Electrophilic Intermediate in the Reaction of Glutathione and Nitrosoarenes

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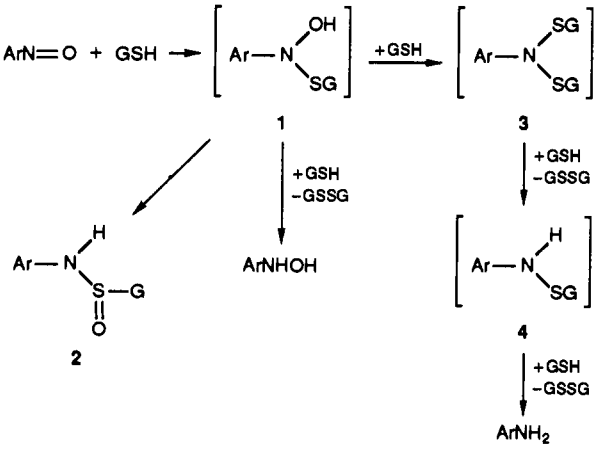
Abstract: A kinetic study is reported of the reaction of glutathione (γ-L-glutamyl-L-cysteinylglycine, GSH) with nine substituted nitrosobenzenes (3,4-Me_2, 4-Me, 3,5-Me_2, 3-Me, parent, 3-MeO, 4-Cl, 3-Cl, 3-NO_2). Previous workers have shown that this reaction proceeds in parallel pathways, producing the appropriate *N*-arylhydroxylamine and GSSG or a sulfinanilide adduct ArNHS(O)G; a rapid equilibrium addition to form a common intermediate, a semimercaptal ArN(OH)SG, has also been observed. In the present study, equilibrium constants for the formation of this intermediate from ArNO and GSH have been measured by a kinetic method, and the kinetic behavior of the slower additional reactions of the semimercaptal have been examined in detail. For experiments carried out at constant pH and buffer concentration, the decay of ArN(OH)SG follows the rate law k_2^{GSH}[GSH] + k_2(rearr). A comparison with product ratios previously reported shows that the bimolecular term with GSH represents the process forming ArNHOH and GSSG, while the unimolecular term represents the rearrangement to the sulfinanilide. The former process is found to be proportional to [OH^-] for solutions near neutrality, is not buffer catalyzed, and has a ρ value of +1.4. This suggests a mechanism in which glutathione anion GS^- reacts at the sulfur of the adduct displacing $ArN^-(OH)$ as a leaving group. The rearrangement reaction follows σ^+ with a ρ^+ value of −3.5 and has a rate law containing a pH-independent term and terms for catalysis by H^+ and the acid component of the buffer. An ^{18}O tracer study shows that the S=O oxygen in the sulfinanilide is derived from solvent, not the original N=O group. A mechanism is proposed with rate-limiting N–O cleavage, either uncatalyzed involving direct heterolysis with OH^- as a leaving group or catalyzed by acids with H_2O as the leaving group. The species produced is a cationic intermediate ArN^+SG, a nitrenium ion stabilized by both the aryl ring and the directly attached sulfur atom. Aryl-stabilized nitrenium ions are commonly encountered in Bamberger-like rearrangements of hydroxylamine derivatives. The sulfur atom of PhN(OH)SG is shown to provide an approximately 10^6 rate acceleration for N–O cleavage in a comparison with the Bamberger rearrangement of PhNHOH.

Nitrosoarenes produced through metabolic N-oxygenation of aromatic amines or reduction of nitro aromatics may play an important role in the biological effects observed with such compounds.[1-3] One important target of the nitroso compound is the SH group of cellular thiols such as glutathione (γ-L-glutamyl-L-cysteinylglycine, GSH). For example, the reaction of nitroso aromatics with glutathione results in the rapid excretion of the nitroso compound by liver cells, with concomitant lowering of GSH levels and alteration of bile flow. Similar reactions occurring in blood markedly modulate ferrihemoglobin formation.[1,4,5] Nitrosoarenes are toxic toward isolated rat hepatocytes with the toxicity being lowered or increased if GSH is depleted beforehand, the direction of the effect being dependent on the Hammett constant of the substituents in the aromatic ring.[6] Nitrosoimidazoles derived from reduction of nitroimidazoles are highly toxic[2,3] and react readily with cellular glutathione[7] as well as protein SH groups.[8] These effects may be responsible for the bioreductive toxicity of the parent nitro drugs.[7,8]

Scheme I



The products of the reaction of nitrosobenzene and its derivatives with glutathione have been analyzed in some detail, leading to the proposed mechanism of Scheme I.[1,5,9,10] The parent PhNO follows two parallel pathways, forming the sulfinanilide adduct **2** or *N*-phenylhydroxylamine coupled with an equivalent amount of the oxidized form of GSH, GSSG.[9] The latter products are favored at higher glutathione concentrations as well as at higher pH.[9] There is UV spectroscopic evidence for an intermediate forming reversibly from the two starting materials.[9] This has been assigned the structure of the adduct **1**, termed a "semimercaptal" through analogy with C=O chemistry. Similar products are observed with nitrosoarenes bearing electron-withdrawing substituents, but there is a marked substituent dependence of the sulfinanilide/*N*-arylhydroxylamine ratio, systems with a strongly electron-withdrawing group such as $COCH_3$ giving mainly the latter product.[5] Nitrosoarenes with the strong π-donors 4-NMe_2 and 4-EtO on the other hand are reduced to the aniline level.[5]

(1) Eyer, P. In *Proceedings of the 3rd International Symposium on the Biological Oxidation of Nitrogen in Organic Molecules*; Gorrod, J. W., Damani, L. A., Eds.; Ellis Horwood: Chichester, Great Britain, 1985; p 386.
(2) Noss, M. B.; Panicucci, R.; McClelland, R. A.; Rauth, A. M. *Biochem. Pharmacol.* **1988**, *37*, 2585.
(3) Elhardt, W. J.; Beaulieu, B. B.; Goldman, P. *Biochem. Pharmacol.* **1988**, *37*, 2603.
(4) (a) Eyer, P.; Kampffmeyer, H.; Maister, H.; Rosch-Oehme, E. *Xenobiotica* **1980**, *10*, 499. (b) Eyer, P.; Kampffmeyer, H. *Chem.-Biol. Interact.* **1982**, *42*, 209. (c) Eckert, K.-G.; Eyer, P.; Kampffmeyer, H. *Naunyn-Schmiedeberg's Arch. Pharmacol. Suppl.* **1983**, *322*, R107. (d) Eyer, P.; Lierheimer, E.; Scheller, M. *Biochem. Pharmacol.* **1984**, *33*, 2299.
(5) Diepold, C.; Eyer, P.; Kampffmeyer, H.; Reinhardt, K. In *Biological Reactive Intermediates: 2. Chemical Mechanisms and Biological Effects*; Snyder, R., Parke, D. V., Kocsis, J. J., Jollow, D. J., Gibson, G. G., Witmer, C. M., Eds.; Plenum Press: New York and London, 1982; p 1173.
(6) Silva, J. M.; Jatoe, S. D.; O'Brien, P. J. *Prog. Pharmacol. Clin. Pharmacol.* **1991**, *8*, 164.
(7) (a) Mulcahy, R. T.; Gipp, J. J.; Ublacker, G. A.; Panicucci, R.; McClelland, R. A. *Biochem. Pharmacol.* **1989**, *38*, 1667. (b) Elhardt, W. J.; Goldman, P. *Biochem. Pharmacol.* **1989**, *38*, 1175. (c) Noss, M. B.; Panicucci, R.; McClelland, R. A.; Rauth, A. M. *Int. J. Radia. Oncol. Biol. Phys.* **1989**, *16*, 1015.
(8) Berube, L.; Farah, S.; McClelland, R. A.; Rauth, A. M. *Biochem. Pharmacol.*, submitted for publication.
(9) Eyer, P. *Chem.-Biol. Interact.* **1979**, *24*, 227.
(10) Dolle, B.; Topner, W.; Neumann, H.-G. *Xenobiotica* **1980**, *10*, 527.

