Additional Pathways of S-Conjugate Formation during the Interaction of Thiols with Nitrosoarenes Bearing $\pi$-Donating Substituents

Dieter Gallemann and Peter Eyer

Walther-Straub-Institut für Pharmakologie und Toxikologie der Ludwig-Maximilians-Universität München, München, Germany

During the well-established reaction pathways of nitrosoarenes interacting with thiols, a reactive $N$-(thiol-S-yl)-arylamine cation was implicated in the so-called rearrangement reaction which transforms the semicaprate of the sulfanamide. In the case of nitrosoarenes with $\pi$-donating substituents, this catonic transition state includes resonance structures bearing the positive charge in 2 and 4 position, thereby facilitating the attack of nucleophiles to the aromatic ring (7). During the formation of 4-nitrosoarenes (NOPT) with reduced glutathione (GSH) in chemical systems and human red cells, some glutathione S-conjugates were detected other than the already known sulfanamide and sulfonamides. Three of them were separated by HPLC and identified by mass spectrometry, $^1$H-NMR, and UV-visible spectroscopy, by determination of $pK_a$ values and chemical behavior. The hitherto unknown conjugates are 4-ethoxy-2-(glutathione-S-yl)-aniline, N-(4-ethoxyphenyl)-N'-(glutathione-S-yl)-benzoquinonedimine, and 4-amino-4'-ethoxy-2-(glutathione-S-yl)diphenylamine. In preliminary experiments, some of these conjugates were shown to be highly active in producing fhenhemoglobin. Considerations on the formation pathways of these metabolites lend further support to the electrophiilic N-glutathione-S-vanillylamine cation as a reactive intermediate that may be implicated in nitrosoarene toxicity. — Environ Health Perspect 102(Suppl 6):137–142 (1994)

Key words: nitrosoarenes, thiols, 4-nitrosoarenes, glutathione S-conjugates, N-sulfonylquinoxinediimine, reaction mechanism, $N$-(thiol-S-yl)-arylamine cation

Introduction

Reactions of nitrosoarenes with cellular thiols are gaining increasing toxicologic interest (1). During the well-established reaction pathways leading to $N$-hydroxynarylamines, sulfanamides, and sulfenamides (2–4), reactive intermediates are formed, such as phenylisothiocyanate free radicals (5) and the metastable semicaprates (6–8). Recently, an intermediate $N$-(thioyl-S)-arylamine cation was implicated during the so-called rearrangement reaction which transforms the semicaprate to the sulfanamide (7,9). In the case of nitrosoarenes with $\pi$-donating substituents, this catonic transition state includes resonance structures bearing the positive charge in 2 and 4 position, thereby facilitating the attack of nucleophiles to the aromatic ring (7). During the reaction products of 4-nitrosoarenes and reduced glutathione in chemical systems and human red cells, some glutathione S-conjugates were detected other than the already known sulfanamide and sulfonamides. Three of them were separated by HPLC and identified by mass spectrometry, $^1$H-NMR, and UV-visible spectroscopy, by determination of $pK_a$ values and chemical behavior. The hitherto unknown conjugates are 4-ethoxy-2-(glutathione-S-yl)-aniline, N-(4-ethoxyphenyl)-N'-(glutathione-S-yl)-benzoquinonedimine, and 4-amino-4'-ethoxy-2-(glutathione-S-yl)diphenylamine. In preliminary experiments, some of these conjugates were shown to be highly active in producing fhenhemoglobin. Considerations on the formation pathways of these metabolites lend further support to the electrophiilic N-glutathione-S-vanillylamine cation as a reactive intermediate that may be implicated in nitrosoarene toxicity.

