Reactions of Oxidatively Activated Arylamines with Thiols: Reaction Mechanisms and Biogetic Implications. An Overview

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Aromatic amines belong to a group of compounds that exert their toxic effects usually after oxidative biotransformation, primarily in the liver. In addition, aromatic amines also undergo extrahepatic activation to yield free arylaminyl radicals. The reactive intermediates are potential promutagens and procarcinogens, and responsible for target tissue toxicity. Since thiols react with these intermediates at high rates, it is of interest to know the underlying reaction mechanisms and the toxicologic implications. Phenoxyl radicals from aminophenols and aminyl radicals from phenylenediamines quickly disproportionate to quinone imines and quinone dimers. Depending on the structure, Michael addition or reduction reactions with thiols may prevail. Products of sequential oxidation/addition reactions (e.g., S-conjugates of aminophenols) are occasionally more toxic than the parent compounds because of their higher autooxidizability and their accumulation in the kidney. Even after covalent binding of quinone imines to protein SH-groups, the resulting thioethers are able to autoxidize. The quinoid thioethers can then cross-link the protein by addition to neighboring nucleophiles. The reactions of nitrosoarenes with thiols yield a so-called "semimercaptal" from which various branching reactions detach, depending on substrants. Compounds with strong S-donors, like 4-nitrophenothiazin, give a resonance-stabilized N-Thiol-Syl-arylamine cation that may lead to bicyclic products, thioethers, and DNA adducts. Examples of toxicologic implications of the interactions of nitroso compounds with thiols are given for nitrosoimidazoles, heterocyclic nitroso compounds from protein pyrolysates, and nitrosoarenes. These data indicate that interactions of activated arylamines with thiols may not be regarded exclusively as detoxication reactions. — Environ Health Perspect 102(Suppl 6):123-132 (1994)

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Introduction

Aromatic amines belong to a group of chemicals that exert their toxic effects usually after oxidative biotransformation, primarily in the liver. As a result, C-oxygenated and N-oxygenated products are generated, which can be further activated to give electrophilic intermediates responsible for toxic, allergic, mutagenic, and carcinogenic effects.

The group of aminophenols can be activated in metabolically competent cells to give ultimate reactive intermediates like phenoxyl radicals and quinone imines. The phenyldihydroxylamines are activated by O-esterification (glucuronidation, sulfatation, acetylation) to yield carbonium-nitrenium ions as ultimate reactive compounds that alkylate nucleic acids under preferable formation of N(deoxyguanosine-8-yl)aryl-amine adducts (1). In addition, oxidative activation of the phenylhydroxylamines produces phenyldihydronitroxides which react with cellular sulfhydryls to give thiyl radicals of glutathione (GSH) and protein sulfhydryl groups (2). The nitrosoarenes, formed by either 2-electron oxidation of the phenylhydroxylamines, by 1-electron oxidation of the phenyldihydronitroxides, by disproportionation of the latter, or by reduction of nitro aromatic compounds, exert their toxic effects mainly through their thiol reactivity (3).

Last, aromatic amines also undergo extrahepatic activation to yield free arylaminyl radicals (4,5). This kind of activation received more attention when it was shown that prostaglandin hydroperoxidase mediated the arachidonic acid-dependent cooxidation of arylamines to DNA-bound products (6).

Since thiols usually react at high rates with all the aforementioned electrophilic intermediates, it is of interest to know the underlying reaction mechanisms and the toxicologic implications. This article is intended to focus not only on detoxication reactions, as usually supposed to emergency reactions, particularly of GSH. Evidence is growing that thiol interaction with electrophilic intermediates may produce sufficiently stable transport forms that can cause toxic effects in remote, sensitive target organs.

Mechanisms of Thiol Reactions with Radicals and Quinoid Compounds

Aminyl Radicals

During our investigations on ferrithemoglobin formation by p-phenylenediamines in human red cells, we detected formation of glutathione adducts. The same adducts were formed when the chemically prepared radical cations were allowed to react with GSH. In addition, some GSSG was formed. Since N-centered free radicals of aromatic amines tend to disproportionate at high rates (7), it was difficult to decide whether redox and addition reactions with GSH had originated directly from the radicals or from the quinone dimers.

