An Anticancer Drug and Its Reactivity Toward GSH and Oxygen

Marília O. F. Goulart, a,b Antonio A. de Souza, a Fabiane C. de Abreu, a Francine S. de Paula, a Écio M. Sales, a Wanda P. Almeida, b Olivier Buriez, c and Christian Amatore c

a Instituto de Química e Biotecnologia, Universidade Federal de Alagoas, 57072-970, Maceió, AL Brazil
b Faculdade de Ciências Médicas/Universidade Estadual de Campinas, Campinas, SP, Brazil
c Ecole Normale Supérieure, Département de Chimie, Unité Mixte de Recherche, Centre National de la Recherche Scientifique, “PASTEUR,” Paris Cedex 05 F-75231, France

Electrochemical experiments with methyl 2-[p-nitrophenyl(hydroxy)methyl]acrylate (I) were performed in protic (EtOH + phosphate buffer 1:9, 0.1 mol L⁻¹, pH 6.9; EtOH + phosphate buffer + NaOH 1:9, 0.1 mol L⁻¹ or 0.2 mol L⁻¹, pH 9.4 and EtOH + NaHCO₃ + NaOH 2:8, 0.18 mol L⁻¹, pH 9.6) and aprotic [dimethylformamide (DMF) + tetrabutylammonium perchlorate (TBA), 0.1 mol L⁻¹] media. The primary reduction behavior in aprotic medium was typical of nitroaromatics along with an additional wave related to the reduction of the acrylate function. Kinetic analysis carried out in aprotic and aqueous basic media pointed out to the high stability of the electrogenerated nitro radical anion, especially in DMF + TBA. Reduced (GSH) and oxidized (GSSG) glutathiones in phosphate buffer influenced the reduction behavior of I, due mainly to protonation effects. Direct reduction of I, in the presence of GSH, led to a transient nitroso-GS adduct. In the presence of GSSG, hydrogen-bonding-associated GSSG-hydroxylamine was the main product. Electrochemical studies of I, in the presence of oxygen, showed no chemical reactivity between O₂ and I. These electrochemical results help in the understanding of the anticancer activity of I that can be considered a bioreductive agent with a glutathione depleting function.

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The important toll of cancer on human societies has promoted the discovery and synthesis of a wide number of new antineoplastic agents. Considerable insight has been gained into the mechanisms by which many of these compounds affect cellular growth and this knowledge has been used to the design of new chemotherapeutic drugs.1

Cellular glutathione (GSH) levels were found to be higher in human cancer cell lines than in a normal cell.2,3 Compounds that either inhibit or stimulate GSH synthesis or affect its intracellular concentration may then be of value in cancer chemotherapy.4,5 GSH is one of the most representative endobiotic thiols and plays essential role in cell cycle. It is responsible for a variety of functions, including protection against reactive oxygen and nitrogen species (RONS) and detoxification of xenobiotics.5 The GSH/oxidized glutathione (GSSG) ratio has been considered one of the most important biomarkers for oxidative stress and controls cell proliferation, differentiation, and apoptosis.6 Depletion or increase in GSH concentrations has been applied to control the redox status of cells, thus being useful for the treatment of tumors and diseases characterized by apoptosis.7 Considerable evidence has accumulated to show that agents altering GSH concentration affect transcription of detoxification enzymes, cell proliferation, and apoptosis.8 β-hydroxyacrylates, known as Baylis–Hillman adducts, have been investigated toward biological nucleophiles, with evidence of significant interaction with glutathione rather than with deoxyribonucleosides.3 It may also be possible to target them against molecules such as GSH in tumor cells by coupling them to hypoxia-activated bioreductive molecules, such as nitroaromatics.9 Such coupling can utilize or incorporate groups that have a propensity to oxidize thiols, such as intracellular glutathione. The resulting depletion of glutathione may increase redox-mediated apoptosis. This potential rationale led to the synthesis and testing of Baylis–Hillman adducts.10 The in vitro antiproliferative activity of these compounds [administered at 250, 25, 2.5, and 0.25 μg/mL concentrations in diluted dimethyl sulfoxide (DMSO)] was evaluated using the protocol established by the National Cancer Institute (NCI)7 with significant results.10 In that study, UACC62 (melanoma), MCF7 (breast), NCI460 (lung, nonsmall cells), OVCAR (ovarian), PC03 (prostate), HT29 (colon), 786-0 (renal), and NCI-ADR (breast cells expressing a multiple-drugs resistance phenotype) were investigated. Methyl 2-[p-nitrophenyl(hydroxy)methyl]acrylate (1) (Fig. 1) showed pronounced effects toward melanoma (UACC62), lung (nonsmall cells, NCI460), breast cells expressing a multiple drugs resistance phenotype (NCIADR), ovarian (OVCAR), and prostate (PC03) cancer cells, with IC₅₀ of 3.8, 4.3, 2.0, 3.2, and 4.1 μM, respectively.10 The experimental procedures have been described in detail.10 This compound was assayed as targeted potential antimalarial and displayed activity at micromolar doses.11 The assumed biological activity of nitrocompounds is related to their facile reduction to RNO₂ species promoted in vivo by nitroreductases. Therefore, an optimum and specific activity should be observed when the redox potential of RNO₂ is located between that corresponding to the cellular electron donor agents and the natural biological oxidizing agent O₂/O₂⁻ couple12-14 which deactivates RNO₂ to initiate other specific pathways. Electrochemical techniques thus represent powerful tools for the rationalization of the biological activity of those compounds by detecting and quantifying the interaction of the electrogenerated species, mainly RNO₂ and their targets (endo or xenobiotics of biological significance), for instance GSH.12-16 Such interactions are expected to result in modifications to the current-voltage response,

