 1.25 is in a range characteristic of adduct I.[14]

Photochemical generation. We have previously found that illumination of certain photoactive pigments in aerated organic solvents in the presence of DMPO is an efficient means to generate adduct I.[7] In the present work we used a similar method to produce adduct I in pyridine. When RB/CPC and DMPO (80 mM) in pyridine were illuminated (570 nm) the EPR spectrum shown in Figure 1B was observed ($a_N = 12.6$ G, $a_H^\beta = 10.06$ G, $a_H^\gamma = 1.36$ G). The spectrum is identical with that generated by KO_2 and it was assigned to adduct I. Similar spectra were observed
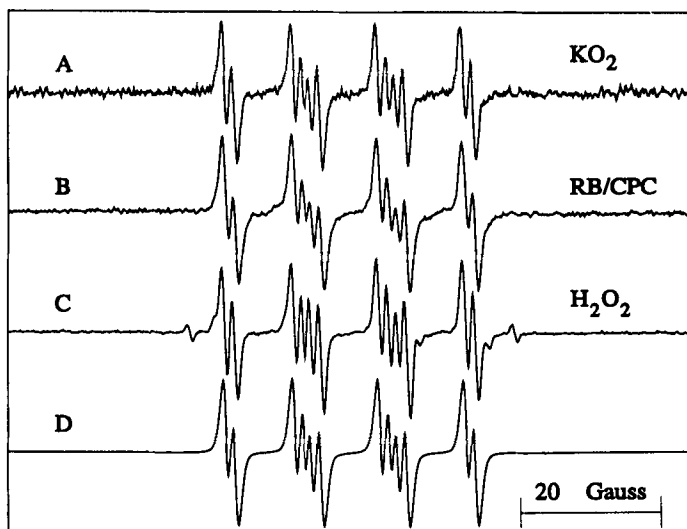
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FIGURE 1   EPR spectra of adduct I in pyridine. (A) Generated by addition of 25 μL of the 18-crown-6 ether-solubilized KO_2 (ca. 4 mg/mL in 0.1 M crown ether) to DMPO (80 mM) in pyridine (475 μL). (B) Generated by illumination (570 nm) of RB/CPC and DMPO (80 mM) in pyridine. Solution was gassed with N_2 for ca. 15 sec prior to the experiment. (C) Generated by addition of 10 μL of H_2O_2 (30%) to 490 μL of pyridine containing DMPO (80 mM). Sample was deaerated with N_2 for ca. 5 min prior to EPR experiment. The spectrum contains lines from adduct I, II and DMPO/R (R—unidentified carbon-centered radical). (D) Simulated spectrum B using the following hyperfine couplings: 12.36 G (N), 9.85 G (H), 1.34 G (H). Instrumental settings: microwave power 20 mW (A, B), 10 mW (C), modulation amplitude 0.33 G, receiver gain 2.5×10^4 (A), 1.6×10^4 (B), 4×10^3 (C); time constant 0.25 sec; scan rate 4 min.

when An (4.4 mM, 420 nm), BZ (4.4 mM, 420 nm), 1,8-DHAQ (2.1 mM, 420 nm), ZnTPP (0.6 mM, 420 nm) were used as sensitizers. This shows that the spin adduct formed upon photolysis of these pigments is independent of the type of sensitizer employed. Control experiments showed that no spin adducts were generated when the pigments were omitted and when DMPO alone in pyridine was exposed to 420 nm or 570 nm radiation.

Comparative analysis. We wished to see how the EPR spectrum of I in pyridine relates to that in other organic solvents. Janzen has previously established that for I, the relationship between a_H^β and a_N is given by Eq. 3.[11] The latter equation was obtained using hf couplings of I in aqueous solution, ethylene glycol, methanol, ethanol, acetonitrile, DMSO and DMF.[11]

$$a_H^\beta = a_N - 2.7 \text{ (in Gauss)} \tag{3}$$

Our revised hf parameters for I in pyridine satisfactorily fit this equation: the calculated value of a_H^β is 9.66 G while our experimental value is 9.85 G for the KO_2 experiment.

