Nitrosobenzene: electrochemical, UV-visible and EPR spectroscopic studies on the nitrosobenzene free radical generation and its interaction with glutathione

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Abstract

This paper reports both the electrochemical characterization and the reactivity of the nitroso radical anion from nitrosobenzene with glutathione. The reduction of nitrosobenzene to the corresponding nitroso radical anion was kinetically characterized in acetonitrile. Free radicals exhibited a natural decay of second order, with a constant value of $k_2 = 15\,555 \pm 321$ M^{-1} s^{-1}. Also, the radicals were characterized by UV-Visible and EPR spectroscopy. Data obtained with these two independent techniques clearly substantiated the formation of the nitrosobenzene radical under our experimental conditions. Furthermore, we have unambiguously demonstrated that glutathione (GSH) scavenged the nitroso radical anion electrochemically generated from nitrosobenzene. The scavenging effect of GSH is supported by the following experimental facts: 1. The parallel decrease of the anodic peak current in the cyclic voltammograms, corresponding to the nitroso radical anion concomitantly with the addition of GSH. 2. The significant decrease of the visible band at 560 nm corresponds to the radical after the addition of GSH. 3. The drop of the EPR signal intensity of the nitroso radical after the addition of GSH. By using a spin trapping technique, thiyl radical (GS·) was detected during the reaction between the nitroso radical anion with GSH.

1. Introduction

Nitroso compounds are among the most potent chemical carcinogens and are widely distributed in the environment. These compounds are also recognized as potentially reactive metabolites of a variety of toxicologically interesting compounds, which after covalent

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binding to proteins and DNA, become carcinogenic and/or mutagenic [1,2].

Many nitroso compounds are reduced nonenzymatically by several endobiotic compounds, such as, NAD (P) H, glutathione, cysteine and hemoglobin with formation of free radical derivatives, as confirmed by their corresponding ESR spectra [3,4]. On the other hand, several studies using electron spin resonance [5–7] have established the presence of radicals in chemical and electrochemical reduction of nitrosobenzene in aqueous and non-aqueous solvent systems.

Several studies [8–10] using cyclic voltammetry and EPR, have demonstrated that thiol compounds, such as glutathione significantly scavenged nitro radicals. However, the interaction between nitroso radical species and thiol compounds has not received an adequate attention up-to date.

In this paper, we examine the interaction between radical species generated from nitrosobenzene and glutathione. From these studies, the electrochemical generation of nitroso radical species from nitrosobenzene has been used. Cyclic voltammetry, UV-Visible and ESR spectroscopy were used to follow the interaction of radical species and glutathione.

2. Materials and methods

2.1. Chemicals

Nitrosobenzene (NB), glutathione (GSH), anhydrous dimethylsulfoxide (DMSO), spectroscopic grade and anhydrous acetonitrile for UV spectroscopy were purchased from Merck. Solutions of NB were prepared in the different solvents, which were presaturated under a pure nitrogen stream. Tetrabutylammonium hexafluorophosphate (TBAHFP) was purchased from Aldrich.

2.2. Drug solutions

Stock solutions of 10 mM NB both in DMSO or acetonitrile were prepared and protected from daylight. Aliquots to obtain final concentrations between 0.05 and 1 mM were taken and diluted in the corresponding solvent, containing 0.1 M TBAHFP as supporting electrolyte.

2.3. Electrochemical measurements

Cyclic voltammetric experiments were carried out in a BAS CV-50 voltammetric analyzer. A routine drug concentration of 1 mM for all the experiments was used. A BAS CGME controlled-growth mercury electrode with a drop surface of 1.90 mm, a glassy carbon or a platinum coil electrode was used as working electrodes. As a counter electrode a platinum wire, was used. All potentials were measured against an Ag/AgCl reference electrode.

2.4. Controlled potential electrolysis

CPE were carried out either on a platinum coil electrode or a glassy carbon electrode at-1200 mV in anhydrous acetonitrile or DMSO containing 0.1 M TBAHFP as supporting electrolyte. Oxygen was removed by pure, dry pre-saturated nitrogen. A three-electrode circuit with Ag/AgCl electrode was used as reference. A Wenking potentiostat model POS 88 was used to electrolyze nitrosobenzene.