To reveal unambiguously the underlying microscopic events, we examined reactions of the stable N-alkyl-p-phenylenediamine radical cations (Wurster’s dyes) with GSH. Of these the N,N-dimethyl-p-phenylenediamine radical cation (DMDP") turned out to be particularly suited for our investigations, because its
Table 1. Apparent second-order rate constants of the reaction of DMPO" with GSH at excess DMPO, pH 7.4, 37°C (6).

|          | Without | 10 × DMPO | 100 × DMPO |
|----------|---------|-----------|------------|
| DMPO"    | 9.4 × 10^4 | 3 × 10^4   | 5 × 10^3   |
| DMQDI+   | 25%     | 5%        | 1%         |
| k (M^-1s^-1)| 3.7 × 10^3 | 6 × 10^5   | 5 × 10^5   |

Abbreviations: DMPO, N,N-dimethyl-p-phenylene-diamine; DMPO\(^{\bullet}\), N,N-dimethyl-p-phenylene-diamine radical cation; DMQDI\(^{\bullet}\), N,N-dimethyl-p-quinone diimine cation; GHS, glutathione.

disproportionation equilibrium is near unity at physiologic pH.

2 DMPO" ↔ DMPO + DMQDI\(^{\bullet}\) + H\(^{\bullet}\) [1]

Figure 1 shows the influence of pH and additional DMPO (10-fold excess) on the disproportionation equilibrium of DMPO" (6). It is obvious that addition of the inactive DMPO increases the radical steady-state concentration 1.8-fold and decreases the quinone diimine concentration to 1/5. By this measure, one can easily decide whether the radical or the quinone diimine (DMQDI\(^{\bullet}\)) is the ultimate reactive compound (Table 1).

From the kinetics (stopped-flow spectroscopy) of disappearance of the bright radical color in the presence of GSH, we calculated an apparent second-order rate constant of about 10^5 M^-1s^-1. Addition of a 100-fold excess of DMPO resulted in a decrease of the reaction rate by a factor of 20. This experiment clearly indicated that the quinone diimine is the reactive intermediate which reacts at pH 7.4 and 37°C with a second-order rate constant of about 5 × 10^3 M^-1s^-1.

The main products formed from DMPO" and GSH were two isomeric thioethers of DMPO, 2- and 3-(gluthionyl-S-yl)-N,N-dimethyl-p-phenylene-diamine, indicating a Michael addition reaction of GSH to the quinoid structure. GSSG formation was negligible. Although the disproportionation reaction of DMPO" is very fast, k = 2 × 10^7 M^-1s^-1, some saturation kinetics was observed when GSH was at high excess. A completely different behaviour was found with the N,N,N',N'-tetramethyl-p-phenylenediamine radical cation (TMPD\(^{\bullet}\)). The reaction velocity with GSH was independent of the GSH concentration below 1 mM. Only at higher concentrations the expected second-order reaction was found. Thioether formation at low concentrations of GSH pointed to a Michael addition reaction to the quinoid compound. When the GSH concentration was increased, the recovery of the thioether decreased. Instead, GSSG formation increased.

Figure 2 compares the different behavior of the two Wurster's radicals. The disparity is a result of the different rates of the disproportionation of the radicals. With TMPD\(^{\bullet}\), the reaction is second-order at low GSH concentrations and yields virtually constant amounts of the thioethers. Only at high GSH concentrations does the disproportionation reaction become rate limiting. This reaction is rather slow with TMPD\(^{\bullet}\) and is obviously rate limiting during thioether formation. At increasing GSH, a direct reaction of GSH with the radical is observed. During this process, GSSG is formed and less quinone diimine is available for addition reactions. Conceivably, at very high GSH concentrations, DMPO" will also react directly with GSH at the expense of the thioethers. Hence, both phenylenediamine radicals show two extremes. Figure 3 schematically compiles the whole continuum. The left part is typical for DMPO", the right for TMPD".

Figure 1. Influence of pH and additional DMPO on the disproportionation equilibrium of DMPO". The actual radical proportion was determined spectroscopically without and with a 10-fold excess of DMPO. Adapted from Störle and Eyer (8).

Figure 2. Dependence of the velocity of radical disappearance and thioether yields on GSH concentration during the interaction of DMPO" and TMPD\(^{\bullet}\), respectively, with GSH. Left panel: DMPO"; right panel: TMPD\(^{\bullet}\). Adapted from Störle and Eyer (8,9,100).

Figure 3. Schematic presentation of the dependence of the different reaction pathways of phenylenediamine radical cations on the GSH concentration.
The tendency of the radicals to react directly with GSH was very weak. With TMPD*+ a rate of 5 M⁻¹sec⁻¹ was determined (9), whereas with DMPD**, the rate is well below 10⁻⁹ M⁻¹sec⁻¹(8).

