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Reaction of Mutagenic Phenacetin Metabolites with Glutathione and DNA

Possible Implications for Toxicity

G. J. MULDER,[1] F. F. KADLUBAR, J. B. MAYS, AND J. A. HINSON

Division of Carcinogenesis Research, National Center for Toxicological Research, Jefferson, Arkansas 72079

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SUMMARY

The direct-acting mutagens, *N*-hydroxy-*p*-phenetidine and *p*-nitrosophenetole, are known to be metabolites of the analgesic phenacetin and may be responsible for its carcinogenic activity. In this study, the potential detoxification of these metabolites by glutathione was examined. Glutathione reacted rapidly with *p*-nitrosophenetole, which was quantitatively converted to a single product as determined by high-pressure liquid chromatography. The analysis of the product by fast atom bombardment mass spectrometry and 500-MHz ^{1}H-NMR spectroscopy established its structure as *N*-(glutathion-*S*-yl)-*p*-phenetidine. The same glutathione conjugate was also formed when *N*-hydroxy-*p*-phenetidine was incubated with glutathione. However, since conjugate formation from *N*-hydroxy-*p*-phenetidine occurred slowly and was decreased in the presence of an argon atmosphere as well as by higher levels of glutathione, it was concluded that the conjugate resulted from oxidation of the *N*-hydroxy arylamine to the nitrosoarene, which subsequently reacted with glutathione. *N*-(Glutathion-*S*-yl)-*p*-phenetidine was semistable in water (half-life, 6–7 hr) and very unstable in the presence of nucleophiles such as 10 mM glutathione (half-life, 7 min), quantitatively decomposing to *p*-phenetidine. The conjugate was also very unstable in acidic buffers (half-life, 17 min, pH 5). Radiolabeled *N*-hydroxy-*p*-phenetidine, but not *p*-nitrosophenetole, was shown to bind covalently to calf thymus DNA *in vitro*, and 4 times more binding was detected at pH 5 than at pH 7. Glutathione did not significantly decrease binding of the *N*-hydroxy derivative at either pH, nor did purified ring-radiolabeled *N*-(glutathion-*S*-yl)-*p*-phenetidine significantly bind to DNA at either pH. Thus, we hypothesize that an important detoxification pathway for phenacetin *in vivo* could involve the facile oxidation of *N*-hydroxy-*p*-phenetidine to *p*-nitrosophenetole, which then reacts rapidly with glutathione to form an excretable conjugate.

INTRODUCTION

The formation of *N*-hydroxylated metabolites is an important factor in the expression of the toxicological properties of many aromatic amines and amides (ref. 1 and references therein). With the model carcinogen 2-acetylaminofluorene, the corresponding *N*-hydroxy arylamide is more potent than the parent compound, and is considered to be a proximate carcinogen. Conversion to an ultimate carcinogen then involves metabolic formation of electrophilic and mutagenic *O*-esters. In contrast, the *N*-hydroxy arylamine metabolite, *N*-hydroxy-2-aminofluorene, can react directly with DNA and has been proposed as an ultimate carcinogen per se (2). Moreover, *N*-hydroxy-2-aminofluorene and its facile oxidation product, 2-nitrosofluorene, are the most mutagenic metabolites of 2-acetylaminofluorene in the *Salmonella* reversion assay (3–6). Since nitrosoarenes can be readily reduced metabolically to their *N*-hydroxy derivatives, both of these metabolites may play an essential role in 2-acetylaminofluorene carcinogenesis.

In comparison, the arylamide drug phenacetin (*p*-ethoxyacetanilide) has been shown to undergo metabolic activation (6–9), and this analgesic has recently been shown to be carcinogenic in experimental animals (10, 11). This finding is consistent with the previous observation that abusers who are sensitive to nephrotoxic effects of the drug have a high incidence of renal pelvic cancer (12). The mechanisms of carcinogenicity may be similar to that of other aromatic amides, since the metabolite *N*-hydroxyphenacetin is more carcinogenic than phenacetin in rats (11, 13). In the *Salmonella* reversion assay, *N*-hydroxyphenacetin is not mutagenic; however, its deacetylation products, *N*-hydroxy-*p*-phenetidine and *p*-nitrosophenetole, are directly mutagenic (5, 8, 14, 15).

The biological potency of mutagenic and carcinogenic

[1] Present address, Department of Toxicology, University of Leiden, Leiden, The Netherlands.

