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SPIN-TRAPPING OF THE SUPEROXIDE RADICAL IN APROTIC SOLVENTS

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The superoxide adduct of 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) has been detected by EPR spectroscopy in aprotic solvents using KO_2 solubilized in 18-crown-6-ether as a source of superoxide. The EPR hyperfine splitting constants of the DMPO-superoxide adduct were as follows: benzene/toluene ($a_N = 12.65\,G$; $a_H^\beta = 10.4\,G$; $a_H^\gamma = 1.3\,G$); heptane ($a_N = 12.49\,G$; $a_H^\beta = 10.29\,G$; $a_H^\gamma = 1.2\,g$); and acetone ($a_N = 12.6\,G$; $a_H^\beta = 10.17\,G$; $a_H^\gamma = 1.3\,G$). The EPR parameters for benzene, toluene and heptane differ significantly from previously reported values. A plot of the hyperfine splitting constants for the DMPO superoxide adduct as a function of solvent polarity (Kosower Z value) indicates that while a_N and a_H^β both decrease by about 1 G on going from water to ethanol, further decreases in polarity do not greatly affect these EPR parameters.

KEY WORDS: EPR, spin trapping, superoxide radical, 5,5-dimethyl-1-pyrroline-*N*-oxide.

INTRODUCTION

Alkoxyl ($RO^\cdot$) and peroxyl ($RO_2^\cdot$) radicals, including superoxide ($^\cdot O_2^-$) and hydroperoxyl ($^\cdot O_2H$) radicals, are of great importance due to their involvement in many biological processes. Electron paramagnetic resonance (EPR) spectroscopy, in conjunction with the spin trapping technique, has become an important tool for studying both the generation and properties of these radicals in various environments.[1-10] The spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) has proved to be particularly useful for identifying oxygen radicals in biological systems because it produces adducts with characteristic spectra upon reaction with $^\cdot OH$ and $^\cdot O_2^-/^\cdot O_2H$ radicals.[1,2,5]

The β-hydrogen hyperfine (hf) splitting of a DMPO adduct is the EPR parameter which is most sensitive to the nature of the radical trapped. In this respect one important limitation of DMPO is that the EPR spectra of its adducts with superoxide and peroxyl radicals are very similar (Table I). However, in aqueous solutions it is possible to distinguish between these two types of adduct by using the enzyme superoxide dismutase (SOD). In those cases in which the superoxide radical is present, the addition of SOD should abolish the EPR spectrum of the $DMPO/^\cdot O_2^-$ adduct, while the $DMPO/^\cdot O_2R$ adduct is not affected.[4] No such problem exists in differentiating between the EPR spectra of $DMPO/^\cdot O_2^-$ and $DMPO/^\cdot OR$ radical adducts (Table I).

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TABLE I
Hyperfine couplings (G) of DMPO/R in aqueous solutions

R	a_N	a_H^β	a_H^γ	Ref.
$^{\cdot}O_2^-$	14.3	11.7	1.25	1
Ethylperoxyl	14.6	11.0	1.25	4
	14.7	11.1	1.25	26
Cumylperoxyl	14.5	10.75	1.7	28
Cumyloxyl	14.8	16.3	-	26
	14.88	15.04	-	6
t-Butoxyl	14.85	16.4	-	26
	14.5	16.0	-	27

It seems reasonable to expect that the EPR spectra of DMPO/$^{\cdot}O_2^-$ and DMPO/$^{\cdot}O_2R$ adducts would also be similar in non-aqueous solvents.* However, the reported hf couplings for these two classes of adducts differ significantly in the aprotic solvents benzene and toluene (Table II and III). For example the a_H^β values for DMPO/$^{\cdot}O_2R$ adducts are in the range 9.0–12.0 G (see Table III for some examples) while for the DMPO/$^{\cdot}O_2^-$ adduct a_H^β values from 6.8 to 6.9 G have been reported (Table II). A comparison of the couplings in Tables II and III shows that published a_H^β values of the DMPO/$^{\cdot}O_2^-$ adduct in benzene and toluene are, contrary to expectation, closer to the couplings of the DMPO/$^{\cdot}$OR radicals.