The mechanism of GSSG formation is still obscure. It may result from the reaction of GSH with both the radical and the quinone diimine. In the latter case, ipso adduct formation is conceivable, as already discussed for other quinone imines (10–12). The situation in the former case is more puzzling.

For thermodynamic reasons, TMPD** with a redox potential of +0.27 V (13) is hardly able to be reduced by GSH with thyl radical formation (redox potential at pH 7.4 of about +0.9 V (14). Hence, the equilibrium for GS' formation is about 4 × 10⁻¹⁰.

$$\text{TMPD}^*+ + \text{GS}^- \leftrightarrow \text{TMPD} + \text{GS}^* \ [2]$$

(On the assumption of an effective pKa of 8.6 (15) for the thiol dissociation, but ignoring other possible prototropic equilibria). Of course, reactions that remove GS' from the equilibrium will drive the reaction to the right. The most important reactions are the conjugation of GS' with oxygen and GS-. The resulting radical conjugates will be quickly eliminated by further reactions:

- $$\text{GS}^- + \text{O}_2 \rightarrow \text{GSOO}^- \ [3]$$
- $$\text{GS}^+ + \text{GS}^- \rightarrow \text{GSSG}^- \ [4]$$
- $$\text{GSSG}^- + \text{O}_2 \rightarrow \text{GSSG} + \text{O}_2^- \ [5]$$
- $$\text{GSSG}^- + \text{TMPD}^* \rightarrow \text{GSSG} + \text{TMPD} \ [6]$$

Because GSSG^- has a very low redox potential (-1.6 V), the overall reaction can in fact result in slow TMPD** reduction and GSSG formation. [For a more in-depth discussion of thyl radical chemistry, refer to Wardman (14)]. The possible involvement of the highly reactive thyl radicals may cause additional oxidative stress, as already reported for p-phenetidine (16–18).

In conclusion, our experiments with N-alkylated p-phenylenediamines indicate that the main reaction with thiols is brought about by the quinone diimines that are usually in a rapid disproportionation equilibrium with the radicals.

**Aminophenoxyl Radicals**

Our first contact with aminophenoxyl radicals dates back to the late seventies, when we analyzed reactions of p-dimethylaminophenol (DMAP) with oxyhemoglobin in the presence of GSH to imitate the red cell milieu. Oxyhemoglobin and DMAP are cooxidized quickly with formation of a transient N,N-dimethylamino-p-phenoxyl radical (19). This radical also was generated by pulse radiolysis and—more conveniently—by reaction with stoichiometric ferricyanide. The DMAP radical is quite stable, although much less than the corresponding DMPD radical. It decays under physiologic conditions with an apparent half-life of about 1 sec with formation of dimethylamine and p-benzoquinone, indicating that the decay proceeds via the quinone imine, which is in a rapid disproportionation equilibrium. Since the radical of DMAP was nearly completely bleached by half an equivalent of GSH, we assume that GSH reacts primarily with the quinone imine. In this reaction, GSSG is hardly formed. Instead, theophyllines are produced as expected from Michael addition reactions to the quinone imine. Hence, the radical of DMAP appears to react very similarly to the corresponding DMPD**. In contrast, the phenoxyl radical of paracetamol (NAPAP) (20) produced in the horseradish peroxidase or prostaglandin H synthetase reaction, was shown to react with GSH under formation of a thyl radical as detected by spin-trapping (17,18).

**Quinone Imines**

The reactions of GSH with the quinone imines from NAPAP, p-aminophenol, and DMAP have been studied in some detail. With all three compounds, theophyllines were produced, but significant amounts of GSSG were found only with NAPAP (21). The mechanism of GSSG formation has been attributed to intermediate ipso adducts which, after attack of a second GSH molecule, yield GSSG and NAPAP (10–12). During the reaction of the quinone imine of p-aminophenol with GSH, sequential oxidation/addition reactions occur as revealed by a variety of polysubstituted thioadducts. GSSG formation was not detected (21). When the quinone imine of DMAP, prepared chemically by oxidation with PbO₂, reacted with GSH, some GSSG was found besides polysubstituted thioadducts. This process also was examined in experiments where the oxidation was brought about by oxyhemoglobin. As shown in Figure 4, DMAP disappeared rapidly from incubates of oxyhemoglobin and glutathione with formation of an intermediate mono-substituted adduct, followed by a bis- and tris-substituted conjugate. Interestingly, GSSG formation proceeded only after a lag phase. With the isolated mono- and bis-glutathione S-conjugates, we found that significant GSSG formation occurred only during the reaction of 2,6-bis(glutathione-S-yl)4-
dimethylaminophenol with oxyhemoglobin and glutathione. Similar substituent effects on reduction versus arylation have been reported for the quinone imines of dimethyl ring-substituted NAPAP: the quinone imine disubstituted ortho to the keto function gave only GSSG, whereas the compound disubstituted meta to the keto function gave only the glutathione adduct (22). These data indicate that substituents that influence the electron density at the quinoid system will govern differently the rates of arylation and reduction (23). Whether thiol radicals are involved also, leading ultimately to GSSG, remains to be established.