![Structure of compound 1](image_url)
measured as shifts in redox potential or changes of peak current height, or alteration of RNO2 lifetime in the presence of the target.1,3

The present work aims to provide specific information about the activity of compound 1 based on a series of electrochemi- cal experiments (cyclic voltammetry (CV), differential pulse voltammetry (DPV), square wave voltammetry (SWV), and controlled potential electrolysis (CPE)) performed with 1 in protic (EtOH + phosphate buffer 1:9, 0.1 mol L−1, pH 6.9; EtOH + phosphate buffer + NaOH 1:9, 0.1 or 0.2 mol L−1, pH 9.4; and EtOH + NaHCO3 + NaOH 2:8, 0.18 mol L−1, pH 9.6) and aprotic [dimethylformamide (DMF) + tetrabutylammonium perchlorate (TBAP), 0.1 mol L−1] media, using a glassy carbon electrode, in the absence and presence of GSH, in saturated O2 solutions and in degassed solutions by N2 bubbling, evidencing redox properties of 1 under different conditions, and searching its resulting effects as a possible thiol scavenger and/or oxygen reducing agent.

Experimental

Chemicals.— Compound 1 was synthesized following published procedures.1,3 Its analytical and physicochemical data are in total agreement with the proposed structure.1,3,17 GSH and GSSG were obtained from Sigma Chemical Co. (St. Louis, MO). The buffers were prepared using analytical-grade reagents and purified water from a Millipore Milli-Q system (conductivity <0.1 μS cm−1). All experiments were performed at room temperature (24 ± 2°C). In reduction, degassing, by purging an inert gas (N2) through the solution, was performed.

Apparatus and procedures.— The electrochemical experiments (CV, DPV, and coulometry) were recorded using a potentiostat/ galvanostat BAS 100B or a PGSTAT AUTOLAB 20. The working electrode was a glassy carbon electrode BAS (d = 3 mm), the counter electrode was a Pt foil, and the reference electrode was an Ag/AgCl, Cl− (0.1 mol L−1), all contained in a one-compartment electrochemical cell, with a volumetric capacity of 10 mL. The glassy carbon electrode was polished with alumina on a polishing felt (BAS polishing kit), resulting in a surface with a mirrorlike appearance. UV-visible (UV-vis) spectra were obtained on a Shimadzu Multi-Spec-1501 spectrophotometer.

Electrochemical studies were performed in protic (EtOH + phosphate buffer 1:9, 0.1 mol L−1, pH 6.9; EtOH + phosphate buffer + NaOH 1:9, 0.1 or 0.2 mol L−1, pH 9.4; and EtOH + NaHCO3 + NaOH 2:8, 0.18 mol L−1, pH 9.6) and aprotic (DMF + TBAP, 0.1 mol L−1) media. DMF was distilled under reduced pressure after stirring with anhydrous copper sulfate. Gluta- thione (GSH) was weighted and added as a powder directly into the cell’s solution. GSH (pKaCOOH 2.126, pKCOOH 3.512, pKaSH 8.736, pKNaSH 9.655)18 and GSSG (pKaCOOH 3.15, pKCOOH 4.03, pKNaSH 8.57, pKNaSH 9.54) (Aldrich) were added to pH 9.4, phosphate buffer + NaOH, in different proportions. Upon addition of GSH or GSSG, pH values changed, when the ionic strength was not enough for leveling the effect of the acidic groups. In EtOH + phosphate buffer 0.1 mol L−1 (1:9), initial pH of 9.4, the pH decreased to 8.8 and 7.7, after the addition of 1 and 4 mmol L−1 GSH, respectively. Upon the addition of GSSG (1 mmol L−1), there was a decrease in pH to 7.6. Bicarbonate buffer was also used, and in this buffer GSH or GSSG addition did not provoke any significant pH modification.

The interaction between compound 1 and O2 was evaluated using UV-vis and coulometry. Compound 1 was added to the electrochemical cell containing sodium bicarbonate + NaOH, pH 9.6, and the solution was adequately degassed with N2. O2 was bubbled into the cell until saturation and its concentration was obtained through the use of an oxygen sensor (oxygenmeter DM-4 Digimed). The concentration of oxygen in this medium is ca 0.25 mmol L−1. After each addition and before recording a new voltammogram, O2 was bubbled into the cell.