Materials and Methods

NOPT (10) (containing 0.7% 4-nitrosoarenes), [u-ring-$^{13}$C]-NOPT (11) (18.7 Kbp/μmole; containing 2% 4-nitrophenol, 2% azoxyphenol, and 0.4% 4-ethoxy-4'-nitrosodiphenylamine), N-(glutathione-S-yl)-4-phenetidine (4) (sulfenamide), and 4-ethoxy-4'-nitrosodiphenylamine (12) were prepared as described previously. 4-Amino-4'-ethoxydiphenylamine (11) (NH$_2$EDPA) was obtained by reduction of 4-ethoxy-4'-nitrosodiphenylamine in CH$_3$CN with 5-fold excess ascorbate under sonication for 2 min. All other reagents were from commercial sources at the purest grade available.

Syntheses:

4-Ethoxy-2-(glutathione-S-yl)-aniline. GSH (0.25 mmole; 100 mM dissolved in phosphate buffer, pH 7.0) was mixed with NOPT (0.025 mmole; 1 M in MeOH) and allowed to react for about 5 min at 20°C (raw yield: 11% of theory, as detected with [u-ring-$^{13}$C]-NOPT). Lipophilic metabolites were removed by ether extraction (5 × vol), hydrophilic ones by semipreparative HPLC. The colorless compound was stable as solid and in aqueous solutions for months.

4-Amino-4'-ethoxy-2-(glutathione-S-yl)-diphenylamine. NH$_2$EDPA (24 μmole; 9 mM in MeOH/ phosphate buffer, pH 7.4 [vol/vol]) was oxidized with PbO$_2$ (0.5 mmole) during 1 min sonication. LC-MS analysis of the supernatant showed a molecular ion being 2 mu smaller than NH$_3$EDPA, indicating the formation of N-(4-ethoxyphenyl)-4-benzoquinone diimine. The orange solution discolored within 1 min upon addition of GSH (125 μmole; 0.5 M in phosphate buffer, 0.2 μM, pH 7.4). The product was purified by semipreparative HPLC (yield: 12.2 μmole, 52% of theory).

N-(4-Ethoxyphenyl)-N'-(glutathione-S-yl)-4-benzoquinone diimine. 4-Ethoxy-4'-nitrosodiphenylamine (0.1 mmole in 50 ml MeOH and 40 μM ammonium carbonate buffer, 0.1 M, pH 10) was mixed quickly with GSH (0.1 mmole in 10 ml ammonium carbonate buffer) and allowed to react for about 5 min at 20°C. Excess educt and the reduction product NH$_2$EDPA were removed by ether extraction (4 × 1 vol). The red-brown product was chromatographically pure (yield: 77 μmole, 77% of theory).

The corresponding β-mercaptoethanol and α-butyramcaptop derivatives were syn-
thesized similarly at pH 11. t-Butylmercapto-
capran reacted only during refluxing at 100°C for 17 hr. The products were purified by thin-layer chromatography on silica gel
plates 60-F254, 0.25 mm thickness from E. Merck (Darmstadt, Germany), using chlo-
roform/hexane mixtures as mobile phase.