**Toxicologic Impacts of the Reactions of Thiols with Quinone Imines**

Alteration will occur in the glutathione status of those cells that are involved in the activation of aromatic amines leading to arylamyl and aminophenoxyl radicals, and ultimately to quinoid compounds. Moderate GSSG formation usually is well tolerated by cells with intact glutathione reductase and glucose-6-phosphate dehydrogenase activities. Patients with glucose-6-phosphate dehydrogenase deficiency and with the rare glutathione reductase deficiency, however, are remarkably more sensitive. A typical example is the hemolytic disorder (i.e., hemolysis) resulting from the *vicia faba* aglycons divicine and isouramil. As revealed by Winterbourn and coworkers (Figure 5) (24), the highly autoxidizable hydroxypyrimidines form radical intermediates and quinoid derivatives in an autocatalytic manner, because the disproportionation reaction amplifies the autoxidizable radicals. It is the particular interaction of GSH that prevents the exponential formation of the radicals. Since the fully substituted hydroxypyrimidines do not allow Michael addition reactions and therefore are not eliminated, GSH acts only as a reductant.

Aminophenols, like the more intensively investigated 4-dimethylaminophenol, exert their toxic actions mainly as a result of arylation of cellular sulfhydryls. The particular nephrotoxicity of DMAP is mainly due to depletion of essential thiols within the mitochondria. DMAP depletes GSH and, more seriously, coenzyme A levels with formation of tri-substituted mixed thioethers composed of coenzyme A and GSH residues (25). Besides, enzymes with SH groups essential for catalytic activity are inhibited (26). A particularly illustrative example of the consequences of covalent binding of a quinoid compound to protein sulfhydryls was detected in hemoglobin (Figure 6) (27). DMAP, after activation by oxyhemoglobin, binds to the reactive SH groups in cysteine 93 of the β-chains. The resulting thioether autoxidizes to give the quinoid derivative, which partly loses the dimethylamino group on hydrolysis. In the last step, an imidazol nitrogen of the C-terminal histidine residue binds to the quinoid thioether. By this process the protein is cross-linked. The Perutz mechanism of conformation changes upon ligation is blocked, and hemoglobin has lost its most important physiologic function: it no longer liberates oxygen to the tissue, because of a 10-fold increased oxygen affinity.

What happens with the different thioethers that are produced with glutathione? Glutathione S-conjugates produced in the liver are excreted predominantly in the bile. Thus, the NAPAP thioether has been isolated and characterized from rat bile (28). Glutathione S-conjugates from DMAP are formed primarily in erythrocytes (29), actively exported through the red cell membrane (30), and excreted in urine as premercapturic acid (31,32). Similarly, p-aminophenol is activated in red cells by oxyhemoglobin with formation of various glutathione S-conjugates, which are excreted into the plasma (33). A variety of the glutathione S-conjugates of 4-aminophenol and DMAP are still active in ferrihemoglobin formation, produce reactive oxygen species, and are able to bind covalently to hemoglobin (29,34,35). Recently, attention has been focused on the particular target toxicity of glutathione S-conjugates in the kidney. When glutathione synthesis was inhibited, 4-aminophenol was less nephrotoxic to rats, and cannulation of the bile duct protected against nephrotoxicity (36). Circumstantial evidence has been presented that 4-amino-2-(glutathione-S-yl)phenol is excreted in the bile after administration of 4-aminophenol to rats (37). This thioether autoxidizes rapidly (21,34,38) and produces more ferrihemoglobin than the parent 4-aminophenol (35). Recently, it has been shown that another isomeristic thioether, 4-amino-3-(glutathione-S-yl)phenol is markedly more nephrotoxic in Fischer 344 rats than 4-aminophenol (39). The kidney-specific toxicity of autoxidizable glutathione S-conjugates results from the capability of the proximal tubules to accumulate and process these conjugates (37,40). Conceivably, oxidative stress by redox cycling and arylation of cellular sulfhydryls (23,41) by the concentrated quinoid compounds are the tubular cells' doom. It remains to be established whether glutathione S-conjugates of aminophenols and phenylenediamines are also genotoxic and contribute to renal carcinogenicity (40).