With the exception of benzene, toluene and heptane the a_H^β values for the DMPO/$^{\cdot}O_2^-$ adduct in solvents of various polartites and proton-donating abilities are in the expected range 9.0–12.0 G.[3] Because the anomalous values of this parameter were observed chiefly for benzene and toluene (there are no literature data on hf couplings for DMPO/$^{\cdot}O_2R$ adducts in heptane and acetone to compare with those of DMPO/$^{\cdot}O_2^-$ in these solvents) it became important to re-examine the EPR spectra of the DMPO/$^{\cdot}O_2^-$ spin adduct in benzene and toluene.

Recently it has been realized that there are important differences between solvents in their reactivities toward $^{\cdot}O_2^-$. For example, both the nucleophilicity and basicity of the superoxide radical are markedly enhanced in aprotic media. Indeed, superoxide is capable of abstracting protons from many, hitherto believed-to-be-inert, solvents and solutes.[12,13] According to some workers[12] only dry benzene and toluene can be considered as truly inert solvents. We have therefore undertaken a systematic EPR/

TABLE II
Literature values for the hyperfine coupling constants of DMPO/$^{\cdot}O_2^-$ in aprotic solvents

Solvent	a_N	a_H^β	a_H^γ	Source of radicals (Ref.)
Benzene	12.9	6.9	-	visible light illumination of phthalocyanine or CdS and DMPO; KO_2/crown ether/DMPO; (3)
Heptane	12.9	6.8	-	same as above, (3)
Acetone	13.1	9.1	-	same as above, (3)
Benzene	12.8	6.9	1.7	UV photolysis of benoxaprofen + DMPO; (24)
Toluene	12.8	6.0	2.0	KO_2/crown ether/DMPO at −50°C; (15)

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*We will generaly denote the reduced molecular oxygen radical as $^{\cdot}O_2^-$, without necessarily implying that the protonation state in aprotic solvents is known.

TABLE III
Hyperfine couplings (G) of DMPO/R radicals in toluene[a]

R	A_N	a_H^β	a_H^γ
Cumylperoxyl	13.92	11.2	-
t-Butylperoxyl	12.72	9.36	1.44
Cumyloxyl	13.08	8.88	1.68
t-Butoxyl	13.08	7.44	1.68
Ethoxyl	13.22	6.96	1.89[b]
Butoxyl	13.61	6.83	2.06[b]

a) From Reference 8; b) in benzene, from Reference 11.

spin trapping study of the superoxide radical in dry benzene and toluene using KO_2/18-crown-6 ether as a source of ${}^{\cdot}O_2^-$, and DMPO and 3,3,5,5-tetramethyl-1-pyrroline-*N*-oxide (Me_4PO) as the spin trapping agents. The DMPO/${}^{\cdot}O_2^-$ adduct has also been generated in acetone and heptane. Finally the EPR spectra of DMPO/${}^{\cdot}O_2^-$ generated chemically and photochemically have been compared.

MATERIALS AND METHODS

Potassium superoxide, DMPO, 3,3,5,5-tetramethyl-pyrroline-*N*-oxide (Me_4PO), and 18-crown-6 ether were purchased from Aldrich Chemical Co. (Milwaukee, Wi). DMPO was purified by distillation under reduced pressure and stored at −20°C before use. Toluene was dried by distillation and benzene with sodium-lead alloy (dri-Na, Baker). n-Heptane and acetone (Aldrich) were used as received.

A stock solution of KO_2 was prepared as follows. Finely powdered potassium superoxide (ca. 3.8 mg) was added to 1 mL of toluene (or benzene) containing 0.1 M crown ether and the mixture was shaken vigorously or agitated on a vortex mixer for 30 sec, or dispersed using magnetic stirrer for 6 hours. Occasionally sonication (ca. 15 sec) was applied if the KO_2 failed to disperse. All these procedures produced similar results. Samples for EPR studies were prepared by adding an aliquot of the KO_2 suspension, shaken well before pipeting, to 500 μL of deaerated benzene (gassed with N_2 for 10 min) containing 0.1 M crown ether and DMPO, ca. 80 mM. Toluene, acetone and heptane solutions were prepared in a similar manner. Subsequently the sample was transfered quickly to a flat EPR cell and the first scan was started in less than 2 min from the moment of KO_2 addition.