2.5. UV-Visible spectroscopic studies

In order to obtain further information on the mechanism, either the progress of the electrolysis or the reactivity of the product of nitrobenzene electrolysis with glutathione in DMSO, a UNICAM UV-3 spectrophotometer was used. UV-Visible spectra were recorded in the 220–700 nm range at different intervals. Acquisition and data treatment were carried out with a Vision 2.11 software. An electrolytic cell of our own construction [10], based on a 1 cm UV cuvette, with a platinum coil or a glassy carbon as a working electrodes were used for the in situ generation of the reduction species. The experiments were conducted under constant stirring, which was stopped before each measurement.

2.6. EPR measurements

The nitro radical anion from nitrosobenzene was generated in situ in the EPR cavity by electrochemical reduction (− 1200 mV) on glassy carbon at room temperature. The flat electrolytic cell Wilmed WG-810 (Wilmed Glass, Route 40 and Oak Road, Buena, NJ 08310 USA) includes a pair of platinum electrodes and an Ag/AgCl reference electrode. A 5 mM solution of nitrosobenzene containing 0.1 M TBAHFP in DMSO, was purged with nitrogen for 10 min, reduced and immediately its EPR spectrum was recorded in the microwave band X (9.85 GHz) in a Bruker ECS 106 spectrometer, using a rectangular mode cavity with a 50 kHz field modulation.

Hyperfine splitting constants were estimated to be accurate within 0.05 G. GSH was dissolved in tetrabutylammonium hydroxide to obtain final concentrations varying between 0.1 and 15 mM. To test the thiyl radical formation, 120 mM 5,5-dimethyl-1-pyrroline N-oxide (DMPO, Aldrich) was used.
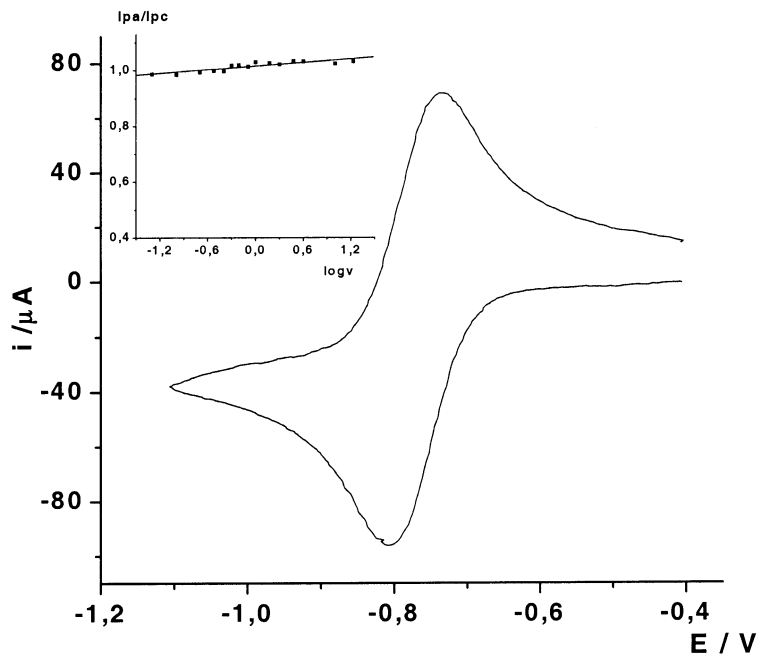
Fig. 1. Cyclic voltammogram of 5 mM nitrosobenzene on glassy carbon electrode in DMSO + 0.1 M TBAHFP. Sweep rate: 0.1 V s^{-1}. Inset: dependence between I_{pa}/I_{pc} ratio with the logarithm of sweep rate.

3. Results and discussion

The main goal of this paper was to assess the possible scavenging effect of the radical's species from nitrosobenzene by glutathione. For this purpose electrochemical, UV-Visible and ESR spectroscopic techniques were used. Because the reduction mechanism is strongly affected by the solvent, studies were performed in two different solvents, i.e. DMSO and acetonitrile.

3.1. Electrochemical generation of radical species from nitrosobenzene in aprotic media

3.1.1. Cyclic voltammetry
Firstly, reduction studies on nitrosobenzene using DMSO + 0.1 TBAHFP as supporting electrolyte on glassy carbon were conducted. As can be seen from Fig. 1, in these experimental conditions nitrosobenzene is reduced to give the nitrosobenzene radical anion at $E_{pa} = -732$ mV and $E_{pc} = -807$ mV with a separation between the anodic and cathodic peaks of 72 mV. The reduction was completely reversible as evidenced by the unity value for I_{pa}/I_{pc} ratio in all range of the sweep rate studied (inset Fig. 1).