**Mechanisms of the Reactions of Nitrosamines with Thiols**

The reactions of thiols with nitrosamines are complex, and product formation is dependent on thiol concentration, pH, and substituent effects (42-45). With the model thiol, 1-thioglycerol (46,47), we could confirm the reversible formation of a semicarbazone-like intermediate, the existence of which had been supposed already.
from kinetic measurements (43). Meanwhile, the semimercaptal of 4-nitrosonitrobenzene and GSH also has been isolated and characterized by NMR spectroscopy (48). The transient semimercaptal can react in several ways (Figure 7):

a) The intermediate loses the hydroxyl group with formation of an N-(thiol-S-yl)-arylamine cation that may be trapped by reaction of a solvent water molecule at the sulfur atom, giving rise to a sulfenamide structure. This sequence has been confirmed with 18O-tracer experiments (47,49).

b) A second thiolate anion reacts at the sulfur of the intermediate semimercaptal, displacing the phenylhydroxylamine anion with formation of GSSG. This reaction has been postulated repeatedly in agreement with reaction kinetics and product pattern (43,44,46,48,49). Until now, however, we have had no definite proof for this proposed reaction mechanism (47).

It should be noted that formation of phenylhydroxylradical was detected when nitrosobenzene was reacted with GSH, and it has been hypothesized that nitrosobenzene is reduced to the phenylhydroxylradical with formation of the thyl radical (2,50). In fact, thyl radical formation was detected when low concentrations of nitrosobenzene (0.1 mM) were mixed with 30 mM GSH in the presence of 100 mM DMPO as spin trap (K Maples, personal communication). These results, however, cannot rule out definitively that the phenylhydroxylradical are formed by comproportionation of nitrosobenzene and phenylhydroxylamine (51) and that thyl radical formation occurs during the reaction of the phenylhydroxylradical with GSH.

c) Formation of a sulfenamide, N-(thiol-S-yl)-arylamine, was detected with 2-nitrosourea and GSH (52), with nitrosobenzene and 1-thioglycerol (46), and with 4-nitrophenol and GSH (53,54). Semimercaptal formation was proposed initially to occur through a mercaptal intermediate, ArN(SR)2, followed by thiolysis cleavage (44). Such a reaction became less probable later on, because reaction of ArN(OH)SR with different thiol R'SH did not yield ArNHSR2 but always ArNH2SR (46). Kazandis et al. (49) in their brilliant work offer an attractive explanation for semimercaptal formation from the N-(thiol-S-yl)-arylamine cation: nucleophilic addition to the ring, particularly when π-donors are in para position, can lead to a thioadduct incapable of aromatization. This adduct can be cleaved thiolatically and forms the sulfenamide. The latter can be cleaved thiolatically once more with formation of the arylamine. In the resonance-stabilized N-(thiol-S-yl)-arylamines with strong π-donors, the positive charge is highly delocalized to the ring carbon atoms thereby facilitating other addition reactions. We have isolated such products from 4-nitrophenol and GSH, 4-ethoxy-2-(glutathione-S-yl)-aniline and a variety of polycyclic compounds (47,54,55).

Investigation of substituent effects have indicated clearly a Hammett correlation of the reactivity and the product pattern of p-substituted nitrosoarenes in their reactions with GSH (45,49,55). Thus, 4-nitrosoarenes gives only the phenylhydroxylamine via the semimercaptal (48). The same holds true for another nitrosoarenes with strong electron-withdrawing groups—4-nitrosoacetophenone (45). The intermediary nitrosobenzene gives phenylhydroxylamine, the sulfanamide, and the sulfenamide. On the other hand, the Hammett scale, 4-nitrophenol and 4-nitroso-N,N-dimethylaniline with their strong π-donating substituents yield no phenylhydroxylamine, but ultimately yield the amine besides the other adducts mentioned above (45,47,54,55).