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This type of reaction is also observed in part with 4-nitrosotoluene.[10] It has been proposed that the semimercaptal can follow a third pathway: reaction with another equivalent of GSH to produce the full mercaptal **3**, followed by sequential S–S coupling with 2 equiv of GSH to give the aniline.[1,5,10] There is no direct evidence for a species such as **3**, although unstable sulfenamides **4** have been characterized in the reactions of thiols with 2-nitrosofluorene and 4-nitrosophenetole.[11]

In this paper, we present a kinetic study of the reaction of glutathione with nine substituted nitrosobenzenes (3,4-Me_2, 4-Me, 3,5-Me_2, 3-Me, H, 3-MeO, 4-Cl, 3-Cl, 3-NO_2). Of particular concern in this investigation are the detailed mechanisms by which the semimercaptal intermediate is converted into the arylhydroxylamine and the rearranged sulfinanilide. There appears to have been little discussion of these mechanisms, especially that of the rearrangement. On the basis of these results, the sulfinanilide is proposed to arise via a dissociative process involving the formation of a cationic intermediate, a nitrenium ion stabilized jointly by an aryl ring and a sulfur atom.

Results

The reaction of nitrosobenzene with excess glutathione at neutral pH results in UV spectral changes that show two components. The first phase involves a rapid decay of the absorbance of nitrosobenzene (λ_{max} = 305 nm). Under most conditions, this process is too fast to be observed by conventional spectroscopy, but it can be followed with a stopped-flow apparatus, where it is found to obey exponential kinetics. At the completion of this phase in experiments with relatively high GSH concentrations, there is little residual optical density at 305 nm. There is, however, a slower subsequent spectral change in the region around 260 nm, also following exponential kinetics. Similar two-component behavior is observed at lower GSH concentrations, with the exception that after the initial fast decay at 305 nm there is residual optical density at this wavelength corresponding to unreacted nitrosobenzene. This now decays in the slower process, with a rate constant that is equal to that obtained by following the change at 260 nm.

These observations are identical to those previously reported by Eyer[9] and constitute the principal piece of evidence for the kinetic scheme

$$\mathrm{ArN{=}O + GSH} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{Ar{-}N(OH)(SG)} \xrightarrow{k_2} \mathrm{PRODS} \qquad (1)$$

where the initial spectral change involves the establishment of the equilibrium with the semimercaptal, and the second change corresponds to the formation of the ultimate products through the further reactions of this intermediate. Such behavior was observed for all of the systems investigated in this study, with the two components in general being sufficiently different in rate that each could be analyzed separately by single-exponential kinetics.[12]

For the above kinetic model, the initial kinetic phase represents an approach to an equilibrium, and under conditions of GSH in excess over ArNO, an observed first-order rate constant is expected that is the sum of rate constants for the forward and reverse reactions.

$$k(\mathrm{fast}) = k_1[\mathrm{GSH}] + k_{-1} \qquad (2)$$

According to this relation, the individual rate constants k_1 and k_{-1} can be obtained as the slope and intercept, respectively, of a plot of k(fast) versus [GSH], with the equilibrium constant K_1 = [semimercaptal]/[GSH][ArNO] being calculated as the ratio k_1/k_{-1}. Such plots for the parent system are shown in Figure 1.

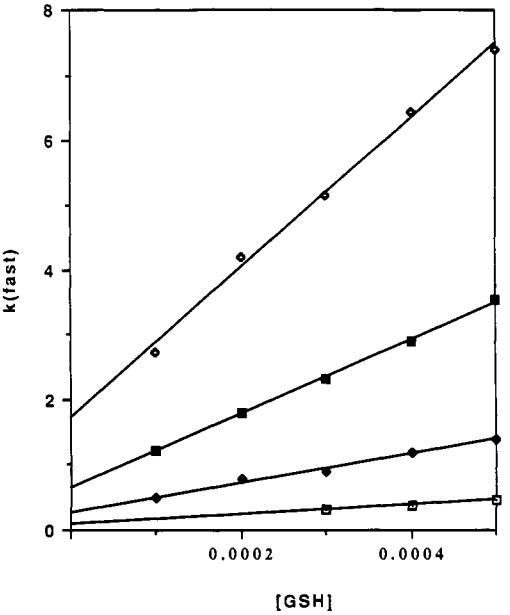


Figure 1. Observed rate constants (s^{-1}) for the fast kinetic component in the reaction of nitrosobenzene and glutathione. Reactions were carried out at 25 °C and ionic strength 1.0 M in phosphate buffers with pH 6.61 (□), 7.05 (◆), 7.49 (■), and 7.87 (◇). Rate constants were obtained at 305 nm in solutions with [PhNO] = 1.0×10^{-5} M. Slopes k_1 (M^{-1} s^{-1}), intercepts k_{-1} (s^{-1}), and the slope/intercept ratio K_1 (M^{-1}) are as follows: for pH 6.61, 9.7×10^{-2}, 7.5×10^2, 7.7×10^3; for pH 7.05, 2.7×10^{-1}, 2.3×10^3, 8.5×10^3; for pH 7.48, 6.4×10^{-1}, 5.7×10^3, 9.0×10^3; for pH 7.87, 1.7×10^0, 1.16×10^4, 6.8×10^3.

Table I. Rate Constants k_1 and k_{-1} and Equilibrium Constants K_1 for the Formation of Semimercaptals from Glutathione and Substituted Nitrosobenzenes[a]

substituent	k_1, M^{-1} s^{-1}	k_{-1}, s^{-1}	K_1,[b] M^{-1}
3-NO_2	$(1.17 \pm 0.08) \times 10^5$	[c]	
3-Cl	$(2.92 \pm 0.03) \times 10^4$	[c]	
4-Cl	$(1.22 \pm 0.06) \times 10^4$	0.27 ± 0.10	4.5×10^4
3-MeO	$(7.92 \pm 0.06) \times 10^3$	0.56 ± 0.15	1.41×10^4
H	$(5.69 \pm 0.10) \times 10^3$	0.60 ± 0.08	9.5×10^3
3-Me	$(4.83 \pm 0.04) \times 10^3$	0.77 ± 0.05	6.3×10^3
3,5-Me_2	$(3.69 \pm 0.04) \times 10^3$	0.85 ± 0.05	4.3×10^3
4-Me	$(1.87 \pm 0.02) \times 10^3$	1.18 ± 0.11	1.58×10^3
3,4-Me_2	$(1.72 \pm 0.01) \times 10^3$	1.37 ± 0.05	1.25×10^3

[a] Conditions: 25 °C and ionic strength 1.0 M in phosphate buffer (0.2 M) with pH 7.49. [b] Calculated as the ratio k_1/k_{-1}. [c] Could not be determined since intercepts in plots of k(fast) versus [GSH] were not statistically significant.

Since K_1 was large, relatively low concentrations of glutathione (and correspondingly lower concentrations of nitrosobenzene) were required to produce statistically accurate intercepts, but this did prove possible and k_{-1} values of ±10% precision could be obtained. Experiments at constant pH with changing buffer concentration showed that there was no effect on either rate constant. However, as shown in the figure, both k_1 and k_{-1} increase with increasing pH,[13] as plots of log k versus pH are linear with slopes of 0.95 and 0.98, respectively, for the data of Figure 1. The equilibrium constant is within experimental uncertainty, unchanged. This lack of dependence is not surprising since the SH group of glutathione has a pK_a value of ~9[14] and thus remains essentially un-ionized at the pH involved in these experiments.

(11) (a) Mulder, G. J.; Unruh, L. E.; Evans, F. E.; Ketterer, B.; Kadlubar, F. F. *Chem.-Biol. Interact.* **1982**, *39*, 111. (b) Mulder, G. J.; Kadlubar, F. F.; Mays, J. B.; Hinson, J. A. *Mol. Pharmacol.* **1984**, *26*, 342–347. (c) Klehr, H.; Eyer, P.; Schafer, W. *Biol. Chem. Hoppe-Seyler* **1985**, *366*, 755–760; (d) **1987**, *368*, 895–902.

(12) This was not true for 3,4-dimethyl- and 4-methylnitrosobenzenes in the experiments with GSH concentrations less than 1 mM. However, with these compounds, the second phase showed little dependency on [GSH], and at higher concentrations the rates of the two processes were well separated.

(13) Eyer made this same observation for k_1,[9] but did not investigate k_{-1}.

