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ELECTROCHEMICAL STUDIES OF *S*-NITROSOTHIOLS

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Abstract: *S*-nitrosothiols (RSNO), such as SNAP, GSNO, and Glc-SNAP-1 exhibited a single, totally irreversible, diffusion controlled reduction peak at potentials of -0.97 V, -0.98 V and -0.91 V, respectively, vs. Ag/AgCl (3 M NaCl) reference electrode. This corresponded to the nitric oxide (NO) release from the *S*-nitrosothiols. A possible mechanism is proposed for the reduction process.

Nitric oxide (NO)–a simple, relatively stable, potentially toxic, diatomic radical, synthesized enzymatically *in vivo* from l-arginine by NO synthase–has been implicated in a range of physiological processes, including vasodilatory and antiplatelet effects, macrophage-induced cytotoxicity, and neurotransmission.[1] *S*-nitrosothiols[2] are believed to play an important role in storing, transporting, and releasing NO.[3] A variety of biological functions of *S*-nitrosothiols have been continuously uncovered. For example, it was recently reported that blood flow is regulated by *S*-nitrosohemoglobin via the release of NO,[4] and that specific *S*-nitrosylation of certain thiol groups on the calcium release channel may regulate the channel and allow specific control of various channel functions.[5] Thus, the mechanism of *S*-nitrosothiol decomposition is of great significance to the understanding of NO biochemistry.

It is known that the decomposition of *S*-nitrosothiols occurs thermally and photochemically to give the disulfide and NO.[6-8] In solution, trace reduced metal ions such as Cu^{+} and Fe^{2+} can catalyze the decomposition of *S*-nitrosothiols more rapidly than oxidized metal ions such as Cu^{2+}.[10] Reducing agents such as thiols can stimulate decomposition of *S*-nitrosothiol by chemical reduction of metal ions.[7,10,11] Transnitrosation can also stimulate *S*-nitrosothiol decomposition if the product *S*-nitrosothiol is more susceptible to transition metal ion-catalyzed decomposition than the parent *S*-nitrosothiol.[12] Furthermore, it was shown that transnitrosation even between the same thiols can cause the decomposition of *S*-nitrosothiol. Using a model transnitrosation reaction between *S*-nitroso-glutathione (GSNO) and glutathione, [illegible] *et al.*[13] have shown that the GSNO was decomposed to oxidized glutathione, nitrite, nitrous oxide, and ammonia. More interestingly, the function of glutathione peroxidase (EC 1.11.1.9, GPx), an essential selenium-containing antioxidant enzyme,[14] has [illegible] with nitric oxide. We found that simple selenium compounds such as selenocystamine and [illegible] can catalyze the decomposition of *S*-nitrosothiols to produce NO in the presence of thiols.[15] To further elucidate the electron transfer mechanism of *S*-nitrosothiol decomposition, we carried out electrochemical studies on three commonly used *S*-nitrosothiols: *S*-nitroso-*N*-acetyl-penicillamine (SNAP), GSNO and *N*-(β-D-Glucopyranosyl)-N^2-acetyl-*S*-nitroso-D-penicillaminamide (Glc-SNAP-1).[16]

SNAP GSNO Glc-SNAP-1

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1. Cyclic voltammetry of *S*-nitrosothiols SNAP, GSNO, and Glc-SNAP-1 were synthesized and cyclic voltammetric studies were carried out using Pt, Au, and glassy carbon electrodes. It was found that SNAP, GSNO, and Glc-SNAP-1 exhibited only single reduction peak at potentials of -0.97 V, -0.98 V, and -0.91 V, respectively, vs. Ag/AgCl (3 M NaCl) reference electrode in a phosphate buffer (0.1 M, pH 7.4, diethylenetriaminepentaacetic acid (DTPA), 2 mM) at a scan rate of 100 mV/s (Fig. 1). The reduction peaks were broad and small. Increasing the scan rate shifted the reduction wave to more negative potentials, indicative of slow heterogeneous electron transfer. Increasing the scan rate up to 50 V/s and changing from an aqueous to an organic medium (0.1 M tetrabutylammonium hexafluorophosphate ($TBAPF_6$) in CH_3CN) did not result in a reverse wave. This observation indicates that the reduction of *S*-nitrosothiols is a totally irreversible process. Plotting of peak current (i_p (c)) vs. square root of scan rate ($v^{1/2}$) (Fig. 2) shows a linear relationship, indicating a diffusion controlled reduction.