Controlled potential electrolysis of 1 was held in a divided cell (100 mL), in EtOH + phosphate buffer (2:8) pH 6.9, using carbon felt as the working electrode, Pt as auxiliary vs Ag/AgCl, and Cl− (0.100 mol L−1) as a reference electrode. The cell was degassed by purging N2 through it. Pre-electrolysis was performed at Eapp = −1.0 V. After the addition of compound 1 (0.050 g, 2.6 mmol), Eapp was adjusted to −0.950 V. UV-vis and CV were used to follow the status of electrolysis. After the elimination of EtOH and extraction with chloroform, the reaction mixture was dried, leaving a brownish solid (0.035 g). It was submitted to analysis by thin-layer chromatography (TLC) in silica gel (CHCl3 + MeOH 9:1), leaving one major spot. The products were not purified. The structures of compounds 2 and 3, in mixture, were elucidated by the use of mass spectrometry (MS), 1H nuclear magnetic resonance (NMR), and 13C NMR.

Compounds 2 and 3 are as follows: Bis-N,N-2-[p-hydroxymaminophenyl(hydroxy)methyl]acrylate (2) and 1-(2-hydroxy-4-[1-(hydroxy-2-methoxy-carbonyl-allyl]-phenyl)-ONN-azonu]-[phenyl]-methyl)-acrylic acid methyl ester (3):

MS [m/z (%): M2+444 (61)](2), 427 (M2+17 or M3+1), 426 (M2+18 or M3+1) = 15 (15), 411 (40), 225 (40), 208 (20), 190 (100) (C124H128N4O6). 1H NMR (250.13 MHz, CDC13): 8: 8.6 Hz, 8.1 (d, Ar, J 8.6 Hz), 7.48-7.37 (m, Ar), 6.29 and 5.8 (2bs, = CH2), 5.52 (d, J 6.3 Hz, CH2O), 3.64 (s, OCH3), 3.37 (bs, OOH), 2.52 (s, OH). 13C NMR (62.90 MHz, CDCl3): δ: 166.7 (CO), 148.2 (Cq, CHAr = N), 145 (CHAr), 142 (CH2), 133.4 (Cq, CHAr = C), 128 (CH2), 125.0 (CH2), 122 (Cq, C = CH2), 72.9 (CHOH), 52.0 (CH2).

Controlled potential electrolysis of methyl 2-phenyl(hydroxy) methyl acrylate was held in a divided cell (35 mL) in DMF + TBAP 0.1 mol L−1 (10 mL) using carbon felt as the working electrode, Pt as auxiliary vs Ag/AgCl, and Cl− (0.100 mol L−1) as a reference electrode. The cell was degassed by purging N2 through it. Pre-electrolysis was performed at Eapp = −2.0 V. After the addition of the acrylate (0.010 g, 0.05 mmol). Eapp was adjusted to −2.3 V. UV-vis and CV were used to follow the status of electrolysis, which was interrupted after the consumption of 2 mol electron mol−1. After the addition of water and extraction with ethyl acetate, the reaction mixture was dried, leaving a white solid (0.005 g), related to the reduced olefin (4), named methyl-3-phenyl-3-hydroxy-2-methylpropanoate. The structure of the reduced compound was elucidated by the use of 1H NMR (data not shown). The signals at δ 6.29 and 5.8 (2bs, = CH2), were not observed, substituted by a signal at δ 1.13 (d, J 6.6 Hz) and a multiplet at δ 2.51–2.72 (1H), related to –CH2=O.10

Controlled potential electrolysis of 1 (0.02 g, 2.11 × 10−3 mol L−1), in the presence of GSH (0.038 g, 3.16 × 10−3 mol L−1) was held in a divided cell (80 mL) and performed as already described, in EtOH + phosphate buffer (2:8) pH 9.4, using carbon felt as the working electrode, Pt as auxiliary vs Ag/AgCl, and Cl− (0.1 mol L−1) as a reference electrode, using Eapp of −0.9 V. The electrolysis was followed by spectrophotometry and CV and was interrupted after consumption of around 3 mol electron mol−1. The solution, initially colorless, turned light yellow at the end of electrolysis. After elimination of EtOH, the solution was extracted with CHCl3, followed by n-BuOH, but the extraction was unsuccessful.