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derivatives of aromatic amines is considered to be a function of metabolic activation and detoxification (1, 16). In this regard, a number of nitrosoarenes have been shown to form conjugates with GSH. Eyer (17) reported that nitrosobenzene reacts with GSH to produce phenylhydroxylamine, oxidized glutathione, and a GSH conjugate identified as glutathionesulfinanilide [(*N*-(glutathion-*S*-yl)-aniline *S*-oxide]. Similarly, Dölle *et al.* (18) found that 4-nitrosostilbene, 4-nitrosobibenzyl, 2-nitrosofluorene, and *p*-nitrosotoluene also reacted with GSH. Three types of products were characterized: the primary arylamines, their *N*-hydroxy derivatives, and glutathione adducts believed to be the corresponding *N*-substituted *S*-oxides. This laboratory has also reported that 2-nitrosofluorene, as well as *N*-hydroxy-2-aminofluorene, reacts with GSH to produce two conjugates (16). Identification of the conjugates by NMR and FAB[2] mass spectrometry established their identity as *N*-(glutathion-*S*-yl)-2-aminofluorene and its corresponding *S*-oxide. Diepold *et al.* (19) presented evidence that GSH reacts with *p*-nitrosophenetole to form *p*-phenetidine and a GSH conjugate. In this paper, we show that *p*-nitrosophenetole (4-ethoxynitrosobenzene) reacts rapidly with GSH to form *N*-(glutathion-*S*-yl)-*p*-phenetidine. Synthetic *N*-hydroxy-*p*-phenetidine was also prepared and shown to react directly with DNA but not with GSH. The role of GSH in the detoxification of these metabolites is discussed.

MATERIALS AND METHODS

Chemicals. GSH and *p*-aminophenol were obtained from Sigma Chemical Company (St. Louis, Mo.), and *p*-phenetidine (4-ethoxyaniline) and 2-hydroxyphenacetin were purchased from Aldrich Chemical Company (Milwaukee, Wis.). [*ring*-^{14}C]*p*-Nitrophenetole and [*ring*-^{3}H] *p*-phenetidine (both greater than 98% pure) were obtained from Dr. R. Roth, Midwest Research Institute (Kansas City, Mo.). [*Gly*-2-^{3}H]GSH was purchased from New England Nuclear Corporation (Boston, Mass.) (greater than 95% pure) and diluted to a specific activity of 0.05 mCi/mmol. Other radiolabeled compounds were synthesized by procedures described below for their nonradioactive derivatives. *p*-Nitrophenetole was a product of Pfaltz and Bauer, Inc. (Stamford, Conn.) and was purified as follows. The compound was dissolved in ethyl acetate, extracted four times with an equal volume of 1 M sodium hydroxide, and subsequently extracted twice with an equal volume of water. The ethyl acetate was dried with anhydrous sodium sulfate and evaporated under reduced pressure, and the product was further purified by recrystallization in diethyl ether. *N*-Hydroxy-*p*-phenetidine (4-ethoxyphenylhydroxylamine) was synthesized as described by Rising (20). *p*-Nitrosophenetole was synthesized by oxidation of *p*-phenetidine with *m*-chloroperbenzoic acid in chloroform (21). *N*-Hydroxy-*p*-phenetidine was prepared by deacetylation of *N*-hydroxy-*p*-phenacetin with carboxylesterase and was purified by HPLC as previously described (22).

The *N*-(glutathion-*S*-yl)-*p*-phenetidine was prepared by addition of 10 μmol of *p*-nitrosophenetole or *N*-hydroxy-*p*-phenetidine in 20 μl of Me_2SO to a 2-ml incubation containing 0.05 M Tris-HCl buffer (pH 7.4) and 0.01 M GSH. One minute after addition of *p*-nitrosophenetole or 10 min after addition of *N*-hydroxy-*p*-phenetidine, the entire sample was fractionated by HPLC on a μBondapak-C_{18} semipreparative column. The conjugate was eluted in 7 min using a solvent program consisting of a linear 5-min gradient of 0–50% methanol in water followed by isocratic elution at 50% methanol. For spectroscopic analyses, purified conjugate fractions from five separate incubations were combined, and the volume was reduced on a rotary evaporator. This sample was again purified by HPLC using the system described above.