\[ ^1H \text{-NMR spectra were recorded with an AM-400 MHz or an AM-500 MHz instrument from Bruker (Rheinstetten-} 
\text{Forschheim, Germany) with } \text{H}_2\text{O set to 4.80 ppm. Electron impact mass spectra (EI-MS) and fast atom bombardment-} 
\text{mass spectra (FAB-MS) were recorded with a CH-7A instrument from Varian or a MAT 312 instrument, respectively, each} 
\text{coupled with a data system SS 200 MS from Finnigan MAT (Bremen, Germany). Liquid chromatography mass spectra (LC-} 
\text{MS) were prepared with a thermospray LC-MS HP 5988a coupled with an HP 1050 HPLC system from Hewlett Packard} 
\text{(Bad Homburg, Germany) on Lichrosorb 100 RP 18 (125 mm } \times \text{ 4.6 mm i.d.), Merck} \text{ and isocratic elution with 60% MeOH/} 
\text{40% ammonium acetate buffer (0.08 M, pH 7.4). HPLC was performed with a gradient system consisting of a low pressure} 
\text{gradient former L 6200 from Merck-} 
\text{Hitachi (Darmstadt, Germany), a UV-visible diode-array detector SPDM6A from Shimadzu (Egliing, Germany), coupled with a personal computer 8810 M 45 from Nixdorf (München, Germany) and a NEC P7-} 
\text{plus pinwriter (München, Germany). Analytical separations were carried out on Novapak C_{18} (15 cm } \times \text{ 4 mm i.d.; Waters,} 
\text{Milford, MA) with MeOH/sodium phosphate buffer (10 mM, pH 7) gradients and detection, if not otherwise stated, at two} 
\text{wavelength windows (230-250 nm and 300-400 nm); for semipreparative separations, } \mu \text{-Bondapak C_{18} columns (30 cm } \times 
\text{ 8 mm i.d., Waters) and MeOH/ammonium hydrogen carbonate buffer (10 mM, pH 7) gradients were used. Peaks were identified by} 
\text{comparing the UV-visible spectra (220-600 nm) and retention times with that of authentic compounds. } pK_a \text{ values were} 
\text{determined by spectroscopic titration. Primary aromatic amino groups were detected by their reaction with Echtblaulsalz} 
\text{B (13). Radioactivity was measured in Bray’s solution (14) with a LKB Rackbeta 1217 liquid scintillation} 
\text{counter. Hum} \text{an human hemoglobin was obtained from outdated} 
\text{blood and purified by column chromatography as described (15). The ferr} 
\text{rihemoglobin content was less than 5% throughout. Free SH groups (β93 Cys} 
\text{were alkylated with N-ethylmaleimide (16) and excess reagent removed by dialysis against phosphate buffer. Experiments on} 
\text{ferrihemoglobin formation by NOPt metabolites were carried out in sodium phosphate buffer (0.2 M, pH 7.4) at 37°C under free} 
\text{access of air. The volumes of added metabolite solutions were less than 3% throughout. Hemoglobin, ferrithemoglobin,} 
\text{and glutathione were determined as described previously (16).} 

\text{Results and Discussion} 

\text{During the HPLC separation of NOPt/} 
\text{GSH incubates, some hydrophilic metab} 
\text{olites were detected other than the already} 
\text{known sulfenamide and sulfinate (Figure 1).} 

\text{4-Ethoxy-2-(glutathione-S-yl)-aniline} 

\text{This compound was obtained in optimized yield from incubates of NOPt with 10-fold excess GSH (pH 7.0, 20°C). Positive} 
\text{reactions with Echtblaulsalz B and with ninhydrin} 
\text{indicated a primary aromatic amine containing a glutathione residue. Its FAB}^+ \text{ mass spectrum showed the molecular ion at } 
m/z = 443 (M+1) \text{ and main fragments at } 
m/z = 368 (M-Gly), 314 (M+2-Glu), \text{and 168 (M-Glu-Ala-Gly), indicating a gluta} 
\text{thione substituted phenetidine. The} 
\text{UV-visible spectrum (Table 1) differed distinctly from that of the} 
\text{sulfenamide N-(glutathione-S-yl)-4-ethoxyaniline (6,9} 
\text{but agreed with that of 2-(thiol-S-yl)-4-hydroxyaniline (17). The } \text{1H-NMR spec} 
\text{trum showed aliphatic signals belonging to the} 
\text{ethoxy group and the glutathionyl} 
\text{substituent and aromatic signals of three pro} 
\text{tons indicating a 1,2,4-substituted aromatic} 

\text{Table 1. } pK_a \text{ values and UV-visible data of some 4-nitrosothiophenol metabolites.} 