Secondly, the stability of radical species of nitrosobenzene was also assessed in acetonitrile + 0.1 M TBAHFP. In Fig. 2, the electroreduction of nitrosobenzene in this aprotic medium is shown. As can be seen from this Figure, different reduction processes were

evidenced: (a) a first couple appears at potentials of: $E_{pc} = -780$ mV and $E_{pa} = -890$ mV), which corresponds to the one-electron transfer resulting in the generation of the nitroso radical anion (b) a second redox couple also appears at $E_{pc} = -1048$ mV and $E_{pa} = -1159$ mV, which corresponds to the reduction of the protonated radical anion (R-NHO$^{\cdot}$). As is shown in the inset of Fig. 2, after the addition of solid NaOH to the solution a complete disappearance of these sig-
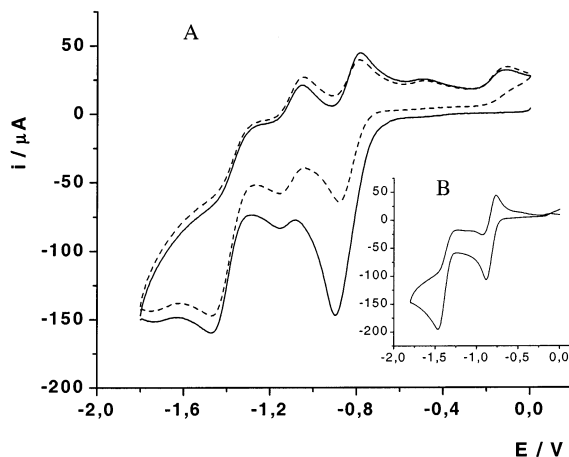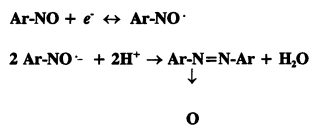

Fig. 2. Cyclic voltammograms of 5 mM nitrosobenzene on glassy carbon electrode in acetonitrile + 0.1 M TBAHFP). (—) First sweep; (---) second sweep. Sweep rate: 0.1 V s^{-1}.

nals occurred, conforming the chemical nature of the species involved in the reduction (c) finally, a third redox couple appears at potentials of: $E_{pa} = -1260$ mV and $E_{pc} = -1470$, which corresponds to the reduction of azoxybenzene [11]. However, other reduction process becomes evident at potentials near to -113 mV. By changing the cathodic switching potential from -1800 to -1100 mV it was readily deduced that this redox process arises from a product of the reduction of nitrosobenzene rather than from the reduction of the species at -1260 mV. In addition, it also was observed qualitatively that the rates of the disappearance of the nitrosobenzene reduction process and the formation of the redox couples at -113 and -1260 mV were enhanced by an increase in the concentration of the parent electroactive species.

Changing the electrochemical conditions, i.e. the scan rate, the switching potential, the stability of the species corresponding to the couple that appears at -780 mV was assessed. Results of these experiments show that as the scan rate increased, the I_{pa}/I_{pc} increased towards unit, a typical behavior for an irreversible chemical reaction following a charge-transfer step, an EC_i type of process [12]. Furthermore, ΔE_p for the couple suggests that one-electron is involved in the reduction. To check the order of the following chemical reaction, I_{pa}/I_{pc} ratio dependence on concentration of nitrosobenzene was studied. Results of those studies indicated that an increase in the nitrosobenzene concentration at a constant sweep rate, resulted in a decreased I_{pa}/I_{pc} value according to the predicted by Olmstead [12]. In consequence, these data permit us to conclude that the chemical reaction was of second order. Confirming the second order character, plots of kinetic parameter, ω, versus time constant, τ, were linear, with average correlation coefficients of 0.98. Experimental k_2 value calculated in acetonitrile according to Olmstead procedure was $15\ 555 \pm 320\ 1\ mol^{-1}s^{-1}$.