(14) (a) Huckey, T. N.; Tudor, A. J.; Dawber, J. G. *J. Chem. Soc., Perkin Trans 2* **1985**, 59. (b) Cheeseman, B. V.; Arnold, A. P.; Rabenstein, D. L. *J. Am. Chem. Soc.* **1988**, *110*, 6359. (c) The ionization state of GSH at higher pH becomes somewhat complicated since the glutamic acid NH_3^+ group (macroscopic pK_a = 9.12) is changing in the pH range in which the SH is ionized (macroscopic pK_a = 8.66). At the lower pH where these experiments were carried out, however, these microscopic ionizations were relatively unimportant in determining the concentration of the reactive thiolate form.

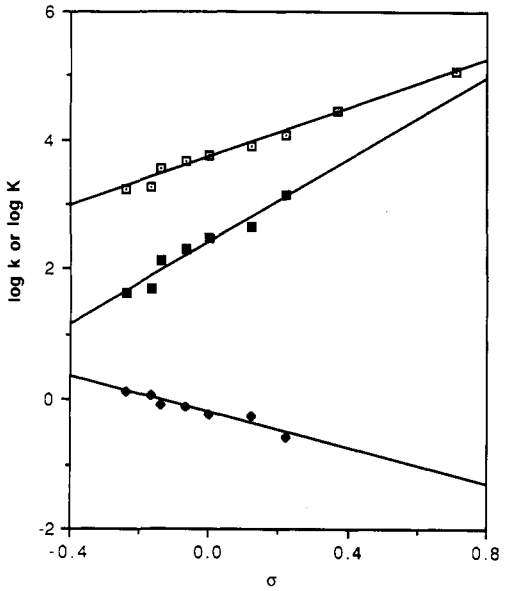


Figure 2. Hammett plots for rate constants k_1 (□) and k_{-1} (◆) and the equilibrium constants K_1 (■) for the formation of semimercaptals from glutathione and substituted nitrosobenzenes. log K_1 has been displaced downward by 1.5 log units. Units are M^{-1} s^{-1} for k_1, s^{-1} for k_{-1}, and M^{-1} for K_1.

Eyer evaluated the equilibrium constant for the parent system using the relative absorbance changes of the fast and slow kinetic phases as a measure of the amount of nitrosobenzene that reacted in each.[9] We have some reservations about this procedure since even under conditions where the initial equilibrium is >99% shifted toward the semimercaptal there is a slow component at 305 nm associated with a small amount of residual absorbance due to the semimercaptal. The result is that values of K_1 calculated from optical density changes decrease in a regular fashion at higher GSH concentration. In any event, the value of K_1 calculated spectroscopically by Eyer, 2.6×10^3 M^{-1} at 37 °C,[9] and that obtained kinetically in our experiments, 8.0×10^3 M^{-1} at 25 °C, are in reasonable agreement, particularly considering the different temperatures.

With the other nitrosobenzenes, the values of the two rate constants and the equilibrium constant calculated as their ratio were obtained in a common solution at one pH, 7.49. The results are listed in Table I, with Hammett plots based upon the numbers being shown in Figure 2. The latter plots were constructed with Hammett σ constants as the use of other scales such as σ^+ did not improve the fit. The ρ values obtained from linear regression are +1.9 (log k_1),[15] −1.4 (log k_{-1}), and +3.2 (log K_1).

First-order rate constants for the slow kinetic component, defined as k(slow), were obtained at 255–265 nm where there was a significant decrease in optical density with all of the systems studied. In general, higher concentrations of GSH were employed to drive the initial equilibrium to the semimercaptal side, although in many cases a correction was still necessary (see below). As with the fast component, the entire set of nitrosobenzenes was studied in one common phosphate buffer of pH 7.49. Plots of the dependence of k(slow) on GSH concentration illustrating extremes of behavior are shown in Figure 3. With 3-nitro- and 3-chloronitrosobenzene, excellent linearity is observed in this plot, with the intercept being statistically insignificant. With 4-chloronitrosobenzene and derivatives with more electron-donating substituents, however, significant intercepts are obtained. Moreover, the plots using the observed k(slow) become curved, an effect that is particularly noticeable for the rate constants measured at low GSH concentration and with the nitrosobenzenes with the more electron-donating substituents (see, for example, the uncorrected data for 4-methyl in Figure 3). This curvature is not unexpected, since it is observed under conditions where the initial equilibrium forming the semimercaptal is incomplete, as demonstrated by calculations using values of K_1. In such a case, the observed rate constant does not equal k_2, the actual rate constant for the further reaction of the semimercaptal intermediate, but rather is given by the equation

$$k(\text{slow-observed}) = k_2\left(\frac{[\text{GSH}]}{K_1 + [\text{GSH}]}\right) \qquad (3)$$

which is derived with the assumption that the equilibration step is much faster than the further reaction of the semimercaptal. As shown by the corrected data for 4-methyl in Figure 3, values of k_2 obtained from the observed k(slow) using the appropriate K_1 produce excellent linear plots in GSH concentration.

The rate constants obtained as slopes and intercepts in the plots of k(slow) or k(slow-corrected) versus [GSH] are given under the headings k_2^{GSH} and k_2(rearr), respectively, in Table II. Hammett plots for these rate constants are shown in Figure 4. The constant k_2^{GSH} gives a reasonable correlation with σ, while k_2(rearr) is better correlated with σ^+. Slopes of the linear regression lines are $\rho = +1.4$ for k_2^{GSH} and $\rho^+ = -3.5$ for k_2(rearr). Measurements with the parent system were made at two additional temperatures, and activation parameters were obtained. These are as follows: for k_2^{GSH}, $\Delta H^\ddagger = 8.6$ kcal mol^{-1} and $\Delta S^\ddagger = -25$ cal deg^{-1} mol^{-1}; for k_2(rearr), $\Delta H^\ddagger = 20.3$ kcal mol^{-1} and $\Delta S^\ddagger = +2$ cal deg^{-1} mol^{-1}.

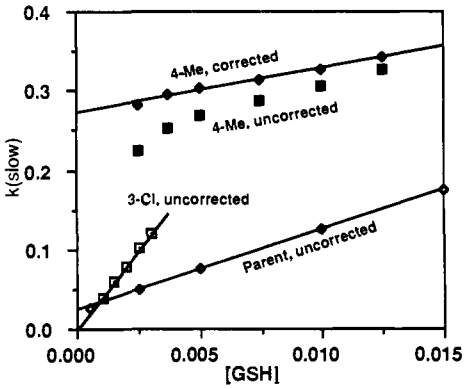


Figure 3. Rate constants (s^{-1}) for the slow kinetic component in the reaction of nitrosobenzenes and glutathione. Reactions were carried out at 25 °C and ionic strength 1.0 M in phosphate buffer, pH 7.49. Uncorrected rate constants are those directly observed for the slow decay. Corrected rate constants account for incomplete formation of the semimecaptal in the initial equilibrium, k(slow-corrected) = k(slow-uncorrected)[(K_1 + [GSH])/[GSH]] using values of K_1 in Table I. For the parent system, the points are uncorrected, while the line is drawn on the basis of linear regression using the corrected rate constants.

Table II. Rate Constants k_2^{GSH} for the Bimolecular Reaction with Glutathione and k_2(rearr) for Rearrangement to the Sulfinanilide for Semimercaptals Derived from Glutathione and Substituted Nitrosobenzenes[a]

substituent	k_2^{GSH}, M^{-1} s^{-1}	k_2(rearr), s^{-1}
3-NO_2	$(1.21 \pm 0.01) \times 10^2$	[b]
3-Cl	$(4.11 \pm 0.04) \times 10^1$	[b]
4-Cl	$(2.47 \pm 0.03) \times 10^1$	0.0070 ± 0.0007
3-MeO	$(1.71 \pm 0.02) \times 10^1$	0.0099 ± 0.0008
H (25.0 °C)	$(1.01 \pm 0.02) \times 10^1$	0.024 ± 0.001
H (37.0 °C)	$(1.84 \pm 0.12) \times 10^1$	0.094 ± 0.004
H (50.0 °C)	$(3.39 \pm 0.34) \times 10^1$	0.37 ± 0.03
3-Me	$(9.34 \pm 0.07) \times 10^0$	0.042 ± 0.002
3,5-Me_2	$(9.61 \pm 0.12) \times 10^0$	0.062 ± 0.001
4-Me	$(5.54 \pm 0.30) \times 10^0$	0.27 ± 0.01
3,4-Me_2	$(2.86 \pm 0.70) \times 10^0$	0.42 ± 0.01

[a] Conditions (unless otherwise specified): 25 °C and ionic strength 1.0 M in phosphate buffer (0.2 M) with pH 7.49. [b] Could not be determined since intercepts in plots of k(slow) versus [GSH] were not statistically significant.