EPR spectra were run on Varian EPR spectrometer, E-Line Century Series, using the following parameters: microwave power 10 mW, modulation amplitude 0.165 G, time constant 0.128 or 0.25 s, scan range 100 G, scan time 4 or 8 min. The kinetics of the decay of EPR signal were followed by monitoring the low-field doublet of the spectrum as a function of time. All experiments were performed at room temperature.

RESULTS AND DISCUSSION

EPR spectrum of the DMPO/${}^{\cdot}O_2^-$ adduct

Preliminary experiments showed that the EPR spectra obtained when KO_2, solubilized with crown ether, was addd to DMPO in benzene were dependent upon the

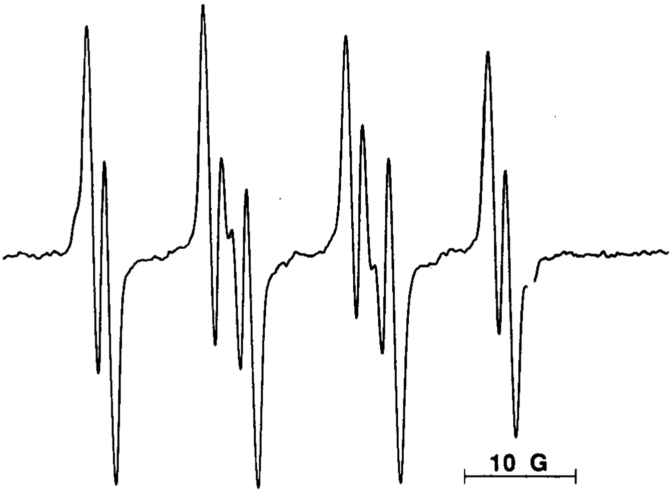

FIGURE 1 EPR spectrum of the DMPO/$^{\cdot}O_2^-$ adduct in benzene. Sample was prepared by adding 20 μL of KO_2 stock solution to 500 μL of deaerated benzene containing 18-crown-6 ether (0.1 M) and DMPO (80 mM). Recording parameters: microwave power 10 mW, modulation amplitude 0.165 G, gain 1.6×10^3, scan range 100 G, time constant 0.128 G, scan time 4 min.

concentrations of the reactants and the time after mixing. However, when 20 μL of KO_2 suspension was added to 500 μL of deaerated benzene as described in Materials and Methods, the EPR spectrum seen in Figure 1 was reproducibly observed (adduct I). Kinetic measurements showed that the signal decayed realtively slowly reaching 50% of the initial value in less than 10 min from the start of the experiment (i.e. KO_2 addition). The kinetics did not fit to either a first or second order reaction. This may be a result of the simultaneous production and decay of the spin adduct. Identical results, both in terms of EPR spectrum and kinetic behavior, were observed when toluene was used as solvent.

The spectrum in Figure 1 is tentatively assigned to the DMPO/$^{\cdot}O_2^-$ spin adduct (see Table IV for hf constants). Unfortunately, no SOD-like test exists for superoxide in non-aqueous solvents, so this assignment cannot be verified in that way. However, based both on the values of hf couplings, which are characteristic of a DMPO/$^{\cdot}O_2R$ spin aduct[8] and the probable chemistry involved,[**] the correctness of this assignment is almost certain. The only possible uncertainty in this assignment stems from the observations of Forrester and Purushotham[15] and ourselves that, under certain experimental conditions, various types of carbon-centered radicals ($R^{\cdot}$) can be produced in a system containing DMPO, KO_2 and crown ether. If molecular oxygen is present (either from air or decomposition of the KO_2), these radicals could produce $RO_2^{\cdot}$ type radicals, which may generate DMPO adducts that are indistinguishable from those derived from the superoxide ion.