Kinetic characterization of the reduction mechanism of 1 in basic aqueous media and the ensuing determination of the bimolecular rate constants were performed on the basis of the variation of 1-pfp of the reduction waves Jc/Ja (ArNO2/ArN2O4). For this, the method proposed by Olmstead and Nicholson19 was used. However, any application of such method requires precise experimental measurements of 1-pfp values. Bard and Faulkner20 proposed a method to measure experimentally these values, yet its experimental
The limit of the linear relationship described by Eq. 1 presents the usual reduction and oxidation waves characteristic of each individual CV according to Nicholson’s procedure. The scan approach of Olmstead and co-workers for disproportionation, was inserted into a working curve to determine the fraction of cathodic current that adds to the anodic one than that required to scan the full wave back and forth. Then, we can posit that the fraction of cathodic current that adds to the anodic one at Epfa is a fraction of Ipc. Let γ be this fraction when the experimental measurement of Ipc is performed, for example, from the baseline of the voltammetric forward trace. Therefore, the real value of Ipc is given by \[ I_{pc\text{corr}} = I_{pc\text{meas}} - \gamma I_{pc\text{meas}}, \] so that \[ I_{pa/Ipc\text{corr}} = \frac{I_{pc\text{meas}} + \gamma}{I_{pc\text{meas}}}. \] This equation is strictly valid, yet γ is unknown. However, for any wave close to its chemical reversibility, the limit of \[ I_{pa/Ipc\text{corr}} \] at high scan rates must be unity by definition of \[ I_{pa/Ipc}. \] We took advantage of this intrinsic property (which is independent of the mechanism operating) to determine γ. Thus, \[ \gamma = 1 - \lim_{vmass} \frac{I_{pa/Ipc\text{corr}}}{I_{pa/Ipc\text{meas}}}, \] where \[ \lim_{vmass} \frac{I_{pa/Ipc\text{meas}}}{I_{pa/Ipc\text{meas}}} \] is the plateau value obtained experimentally for \[ I_{pa/Ipc\text{meas}} \] at large scan rates. This procedure was used to correct all experimentally measured values.

The parameter \[ I_{pa/Ipc\text{corr}} \] reveals the tendency of an electrochemically generated species, i.e., the nitro radical anion, to undergo chemical-following reactions. This measurement was carried from each individual CV according to Nicholson’s procedure. The scan rate was varied between 0.100 and 5 V s\(^{-1}\). Using the theoretical approach of Olmstead and co-workers for disproportionation, the values \[ I_{pa/Ipc\text{corr}}, \] experimentally measured at each scan rate, were inserted into a working curve to determine the ω parameter, which incorporates the effects of rate constant, analyte concentration, and scan rate. A plot of \[ \log \omega \text{ vs } \log \tau + 0.047(aT - 4) \]
\[ \log \omega = \log k_2 C_0 \tau + 0.047(aT - 4) \quad [1] \]
where \( k_2 \) is the second-rateconstant for the decomposition of \( \text{ArNO}_2^- \), \( C_0 \) is the nitro compound concentration, \( \tau = (E_0 - E_1/2)/v \), where \( E_0 \) is the switching potential, \( E_1/2 \) is the half-wave potential, \( v \) is the scan rate, and \( a \) is equal to \( nFv/RT \) ( \( n \) is the number of electrons involved in the rate-determining step and \( F \) is the Faraday constant). The second-rate constant \( k_2 \) can be obtained directly from the graph by intercept on the \( \log \omega \) axis.\(^{19} \)

**Results and Discussion**

**Electrochemical studies in aprotic medium.—** CV of compound I, at a glassy carbon electrode, in DMF + TBP, 0.1 mol L\(^{-1}\) (Fig. 2), presents the usual reduction and oxidation waves characteristic of nitroaromatic compounds. In this medium, three reduction waves are observed in the CVs of compound I. The first two [Ic \((-1.122 \text{ V}), \text{Ilc} \((-1.917 \text{ V})\)] and the third one [IIc \((-2.311 \text{ V})\)] are related to the nitroaromatic function and the acrylate moiety, respectively (Fig. 2a). The generation of the stable nitro-anion radical is observed (system IIc/Ia) (Fig. 2a), followed by a second wave, related to the generation of the hydroxylamine that suffers oxidation at Ila \((-0.655 \text{ V})\) (Fig. 2a). At scan rates slower than 1.00 V s\(^{-1}\), there is no corresponding response on the second negative scan (Fig. 2a, inset), related to the reversible 2e\(^{-}\)/2H\(^{+}\) RNO/RNHOH couple, establishing the chemical instability of the electrogenerated hydroxylamine (Fig. 2a, inset), in this medium. As scan rate increases (\( v \geq 1.00 \text{ V s}^{-1}\)), the cathodic wave \( \Gamma_c \) related to the reduction of the product electrogenerated from the oxidation of the hydroxylamine begins to appear (Fig. 2b).

Figure 2 displays the possible reduction mechanism for compound I in aprotic medium (Eq. 2 and 3). The third wave is similar to the one obtained in the same conditions for methyl 2-[phenyl(hydroxy)methyl]acrylate and is related to the reduction of the acrylate system. Electrolysis held in aprotic medium with this nonsubstituted acrylate led to the consumption of 2 mol electron mol\(^{-1}\) (Fig. 4). The olefin group was reduced, as demonstrated by \(^{1}H\) NMR, through the absence of methylene signals (6 6.29 and 5.8, two broad signals) and the presence of a methyl group signal (6 2.13) and a multiplet (6 2.51–2.72) related to the α-carbonyl methylic hydrogen.