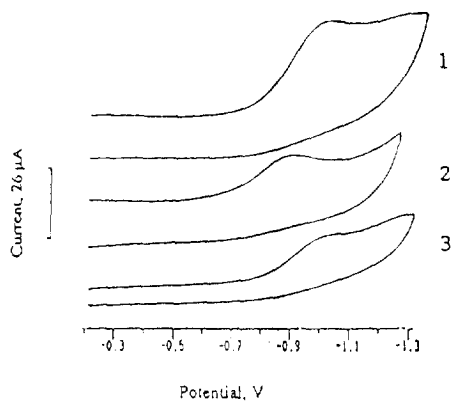

Figure 1. Cyclic voltammograms of SNAP (1), Glc-SNAP-1 (2), and GSNO (3)

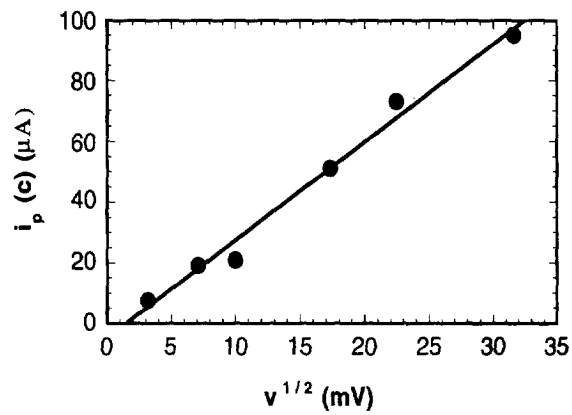

Figure 2. Plot of reduction peak current (i_p (c), µA) vs. square root of scan rate ($v^{1/2}$, $(mV/s)^{1/2}$).

2. NO formation from the reduction of *S*-nitrosothiols. Since one totally irreversible reduction peak was found for the *S*-nitrosothiols, it was expected that NO would be cleaved from the *S*-nitrosothiols during the reduction process. Using a glassy carbon with a 3.2 mm diameter radius as the working electrode, bulk electrolysis was conducted with 2 mM SNAP in phosphate buffer (0.1 M, DTPA 2 mM) at different reduction potentials for a period of 60 s. Results clearly confirmed that NO was formed during the bulk electrolysis of *S*-nitrosothiols. From potentials between -500 mV and –800 mV, there was hardly any NO generated during bulk electrolysis. However, at a potential between -800 mV and -1100 mV, the amount of NO generated correlated linearly with the bulk electrolysis potentials (Fig. 3). The result was consistent with the cyclic voltammetric result, indicating that indeed *S*-nitrosothiols were reduced and NO was generated.

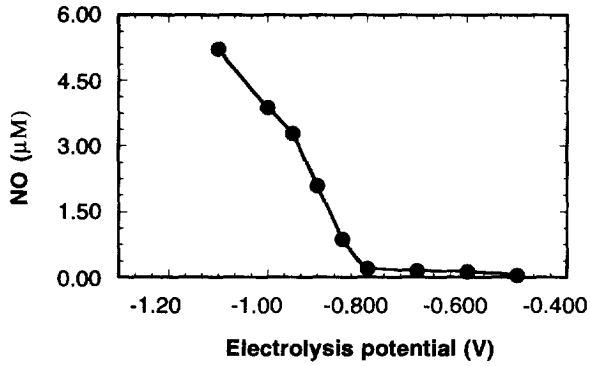

Figure 3. Nitric oxide generated from the electrochemical reduction of SNAP at different potential