(15) Eyer and co-workers reported a ρ value of +2.1 for this rate constant,[5] but did not investigate the dependence of k_{-1} and K_1.

(16) Bolton, J. L.; McClelland, R. A. *J. Am. Chem. Soc.* **1989**, *111*, 8172.

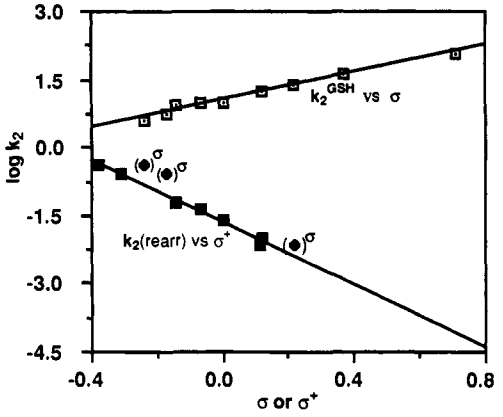
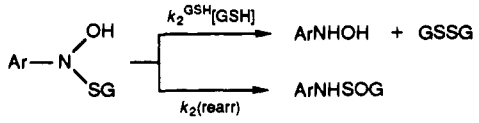


Figure 4. Hammett plots for rate constants k_2^{GSH} and k_2(rearr) for the reactions of semimercaptals. log k_2^{GSH} has been plotted against σ and log k_2(rearr) versus σ^+. The bracketed points represent 3,4-Me_2, 4-Me, and 4-Cl, showing the location if σ is employed.

The constants k_2^{GSH} and k_2(rearr) can be attributed to the two pathways in which the semimercaptal reacts with another molecule of GSH or undergoes rearrangement to the sulfinanilide:



(4)

This can be demonstrated through a comparison of the absolute rate constants measured in this work with ratios obtained previously through analysis of products. With the latter, data measured in a particular buffer were found to correlate with eq 5.[5,9]

$$\frac{\text{ArNHOH formed}}{\text{ArNO added}} = \frac{p[\text{GSH}]}{1 + p[\text{GSH}]} \qquad (5)$$

This is the equation for a simple competition, and the parameter p is the ratio k_2^{GSH}/k_2(rearr). For nitrosobenzene and GSH in a phosphate buffer of pH 7.4 at 37 °C, p is found to be 140 M^{-1},[9] which can be corrected to 172 M^{-1} at pH 7.49 from the pH dependence of the two rate constants (see below). This number is in excellent agreement with the ratio k_2^{GSH}/k_2(rearr) = 196 M^{-1} calculated from the kinetics under identical conditions. Agreement can also be noted in the dependence on aromatic substituent. Thus, values of p were also obtained at 37 °C, pH 7.40 for the 4-Cl derivative, 900 M^{-1}, and for 3,4-Cl_2, 20000 M^{-1}.[5] With the three substituents, the Hammett plot for log p has a slope of +3.7 if the σ scale is employed and +4.4 for σ^+. Using a wider range of substituents and recognizing that the individual rate constants follow different scales, the number directly measured is +4.9 ($\rho(k_2^{GSH}) - \rho^+(k_2(\text{rearr}))$). The excellent agreement between the products and rate constants shows that for these systems one is dealing with a simple competition for the intermediate semimercaptal. In particular, the k_2^{GSH} term represents the reaction of the semimercaptal to form the hydroxylamine and 1 equiv of the disulfide. It is possible that with systems with more electron-donating substituents, where full reduction to the aniline is also observed, there is some contribution of this reaction to k_2^{GSH} These systems are the subject of current research.

The effects of pH and buffer on the semimercaptal reactions were examined for the parent system. As shown by the data in Figure 5, the slope k_2^{GSH} of plots of k(slow-corrected) versus [GSH] are within experimental error, independent of buffer concentration in experiments at constant pH, but there is a significant effect on the intercept k_2(rearr). The rate constants k_2^{GSH} vary with pH, with values at 25 °C of 1.89 M^{-1} s^{-1} at pH 6.61, 4.53 at pH 7.05, 10.1 at pH 7.49, and 22.1 at pH 7.84; a plot of log k_2^{GSH} versus pH for these data has a slope of 0.9. With the rate constant k_2(rearr), the experiments at different pH reveal that it is the acid component of the phosphate buffer, $H_2PO_4^-$, that is the species responsible for the rate acceleration. However,

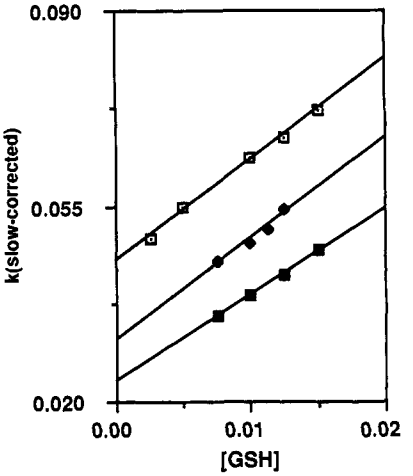


Figure 5. Rate constants (s^{-1}) for the slow kinetic component in the reaction of nitrosobenzene and glutathione in a 1:1 $H_2PO_4^-/HPO_4^{2-}$ buffer (pH ≈ 6.6), with total buffer concentration of 0.50 (□), 0.25 (◆), and 0.125 (■). The temperature was 25 °C and ionic strength 1 M. The rate constant has been corrected as described in the text for incomplete formation of semimercaptal in the initial equilibrium stage. Slopes, k_2^{GSH}, obtained by linear regression analysis are 1.82 M^{-1} s^{-1}, 1.81 M^{-1} s^{-1}, and 1.56 M^{-1} s^{-1} from top to bottom, with intercepts k_2(rearr) 0.045 s^{-1}, 0.031 s^{-1}, and 0.023 s^{-1}, respectively.

over the region from pH 6.6 to pH 7.8, rate constants obtained by extrapolation to zero buffer concentration are constant at 0.020 ± 0.003 s^{-1}. The consequence of these combined effects is that in phosphate buffers of pH 6–8 the sulfinanilide product becomes more favored over the arylhydroxylamine, i.e., p decreases at lower pH and higher $H_2PO_4^-$ concentrations. Such behavior was noted previously in the analysis of the products,[9] although a dissection into the individual effects of pH and buffer concentration was not carried out.

That H^+ accelerates the formation of the sulfinanilide product was suggested previously in a pH-jump experiment in which the semimercaptal was formed at pH 8.2 and the solution then acidified (see Figure 5 of ref 9). To accurately measure a rate constant for the H^+ reaction and to further study the reaction catalyzed by buffer acids, we have repeated this approach, in our case by adding nitrosobenzene to a 1 mM solution of GSH in 4 mM phosphate buffer of pH 7.3, followed by mixing with acid solutions using a stopped-flow apparatus. In our experiments, the time that elapsed between the initial addition of the nitrosobenzene to the neutral GSH solution and the acidification was 15–25 s. This is easily sufficient time for the semimercaptal to have formed in the initial equilibrium; it will have decayed about 50% to products. Acidification results in more rapid decay and also freezes the back reaction to nitrosobenzene and glutathione, since this is base dependent. Rate constants for the decay in the acid solutions were measured by following an absorbance decrease at 260 nm. In experiments in which the acidification was carried out with HCl, excellent linearity was observed in the $[H^+]$ concentration of the final solution, as measured with a pH meter. Experiments were also conducted by acidifying with carboxylic acid buffers, and in each case the observed rate constants measured at constant pH were linear in [RCOOH]. Combining the results of both the direct measurements in phosphate buffers and the pH-jump experiments gives first-order rate constants for the conversion of semimercaptal to sulfinanilide following the equation

$$k_2(\text{rearr}) = k_2^{H}(\text{rearr})[H^+] + k_2^{HA}(\text{rearr})[HA] + k_2^{0}(\text{rearr}) \qquad (6)$$

with terms showing catalysis by H^+ and added general acids as well as a pH-independent term. In the absence of buffer, or at low buffer concentrations, the last term dominates in solutions with pH > 5, i.e., the rearrangement is pH independent. Values of the individual rate constants are listed in Table III. A Bronsted plot is shown in Figure 6. The five acid catalysts, including H^+, fall reasonably well on a single line, with a slope, α, of 0.6.