TABLE I

Experimental and calculated (using Eqs. 4 and 5) values of hyperfine coupling constants of adduct **I**

Solvent	a_N (G) measured	a_N (G) calculated	a_H^β (G) measured	a_H^β (G) calculated
Pyridine	12.36	12.59	9.85	10.15
DMF[a]	12.8	12.65	9.9	10.19
Acetone[a]	12.35	12.54	8.87	10.12

[a] From reference 7.

Recently we have shown that the hf couplings of **I** are related to the solvent acceptor number AN by equations (4) and (5) (ref. 7).*

$$a_N \ (G) = (0.032 \pm 0.004) \times AN + (12.14 \pm 0.11) \qquad (4)$$

$$a_H^\beta \ (G) = (0.018 \pm 0.006) \times AN + (9.9 \pm 0.16) \qquad (5)$$

Equations (4) and (5) were derived using hf couplings for **I** in the following solvents: water (54.8), methanol (41.3), ethanol (37.1), acetonitrile (19.3), dimethylsulfoxide (19.3), N,N-dimethylformamide (16.0), acetone (12.5), benzene (8.2), n-heptane (0.0), (values in parentheses show AN for a given solvent[15]). The pyridine AN value of 14.2 (ref. 15) indicates that in this solvent a_N and a_H^β couplings for **I** should be between those determined in DMF and acetone.[7] Table 1 summarizes experimental and calculated hf constants. Figure 2 shows the relationship between the hf couplings of **I** and AN for several solvents. It can be seen that the experimental values for pyridine (data point #7) are indeed close to those in DMF (#6) and acetone (#8), as expected. Thus, the newly determined hf couplings of the DMPO adduct produced with KO_2 and by photolysis of pigments in pyridine conform to parameters expected for **I** in this solvent. This analysis also indicates that the spectra produced by $TPAH/H_2O_2$ in pyridine[8,9] cannot be assigned to the DMPO/superoxide adduct, **I**.

*EPR spectrum of DMPO/·OH adduct (**II**) in pyridine*

Because it was previously reported[8,9] that there was no evidence for the formation of adduct **II** in pyridine (although no specific data for the latter species were given), we decided to determine the hf couplings of **II** in this solvent. For this purpose adduct **II** was first generated in aqueous solution by UV photolysis of H_2O_2 (90 mM) in the presence of DMPO (80 mM). A small aliquot of the irradiated solution (25 μL) was then added to pyridine (475 μL) and the sample was deoxygenated with nitrogen gas for 1 min. The observed EPR spectrum of adduct **II** in pyridine (5% H_2O) is shown in Figure 3A ($a_N = 13.53$ G, $a_H^\beta = 11.38$ G, $a_H^\gamma = 0.78$ G). This spectrum is very similar to that previously attributed to **I**[8,9] although our spectrum has narrower lines and additionally exhibits a γ–H coupling. Control experiments indicated that these differences are due to the oxygen-broadening effect, which in our samples has been reduced by saturation of the sample with N_2, and due to overmodulation of the spectral lines (0.33 G and 0.165 G *versus* 1 G). The presence of coupling to γ–H