**Figure 7.** Reaction pathways of nitrosoarenes and glutathione.

**Toxicologic Impacts of the Reactions of Thiols with Nitroso Compounds**

**Nitrosoimidazoles**

Nitroheterocyclic compounds like the nitroimidazoles are used worldwide as antimicrobial, antiparasitic, and radiosensitizing agents. Metabolic activation, mainly by nitro group reduction, seems to be a prerequisite for the biologic effects (56). The influence of thiols, mainly GSH, on the toxicity, mutagenicity, and DNA damage has revealed complex mechanisms. The 2-hydroxylimidazoles conjugates which in the presence of excess GSH produce two stable 4-C- and 5-C-(glutathione-S-yl) conjugates, possibly with a nitrenium ion as intermediate (57–60). These data indicate that thiols may both activate and inactivate penultimate reactive nitroimidazole compounds.

GSH and cysteine enhanced the incorporation of labeled 1-methyl-4-phenyl-5-nitroimidazole into lambda DNA and polynucleotides (67). The authors concluded that a semimercaptal-like intermediate might yield a highly reactive cation that binds to DNA. These data suggest that the nitroimidazole might still be a penultimate reactive intermediate in the bioactivation of
nitroimidazoles, which by interaction with thiols gives the ultimate reactive species that binds to DNA.

1-Methyl-2-nitrosoimidazole was two orders of magnitude more toxic to CHO cells than the nitro and hydroxylamine compound. Circumstantial evidence suggested that GSH may reduce cytotoxicity (62). Similar observations were reported with HT-29 human colon cancer cells. Depletion of cellular GSH with buthionine sulfoximine before incubation with the nitrosoimidazole resulted in enhanced susceptibility (63). In chemical systems, 1-methyl-2-nitrosoimidazole reacted rapidly with GSH, with formation of GSSG, the hydroxylamine, and imidazole-GSH adducts. Mixtures of the nitrosoimidazole with GSH gave rise to DNA strand breaks in the plasmid assay (64). Since the DNA damage by the nitrosoimidazole in HT-29 colon cancer cells probably was not a result of a direct interaction of the nitroso compound, a possible activating effect of GSH to yield the ultimate electrophile (which is not the hydroxylamine) was discussed (65). All these hypotheses have to be confirmed, but it appears that GSH might have an ambiguous role in the reactions with nitrosoimidazoles. Probably, steady state concentrations of both the metastable intermediates and GSH at sensitive targets critically control activating and inactivating reactions.

Heterocyclic Nitroso Compounds from Protein Pyrolysates

Sugimura and co-workers have found numerous mutagenic compounds in broiled fish and meat, and in pyrolysates of protein and amino acids. These mutagens are heterocyclic amines and exhibit mutagenicity in the presence of S9 mix (66). All these mutagens, derivatives of pyrindinoled (Trp-P-1 and Trp-P-2), pyridoimidazole (Glu-P-1 and Glu-P-2), and imidazoquinoline (IQ) are N-hydroxylated at an exocyclic amino group to form proximate reactive compounds.

Thiols, particularly GSH, modify the mutagenic activity and covalent binding to DNA by at least two mechanisms. The first one involves glutathione S-transferase, which in the case of N-OH-Trp-P-2 produces a stable C-conjugate and two labile N-conjugates, a sulfenamide and, to the best of our knowledge, a hitherto unidentified derivative that easily liberates the parent N-OH-Trp-P-2. This last compound was found to be outstandingly mutagenic (67,68). The second pathway starts with the nitroso derivative leading to the putative semimercaptal-like intermediate (69), a sulfenamide, and a sulfinamide (68). Most interestingly, also a N-hydroxysulfonamide from NO-Glu-P-1 was apparently formed (70), which may have derived from reaction of the (liberated) glutathione sulfenic acid with NO-Glu-P-1. Such a reaction has been reported to occur with nitrosoureas and aresensulfenic acids (71). A direct reaction of nitroso compounds of interest with authentic glutathione sulfenic acid under physiologic conditions, however, remains to be established. No reaction between N-OH-Glu-P-1 and GSH was detected (70).

At present, it appears that the interaction of GSH with the nitroso heterocyclic pyroles is mainly a detoxication reaction.

Nitrosoarenes

A chemical intermediate widely used in the synthesis of certain dyes and explosives, m-dinitrobenzene has been found to cause testicular toxicity, with the Sertoli cells being the initial target. The toxicity is probably mediated through m-nitrosodinitrobenzene. Depletion of cellular GSH with diethylmaleate made the cultured rat Sertoli cells more susceptible to damage by m-dinitrobenzene and m-nitrosodinitrobenzene, while cysteamine reduced the toxicity. These data suggest that GSH interferes with metabolically formed reactive species, probably by scavenging the nitrosoarene (48,72).