[**] In solvents in which KO_2 is solubilized with the agency of the crown ether (C), the superoxide radical could exist as a free ion $^{\cdot}O_2^-$, or in the form of an ion pair with the potassium cation-crown ether complex,[12] $C[K^+]^{\cdot}O_2^-$. Certain experimental data suggest that in benzene $^{\cdot}O_2^-$ exists in the latter form (Ref. 12 and references quoted therein). If this would be the case, then in the presence of DMPO adduct I could be formed according to the following reaction: $C[K^+]^{\cdot}O_2^- + \mathrm{DMPO} \rightarrow C[K^+] + \mathrm{DMPO}/^{\cdot}O_2^-$. This would indicate that the binding of $^{\cdot}O_2^-$ within the ion pair is sufficiently weak to allow a nucelophilic addition of the radical to the spin trap.

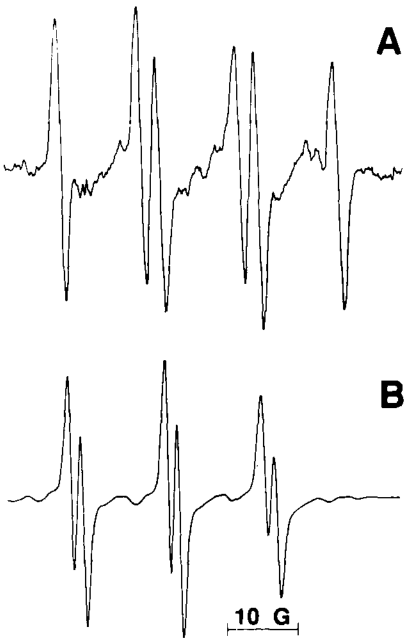

FIGURE 2 EPR spectra of DMPO adducts from deaerated samples consisting of: A – 400 μL of 0.1 M crown ether in benzene + 100 μL KO_2 stock solution + DMPO (80 mM); B – 500 μL of 0.1 M crown ether in benzene + DMPO (80 mM) to which a few mg. KO_2 powder was added.

Effect of the KO_2 concentration on EPR spectra of DMPO adducts

The procedure for the generation of adduct I, described above, gives reproducible results. The KO_2 stock solution, prepared as described in Materials and Methods, is a very efficient and long-lived source of superoxide radicals.[14] However, the type of EPR spectrum appeared to depend on the amout of KO_2 added. For example, Figure 2A shows spectrum of another adduct (II; $a_N = 13.7\,G$, $a_H^{\beta} = 11.2\,G$), recorded when 100 μL of the KO_2 stock solution (5-times more than in the case shown in Figure 1.) was added to 400 μL of benzene. Figure 2B shows spectrum of a third adduct (III; $a_N = 13.23\,G$, $a_H = 1.6\,G$), observed when a few milligrams of the KO_2 powder were added directly to benzene containing the crown ether and DMPO. When these higher concentrations of the KO_2 were used the DMPO/$^{\cdot}O_2^-$ spin adduct was observed either as a short-lived transient (the first scan gave spectrum of the adduct I, but the second and subsequent scans produced totally different specta) or was not observed at all. Although not studied in detail, it may be concluded that there is a rather narrow range of concentrations of the KO_2/crown ether complex, over which the DMPO/$^{\cdot}O_2^-$ adduct can be observed.