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* The solvent acceptor number, AN, is defined as a dimensionless number related to the relative chemical shift of ^{31}P in triethylphosphine oxide in the particular solvent.[15]
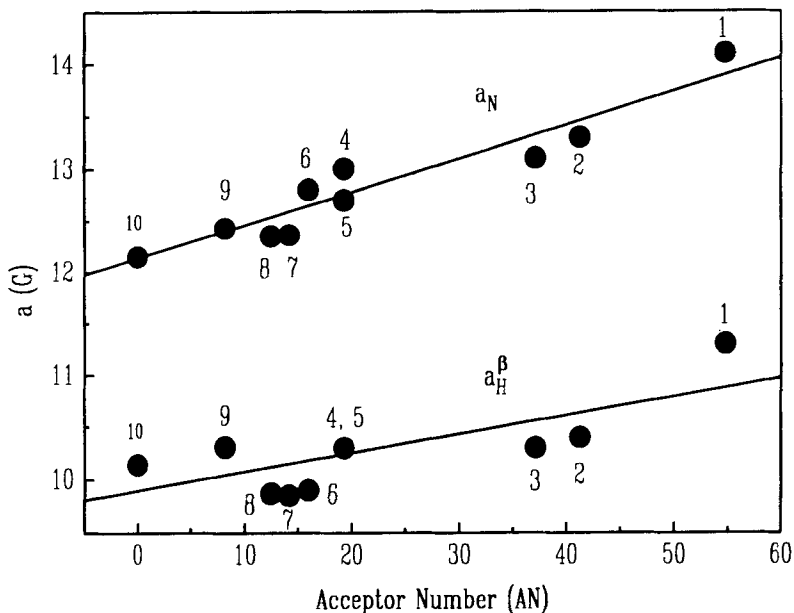
FIGURE 2   Relationship between hyperfine couplings a_N and a_H^β and the solvent acceptor number AN (from ref. 7). 1—H_2O, 2—MeOH, 3—EtOH, 4—acetonitrile, 5—DMSO, 6—DMF, 7—pyridine, 8—acetone, 9—benzene, 10—n-heptane.
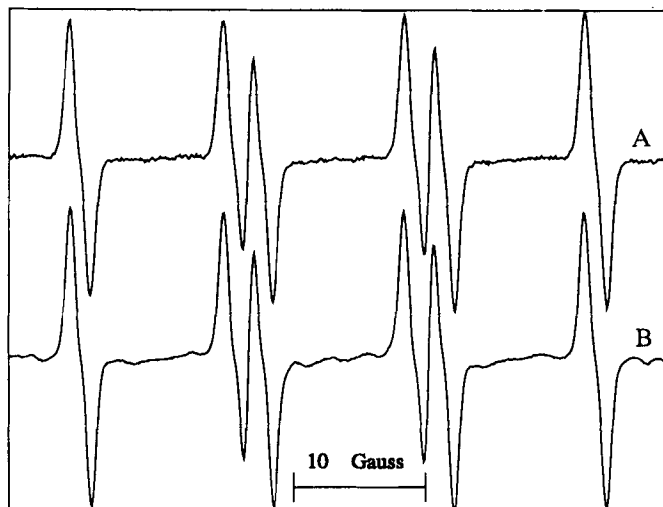

FIGURE 3   EPR spectra of adduct II in pyridine. (A) Adduct II was produced by UV photolysis (2 min) of H_2O_2 (90 mM) in the presence of DMPO (80 mM) in aqueous solution in a pyrex test tube. Then 25 μL of the photolyzate has been added to 475 μL of pyridine followed by deaeration with N_2 for 1 min prior to EPR experiment. (B) Adduct II from a pyridine solution containing TPAH (1 mM), H_2O_2 (45.5 mM) and DMPO (ca. 80 mM). Sample was gassed with N_2 for 30 sec prior EPR measurement. Instrumental settings: microwave 20 mW, modulation amplitude 0.33 G (A) and 0.165 G (B), receiver gain 5×10^3 (A) and 4×10^2 (B), time constant 0.128 sec and scan rate 4 min.

in the EPR spectrum of **II** has been previously reported in a 50:50 EtOH/H_2O mixture.[16]

Next, we investigated the radicals formed in a system consisting of TPAH, H_2O_2 and DMPO in pyridine. We found that over a wide range of concentrations of TPAH (0.2 to 100 mM) and H_2O_2 (9 to 100 mM) all observed EPR spectra were similar to that shown in Figure 3B ($a_N = 13.53$ G, $a_H^\beta = 11.39G$, $a_H^\gamma = 0.79$ G) and therefore they were also assigned to adduct **II**. Because these spectra are also similar to that shown in Figure 3A we believe that the EPR spectra observed by Damerau[8] and Haseloff *et al.*[9] belong to adduct **II** and not adduct **I**. Thus, addition of DMPO to a superoxide-generating system does not guarantee formation of the anticipated adduct **I** because both the spin trap and the adduct may participate in other radical and non-radical processes. (For example it has been reported that DMPO undergoes degradation and oxidative ring opening when exposed to some chemical sources of superoxide radicals.[10,17])