The reduction of I was also investigated by SWV (Fig. 5) and the primary reduction mechanism described above was confirmed. SWV forward and backward currents for the second scan clearly show the reversibility of the electrogenerated nitro radical anion [Ia and Ic \((-1.057 \text{ V})\)] and the irreversibility of the following waves [IIc \((-1.817 \text{ V})\) and IIlc \((-1.919 \text{ V})\)].

**Electrochemical studies in protic medium.—** Neutral pH.— CV of I shows a reduction behavior typical of nitroaromatics (Eq. 6, Fig. 9), involving a first diffusional (Ipc vs \( \nu^{1/2} \)) and irreversible (EpIc dependent on \( \nu \)) wave (Ic, \( EPc = -0.742 \text{ V} \)), in agreement with the generation of the derived hydroxylamine, through a \( 2e^-/2H^+ \) process (Fig. 6). The quasi-reversible system Ia (EpIa
= 0.154 V/I’c (EpI’c = −0.213 V) is related to the derived NHOH → NO transformation (Fig. 6, inset). Though this is not apparent in CV, SWV suggests a certain degree of reversibility of the nitro reduction (Fig. 6).

Electrolysis, performed in EtOH + phosphate buffer (1:9), pH 6.9, with $E_{\text{app}} = −0.950$ V, was followed by CV (Fig. 7a) and led to the consumption of 4 mol electron mol$^{-1}$. Figure 7b shows the UV-vis spectra of 1 and its reduction products. Workup of the electrolysis product allowed the separation of a dark brown product, which was submitted to analysis by usual methods (MS, $^{13}$C NMR, and $^1$H NMR) for structure determination. This led to dimeric structures 2 and 3 (Fig. 8) for these reduction products. Such dimers evidence a reaction between nitroso and hydroxylamine derivatives in the nitroanion radical can also be produced by a homogeneous electron transfer represented in Eq. 11. The second wave IIc, $E_{\text{pIIc}} = −1.311$ V corresponds to the production of the hydroxylamine derivative that suffers oxidation to the nitroso at wave IIa, $E_{\text{pIIa}} = 0.167$ V, the latter being reduced at wave IIc’ (EpIc’ = −0.423 V), during the following scan. The reduction mechanism is similar to the one obtained in aprotic medium (Fig. 2 and 3) but occurred at less negative potentials, reflecting the strongest solvation of the anionic species. In bicarbonate buffer, pH 9.6, the same behavior as in phosphate buffer was obtained (figure not shown).

Determinative and kinetic parameters.— As this paper is mostly concerned with the characterization of the reactivity of the nitro radical anion, we have centered our study on the first reversible reaction between nitroso and hydroxylamine derivatives as shown in Eq. 8–10 or, alternatively, by Eq. 11 and 12 (Fig. 9). SWV has shown a certain degree of reversibility on the reduction process. The azoxy derivative 3 can be formed by dehydration of the dimer 2 (Eq. 13) or by the condensation between hydroxylamine and nitroso derivative (Eq. 14, Fig. 9).

Alkaline pH (9.4).— In EtOH + phosphate buffer + NaOH (1:9), 0.1 mol L$^{-1}$, pH 9.4, the single signal observed in neutral media (Fig. 6a), splits into two different signals. The electron transfer to the nitro group is fast and the process ArNO$_2$/ArNO$_2^-$ turns quasi-reversible (Fig. 10). The second wave (IIc, $E_{\text{pIIc}} = −1.311$ V) corresponds to the production of the hydroxylamine derivative that suffers oxidation to the nitroso at wave IIa ($E_{\text{pIIa}} = 0.167$ V), the latter being reduced at wave IIc’ ($E_{\text{pIIc’}} = −0.423$ V), during the following scan. The reduction mechanism is similar to the one obtained in aprotic medium (Fig. 2 and 3) but occurred at less negative potentials, reflecting the strongest solvation of the anionic species. In bicarbonate buffer, pH 9.6, the same behavior as in phosphate buffer was obtained (figure not shown).
A couple obtained in aprotic and protic media, at pH values $>9$. By using a narrow potential window it was possible to sample this couple in our voltammetric studies, though this led to some inconvenience on the determination of $I_{pa}$, the current peak intensity of wave Ia.

For phosphate + NaOH, bicarbonate + NaOH buffers, the ratio $I_{pa}/I_{pc}$ increases with the scan rate and turns constant beyond $0.300 \text{ V s}^{-1}$, showing a high stability of the nitro radical anion on the time scale of CV, under these conditions. Note that because of the presence of successive cathodic waves, $I_{pa}/I_{pc}$ values determined by the classical method were altered by residual currents. For this reason, a meaningful comparison required the use of corrected measurements along the method presented in the Experimental section. After this treatment, corrected $I_{pa}/I_{pc}$ values were obtained and analyzed in terms of their scan-rate dependence (Fig. 11).