Table III. Rate Constants for Rearrangement to the Sulfinanilide of the Semimercaptal Derived from Glutathione and Nitrosobenzene[a]

catalyst	k_2(rearr), M^{-1} s^{-1}	k_{INHOH}, M^{-1} s^{-1} [b]
H^+	8.2×10^2	4.0×10^2
$CH_2ClCOOH$	6.9×10^0	
$CH_2OMeCOOH$	3.0×10^0	
CH_3COOH	6.2×10^{-1}	4.1×10^{-2}
$H_2PO_4^-$	1.2×10^{-1}	8.6×10^{-3}
none (s^{-1})	2.0×10^{-2}	1.5×10^{-2}

[a] Conditions: 25 °C, μ = 1.0 M. [b] Rate constants for the Bamberger-like rearrangement of 1-methyl-2-(hydroxyamino)imidazole; from ref 16.

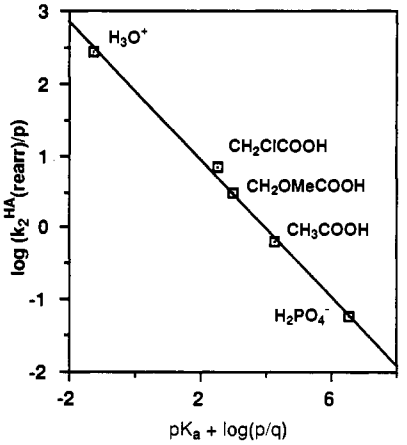
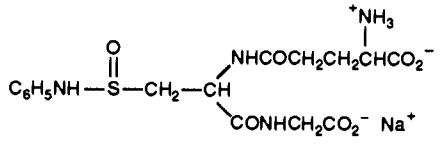


Figure 6. Bronsted plot for the rearrangement to the sulfinanilide of the semimercaptal of glutathione and nitrosobenzene.

Our final experiment was performed to determine the origin of the oxygen in the sulfinanilide product.[17] This was carried out by adding nitrosobenzene to an unbuffered solution of pH 5 containing a slightly excess of glutathione, waiting 20 min, and removing the water by lyophilization. Calculations with k_2^{GSH} and k_2(rearr) showed that the sulfinanilide product should account for 95% of the nitrosobenzene at this pH and the concentration of GSH employed. This was confirmed in an NMR spectrum of the sample, which showed only signals due to the sulfinanilide (and unreacted GSH). FAB mass spectra were then recorded. At the pH of the experiment, the sulfinanilide is expected to be isolated as a monosodium salt P, the sodium being derived from the NaOH employed to neutralize the glutathione in obtaining the pH 5 solution. For the sample obtained from normal water,



P

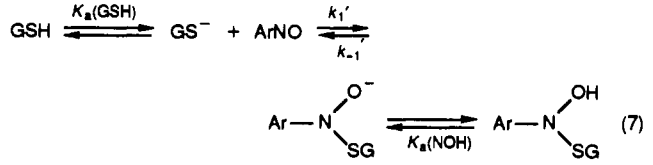
the mass spectrum obtained with positive ion detection shows a strong peak at m/e 437 corresponding to P, with a weaker signal at m/e 415 for P–Na^+ + $2H^+$. The spectrum with negative ion detection shows two peaks of about equal intensity, corresponding to P–$2H^+$ (m/e 435) and P – Na^+ – H^+ (m/e 413). Exactly the same patterns are observed with a sample obtained in a reaction in which water contains 90% oxygen-18, except that all of the peaks are shifted to 2 higher mass units, indicating the incorporation of one oxygen-18. Control experiments demonstrate that nitrosobenzene does not exchange its oxygen with water under these experimental conditions and that addition of the sample of sulfinanilide isolated from labeled water to normal water results in no loss of label. The former control rules out the possibility that the oxygen-18 in the product arises from exchange of starting material before reaction with glutathione. The latter control shows that exchange does not occur after the product is formed and also shows, not unexpectedly, that there is no exchange into the amide and carboxylate groups of the glutathione portion. Thus, the oxygen-18 that appears in the product from the reaction in labeled solvent is present in the S=O group, and moreover this oxygen enters during the rearrangement process, not before or afterward. The conclusion is that this oxygen is derived from solvent and is not the oxygen of the starting nitrosobenzene.

(17) This experiment has also been carried out by Eyer and Klehr. Klehr, Ph.D. Thesis, Ludwig-Maximilians-Universitat, 1988.

Discussion

Formation of Semimercaptal. The addition of a thiol to the nitroso double bond is obviously directly analogous to the reaction forming a hemithioacetal from a thiol and an aldehyde, and it is interesting to compare the two systems. From an equilibrium point of view, the addition to the nitroso group is more favorable. This conclusion is reached on comparing K_1 for GSH adding to *p*-chloronitrosobenzene, 4.5×10^4 M^{-1}, with the equilibrium constants 2.3 and 3.7 M^{-1} for the addition of 2-mercaptoethanol and mercaptoacetic acid to the carbonyl analogue *p*-chlorobenzaldehyde.[18] There also appears to be a greater dependence on substituent for the addition to the nitrosoarenes, the ρ value of +3.2 for the equilibrium addition of GSH to ArNO being about twice the values for ArCHO adding water, ρ = +1.7,[19a] and HCN, ρ = +1.5.[19b] This may be associated with a greater interaction of the N=O group and substituents in the nitroso form.

From a mechanistic point of view, the following scheme can be proposed

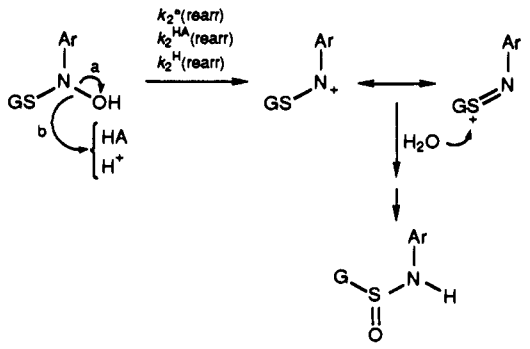


in which the thiol anion adds to the nitroso group to give initially the conjugate base of the semimercaptal. In the range where the kinetics is studied, pH 6.5–8, both glutathione and the semimercaptal are predominantly in their neutral forms, so that the observed rate constants in the two directions are proportional to the concentration of hydroxide ion, while the overall equilibrium is pH independent. The absence of general acid–base catalysis implies that the rate-limiting step is the central one involving S–N bond formation and cleavage.

An exactly analogous mechanism is observed for the formation of hemithioacetals, where the C–S bond is formed in a reaction involving the addition of a thiol anion to the carbonyl group to give initially the conjugate base of the adduct.[20] With this system, there are some examples of buffer catalysis arising through trapping or preassociation mechanisms. These are explained in terms of kinetic schemes where the lifetime of the hemithioacetal anion becomes extremely short with respect to C–S cleavage, so that catalysis becomes enforced.[20] The step which determines the mode of catalysis in hemithioacetal formation is analogous to the breakdown step k_{-1}' in eq 7. An estimate showing that semimercaptal anions are much longer lived can be made for this rate constant. To do this a value for the acidity constant K_a(NOH) is required, and this can be estimated as follows. Starting with the hydroxylamine CF_3CH_2NHOH (pK_a = 11.8),[21] a value of 13.7 for CH_3CH_2NHOH is obtained assuming that the CF_3 group has the same effect as seen in comparing CF_3CH_2COOH (pK_a = 3.1) and CH_3CH_2COOH (pK_a = 4.8), two compounds where the OH group is equally as far removed from the CF_3 substituent. A value of 13.1 for PhNHOH can then be estimated, assuming

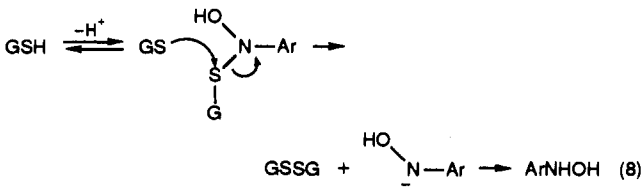
(18) Sanders, E. G.; Jencks, W. P. *J. Am. Chem. Soc.* **1968**, *90*, 6154.
(19) (a) McClelland, R. A.; Coe, M. *J. Am. Chem. Soc.* **1983**, *105*, 2718. (b) Ching, W.-M.; Kallen, R. G. *J. Am. Chem. Soc.* **1978**, *100*, 6119.
(20) Gilbert, H. F.; Jencks, W. P. *J. Am. Chem. Soc.* **1977**, *99*, 7931.
(21) Acidity constants were taken from the following: Kortum, C.; Vogel, W.; Andrussov, K. *Pure Appl. Chem.* **1960**, *1*, 189. Serjeant, E. P.; Dempsey, B. In *Ionisation Constants of Organic Acids in Aqueous Solution*; IUPAC Chemical Data Series 23; Pergamon Press: Oxford, 1979.