3. Proposed mechanism of electrochemical reduction of *S*-nitrosothiols. An exhaustive bulk electrolysis was carried out with SNAP in an organic solvent (0.1 M $TBAPF_6$ in CH_3CN) using a Pt mesh working electrode. The organic solvent was chosen because an aqueous solution can not be used at a more negative potential than -0.5 V vs. Ag/AgCl (3 M NaCl) with a Pt electrode. From the current integration of the electrolysis, it was determined that one electron per equivalent was transferred during the bulk electrolysis. The reaction products were extracted and separated. NMR analysis of the products confirmed that *N*-acetyl-penicillamine and its disulfide were formed during the bulk electrolysis. It is known that a thiol anion can nucleophilically attack the *S*-nitrosothiol to form a disulfide and NO^-, the final product of NO^- is N_2O and water.[17] Stamler and Loscalzo *et al.*[18-20] reported that *S*-nitrosothiols can be reduced to form free thiols and NO by using a HPLC and electrochemical detector (ECD) technique with a dual Au-Hg electrode set. Based on our experiment and literature results, we propose a mechanism for the electrochemical reduction of the *S*-nitrosothiols (Scheme 1).

$$RSNO + e^- \longrightarrow RS^- + \cdot NO \quad (1)$$

$$RS^- + H^+ \rightleftharpoons RSH \quad (2)$$

$$RSNO + RS^- \longrightarrow RS\text{-}SR + NO^- \quad (3)$$

$$2NO^- + 2H^+ \longrightarrow N_2O + H_2O \quad (4)$$

Scheme 1. A proposed mechanism for the electrochemical reduction of *S*-nitrosothiols

Initially, *S*-nitrosothiols accept one electron from the working electrode to result in RS^- and NO (eq 1). RS^- can obtain a proton from the aqueous medium to form a free thiol (eq 2), or proceed via a nucleophilic attack to RSNO to produce a disulfide, RSSR and NO^-(eq 3). Two molecules of NO^- then combine to give N_2O as the

final product (eq 4). The rate of the reaction (eq 3) is dependent on thiol structure, concentration and pH. Göbitz *et al.*have conducted an *ab initio* calculation for SNAP.[21] They found that due to the steric hindrance of the two gem methyl groups, formation of disulfide, RSSR, is not favored. As a matter of fact, less than 5% of RSSR was formed during electrochemical reduction of SNAP.

4. Linear relationship between peak potential of *S*-nitrosothiols and pK_a of thiols. We conducted cyclic voltammetric studies of a series of *S*-nitrosothiols with different pKa's of the corresponding thiols.[22] It was found that different RSNO has different reduction peak potential. The experimental results are summarized in Table 1.

S-nitrosothiols (RSNO)	pK_a of thiols	Ep (c) of RSNO, V
S-nitroso-l-cysteine ethyl ester	6.5	-0.80
S-nitroso-l-cysteine	8.3	-0.99
S-nitroso-glutathione (GSNO)	8.75	-0.98
S-nitroso-mercaptoethanesulfonate	9.1	-1.05
S-nitroso-*N*-acetyl-l-cysteine	9.52	-1.14
S-nitroso-3-mercaptopropionate	10.27	-1.08
S-nitroso-mercaptoacetic acid	10.31	-1.14