2-Nitrosofluorene is a potent direct-acting mutagen that is derived from biotransformation of the well-known carcinogen N-acetyl-2-aminofluorene (73). The mutagenicity of 2-nitrosofluorene is known to be inhibited by inclusion of GSH in the assay media. Interestingly, the reaction of 2-nitrosofluorene with GSH, which yields a water-soluble product that liberated 2-aminofluorene under acidic conditions, is probably the first example of this class of reactions reported in the literature (74). Later on, others have elucidated the reactions more intensively (44,52) and found hydroxylamine, sulfinamide, and sulfenamide formation. In addition, slow formation of the sulfinamide from the interaction of N-hydroxy-2-aminofluorene with GSH was reported, and a nucleophilic displacement reaction was suggested to be underlying (52). Because this reaction was also observed in the presence of ascorbic acid, interference of 2-nitrosofluorene via autooxidation or disproportionation was excluded. Conceivably, N-O cleavage is facilitated by the stabilizing effect of the phenyl residue that may act as a strong π-donor (49). The resulting nitrenium ion may then react with GSH to form the sulfinamide. In fact, N-hydroxy-2-aminofluorene is quite electrophilic and reacts readily with DNA and deoxyguanosine, even at neutral pH (FF Kadlubar, personal communication, 1984). 4-Nitrosophenetol bound directly to tRNA (75) but only weakly to calf thymus DNA, whereas N-hydroxy-4-phenetidine bound readily (53). GSH was without effect on binding of 4-nitrosophenetol.

Studying toxic effects of p-substituted nitrosoarenes with widely varying Hammett constants that induce cellular damage in isolated rat hepatocytes, O'Brien and co-workers detected dichotomic modulating effects of GSH (41). GSH depletion by diethylmaleate decreased cytotoxicity by nitrosobenzene and 4-chloronitrosobenzene but increased cytotoxicity with 4-nitrosacetophenone and 4-nitroso-N,N-dimethylaniline. From these data, it was concluded that those nitrosoarenes capable of forming sulfanimides with GSH (45) may produce particularly toxic intermediates (e.g., the sulfimide itself) (76) or, more likely, the electrophilic arylnitrenium cations as proposed by McClelland's group (49). Hence, these observations point to mechanisms whereby GSH enhances toxicity.

The protective effect of GSH on 4-nitrosacetophenone toxicity might be a result of the very rapid reduction rates with formation of the hydroxylamine and GSSG [k = 10³ M⁻¹sec⁻¹ (45)]. A similar mechanism in 4-nitroso-N,N-dimethylaniline toxicity is less likely, since the reaction with GSH is sluggish [k = 1.5 M⁻¹sec⁻¹ (45)]. In this particular case, another detoxication mechanism appears to act: conjugation with GSH is brought about by hepatic glutathione S-transferase (77).

A peculiar toxic action of nitrosoarenes was observed during liver perfusion. 4-Nitrosophenetol (78) and 4-nitroso-N,N-dimethylaniline inhibited glutathione and GSSG excretion into bile, while nitrosobenzene and 4-nitrosacetophenone were inactive (3). Such an inhibition has been observed also in erythrocytes, where 4-nitrosophenetol inhibited the export of the model glutathione S-conjugate, S-(2,4-dinitrophenyl)-glutathione, in a competitive manner. The competition is probably caused by glutathione S-conjugates of 4-nitrosophenetol (55). 4-Nitrosophenetol and the bicyclic derivative 4-ethoxy-4'-nitrosodiphenylamine have been shown to be cytotoxic to isolated rat hepatocytes with GSH depletion (79).

The most obvious effects of GSH on cytotoxicity of nitrosoarenes are observed

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in the blood. The methemoglobinemia and hemolysis brought about by nitrosoarenes in the Kiese cycle, where nitrosoarenes are enzymically reduced and the phenylhydroxylamines are cooxidized to yield ferric hemoglobin and the parent nitrosoarene (80–82), are markedly influenced by reactions with thiols. GSH decreases the extent of ferrihemoglobin formation and increases the clearance of the blood from the nitrosoarenes (3,83–86). Such an example is shown in Figure 8, where GSH markedly diminished ferrihemoglobin formation by eliminating 4-nitrosophenol from the Kiese cycle.