Scheme II



that the phenyl group has the same acidifying effect as found in alcohols, for example, in comparing $PhCH_2OH$ (pK_a = 15.4) and CH_3CH_2OH (pK_a = 16.0). The sulfur atom in a hemithioacetal appears to have an acidifying effect worth about 3 pK_a units on the basis of a comparison of HCH_2OH (pK_a = 15.7) and ($HOCH_2CH_2S$)CH_2OH (pK_a = 12.4).[20] With the assumption that this difference also pertains to NOH compounds, pK_a(NOH) for PhN(SG)OH is estimated as being around 10. For the mechanism of eq 7, the observed rate constant k_{-1} is given by $(k_{-1}'K_a(NOH))/(K_a(NOH) + [H^+])$. A value of k_{-1}' of $\sim 3 \times 10^2$ s^{-1} is therefore obtained from this pK_a and the value of k_{-1} observed for breakdown of PhN(SG)OH at pH 7.49. This rate constant is orders of magnitude lower than those associated with the breakdown of hemithioacetal anions, which are around 10^7–10^8 s^{-1} for acetaldehyde derivatives with leaving groups similar to GS^-.[20] The kinetic stability of the semimercaptal anion with respect to breakdown means that it will always be in equilibrium with its conjugate acid as equilibrium is established, and no general catalysis is therefore expected.

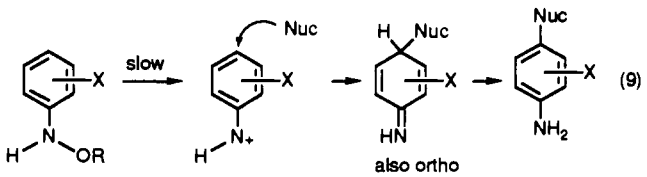
Conversion of Semimercaptal to Hydroxylamine. The formation of the *N*-arylhydroxylamine corresponds to a net two-electron reduction of the starting nitroso compound. The reaction proceeds sequentially, with adduct formation followed by a bimolecular reaction with the second equivalent of glutathione. This latter stage is characterized by a dependence on hydroxide ion concentration in solutions near neutrality, a substantially negative entropy of activation, and a positive ρ value for substituent change in the aromatic group. These observations are consistent with the mechanism



in which GS^- reacts at the sulfur of the adduct displacing an *N*-hydroxyaniline anion. Assistance of the departure of this group by a buffer acid can be ruled out on the basis of the absence of buffer catalysis. On the other hand, a mechanism in which the solvent is simultaneously transferring a proton to nitrogen is possible and in fact is reasonably likely considering that the strongly basic nitrogen anion will be protonated by water at the encounter limit. The positive ρ value does, however, imply substantial buildup of negative charge adjacent to the ring, so that in the case where water is assisting the departure the transition state would be described as having $ArN^-(OH)$ character, with proton transfer lagging behind S–N bond breaking.

Rearrangement of Semimercaptal to Sulfinanilide. The proposed mechanism for the rearrangement reaction, shown in Scheme II, involves dissociative cleavage of the hydroxyl group, either in an unassisted manner where hydroxide ion itself is the leaving group (path a) or with catalysis by buffer acids or H^+ in which H_2O is the product (path b). This is the rate-limiting step in the reaction, and the three types of cleavages give rise to the three terms in the rate law of eq 6. The product of this cleavage is a resonance-stabilized cationic intermediate ArN^+SG that is trapped by reaction of a solvent water molecule at the sulfur atom. Subsequent loss of one of the H_2O protons and transfer of the second from the oxygen to nitrogen (probably by way of the solvent) gives rise to the sulfinanilide product. In addition to satisfying the pH and buffer dependence observed in the kinetics, this mechanism is consistent with the ^{18}O-tracer experiment that shows the S=O oxygen to be derived from solvent. The ρ^+ value of −3.5[22] and the improved fit with σ^+ constants provide further justification, indicating that there is substantial buildup of positive charge adjacent to the aromatic ring in the transition state.

The semimercaptal intermediate is an *N*-arylhydroxylamine, and the N–O cleavage step is indentical to the rate-limiting step of the Bamberger[23] and Bamberger-like[24] rearrangements common to this class of compounds. These latter reactions also proceed with an initial N–O cleavage to produce an intermediate arylnitrenium ion. This reacts with water and added nucleophiles at the para and ortho ring carbons, followed by aromatization (in most cases)[25] to give ring-substituted anilines as products.



With the parent *N*-phenylhydroxylamine, the Bamberger reaction proceeds only under acidic conditions,[23] although noncatalyzed versions can occur after esterification of the OH produces a better leaving group.[24] The second-order rate constant for H^+ catalysis of the rearrangement of PhNHOH itself is 10^{-3} M^{-1} s^{-1} at 25 °C.[26] The constant k_2^H(rearr) for the analogous semimercaptal PhN(SG)OH is 8.2×10^2 M^{-1} s^{-1} (Table III), showing a 10^6 rate acceleration associated with the introduction of the sulfur atom. This of course is due to the ability of the sulfur to stabilize the positive charge, as shown by the second resonance contributor of Scheme II. A further indication of the importance of this interaction comes in comparing the ρ^+ values of −6 to −7 typically found for Bamberger-type reactions[23b,24a,c-e,27] with the value of −3.5 for the semimercaptal rearrangement. While the latter does indicate significant delocalization of the developing positive charge into the aromatic ring, this effect has clearly been attenuated by the competing resonance toward sulfur.

The Bamberger rearrangement of PhNHOH is specific-acid-catalyzed,[28] indicating that complete proton transfer to the OH leaving group is required for sufficient activation of the N–O cleavage reaction. General acid-catalyzed N–O cleavage is,

(22) The ρ^+ value of −3.5 calculated from the k_2(rearr) data in Table I principally reflects the k_2^0(rearr) process, i.e., heterolytic cleavage to the cation and hydroxide ion. There is a small contribution from $H_2PO_4^-$ catalysis, but at the buffer concentration employed in these particular experiments this amounts to no more than 15% of the total k_2(rearr).

(23) For recent reference, see: (a) Sone, T.; Tokudo, Y.; Sakai, T.; Shinkai, S.; Manabe, O. *J. Chem. Soc., Perkin Trans. 2* **1981**, 298–302. (b) Sone, T.; Hamamoto, K.; Seiji, Y.; Shinkai, S.; Manabe, O. *Ibid.* **1981**, 1596–1598. (c) Kohnstam, G.; Petch, W. A.; Williams, D. L. H. *Ibid.* **1984**, 423–427.

(24) For recent references, see: (a) Gassman, P. G.; Granrud, J. E. *J. Am. Chem. Soc.* **1984**, *106*, 1498. (b) Scott, C. M.; Underwood, G. R.; Kirsch, R. B. *Tetrahedron Lett.* **1984**, *25*, 499. (c) Novak, M.; Pelecanou, M.; Roy, A. K.; Andronico, A. F.; Plourde, F. M.; Olefirowicz, T. M.; Curtin, T. J. *J. Am. Chem. Soc.* **1984**, *106*, 5623. (d) Novak, M.; Pelecanou, M.; Pollack, L. *Ibid.* **1986**, *108*, 112. (e) Novak, M.; Lagerman, R. K. *J. Org. Chem.* **1988**, *53*, 4762.

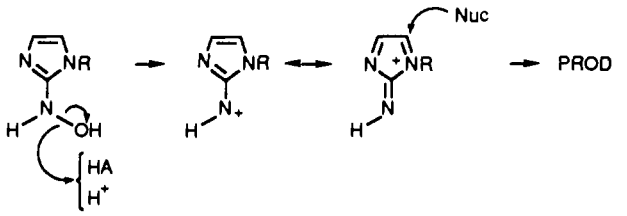
(25) (a) Nonaromatic products have also been observed. (b) Gassman, P. G.; Granrud, J. E. *J. Am. Chem. Soc.* **1984**, *106*, 2448. (c) Novak, M.; Roy, A. K. *J. Org. Chem.* **1985**, *50*, 571. (d) Panda, M.; Novak, M.; Magonski, J. *J. Am. Chem. Soc.* **1989**, *111*, 4524.