\begin{tabular}{|l|l|l|l|}
\hline 
Metabolite & \( pK_a \) & Solvent & \( E_{	ext{max}} \) & \( \varepsilon \) \\
& & & nm & mM \text{ cm}^{-1} \\
\hline 
GS-NH_2Pt & 3.7 & MeOH/Na-P_2O_7 (10 mM, pH 6.5), 20/80 (V/V) & 236 & 10.4 \\
& & & 314 & 3.8 \\
NH_3Pt & 5.3 & MeOH/Na-P_2O_7 (0.2 M, pH 7.4), 20/80 (V/V) & 232 & 9.9 \\
& & & 235 & 2.1 \\
GS-NH_2EDPA & 5.2/d0 & MeOH/Na-P_2O_7 (10 mM, pH 7.0), 25/65 (V/V) & 246 & 13.1 \\
& & & 289 & 12.3 \\
NH_3EDPA & 5.4/0.3 & MeOH/Na-P_2O_7 (10 mM, pH 7.4), [V/V] & 284 & 14.2 \\
& & & 287 & 17.3 \\
GS-EPQDI & 4.9 & MeOH & 412 & 21 \\
& & & Na-P_2O_7 (5 mM, pH 7.11) & 418 & 19.2 \\
& & & HCl (pH 1.8) & 432 & 28 \\
\hline 
\end{tabular} 

Abbreviations: GS-NH_2Pt, 4-ethoxy-2-(glutathione-S-yl)-aniline; NH_3Pt, 4-phenetidinol; GS-NH_2EDPA, 4-amino-4'-
ethoxy-2-(glutathione-S-yl)-diphenylamine; NH_3EDPA, 4-amino-4'-ethoxydiphenylamine; GS-EPQDI, N-(4-
ethoxyphenyl)-N'-(glutathione-S-yl)-4-benzoquinone diimine. 

\text{Figure 1. HPLC separation of hydrophilic metabolites of 4-nitrosothiophenol after reaction with GSH.} [\text{a}-ring-
\text{14C}]\text{-NOPT (5 mM)} \text{ added to GSH (in sodium phosphate buffer, 0.2 M, pH 7.4) and incubated for 0.5 hr at} 
37°C. Lipophilic products were removed by ether extraction. Samples were separated by HPLC on Novapak C_{18} 
with MeOH/sodium phosphate buffer (pH 7) gradient, detection at 220 to 320 nm (for structure see Figure 2). (A) 25 mM GSH; 
the hydrophilic metabolites amounted to 15% of total activity. (B) 1 mM GSH; the hydrophilic metabolites amounted to 5% of total 
activity. See Table 1 for abbreviations. 

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ring. From the signal shifts in acidic solution it was derived that the ortho position to the amino group is substituted by glutathione (Table 2). This structure was confirmed by the distinctly lower $pK_a$ of GS-NH$_2$Pt compared to the parent amine NH$_2$Pt (Table 1). It has been shown previously that the $pK_a$ of primary aromatic amino groups is considerably decreased by ortho-substitution with thiols but hardly by meta-substitution (17).

### Table 2. $^1$H-NMR data of 4-ethoxy-2-(glutathione-S-yl)-aniline (GS-NH$_2$Pt) and 4-phenetidine (NH$_2$Pt) in neutral (D$_2$O) and acidic solution (DCI, pD=1).