An EPR spectrum similar to that of adduct III (Figure 2B), with hf couplings $a_N = 13.2\,G$, $a_H^{\beta} = 1.6\,G$, has been previously observed by Forrester and Purushotham[15] 30 min after the addition of KO_2 or potassium ozonate to toluene or benzene solutions containing DMPO and crown ether. Since these couplings are characteristic of 2-methyl-2-nitrosopropane (MNP)-type spin adducts, Forrester and Purushotham have suggested[15] that adduct III is an *s*-alkyl-*t*-alkyl nitroxide formed by the reaction of a DMPO-derived nitroso compound with a carbon-centered radical from the crown ether. A spectrum similar to that of adduct III was obtained by these workers when a solution of the 18-crown-6-ether and MNP in benzene was treated with the *t*-butoxy radical source di-*t*-butyloxate.[15] Mottley and Mason[10] have also observed an adduct similar to III when acetylacetone was treated with the horseradish peroxidase/H_2O_2 system in the presence of oxygen and DMPO. These workers suggested that a spin adduct "imposter" was formed by the opening of the DMPO ring to yield a MNP-type adduct. Hill and Thornalley[16] have reported that DMPO ring opening occurs during the reaction of this spin trap with coordinated superoxide in phosphate buffer pH 7.0 and is enhanced by the presence of oxygen. These workers proposed that 5,5-dimethyl-2-pyrrolidone-1-oxyl was a key intermediate in the reaction.

The identity of spin adduct II (Figure 2A) is less clear, although its hf couplings, suggest that it is the DMPO adduct of an oxygen-centered radical. A survey of the liteature[17,18] yields three examples of DMPO spin adducts with hf couplings reasonably close to adduct II. Two of them have been attributed to the DMPO/$^\bullet$OH adduct in toluene; their hf couplings are: $a_N = a_H^\beta = 13.0\,G$[15] and $a_N = 13.75\,G$, $a_H^\beta = 12.10\,G$.[19] While the formation of the $^\bullet$OH radical, via a superoxide driven Fenton reaction, and its trapping by DMPO is known to occur in aqueous solutions[20] formation of a similar adduct in toluene (or benzene) is unlikely in view of the very high rate constant ($\sim 5 \times 10^9\,M^{-1}s^{-1}$) for the addition of the $^\bullet$OH radical to toluene[21] and benzene[22] and the high concentrations of these solvents relative to the spin trap. An alternative non-radical mechanism, at least in aqueous solutions, is the transformation of the DMPO/$^\bullet O_2^-$ adduct into the DMPO/$^\bullet$OH adduct.[23] The third example is the DMPO/cumyl peroxyl adduct in toluene, with $a_N = 13.92\,G$ and $a_H^\beta = 11.2\,G$.[8] These hf couplings, as well as the absence of a γ-hydrogen splitting, are characteristic of an adduct formed by the reaction of DMPO with a bulky peroxyl radical and they are totally different from those assigned to the DMPO/$O_2^{\bullet -}$ adduct. Although the nature of this radical is not known, one may speculate that in our system a similar species could be formed by oxygen attack on carbon-centered radicals derived from the crown ether or the spin trap itself.

Correlation between hf couplings of DMPO/$^\bullet O_2^-$ and solvent polarity

Harbour and Hair[3] have examined the relationship between the hf coupling constants of the DMPO/$^\bullet O_2^-$ adduct and solvent polarity as estimated by the Kosower Z value. These workers have reported that, while a_N values showed a small decrease as solvent polarity decreased, the a_H^β values declined significantly as the dielectric constant of the medium was lowered on going from water to benzene. Figure 3 shows the data of Harbour and Hair[3] together with our revised hf constants for the DMPO/$^\bullet O_2^-$ adduct in benzene. In addition we re-determined the hf constants for acetone and heptane using the procedure described in Methods for benzene (Table IV). It may be seen from Figure 3 that when the new values are used (data for heptane are not plotted since no *Z* value is available) the plot of a_H^β vs *Z* parallels that for a_N vs *Z*, and that for solvents with *Z* values below ca 75 kcal/mol the couplings are very similar.