(26) From ref 23b. The authors of this work reported rate constants k_N for the conversion of the N-protonated hydroxylamine to the rearrangement product, and this has been converted to the H^+-catalyzed rate constant by dividing k_N by the acidity constant K_a that was also determined.

(27) Gassman, P. G.; Campbell, G. *J. Am. Chem. Soc.* **1971**, *93*, 2567; **1972**, *94*, 3891.

(28) Fishbein, J. C.; McClelland, R. A. Unpublished results.

however, observed in the Bamberger-like reactions of imidazole derivatives.[16]

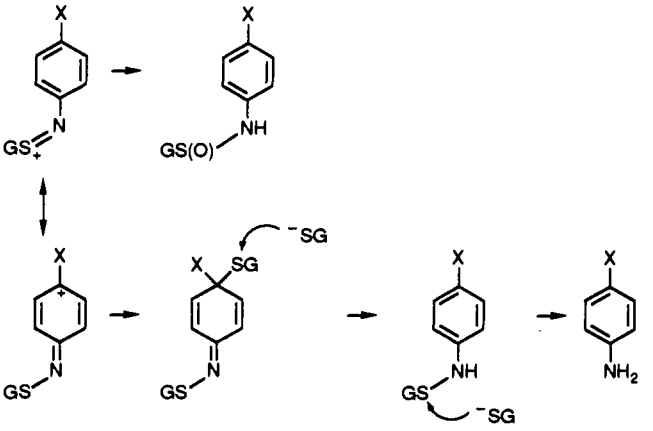


Unlike the PhNHOH, these hydroxylamines also exhibit a noncatalyzed N–O cleavage which becomes the predominant reaction in dilute phosphate buffers at neutral pH. The difference between PhNHOH and the imidazole derivatives can be explained[16] in terms of the relative stabilities of the intermediate nitrenium ions, as an imidazole group is much more stabilizing than phenyl when there is a cationic center adjacent to the ring.[29] The consequence is that full proton transfer is not required in the transition state for N–O cleavage with the imidazole system, and there is even enough "push" from the imidazole ring for this reaction to occur without assistance. The general behavior of the arylhydroxylamines in their N–O cleavage is in fact analogous to that observed in the C–O cleavage of acetals and ortho esters. Here also specific-acid-catalyzed reactions give way to general acid-catalyzed reactions when the intermediate cation, an oxo carbocation, becomes highly stabilized; moreover, when the change to general acid catalysis occurs, a noncatalyzed component to the C–O cleavage is invariably also observed.[30]

The detailed nature of the transition states for the N–O cleavage reactions is considered in the analysis of the imidazole system,[16] with the conclusion that the H^+-catalyzed and buffer acid-catalyzed reactions proceed with proton transfer concerted with bond breaking, while the uncatalyzed reaction proceeds with loss of hydroxide ion and not general acid catalysis by water. As shown in Table III, rate constants for N–O cleavage of 1-methyl-2-(hydroxyamino)imidazole[16] and the semimercaptal PhN(SG)OH are very similar. This is coincidental, the implication being that the combined stabilizing effects of a phenyl ring and a sulfur are very nearly equivalent to that of a single imidazole ring. The close similarity in the rate constants does, however, suggest that the two systems follow identical mechanistic behavior.

The possibility that sulfur-stabilized nitrenium ions are intermediates in this or other types of reactions appears not to have been considered previously. There is, however, evidence for oxygen-stabilized analogues,[31] for example, in the solvolysis of *N*-acetoxy-*N*-alkoxybenzamides. Nitrenium ions RNH^+ substituted with phenyl, OH, and SH have been considered at the theoretical level, with the conclusion that all three substituents have comparable stabilizing effects.[32] This is interesting with respect to the cation derived from PhN(SG)OH, since it implies that the positive charge will be delocalized to a comparable extent in both the aromatic ring and on the sulfur atom.[33] This idea

Scheme III



is consistent with the substituent dependence that is observed experimentally. As discussed previously, this shows that there is substantial charge in the aromatic ring, but the magnitude is reduced compared to nitrenium ions lacking the sulfur atom.

We offer here a simple explanation as to why water addition to PhN^+SG occurs at sulfur, and not at carbons in the phenyl ring as in a Bamberger-like reaction. Arylnitrenium ions such as $ArNH^+$ are significantly longer-lived in water when compared with analogous arylcarbenium ions.[16] The carbenium ions, however, react with water at the external carbon so that aromaticity is not lost, while the nitrenium ions react in the ring forming a nonaromatic intermediate. This suggests that the relative kinetic stability of the nitrenium ions is due to a larger intrinsic barrier associated with this loss of aromaticity. The cation PhN^+SG obviously offers an alternative site at which water can react, the sulfur atom, and this site has the advantage that the addition does not destroy aromaticity.

As shown in Scheme III, nucleophilic addition to the ring could account for the aniline products observed in the reactions of glutathione and 4-substituted nitrosoarenes bearing strong π donors such as 4-NMe_2, 4-MeO, and phenyl (as in a 2-fluorenyl derivative). The negative ρ^+ value for N–O cleavage shows that this reaction will be particularly facile with these substituents present. Moreover, the cation will have considerable positive charge at the para position of the aromatic ring next to the substituent, and this could drive nucleophilic addition to occur at this position. The adduct that forms is incapable of aromatization, and with glutathione as the nucleophile, the sequence shown in Scheme III can be envisaged where the aniline is eventually produced along with 2 equiv of GSSG. Interestingly, the literature reports of the products from 4-nitroso-*N*,*N*-dimethylaniline and 4-nitrosophenetole showed a poor mass balance, and the HPLC analysis revealed the presence of unstable species.[5] These were not characterized, but one possibility is that they are due to nucleophilic addition to the ring of the cation ArN^+SG.[34] The full mercaptal **3** of Scheme I $\{ArN(SG)_2\}$ can obviously also arise from this cation by reaction of glutathione at the nitrogen atom.[35] This possibility cannot be ruled out on the basis of the present evidence. However, this study does suggest that such a compound will be unstable with respect to the reverse of the addition: loss of GS^- to go back to ArN^+SG. This is an analogous process to the one where the semimercaptal loses ^-OH, and GS^- should be a much better leaving group.

Nitrenium ions obtained upon N–O heterolysis of esters of *N*-arylhydroxylamines are commonly proposed as the DNA-binding intermediates responsible for the mutagenicity and car-

(29) (a) Noyce, D. S.; Stowe, G. T. *J. Org. Chem.* **1973**, *38*, 3762. (b) Noyce, D. S.; Sandel, B. B. *Ibid.* **1976**, *41*, 3640. (c) Bolton, J. L.; McClelland, R. A. *THEOCHEM* **1988**, *165*, 379.

(30) (a) Fife, T. H. *Acc. Chem. Res.* **1972**, *5*, 264. (b) Fife, T. H.; Jao, L. K. *J. Am. Chem. Soc.* **1968**, *90*, 4081. (c) Fife, T. H.; Brod, L. H. *Ibid.* **1970**, *92*, 1681.

(31) (a) Glover, S. A.; Goosen, A.; McCleland, C. W.; Schoonrad, J. L. *J. Chem. Soc., Perkin Trans 2* **1984**, 2255; (b) *Tetrahedron* **1987**, *43*, 2577. (c) Gerdes, R. G.; Glover, S. A.; ten Have, J. F.; Rowbottom, C. A. *Tetrahedron Lett.* **1989**, *30*, 2649. (d) Glover, S. A.; Rowbottom, C. A.; Scott, A. P.; Schoonrad, J. L. *Tetrahedron* **1990**, *46*, 7247. (e) Campbell, J. J.; Glover, S. A.; Rowbottom, C. A. *Tetrahedron Lett.* **1990**, *31*, 5377.

(32) Glover, S.; Scott, A. P. *Tetrahedron* **1989**, *45*, 1763.