| CH$_3$- | GS-NH$_2$Pt | GS-NH$_2$Pt | NH$_2$Pt | NH$_2$Pt |
|--------|-------------|-------------|----------|----------|
| d$_1$J | 1.47 $^{<2a}$ | 1.46 $^{<2a}$ | 1.43 $^{<2a}$ | 1.43 $^{<2a}$ |
| d$_1$J | (6.8) | (6.6) | (7.0) | (7.0) |
| $-CH_2$O- | 4.21 $^{<2a}$ | 4.22 $^{<2a}$ | 4.14 $^{<2a}$ | 4.17 $^{<2a}$ |
| d$_1$J | (6.8) | (6.6) | (7.0) | (7.0) |
| H$_2$ | 7.21 $^{<1d}$ | 7.49 $^{<1d}$ | 6.91 $^{<2d}$ | 7.15 $^{<2d}$ |
| d$_1$J | (9.0) | (8.0) | (9.0) | (7.8) |
| H$_4$ | 7.07 $^{<2d}$ | 7.14 $^{<1d}$ | 6.98 $^{<2d}$ | 7.06 $^{<2d}$ |
| d$_1$J | (9.0) | (8.0) | (9.0) | (7.8) |
| H$_3$ | 7.28 $^{<1d}$ | 7.36 $^{<1d}$ | (NR) | (NR) |
| d$_1$J | (2.6) | (NR) | (NR) | (NR) |
| Cys B$_1$ | 3.37 $^{<1c}$ | 50 | 50 | 50 |
| Cys B$_2$ | 3.44 $^{<1c}$ | 50 | 50 | 50 |
| Cys $\alpha$ | 4.62 $^{<1c}$ | 4.69 $^{<1c}$ | 4.69 $^{<1c}$ | 4.69 $^{<1c}$ |
| Gly | 3.76 $^{<2c}$ | 3.90 $^{<2c}$ | (NR) | (NR) |
| Glu $\gamma$ | 2.55 $^{<2c}$ | 2.56 $^{<2c}$ | (NR) | (NR) |
| Glu $\beta$ | 2.19 $^{<2c}$ | 2.24 $^{<2c}$ | (NR) | (NR) |
| Glu $\alpha$ | 3.94 $^{<2c}$ | 4.10 $^{<2c}$ | (NR) | (NR) |

Abbreviations: NR, not resolved; SO, signal overlapped with MeOH; d, double; dd, double double; t, triple, q, quartet.

### Table 3. $^1$H-NMR data of GS-NH$_2$EDPA and NH$_2$EDPA in neutral (D$_2$O) and acidic solution (DCI, pD=1).

| CH$_3$- | GS-NH$_2$EDPA | GS-NH$_2$EDPA | NH$_2$EDPA |
|--------|---------------|---------------|------------|
| d$_1$J | 1.46 $^{<2a}$ | 1.46 $^{<2a}$ | 1.45 $^{<2a}$ |
| d$_1$J | (6.6) | (6.9) | (6.9) |
| $-CH_2$O- | 4.21 $^{<2a}$ | 4.21 $^{<2a}$ | 4.20 $^{<2a}$ |
| d$_1$J | (6.6) | (6.9) | (6.9) |
| H$_2$ | 7.22 $^{<1d}$ | 7.25 $^{<1d}$ | 7.26 $^{<1d}$ |
| d$_1$J | (NR) | (8.5) | (8.1) |
| H$_4$ | 7.09 $^{<2d}$ | 7.10 $^{<2d}$ | 7.09 $^{<2d}$ |
| d$_1$J | (8.0) | (8.5) | (8.1) |
| H$_3$ | 7.12 $^{<1d}$ | 7.15 $^{<1d}$ | 7.18 $^{<1d}$ |
| d$_1$J | (NR) | (8.8) | (8.0) |
| H$_5$ | 7.21 $^{<1d}$ | 7.28 $^{<1d}$ | 7.35 $^{<1d}$ |
| d$_1$J | (NR) | (NR) | (NR) |
| H$_6$ | 7.53 $^{<1c}$ | 7.63 $^{<1c}$ | (NR) | (NR) |
| Cys B$_1$ | 3.28 $^{<1c}$ | 3.25 $^{<1c}$ | 3.25 $^{<1c}$ |
| Cys B$_2$ | 3.47 $^{<1c}$ | 3.47 $^{<1c}$ | 3.47 $^{<1c}$ |
| Cys $\alpha$ | 4.50 $^{<1c}$ | 4.45 $^{<1c}$ | 4.45 $^{<1c}$ |
| Gly | 3.71 $^{<1c}$ | 3.96 $^{<1c}$ | 3.96 $^{<1c}$ |
| Glu $\gamma$ | 2.51 $^{<2c}$ | 2.52 $^{<2c}$ | (NR) | (NR) |
| Glu $\beta$ | 2.11 $^{<2c}$ | 2.13 $^{<2c}$ | (NR) | (NR) |
| Glu $\alpha$ | 3.76 $^{<1c}$ | 4.01 $^{<1c}$ | (NR) | (NR) |