The importance of the clearing function of blood to protect other sensitive target organs has been postulated earlier (84) and confirmed in the case of nitrosochloramphenicol. This bacterial metabolite of chloramphenicol has been accused to produce the occasionally observed stem cell damage (87). However, nitrosochloramphenicol reacts very rapidly with GSH in chemical systems (88) and in the blood (85), so that the reactive compound was calculated to be degraded within a few seconds. Hence, nitrosochloramphenicol, formed by microorganisms in the intestine or produced in the liver, will be degraded in blood before it can reach the bone marrow (85,89). Interestingly, another bacterial chloramphenicol metabolite emerged as a favorite proximate toxic candidate, dehydrochloramphenicol, a compound with a propiophenone moiety (89) (Figure 9). This compound is fairly stable in the blood and can reach the bone marrow cells. Dehydrochloramphenicol itself inhibits myeloid colony growth. Perhaps the most important aspect of dehydrochloramphenicol is that, in contrast to chloramphenicol, it is readily reduced by human bone marrow homogenates, even under aerobic conditions (90). One can assume that the 4-nitrosopropiophenone will react with GSH similarly to 4-nitrosoacetophenone to give the hydroxylamine and GSSG, thereby inducing a marked oxidative stress. Further investigations will show whether this metabolic pathway is indeed responsible for the chloramphenicol induced aplastic anemia.

A variety of arylamine drugs induce idiosyncratic reactions like lupus erythematoses and agranulocytosis. Procainamide is associated with a relatively high incidence of both idiosyncrasies. It has been suggested that myeloperoxidase-mediated reactions might be involved. The hydroxylamine and nitroso derivatives of procainamide were produced by activated human neutrophils and in the presence of myeloperoxidase/hydrogen peroxide. The nitroso compound was found to react rapidly with thiols to give a sulfanamide (91). In addition, covalent binding to proteins was observed. Such a reaction sequence, which produces a hapten-macromolecule adduct, will ultimately induce antibody formation and give rise to an immune system-mediated idiosyncratic drug reaction. Similar reactions may be underlying other idiosyncrasies observed with dapsone, sulfonamides, metoclopramide, and p-aminosalicylic acid (92,93).

Finally, and notably, nitrosoarene reactions with SH groups in human hemoglobin are particularly worthy to mention because of the genius loci. Neumann and coworkers have undertaken many efforts during the last decade to use hemoglobin adducts as a dose monitor in exposure control of aromatic amines and their relationship to risk assessment (44,94–96). The method makes use of sulfanilamide formation of nitrosoarenes with the exposed SH group of cysteine β93 in human hemoglobin. These adducts are rather stable to provide an integral dose monitor for the internal exposure to and the extent of metabolic activation of aromatic amines. The phenylhydroxylamines are cooxidized by oxyhemoglobin, and a fraction of the nitroso compound can react with the hemoglobin sulfhydryls. The precipitated hemoglobin of washed red cells is hydrolyzed, and the liberated amine is determined. This method may also allow the identification of persons with high individual risk (acetylator status, oxidative phenotype, G6PD deficiency). Of course, direct comparison of the hemoglobin binding indices of various aromatic amines (or nitroarenes) has to consider the widely varying bioavailability of the nitrosoarenes for binding, because steric and substituent effects markedly influence the yield of sulfanilamide formation with hemoglobin SH groups (86,97), topics that have already been discussed.

**Conclusion**

The study of the reactions of oxidatively activated arylamines with thiols has revealed a puzzling variety of reaction types. Some pathways lead to rather stable products that are excreted with urine or bile and can be regarded as water-soluble, inactive conjugates. Other pathways lead to metastable derivatives that can be reactivated in particular target cells. Autoxidizable thioethers are concentrated in the kidney;

![Figure 8](image-url). Influence of GSH on the decrease in 4-nitrosophenol (NOPt), ferrihemoglobin (HbFe3+), and phenetidine (NH2Pt) formation in the reaction of nitrosophenol with hemolysate, fortified by a NADPH-regenerating system (3).

![Figure 9](image-url). Structural formulae of chloramphenicol and putative myelotoxic metabolites.
labile thiol adducts of nitroso compounds may liberate carbonium/nitrenium ions that exhibit genotoxic activity. Covalent binding to proteins can inactivate vital enzymes and may lead to haptenization, followed by an immune response. Last, covalently bound adducts to proteins can pose a latent risk when the adducts are degraded, either in the body or in processed food from animals that have been treated with antibiotics (e.g., chloramphenicol, nitrofurans, or nitroimidazoles) or from agricultural products contaminated with herbicides, insecticides, etc. The rather new public interest in the toxicity of (drug) residues bound covalently to cysteine (98,99) will probably help toxicologists remain employed well into the future.

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