(33) (a) It can be noted that theoretical calculations,[29c,33b,c] as well as experiment,[23c] suggest that the positive charge in an arylnitrenium ion is highly delocalized to the ring carbons. In other words, the nitrenium ion resonance contributor $ArNH^+$ would appear to play a relatively minor role, and the cations are better described as carbenium ions or, in the case of the imidazole system, as an iminium ion. Obviously with the sulfur also present, the importance of the nitrenium ion form is further diminished. (b) Ohwada, T.; Shudo, K. *J. Am. Chem. Soc.* **1989**, *111*, 34. (c) Li, Y.; Abramovitch, R. A.; Houk, K. N. *J. Org. Chem.* **1989**, *54*, 2911.

(34) (a) Eyer and co-workers have isolated ring addition products in the case of the reactions of 4-nitrosophenetole and 4-ethoxy-4′-nitrosodiphenylamine and thiols[11d] and have isolated 3-(glutathion-*S*-yl)-4-ethoxyaniline from incubates of 4-nitrosophenetole and glutathione.[34b] (b) Eyer, P.; Galleman, D. Personal communication.

(35) Eyer and co-workers have proved that the full mercaptal cannot be an intermediate in the form of a finding that the reaction of a semimercaptal ArNOHSR with a second thiol R′SH yields only ArNHSR.[11c]

cinogenicity of aromatic amines,[36] and there are two reports indicating that a cation ArN^+SG derived from a semimercaptal may play a similar role. The first involved a study with hepatocytes, with the finding that the cytotoxicity of nitrosobenzene was prevented if glutathione was depleted beforehand, while with 4-nitrosoacetophenone cytotoxicity was increased.[6] Thus, glutathione plays an activating role for the former and a protecting role with the latter. It is the former compound that reacts to form PhN^+SG, while this pathway is relatively unimportant with the latter. The second report involved a study of the interaction of a radiolabeled nitrosoimidazole with DNA, with the finding that covalent binding is enhanced 2–3 orders of magnitude in the presence of physiological concentrations of glutathione.[7b] Again we can speculate that an intermediate such as ArN^+SG is the species responsible.

Whatever the role of these cations, this study does demonstrate that electron-deficient intermediates resembling nitrenium ions can arise at the nitroso level of oxidation after activation by glutathione. Moreover, it is not necessary to further activate the leaving group by esterification as is normally necessary to obtain nitrenium ions under physiological conditions at the hydroxylamine level.

Experimental Section

The nitrosoarenes employed in this study were known compounds and were prepared by standard methods.[5,7,37] They were purified shortly before use by sublimation at reduced pressure.

For kinetic studies, a stock solution of a known concentration of glutathione (~0.2 M) was prepared each day and kept under argon. This solution was adjusted to the pH of the experiment by adding 1 M NaOH. A stock solution of nitrosoarene, 0.1–0.5 M in acetonitrile, was also prepared each day. Kinetic experiments were carried out with a Hi-Tech Scientific SF-51 stopped-flow apparatus interfaced with a Hewlett-Packard Model 300 microcomputer. A small quantity of the acetonitrile solution of the nitrosoarene was added to the appropriate phosphate buffer to give a solution of final concentration 20–100 μM ArNO, and this solution was placed in one drive of the apparatus. The second syringe contained a solution of glutathione prepared in the same buffer by dilution of the stock solution. The buffer solution was kept under argon prior to the addition of the glutathione. There was, however, no attempt to maintain the solutions oxygen-free on the stopped-flow apparatus. To check whether oxidation was occurring, control experiments were carried out in which the nitrosoarene solution was replaced in the stopped-flow apparatus by a solution of Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid), in pH 8 phosphate buffer. These experiments were carried out with solutions where the glutathione concentration was less than 500 μM and the Ellman's reagent was in excess. On mixing, the two reagents underwent a thiol exchange, each GSH quantitatively releasing 1 equiv of 4-nitro-2-carboxybenzenethiol anion,[38] whose absorbance was monitored at 412 nm. Analysis of the amount of this absorbance as a function of time demonstrated less than 5% loss of free GSH over the first 20 min after introduction of its solution to the stopped-flow apparatus. Nitrosoarenes exist partly as dimers in the solid state and in concentrated solutions in organic solvents. The dilute aqueous solutions employed in this work, however, obeyed Beer's law up to the maximum concentration employed, implying that they contained >95% monomer.

The fast kinetic component was monitored at the λ_{max} of the nitrosoarene and the slow component at 260 nm. In general these two processes were sufficiently separate in rate that they could be studied independently with each being fit by the computer to the equation for a single exponential. In a few cases where the rates appraoched more closely, the absorbance–time curves were fit to a double exponential.

For the pH-jump experiments, one syringe of the stopped-flow apparatus was filled with the acidifying solution and the other was left empty. A solution of 1 mM GSH in 4 mM pH 7.3 phosphate buffer was placed in a thermostating bath at 25 °C, and after the solution reached bath temperature, the stock solution of nitrosobenzene in acetonitrile was added so as to give a solution with [PhNO] = 200 μM. The resulting solution was rapidly loaded into the empty syringe of the stopped-flow apparatus, and kinetic experiments at 260 nm were initiated within 15 s of the addition of the nitrosobenzene. The pH of the solutions being mixed on the stopped-flow apparatus was measured by collecting the effluent. In the case of the experiments with HCl, the measured pH was used to calculate the second-order rate constant for H^+ catalysis as the slope of a plot of k_{obsd} versus $[H^+]$. For the carboxylate buffers, the acidifying solutions contained at least 0.2 M buffer, and there was little change in the pH for a given buffer dilution. Plots of k_{obsd} versus [RCOOH] were linear, and the rate constant for the buffer acid was calculated as the slope.

The tracer experiment was carried out by preparing a solution of 20 mM glutathione in water (5 mL) or 90% ^{18}O-water (1 mL). With the pH being recorded by a pH meter, 1 M NaOH was carefully added until the pH reached 5. A solution of nitrosobenzene, 1 M in acetonitrile, was then added by syringe over a 5-min period until the final amount of substrate added corresponded to 15 mM. The pH was monitored during this addition, but remained essentially unchanged (±0.2 pH change at most). The solution was then stirred for an additional 30 min at room temperature, and the water was removed by lyophilization, leaving behind a pale yellow solid. The FAB mass spectra discussed in the results were recorded directly on this sample. ^1H NMR (400 MHz) spectra in D_2O were also recorded. This showed the expected signals due to unreacted GSH, plus additional signals that could be assigned to the sulfinanilide adduct: δ 7.32 (2 H, t, J = 5.5 Hz, *m*-Ar*H*), 7.21 (1 H, t, J = 5.5 Hz, *p*-Ar*H*), 7.11 (2 H, d, J = 5.5 Hz, *o*-Ar*H*), 4.48 (1 H, dd, J = 8.5 and 4 Hz, cysteinyl CH), ~3.6 (glycine CH_2 and glutamyl CH, signals overlap with those of GSH), 2.65 (1 H, dd, J = 10.5 and 8.5 Hz, cysteinyl C*H*H*), 2.44 (1 H, dd, J = 10.5 and 4 Hz, cysteinyl CH*H**), ~2.35 and ~2.0 (glutamyl CH_2CH_2, signals overlap with those of GSH).

In one control experiment, a solution of nitrosobenzene alone (5 mM) at pH 5 was prepared in 10 mL of 10% ^{18}O-water. After standing for 1 h, this was extracted with 10 mL of ether, and the ether was carefully[39] removed with a rotary evaporator. The electron impact MS of this material was identical with that of a sample which had not been treated with the labeled water, indicating that there had been no incorporation of oxygen. In the other control experiment, the product that had been obtained from ^{18}O-water was dissolved in normal water at pH 5, left for 30 min, and the water removed by lyophilization. The FAB MS of this sample was recorded and was found to be unchanged.

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Registry No. GSH, 70-18-8; 3-$NO_2C_6H_4NO$, 17122-21-3; 3-ClC_6H_4NO, 932-78-5; 4-ClC_6H_4NO, 932-98-9; 3-$MeOC_6H_4NO$, 26595-63-1; PhNO, 586-96-9; 3-MeC_6H_4NO, 620-26-8; 3,5-$Me_2C_6H_3NO$, 67083-40-3; 4-MeC_6H_4NO, 623-11-0; 3,4-$Me_2C_6H_3NO$, 38899-22-8.

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(39) Nitrosobenzene is sufficiently volatile that it can also be removed in this procedure.