4-Amino-4'-ethoxy-2-(glutathione-S-yl)-diphenylamine

This metabolite from NOPt/GSH incubates was obtained in much higher yields when NH$_2$EDPA was oxidized to the corresponding benzoquinone diimine and added to GSH. Positive reactions with Echtlausitz B and with ninhydrin pointed to a primary arylamine substituted with glutathione. The FAB$^+$ mass spectrum revealed the molecular ion at m/z = 534 (M+1) and main fragments at m/z = 260 (M+1-Glu-Ala-Gly) and 228 (M+1-GS), indicating a glutathione conjugate of NH$_2$EDPA. The $^1$H-NMR spectrum showed aliphatic signals belonging to the ethoxy and the glutathionyl group; besides the two proton pairs of the ethoxyring, the aromatic signals indicated three single protons, of which two were shifted to lower field during acidification (Table 3).

Therefore, the position of the glutathione substituent was assumed to be meta referred to the primary amino group. Accordingly, the $pK_a$ of this amino group was only slightly decreased upon glutathione substitution (Table 1). [A similar compound has been proposed to occur during the peroxidase-catalyzed oxidation of NH$_2$Pt in the presence of GSH (19)].

### N-(4-Ethoxyphenyl)-N'-(glutathione-S-yl)-4-benzoquinone diimine

The orange compound was immediately formed at maximal yields in incubates of NOPt with about 2.5-fold excess GSH (pH 7.4, 20°C); further GSH addition resulted in discoloring. Interestingly, this NOPt metabolite was obtained in nearly quantitative yield during the reaction of 4-ethoxy-4'-nitrosodiphenylamine with equivalent amounts of GSH at pH 10. Identification of the glutathione derivative by FAB$^+$ and El mass spectra was unsuccessful as the compound discolored instantly, probably by reduction. However, the β-mercaptoethanol and t-butylmercaptan derivatives revealed reasonable mass spectra (in a hardly reducing 3-nitrobenzylalcohol matrix), indicating a benzoquinone diimine substituted with one ethoxyphenyl and one thiol group (Table 4).

$^1$H-NMR spectra of the three derivatives showed the aliphatic signals of the ethoxy group and the respective thiol. The aromatic protons (relative intensity: 7–8 protons), however, revealed a highly complex splitting pattern resulting from the signal overlap of the ethoxyphenyl ring with two cis-trans isomeric forms of the quinone diimine ring (J Sonnenbichler, personal communication, 1992).

In order to clarify the position of the glutathionyl substitution in this metabolite, some chemical experiments were undertaken. Reduction of N-(4-ethoxyphenyl)-N'-(glutathione-S-yl)-4-benzoquinone diimine (GS-EPQD1) with GSH delivered 4-amino-4'-ethoxy-2-(glutathione-S-yl)-diphenylamine (GS-NH$_2$EDPA) as one of the products, indicating that GS-EPQD1 may be the corresponding benzoquinone diimine of GS-NH$_2$EDPA. However,
Table 4. Fast atom bombardment and electron impact mass spectra of the glutathione-, β-mercaptopethanol-, and t-butylmercaptan-derivatives of N(4-ethoxyphenyl)-N’-(thiol-S-yl)-4-benzoquinonediimine.