Photochemical generation of DMPO/$^\bullet O_2^-$ adduct

The detection of the true EPR spectrum of the DMPO/O_2^- adduct in benzene

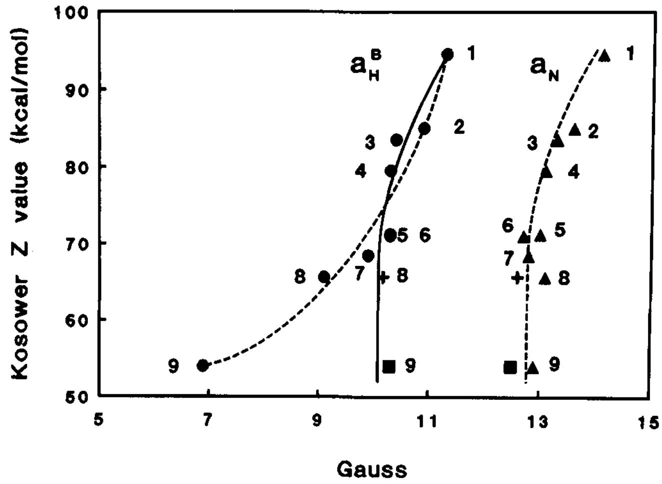

FIGURE 3 Dependence of a_N and a_H^β of the DMPO/˙O_2^- adduct on the Kosower Z values. ---●---, ---▲--- relationship between a_H^β/a_N and Z from reference 3. ——, relationship between a_H^β and Z taking into account the results of this work (+, ■). Solvents: 1, H_2O; 2, ethylene glycol; 3, methanol; 4, ethanol; 5, acetonitrile; 6, dimethylsulfoxide; 7, dimethylformamide; 8, acetone; 9, benzene.

prompted us to repeat our photochemical experiments with the phototoxic anti-inflammatory drug, benoxaprofen.[24] Previously we reported that the superoxide radical is produced upon UV irradiation of this drug in ethanol, DMSO, and benzene solutions. While EPR spectra observed in ethanol and DMSO correspond to those expected for DMPO/˙O_2^- adducts in those solvents, the spectrum observed in benzene (see Table II for hf couplings) now must be viewed with suspicion. In our previous study an aerated benzene solution of benoxaprofen and DMPO was irradiated in a glass test tube outside the EPR spectrometer, saturated with N_2 for several minutes, and then transfered to quartz cell for the EPR measurement. In this study, a benzene solution of benoxaprofen (ca. 1 mg/mL) and DMPO (ca. 80 mM) was purged gently with N_2 for 15 sec, to remove an excess of oxygen, and then irradiated (326 nm) directly inside the microwave cavity of the EPR spectrometer. After a brief exposure a strong signal was generated which contained lines from at least two components (Figure 4A). Incubation of the sample in the dark for ca. 30 min produced the spectrum shown in Figure 4B, previously assigned to the DMPO/˙O_2^- radical. The EPR spectrum in Figure 4C was obtained by adding the true spectrum of the DMPO/˙O_2^- adduct (Figure 1) to that in Figure 4B. The composite spectrum is similar to the one induced photochemically (Figure 4A). Photolysis of the drug and DMPO in deaerated benzene produced only one adduct of a carbon centered radical (weak outer lines from this adduct may be seen in Figure 4A).[24] EPR spectra similar to those

TABLE IV
Hyperfine couplings (G) of DMPO/˙O_2^- in aprotic solvents

Solvent	a_N	a_H^β	a_H^γ
Benzene/Toluene	12.65	10.4	1.3
Acetone	12.6	10.17	1.3
Heptane	12.49	10.29	~1.2