From our experimental data and others reported in the literature [11,13], it can be concluded that the reduction of nitrosobenzene in acetonitrile corresponds to a dimerization reaction with elimination of water and presumably through participation of protons from the solvent. The mechanism can be summarized by the following equations:

$$Ar\text{-}NO + e^- \leftrightarrow Ar\text{-}NO^{\cdot}$$

$$2\ Ar\text{-}NO^{\cdot-} + 2H^+ \rightarrow Ar\text{-}N\text{=}N\text{-}Ar + H_2O$$
$$\downarrow$$
$$O$$

For the studies of the reactivity of nitrosobenzene radical, we prefer DMSO as solvent because of stability of radical species is greater than the other solvent systems tested (DMF, acetonitrile) and the electrochemical process is not complicated with a coupled chemical reaction.
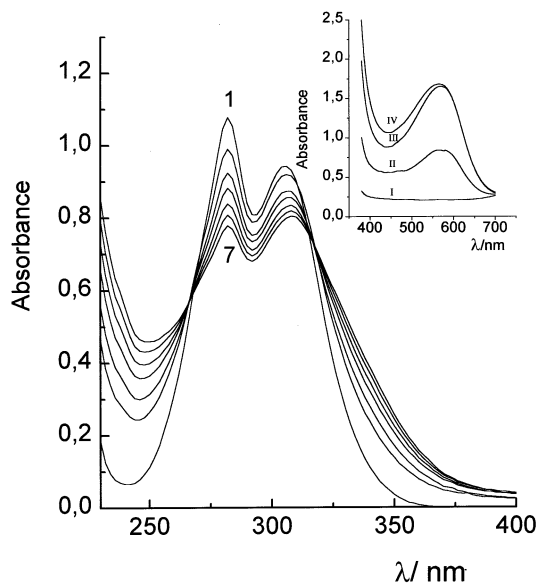

Fig. 3. Time-course of CPE of 0.4 mM nitrosobenzene solution followed by UV-Visible spectrum. $1–7 = 30$ min. Inset: visible spectrum of the nitroso radical anion formation from a 2 mM nitrosobenzene solution.

3.2. Controlled potential electrolysis

CPE of nitrosobenzene in DMSO to obtain the nitrosobenzene radical in bulk solution (big quantity) were carried out. Thus, electrolysis at a fixed potential of -1200 mV using a glassy carbon electrode as a working electrode were carried out and cyclic voltammetric and spectroscopic (UV-Visible and EPR) curves for detection were recorded.

3.3. Cyclic voltammetry

The time-course of electrolyzed 1 mM nitrosobenzene solution indicated that after 15 min the peak currents of the radical decreased, with a complete disappearance of the signals within 30 min of electrolysis. Thus, cyclic voltammetry results to be an appropriate tool to follow the time course-course of electrolysis.

3.4. UV-Visible spectroscopy

UV-Visible curves on the time-course of electrolysis were recorded at different intervals in DMSO (Fig. 3). We can see as the absorption at $\lambda_{max} = 282$ and $\lambda_{max} = 305$ nm decreased during CPE. Two isobestic points at 268 and 317 nm were also observed. This diminution in the λ_{max} indicates that there was reduction of the nitroso group. On the other hand, before the onset of the electrolysis (Fig. 3, inset: spectrum I) no absorption in the visible region is apparent. However, concomi-

tantly with the increase of the time-course of electrolysis, a visible band with a maximum at 560 nm is observed (Fig. 3, inset: spectra II–IV). These facts indicate that there is a new species in solution. To investigate the possible nature of this species, the effect of bubbling of pure oxygen on the visible absorption at 560 nm was assessed. Upon bubbling of O_2, a decrease of the visible band reaching about of 80% of diminution within 30 min was observed. Consequently, this decrease could be ascribed to a reaction between oxygen and a free radical species formed, presumably the nitroso radical anion. The reaction accounts for the decay of the visible band at 560 nm can be explained by the following well-known equation:

$$R\text{-}NO^{\bullet} - + O_2 \rightarrow O_2^{\bullet} - + R\text{-}NO$$
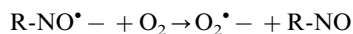
3.5. Electron paramagnetic resonance experiments

The electrochemical reduction of nitrosobenzene to the corresponding nitroso radical anion and its detection by EPR was carried out in aprotic media (DMSO). The spectrum of this radical is shown in Fig. 4 (curve 2). After the interpretation of the EPR spectrum by means of a simulation process (curve 3) led us to the determination of the coupling 1st revised version con-