| Fragment | R=−Glu-Ala-Gly | R=−CH₃CH₂OH | R=−(CH₃)₂CH | R=−CH₂CH₂OH | R=−CH₂CH₂OH |
|----------|---------------|-------------|-------------|-------------|-------------|
| [M]⁺     | 788 (0%)      | 302 (15%)   | 303 (29%)   | 304 (11%)   | 533 (15%)   |
| [M+1]⁺   | 789 (0%)      | 314 (10%)   | 315 (35%)   | 316 (2%)    | 305 (6%)    |
| [M+2]⁺   | 790 (0%)      | 317 (6%)    | 318 (12%)   | 317 (6%)    | 306 (11%)   |
| [M+3]⁺   | 791 (0%)      | 318 (6%)    | 319 (12%)   | 318 (6%)    | 307 (11%)   |
| [M−R]⁻   | 787 (0%)      | 257 (5%)    | 258 (10%)   | 259 (10%)   | 227 (22%)   |
| [M−R−C₂H₅]⁻ | 785 (0%) | 258 (6%)    | 260 (14%)   | 261 (14%)   | 228 (11%)   |
| [M−R−C₆H₅]⁻ | 783 (0%) | 259 (6%)    | 262 (14%)   | 263 (14%)   | 229 (85%)   |
| [M−R−C₆H₅]⁺ | 782 (0%) | 257 (6%)    | 260 (14%)   | 261 (14%)   | 228 (85%)   |
| [M−OH]⁻  | 786 (0%)      | 257 (5%)    | 258 (10%)   | 259 (10%)   | 227 (22%)   |
| [M−OH−C₂H₅]⁻ | 784 (0%) | 258 (6%)    | 260 (14%)   | 261 (14%)   | 228 (11%)   |
| [M−OH−C₆H₅]⁻ | 782 (0%) | 259 (6%)    | 262 (14%)   | 263 (14%)   | 229 (85%)   |
| [M−OH−C₆H₅]⁺ | 781 (0%) | 257 (5%)    | 258 (10%)   | 259 (10%)   | 228 (85%)   |

Abbreviations: FAB⁺-MS, fast atom bombardment mass spectroscopy; El-MS, electron impact mass spectroscopy.

when GS-NH₂EDPA was oxidized with lead dioxide with concomitant color change to orange, no compound with the UV-visible spectrum of GS-EPQDI was observed; reduction with dithionite restored part of GS-NH₂EDPA, indicating that the ring-substituted benzoquinone diimine had actually been formed. Therefore, GS-EPQDI was supposed to contain glutathione not at a ring position but at the second imino nitrogen. This presumption was confirmed as mild reduction of GS-EPQDI with ascorbate liberated glutathione, which was proved enzymically. In the reaction of GS-EPQDI with β-mercaptopethanol, no GS-NH₂EDPA was found, only the β-mercaptopethanol derivative. These findings are in agreement with the proposed structure.

Considerations on the Mechanisms of S-Conjugate Formation

The reaction pathways of nitrosoarenes with aliphatic thiols are known to be distinctly influenced by their aryl substituents.
Recent results indicate that the initially formed semimercaptal undergoes N-O cleavage to give an intermediate sulfenamide cation (7,9). The NOPt metabolites presented here further support that the sulfenamide cation may play a central role in NOPt metabolism (compare Figure 2);

a) The formation of N-hydroxy-4-phenetidine from the semimercaptal was not observed hitherto (20,21). As generally deduced by Kazanian and McClelland (7), the π-donating ethoxy substituent should favor the N-O cleavage with formation of a sulfenamide cation rather than the nucleophilic substitution of GS− at the semimercaptal sulfur with formation of the corresponding hydroxylamine. Accordingly, the semimercaptal that has been detected with 1-thioglycerol (21) is rapidly converted to the resonance-stabilized sulfenamide cation.

b) The formation of appreciable amounts of sulfenamide was only observed when GSH reacted with excess NOPt (9) (Figure 1B). At excess GSH, however, this pathway was not followed (Figure 1A), suggesting that the positive charge is highly delocalized to the ring carbon atoms because of the strong π-donating ethoxy substituent.

c) The formation of GS-NH₂Pt at excess GSH (Figure 1) again indicated the intermediate occurrence of a resonance-stabilized sulfenamide cation, which is prone to nucleophilic ring addition of GSH. The resulting 2,N-bis-(glutathione-S-yl)-4-phenetidine may be rapidly reduced by GSH or hydrolyzed—as observed with other sulfenamides—to give GS-NH₂Pt.