**Figure 7.** (a) CV of 1 during electrolysis in EtOH + phosphate buffer: (---) initial time, $E_i = 0.0$, (---) after passage of 4 mol electron mol$^{-1}$, $E_i = 0.0$, and (...) CV of the reduced product, $E_i = 0.2 \text{ V}$, $v = 0.100 \text{ V}$. (b) UV-vis spectra of 1 and reduced product 2 and 3 $(0.1 \text{ mmol L}^{-1})$.

**Figure 8.** Structures of compound 2 and 3, products from the reduction of 1, and compound 4, obtained from electrolysis of the nonsubstituted phenyl acrylate.

**Figure 9.** Probable mechanisms for the reduction of the nitro group in different conditions, showing also the formation of dimer 2 and azoxy derivative 3.

**Figure 10.** CV of 1, $c_1 = 2 \text{ mmol L}^{-1}$, EtOH + phosphate buffer + NaOH:1:9, 0.1 mol L$^{-1}$, pH 9.4, $v = 0.100 \text{ V s}^{-1}$, successive cycles and different $E_i$. 

For phosphate + NaOH, bicarbonate + NaOH buffers, the ratio $I_{pa}/I_{pc}$ increases with the scan rate and turns constant beyond $0.300 \text{ V s}^{-1}$, showing a high stability of the nitro radical anion on the time scale of CV, under these conditions. Note that because of the presence of successive cathodic waves, $I_{pa}/I_{pc}$ values determined by the classical method were altered by residual currents. For this reason, a meaningful comparison required the use of corrected measurements along the method presented in the Experimental section. After this treatment, corrected (corr.) $I_{pa}/I_{pc}$ values were obtained and analyzed in terms of their scan-rate dependence (Fig. 11).
allowing a qualitative assessment of the nitro anion radical stabilization, in the following media order: aprotic > bicarbonate > phosphate alkaline aqueous media.

In order to investigate the order of the coupled reaction in this medium, we have observed the effect of different concentrations of compound 1 on the current of wave Ic. For a first order chemical reaction, concentration should not affect \( I_{pa}/I_{pc} \) values, though we observed the reverse effect, \( I_{pa}/I_{pc} \) decreases upon increasing the concentration. Similarly, the peak potential of wave I shifted anodically upon increasing the concentration of 1. Both observations indicated that the reactivity of the anion radical of 1 involved a second-order process, pointing to the occurrence of an overall DISP2 process (Eq. 5, Fig. 9) proceeding through the uphill protonation equilibrium of the primary anion radical, followed by the fast homogeneous reduction of the intermediate by a second anion radical.

This rationale was confirmed by the straight lines obtained when using the Olmstead and Nicholson method for a DISP2 (EC2) mechanism\(^ {19,20} \) as seen in Fig. 12a. This allowed the determination of the corresponding overall rate constant \( k_2 \), obtained by the intercept on log \( \alpha \) axis and half-life time \( (t_{1/2}) \), for the nitro radical anion of 1, in phosphate + NaOH + 10% EtOH buffer. These were found to be, respectively, 622.1 M\(^{-1} \) s\(^{-1} \) and 0.8 s (for \( c_1 = 2 \) mmol L\(^{-1} \)). In a similar way, \( k_2 \) and \( t_{1/2} \) values were determined in bicarbonate buffer, being, respectively, 1029 M\(^{-1} \) s\(^{-1} \) and 1.0 s (for \( c_1 = 1 \) mmol L\(^{-1} \)).

An alternative method was used to determine \( k_2 \) through the plot of corrected \( I_{pa}/I_{pc} \) vs \( t \), obtained from the theoretical curve by Nicholson,\(^ {25} \) when the ratio of anodic to cathodic peak current was 0.75 for the second-order process and the rate-constant value was very close to that already presented (\( k_2 = 478.5 \) M\(^{-1} \) s\(^{-1} \) in phosphate + NaOH + 10% EtOH buffer, Fig. 12b).

An addition of endobiotics.—Phosphate buffer.—The direct effect of GSH on the ArNO\(_2/\)ArNO\(_2^\cdot\) redox couple is dependent on the buffer system nature and its ionic strength. This is summarized in Fig. 13. Addition of GSH at different [GSH]/[I] ratios, in phosphate buffer, results in an increase in current response of wave Ic, a concomitant decrease of wave IIc, and a decrease of current response for the RNHOH oxidation wave, IIa, accompanied by the growth of wave IIa', until stabilization at [GSH]/[I] equal to 4. With this thiol concentration, the current increases in agreement with the occurrence of a 2e\(^-\)/2H\(^+\) process, suggestive of the generation of an in situ stable nitroso (ArN) derivative,\(^ {14} \) which otherwise would not be formed. The production of ArNO is not an easy process, once it is reduced easier than the original nitroaromatic compound.\(^ {23,24} \) The removal of IIa (oxidation of the generated ArNHOH) is in agreement with the decrease of wave IIc (practically absent). Furthermore, it can be argued that if any ArNHOH is produced, it should react spontaneously with thiols to give the corresponding adducts.\(^ {26,27} \) Thus, the anodically shifted broad wave II’a may be ascribed to the formed thiol adduct, either with ArNHOH (if any) or ArNO (more probable). No cathodic counterpart is observed of IIa’ (Fig. 13, inset), which indicates the absence of a free ArNO. The addition of GSSG leads to a pH decrease [9.4 to 8.8 (c\(_{GSSG} = 1 \) mmol L\(^{-1} \)) and 7.7 (c\(_{GSSG} = 4 \) mmol L\(^{-1} \))]. This buffering system is not adequate to leveling the acidity of GSH. At this pH, GSH has a negative charge; however, two acidic functions are still associated (–SH and –N\(_2\)H\(_2\)), being a facile source of protons.