Despite our failure to observe **I** in the presence of TPAH and H_2O_2 this adduct can be readily generated by addition of concentrated hydrogen peroxide (30%) to DMPO (80 mM) in pyridine. Figure 1C shows the EPR spectrum observed from such a system. The spectrum is a composite of three species: dominating components come from adduct **I** ($a_N = 12.45$ G, $a_H^\beta = 9.85$ G, $a_H^\gamma = 1.38$ G), and much weaker lines from adduct **II** ($a_N = 13.7$ G, $a_H^\beta = 11.3$ G, $a_H^\gamma = 0.9$ G) and a DMPO adduct of an unidentified carbon-centered radical ($a_N = 14.0$ G, $a_H^\beta = 19.7$ G). While it is not known exactly how adduct **I** might be produced in this system, it may result from nucleophilic addition of H_2O_2 to DMPO, to give hydroxylamine **III**, followed by oxidation to yield nitroxide **I** (Eq. 6).

$$ \tag{6} $$
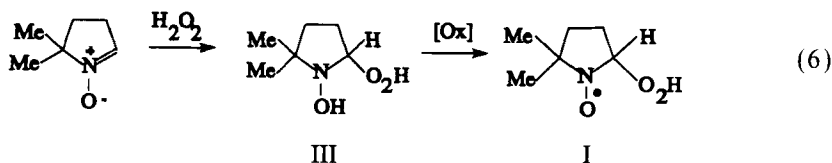
It is known that nitrone spin traps are prone to form conjugates with nucleophiles and this reaction may be enhanced in aprotic solvents. In fact, it was reported that addition of H_2O_2 to PBN in pyridine produces an EPR spectrum with $a_N = 15.20$ G and $a_H^\beta = 3.38$ G indicating the formation of a PBN/$HO_2^\bullet$ adduct whereas no such an adduct was formed by addition of H_2O_2 to PBN in aqueous solution.[18]

Such a non-radical mechanism is known to produce DMPO/·OH *via* addition of water to DMPO to give hydroxylamine form of **II** which upon oxidation yields **II**.[19,20] This process is markedly enhanced by metal ions, e.g., Fe^{3+}, Cu^{2+}.[20] In fact, adduct **II** in the spectrum in Figure 1C may have a similar origin. For a similar process (Eq. 6) to produce adduct **I** in organic media it would be necessary for H_2O_2 to add to DMPO more efficiently than H_2O does. This is likely because H_2O_2 is a more polarizable molecule than H_2O and it is therefore expected to possess stronger nucleophilic properties. The hydroxylamine **III** could be converted into adduct **I** by reacting with another molecule of H_2O_2 or dissolved oxygen.* However, other mechanism(s) cannot be excluded.

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* We observed that addition of H_2O_2 (30%) to DMPO in benzene or toluene also generates adducts **I** and **II** (K. Reszka, R.D. Sik and C.F. Chignell, unpublished observations).

In this work we generated the DMPO/superoxide adduct in pyridine using KO_2 solubilized by 18-crown-6 ether. The same adduct was also generated photochemically using several photoactive pigments. All these approaches produced similar EPR spectra consistent with the formation of adduct I. The identification was further confirmed by comparison of the revised hf couplings of I with those previously determined for the same adduct in other organic solvents. It is shown that the hyperfine couplings of adduct I in pyridine obey the formula relating coupling constants with solvent acceptor number.

We have also compared EPR spectra of DMPO adducts with superoxide and hydroxyl radicals in pyridine and found that these species produce markedly different spectra allowing their easy idenfitication. It is concluded that although the reaction between TPAH and H_2O_2 in pyridine may generate superoxide radicals only the ·OH adduct (II) is observed in the presence of DMPO. It is not known whether the DMPO/·OH adduct is generated by reaction of ·OH with trap or by some other radical or non-radical processes.

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