Table 1. Reduction peak potentials of the different RSNOs

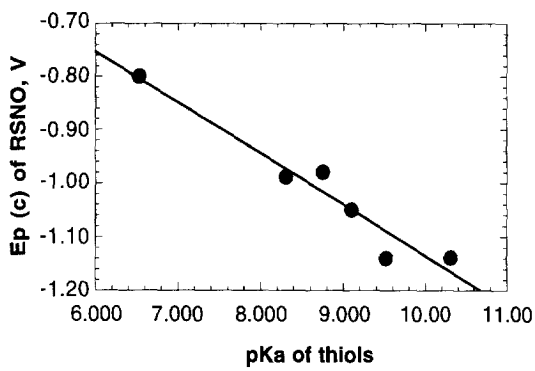

Figure 4. Ep (c) of the *S*-nitrosothiols vs. pK_a of the thiols

Plotting the reduction peak potential (Ep (c)) of RSNOs with pK_a of thiols gives a very good linear relationship (Fig. 4). This suggests that the smaller the pK_a's of the thiols, the less negative the reduction peak potentials of the corresponding *S*-nitrosothiols. In other words, the easier it is for a thiol to form RS^-, the easier it is for the corresponding *S*-nitrosothiol to be reduced electrochemically. By using an NO probe, bulk electrolysis of these RSNOs confirmed the release of NO during the electrochemical reduction. These studies were consistent with eq 1 and further supported our proposed mechanism. Further, our finding may provide a general guide to predict the NO releasing potential from electrochemical reduction of the *S*-nitrosothiols based on the pK_a's of the corresponding thiols.

In conclusion, we found that *S*-nitrosothiols exhibit a single diffusion controlled, irreversible reduction peak, corresponding to the release of NO from the *S*-nitrosothiols. The reduction potentials are far more negative than reduction potential of NAD^+/NADH (-0.3 V) *in vivo*,[23] which may imply that the real mechanism for the *S*-notrosothiols to release NO may not be via this reduction path *in vivo*. Our finding on the linear relation between the reduction potentials of the RSNOs and the pK_a's of the thiols may be used to predict the electrochemical releasing potential of NO.

Experimental

General Procedures. All the reagents were purchased from commercial suppliers and used without further purification. The ^{1}H NMR spectra were recorded on a Varian VXR 400 MHz NMR instrument. Silica gel plates (Merck F254) were used in analytical thin-layer chromatography (TLC).

Electrochemical Measurements. Cyclic voltammetry (CV) and bulk electrolysis (BE) were carried out on a BAS 100 B/W Electrochemical Analyzer (Bioanalytical Systems, Inc.). Exhaustive electrolysis was accomplished with a BAS 100 Electrochemical Analyzer in the hood. Ag/AgCl (3 M NaCl) and Pt were used as a reference and a counter electrode in a three-electrode system, respectively. A phosphate buffer (0.1 M, NaH_2PO_4-Na_2HPO_4, pH 7.4, DTPA 2 mM) was used as a supporting electrolyte in the aqueous solution and 0.1 M $TBAPF_6$ in CH_3CN was used as an organic medium. SNAP, GSNO and Glc-SNAP-1 in 2 mM concentration were used to conduct the CV and BE measurements. A solution of 20 mM of SNAP was used to conduct the exhaustive bulk electrolysis. All solutions were bubbled with argon and all experiments were carried out in the dark.

Nitric Oxide Measurements. The NO probe was inserted through a bypass into the electrochemical cell at a fixed position and NO was measured simultaneously during the bulk electrolysis while the solution was stirred at the maximum speed by using a commercial ISO-NO Mark II Isolated Nitric Oxide Meter, using a Duo. 18 Interface & Software to process the data (both manufactured by World Precision Instruments, Inc. Sarasota, Florida).

Synthesis of RSNOs. SNAP, GSNO and Glc-SNAP-1 were synthesized by the methods of Field *et al.*[24], Hart *et al.*[25] and Ramirez *et al.*[16] respectively. Other RSNOs were prepared according to the following method: solutions of 10 mM thiol and 10 mM sodium nitrite in 0.5 M HCl were used to prepare *in situ* RSNOs at room temperature. After 12 min, DTPA was added and the pH was adjusted to pH ~7.5 with 1 M NaOH. The solution was diluted to 2 mM *S*-nitrosothiol, 1 mM DTPA and used to carry out the cyclic voltammetric study without any further purification. The formation of the *S*-nitroso group was confirmed by the characteristic absorption maxima at 330-370 nm.

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