The addition of GSSG (Fig. 14) to the solution of 1, in phosphate buffer, leads also to a pH variation (from 9.4 to 7.6) and, more importantly for our purpose in this work, to deep modifications on the CV features. The system Ic/Ia loses its reversibility, the current

Figure 11. Comparison of corrected \( I_{pa}/I_{pc} \) vs scan rate in different conditions.

Figure 12. (a) Plot of the kinetic parameter, \( \omega \), with the time constant, \( \tau \), for \( c_1 = 2 \) mmol L\(^{-1} \), in EtOH + phosphate buffer + NaOH (1:9), 0.1 mol L\(^{-1} \), pH 9.4. (b) Variation of \( I_{pa}/I_{pc} \) as a function of \( k_2 \) from Ref. 19, Table XI, for charge transfer, followed by an irreversible chemical reaction mechanism.
Addition of GSSG as exemplified in the case of quinones, despite the use of different reversibility, which is indicative of a hydrogen bonding association, media.

anodic shift hydroxylamine ArNOH. The system II number of acid functions, absence of nucleophilicity attack, rationalizes the reactivity in the presence of GSSG, while a higher degree of protonation, without nucleophilic participation, explains the behavior in the presence of GSH, the reversibility of the system ArNO 2/ArNO2 is not altered, though wave Ic as well as wave IIc suffer a small current decrease (Fig. 14).

Protonation effects coupled with efficient nucleophilic reactions upon the addition of GSH explain the behavior in the presence of GSH, while a higher degree of protonation, without nucleophilic attack, rationalizes the reactivity in the presence of GSSG (higher number of acid functions, absence of nucleophilicity), leading to the hydroxylamine ArNOH. The system II’a/I’c keeps its quasi-reversibility, which is indicative of a hydrogen bonding association, as exemplified in the case of quinones, despite the use of different media.

These series of experiments were repeated with a 0.2 mol L⁻¹ phosphate buffer and the results were quite different (figure not shown), bringing strong confirmations of the acidic properties of GSH and GSSG. The behavior approached that observed in bicarbonate buffer, pH 9.6. GCE, 0.1 mol L⁻¹, pH 9.4, in the presence of GSSG, GCE, E = 0.100 V s⁻¹. The electrolysis process was followed by CV and also by UV-vis absorption at −0.950 V in phosphate buffer + NaOH in the presence of GSSG (initial pH 9.4; final pH 8.0). The electrolysis process was followed by CV and also by UV-vis (Fig. 17a and b). After a consumption of 2.3 mol electron mol⁻¹, the electrolysis was inter-

The CV of I in bicarbonate buffer is similar to the one observed in phosphate buffer; however, this behavior changes slightly upon the addition of GSH and GSSG (Fig. 15a and b, respectively). In GSH, the reversibility of the system ArNO2/ArNO3 is not altered. The electrolysis process was followed by CV and also by UV-vis absorption (Fig. 17a and b). The electrolysis process was followed by CV and also by UV-vis absorption (Fig. 17a and b).

The small decrease in current intensity of the reduction wave Ic, observed in the presence of added GSH, may be explained by the participation of GS⁻, leading to a partial reduction of the nitro group, without affecting the nitro anion radical (wave Ia remains the same) (Fig. 16). The decrease of wave Ic displayed at more negative potentials and that of wave IIa during the anodic scan supports the rationale that some intermediate attack, forming adducts, probably ArNO-thiolates.

This result is compatible with the evidence obtained by Wardman and co-workers that ruled out any detectable reaction between nitro radical anions and GSH under physiologically relevant conditions.

Reduction of I was performed by controlled potential electrolysis at −0.950 V in phosphate buffer + NaOH in the presence of GSH (initial pH 9.4; final pH 8.0). The electrolysis process was followed by CV and also by UV-vis absorption (Fig. 17a and b). After a consumption of 2.3 mol electron mol⁻¹, the electrolysis was inter-

![Image 93x100 to 297x287]

![Image 357x397 to 568x755]

Figure 13. CV of 1, c₁ = 2 mmol L⁻¹, EtOH + phosphate buffer + NaOH(1:9), 0.1 mol L⁻¹, pH 9.4. Eₚₘᵦ = 0.1 V. Addition of GSH (4 mmol L⁻¹) led to the increase of wave Ic (now Ic) and disappearance of wave IIc, v = 0.035 V s⁻¹. (Inset) Same conditions, two cycles.