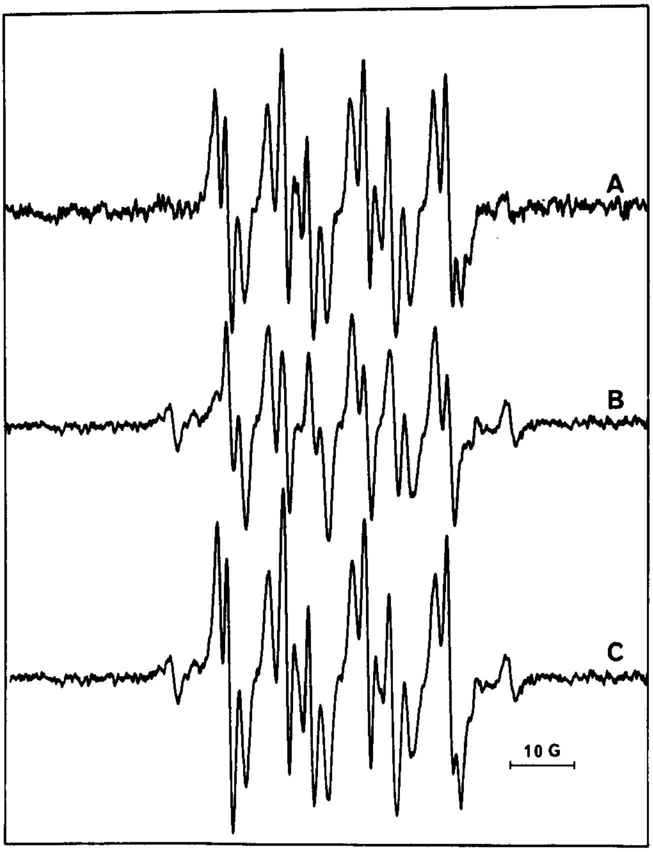

FIGURE 4 EPR spectra of DMPO adducts formed during the UV irradiation (326 nm) of benoxaprofen (ca. 1 mg/mL) and DMPO (80 mM) in benzene. A) During the photolysis; B) Sample A after ca. 30 min incubation in the dark; C) Composite spectrum obtained by adding the spectrum of the DMPO/$^{\cdot}O_2^-$ adduct from Figure 1 to the spectrum in Figure 4B. Recording parameters: microwave power 10 mW, modualtion amplitude 0.165 G, scan range 100 G, time constant 0.25 s, scan rate 4 min and gain 2.5 × 10^4 (A) and 2 × 10^4 (B).

shown in Figure 4A were also observed when phenylbenzoxazole, a simple analog of benoxaprofen, was UV irradiated in the presence of DMPO in benzene or toluene solutions. These findings indicate that both benoxaprofen and phenylbenzoxazole do generate superoxide upon irradiation in benzene solution producing spectra consistent with those from the chemical source of the $^{\cdot}O_2^-$ radical. The failure to observe the true spectrum of the DMPO/$^{\cdot}O_2^-$ adduct from the UV-photolyzed benoxaprofen in the former experiments[24] may be a result of a prolonged deoxygenation after the photolysis, during which time the DMPO/$^{\cdot}O_2^-$ spin adduct decayed away, due to its relatively short lifetime.

Trapping of superoxide with Me_4PO

The spin trap Me_4PO has been used previously to detect $^{\cdot}O_2^-$ in benzene from the

KO_2/crown ether source.[25] In the present work, when Me_4PO (80 mM) was used instead of DMPO, an adduct was detected with $a_N = 12.75$ G and $a_H^\beta = 7.85$ G. These parameters differ slightly from those reported earlier[25] for the $Me_4PO/^{\cdot}O_2^-$ adduct $a_N = 13.38$G and $a_H^\beta = 7.95$ G. When benoxaprofen was irradiated (326 nm) in the presence of Me_4PO in benzene, the resultant EPR spectrum contained contributions from two spin adducts. One of them was identified as the superoxide adduct because it showed hf couplings: $a_N = 12.75$ G and $a_H^\beta = 7.85$ G, which are in a agreement with the spectrum obtained from the chemical source.

CONCLUSIONS

In conclusion, we believe that the EPR spectrum in Figure 1 is that of the $DMPO/^{\cdot}O_2^-$ radical in benzene and toluene solutions. The successful detection of the $DMPO/^{\cdot}O_2^-$ adduct in those solvents, in the presence of the KO_2/crown ether requires the careful preparation of the stock potassium superoxide solution, the control of the concentrations of all reactants and the proper time regime for recording EPR specta. Because no experimental details are given in earlier reports[3,15] it is possible that these factors may have been overlooked. The structure of the DMPO adduct previously assigned to $DMPO/^{\cdot}O_2^-$ (Table II and Figure 4B) is still unknown, although on the basis of its hf coupling constants it is probably formed by the reaction of DMPO with an alkoxy radical. We are currently attempting to identify this adduct by thermospray mass spectrometry.

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