Table 1
Experimental hyperfine constant values for the nitroso radical anion from nitrosobenzene electrochemically generated. Comparison with constant values obtained from Refs. [5] and [7]

	a^N/G	a_O^H/G	a_O^H/G	a_m^H/G	a_p^H/G
This paper	7.64	3.82	2.91	1.00	3.82
Ref. [5]	8.34	4.11	3.02	1.03	3.91
Ref. [7]	7.90	3.90	2.83	1.10	3.90

stants for all magnetic nuclei. In Table 1 a comparison between our values of the hyperfine constant with those obtained in Refs. [5] and [7] are included. As can be seen from this Table, no significant differences between the previously reported hyperfine constants and our values were found. Furthermore, the simulated spectrum (Fig. 4, curve 3) shows good agreement with the hyperfine constant assignments [5,14].

In conclusion, after voltammetric and spectroscopic experiments we were able to generate the nitro radical anion from nitrosobenzene in solution (in vitro) characterizing its main electrochemical and spectroscopic parameters.

3.6. Reactivity of the nitroso radical anion electrochemically generated from nitrosobenzene with glutathione (GSH)

For the studies on the reactivity of GSH with the nitroso radical, several types of experiments were conducted.
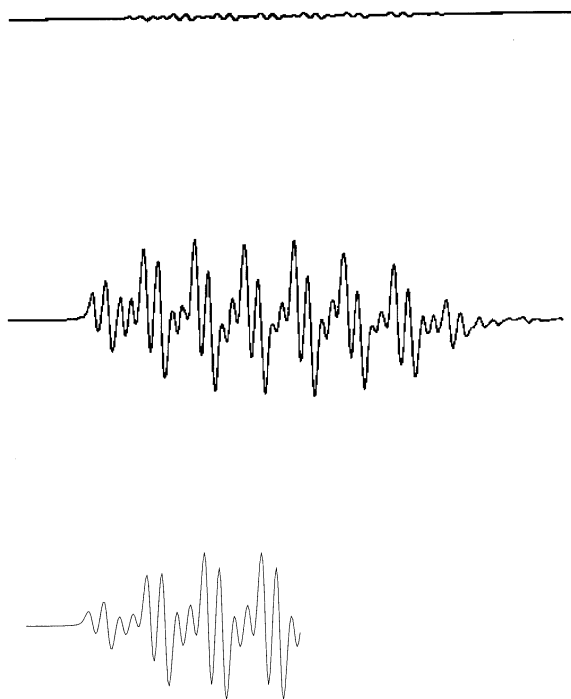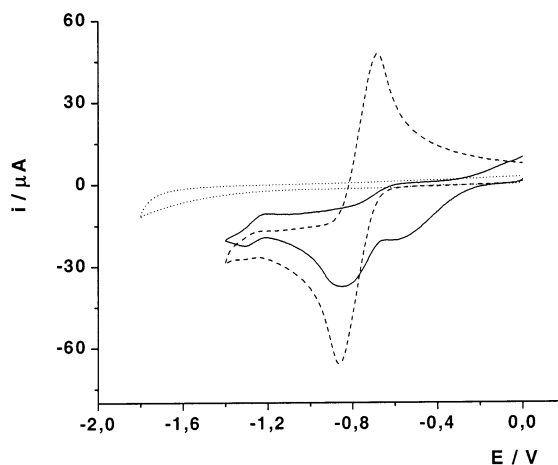

Fig. 4. Experimental EPR spectra of: (1) 0.1 M TBAHFP in DMSO. (2) Nitroso radical anion electrochemically generated from at 1 mM nitrosobenzene concentration. (3) Simulated spectrum of nitrosobenzene radical anion.

Fig. 5. Cyclic voltammograms on glassy carbon in DMSO + 0.1 M TBAHFP of: (----) 5 mM nitrosobenzene; (——) 5 mM nitrosobenzene + 10 mM GSH; (…) 10 mM GSH. Sweep rate: 1 V s^{-1}.