Figure 14. CV of 1, c₄ = 2 mmol L⁻¹, EtOH + phosphate buffer + NaOH(1:9), pH 9.4, in the presence of GSSG, GCE, v = 0.035 V s⁻¹. Addition of GSSG (4 mmol L⁻¹), with successive scans.

Figure 15. CV of 1, c₁ = 1 mmol L⁻¹, in EtOH + NaHCO₃ + NaOH (2:8), pH 9.6. GCE, v = 0.100 V s⁻¹. (a) Addition of GSH (1 mmol L⁻¹). (b) Addition of GSSG (4 mmol L⁻¹).
ruptured. The solution was extracted, in sequence, with ethyl acetate, chloroform, and finally with n-butanol, without success. The electrolysis product remained in the aqueous phase during all of these extractions.

CV of the aqueous electrolyzed fraction showed the peak of the formed nitrosothiol adduct (II’), a small amount of the free ArNO (I’c) and some ArNO₂⁺ (lc) which had not been converted (Fig. 17a). UV-vis evidenced two bands (283 and 310 nm) and a shoulder (242 nm) (Fig. 17b), clear evidence for a structural change similar to previously reported ones, such as that reported by Eyer and more recently by McClelland and Kazanis, who investigated the reaction of nitrosobenzene with excess GSH at neutral pH. The mechanisms proposed by these authors consider the formation of a L-cysteine thiyl radical, the latter dimerizing spontaneously to give the disulfide. Furthermore, such rates of decomposition are expected to strongly depend on the particular structure of thionitrites. Most of them are too unstable in solution or even in solid phase to allow their full structural characterization. In our case, though a certain stabilization was achieved, the impossibility to obtain a precipitate at the end of electrolyses prevented its isolation and thus its full characterization.

Spectroscopic studies were performed in both buffers to examine any possible direct reaction of GSH with the nitroacrylate derivative (1), once it was clear from the electrochemical experiments that GSH reacted with reduced forms of 1. In phosphate buffer, pH 9.4, the characteristic band of the nitrocompound at 275 nm changed very little after 4 h of contact. The same unreactive behavior was observed for compound 1, with GSH in bicarbonate buffer (data not shown).

All these results establish that the reduction of compound 1 in aprotic and alkaline protic media leads to a stable nitro radical anion, with a well-resolved ArNO₂⁺/ArNO₂ redox couple when reduction involved only 1. However, the addition of GSH or GSSG provokes a drastic change in the voltammetric feature, mainly due to protonation of reduced intermediates unless this reaction is examined in strongly buffered basic solution (bicarbonate).

**Figure 16.** CV of 1, ε₁ = 1 mmol L⁻¹. Normalized currents, in EtOH + NaHCO₃ + NaOH (2:8), pH 9.6, GCE, v = 0.075 V s⁻¹.

**Figure 17.** (a) CV of 1, before (---) and after (----) electrolysis, performed in phosphate buffer + NaOH pH 9.4, in the presence of GSH, final pH 8.0. (b) UV-vis spectra of the electrolysis product showing two main components at 283 and 310 nm.

**Figure 18.** Adduct formation between reduced 1 and GS⁻.
tween any of the four species in Eq. 15 (viz., except for the spontaneous disproportionation of $O_2^-$) is ruled out by our observation.

**Conclusion**

Compound I has two electroactive functions; however, the acrylate group suffers reduction at relatively high negative potentials and cannot influence the biological redox activity of I. Many drugs contain nitro groups, though their presence may not always be directly involved in the redox mechanism of biological action. However, the occurrence of an aromatic nitro moiety may be connected to the incidence of toxic side effects. From the present results, it is clear that the redox pathways initiated by the reduction of I strongly depend on the reaction media, as expected.

The nitro anion radical obtained after reduction of compound I, in aprotic and alkaline media, is relatively stable, though the values of its decay rate constants by a DISP2 route could be quantitatively evaluated in alkaline-buffered media. More interesting is that our electrochemical experiments evidenced definite interactions of the reduced intermediates of the nitroaromatic group with targets of biological significance, GSH, in phosphate buffer, induced the generation of the nitroso-thiolate adduct. GSSG addition, in the same medium, led to the generation of hydroxylamine in $4e^-/4H^+$ process, which is characteristic of a pathway initiated by the protonation of the initial anion radical by the acidic GSSG species in such a buffer. It is thus important to test for possible pH variations after any addition of such compounds, viz., bearing many acidic functions, because they may overcome a phosphate buffer. Conversely, in a more efficient bicarbonate buffer, the effect of these thiols was only apparent after an extended reduction of the nitro group.

S-nitrosothiols, whose generation is suggested in the present paper, are signaling molecules that may act via direct exchange of their NO function with thiol groups of low molecular-weight compounds as well as with the many thiol-containing proteins. Yet, in connection with such activity it must be stressed that we observed that GSH depletion is caused by its reaction with the nitro and hydroxylamine species and not with the nitro radical anion, simple deprotonation being excluded.

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