d) Reaction of NOPt with excess GSH mainly led to the sulfenamide which decomposed to NH₂Pt (9). According to the reaction sequences proposed by Kazanian and McClelland (7), addition of GS− to the para position of the sulfenamide cation and subsequent reduction by further GSH or hydrolysis would yield the sulfenamide. Because the para position is thought to have the major partial charge in the sulfenamide cation, the high yields of NH₂Pt would be reasonably explained.

e) The formation of bicyclic metabolites supported the consideration that the para position of the sulfenamide cation may have the lowest electron density. Obviously not only GS− but also the less-nucleophilic NOPt metabolite NH₂Pt undergoes ring addition to this position, delivering the N-sulfenylquinonediimine GS-EPQDI by elimination of EtOH. [This reaction mechanism would be consistent with the acid-catalyzed amination of p-nitrosophenol ethers (12), where the initial attack of a proton at the nitroso oxygen produces a positive charge at the para position of the ring.] Accordingly, the reaction of NOPt with GSH in the presence of authentic NH₂Pt (1:1:1) resulted in immediate production of GS-EPQDI at increased yields. This reaction was shown to occur also with other arylamines but not with alkylamines.

f) Reduction of authentic GS-EPQDI with ascorbate resulted in discoloring and production of a metastable compound—probably the 2e− reduction product N-(4-ethoxyphenyl)−N′-(glutathione-S-yl)-phenylenediamine. This compound decomposed slowly (faster after acidification) to give the already known NOPt metabolite NH₂EDPA (II).

g) In incubates of NOPt with > 2.5 excess GSH, the orange color of GS-EPQDI disappeared after a few minutes. When authentic GS-EPQDI reacted with GSH, the ring-substituted GS-NH₂EDPA was formed besides the reduction product NH₂EDPA. The former pathway would be consistent with a reductive 1,4-Michael addition of GSH to the quinone diimine and subsequent hydrolysis or reduction of the ring substituted bicyclic sulfenamide.

**Ferrihemoglobin-forming Activity of the NOPt Metabolites**

The Kiese cycle was found to play only a minor role during ferrihemoglobin formation by NOPt (10,22). Because in preliminary experiments the identified metabolites also were detected in NOPt-exposed human red cells, we investigated their activity in producing ferrihemoglobin. As shown in Table 5, the mononuclear metabolites hardly formed ferrihemoglobin in solutions of purified human hemoglobin. The bicyclic metabolites GS-EPQDI, NH₂EDPA, and GS-NH₂EDPA, however, were highly active, producing many equivalents of ferrihemoglobin by hitherto unknown mechanisms.

**Conclusion**

The formation of ring-substituted glutathione S-conjugates during the reactions of NOPt with glutathione lend further support to the sulfenamide cation as reactive intermediate. This cation obviously reacted not only with GSH leading to mononuclear metabolites but also with sufficient nucleophilic arylamines producing various bicyclic metabolites. The latter were shown to be highly active in producing ferrihemoglobin, once more indicating that metabolic reactions with GSH should not be considered obligatory detoxication reactions. Despite its toxic action in erythrocytes, the stabilized electrophilic sulfenamide cation may play an important role in the *in vivo* toxicity of π-donor substituted nitrosamines.

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**Table 5. Ferrihemoglobin formation by some 4-nitrosophenol metabolites.** Purified human oxyhemoglobin (3 mM, SH groups blocked) reacted with the respective 4-nitrosophenol metabolite (0.1–0.3 mM) at pH 7.4 and 37°C under free access of air (means, n = 2–6).

| NOPt metabolite | k₂, app [M⁻¹ sec⁻¹] |
|-----------------|-----------------------|
| NH₂Pt⁺          | 0.004 (23)            |
| sulfenamide⁻    | 0.04                  |
| GS-NH₂Pt⁺       | 0.1                   |
| GS-NH₂EDPA⁻     | 1.1                   |
| GS-NH₂EDP⁻      | ≥3.8                  |
| NH₂EDPA⁻        | 6.4                   |

*paneled in CH₃CN, & aqueous solution. The reaction kinetics was pseudo first-order after the second minute, while the reaction proceeded much faster initially. Dissolved in MeOH.

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