3.6.1. Cyclic voltammetry

Fig. 5 shows the cyclic voltammograms of nitrosobenzene in DMSO solutions in the absence and in the presence of GSH. Curve 1 illustrates the CV involving the 1-electron transfer process corresponding to the nitroso radical anion formation. When GSH was added to the medium, the anodic peak practically disappeared concomitantly with a significant decrease of the cathodic peak (Curve 2). Also, GSH produces a significant increase in the cathodic current of the couple that appears at -1230 mV. This effect of GSH can be attributed to a protonation effect on the radical. On the other hand, GSH at the studied concentrations did not interfere the signals corresponding of the radical (curve 3).

These results clearly indicate that the free radical formed from nitrosobenzene was scavenged by glutathione at a nitrosobenzene: GSH ratio of 1:2. Similar results were obtained at other molar ratio, exhibiting a concentration-dependent effect (data not shown).

3.7. UV-Visible spectroscopy

The effect of GSH on the nitroso radical was followed through the intensity changes of the peak at 560 nm. The addition of 1 mM of GSH to reaction mixture produced the total disappearance of the visible band at 560 nm, indicating that the quantity of radical produced during the electrolysis is scavenged by GSH. The time in which thiol is added to the medium, either before start or the end of the electrolysis had not influence on the final results.

3.8. Electron Paramagnetic Resonance experiments

When the nitro radical anion was formed by CPE, GSH was added to the solution. By means of the spectrum we try to establish the scavenger ability of the thiol in this system. In Fig. 6 (curve 2) the spectrum corresponding to the nitroso radical anion is shown. In curve 3, the scavenging effect of GSH on the nitroso radical anion is shown. As illustrated in this Figure, the presence of GSH decreased the intensity of the signal until complete disappearance. This effect was concentration-dependent, from 1 mM concentration of GSH the scavenging is practically complete. GSH concentrations lower than 0.1 mM just exhibits some scavenger effect (almost a 15% diminution in the intensity of the signal).

Finally, to assess the type of the interaction between GSH and the nitroso radical, spin-trapping studies using DMPO were conducted. DMPO was added to the reaction mixture after the electrolytic generation of the nitroso radical anion in the presence of GSH. After 3 min of the addition of the spin trap, a typical ESR spectrum of DMPO appeared. The spectrum had four
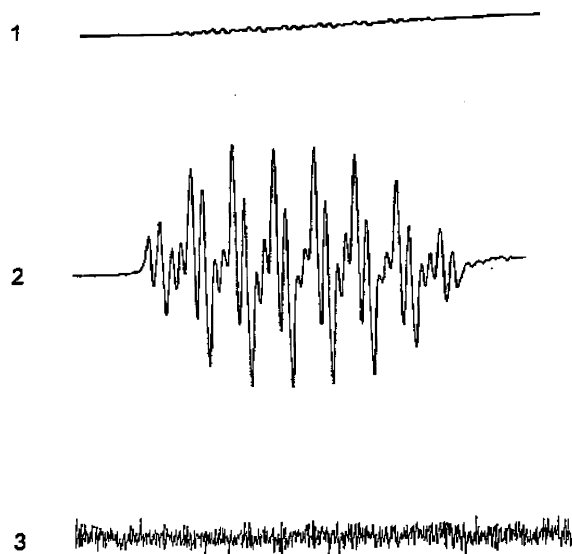

Fig. 6. Experimental EPR spectra of: (1) DMSO + 0.1 M TBAHFP; (2) EPR spectrum of nitrosobenzene anion radical electrochemically generated from a 1 mM nitrosobenzene solution in DMSO + 0.1 M TBAHFP; (3) in the presence of 5 mM GSH.

lines, with a constant value of 15.26 G (aN = aH), a typical value corresponding when the trapping of GS· radical by DMPO occurred [15].

4. Concluding remarks

Our study provides experimental data that demonstrated the nitro radical anion electrochemical generated from nitrosobenzene is scavenged by GSH and could support the view that under hypoxic or anaerobic conditions, is not possible to discard the reactivity of this thiol molecule with this type of species and at least, partially could explain the significant loss of GSH under the above experimental conditions. On the other hand, the generated nitroso radical anion reacted with O_2 (as was demonstrated in UV-Vis experiments) followed by the generation of superoxide anion ($O_2^{\bullet-}$), this reaction could partially document the cellular damage attributed to nitroso compounds in addition to the effects of the nitroso radical.Experimental hyperfine constant values for the nitroso radical anion from nitrosobenzene electrochemically generated. Comparison with constant values obtained from Ref. [5] and [7].

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