To determine the possible covalent binding of *p*-phenetidine derivatives to DNA, incubations (2 ml) were carried out for 4 hr under argon in 10 mM potassium citrate/0.1 mM EDTA buffer (pH 5 or 7) containing DNA (5 mg/ml) and 1 mM [^{14}C]*N*-hydroxy-*p*-phenetidine (0.25 mCi/mmol), [^{3}H]*p*-nitrosophenetole (1.08 mCi/mmol), or [^{14}C]*N*-(glutathion-*S*-yl)-phenetidine (0.25 mCi/mmol, which were synthesized using the procedures indicated above. The DNA isolation and estimation of covalent binding were carried out as previously described (23).

Instrumentation. Analytical or semipreparative HPLC was performed on a Waters Associates Chromatograph that was equipped with two Model 6000A pumps, a U6K injector, an automated gradient controller, a Model 440 absorbance detector (254-nm filter), a μBondapak-C_{18} semi-preparative column, and a Hewlett-Packard 3390A integrator. In some other chromatographic analyses, the latter was replaced with a Hewlett-Packard Model 1040A high-speed spectrophotometric detector. Radioactivity was determined in Scintisol (Isolabs, Inc., Akron, Ohio) using a Searle Analytic Mark III scintillation spectrometer. NMR spectra were obtained using a Bruker WM500 spectrometer.

FAB mass spectrometry was performed on a Varian-Mat CH-5-DF with an Ion-Tech neutral beam FAB source. Glycerol was used as the matrix, and xenon was used as the source of the neutral beam (24).

RESULTS

Reaction of p-nitrosophenetole with GSH. When *p*-nitrosophenetole was incubated with GSH in Tris buffer, a single UV light-absorbing product was detected by reversed-phase HPLC (Fig. 1). The product had a 7-min retention time that distinguished it from the parent compound (24.6 min) and from *p*-phenetidine (17.3 min), *N*-hydroxy-*p*-phenetidine (9.3 min), 2-hydroxy-*p*-phenetidine (11.9 min), or *p*-aminophenol (9.2 min). Analysis of the UV spectrum of the product by a high-speed spectrophotometric detector further indicated that the product was not *p*-phenetidine, *N*-hydroxy-*p*-phenetidine, or *p*-nitrosophenetole (Fig. 2). In addition, when

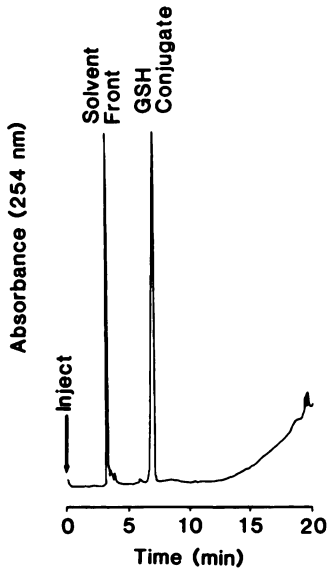

FIG. 1. *HPLC profile of p-nitrosophenetole-GSH incubation mixture*
p-Nitrosophenetole (41.8 nmol) in 10 μl of Me_2SO was added to 2 ml of 0.05 M Tris-HCl (pH 7.4) containing 1 mM GSH at 37°. At the end of 0.5 min, a 25-μl sample was analyzed as described under Materials and Methods.

[2] The abbreviations used are: FAB, fast atom bombardment; HPLC, high-pressure liquid chromatography; Me_2SO, dimethyl sulfoxide.

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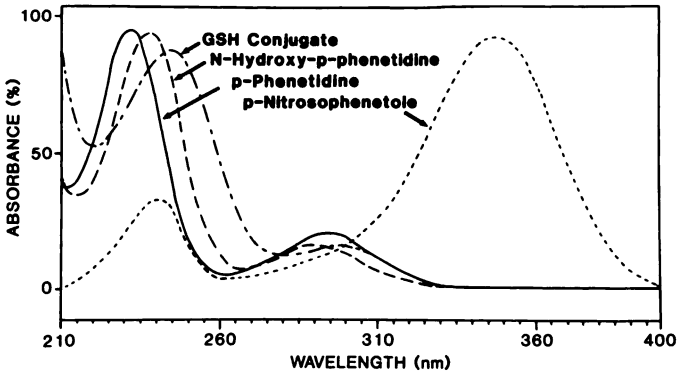

FIG. 2. *UV spectra of various phenacetin metabolites*
A Hewlett-Packard 1040A spectrophotometric detector was used with the HPLC and the absorbance was plotted by the detector. The molar extinction coefficient of the conjugate in methanol at 243 nm was 13,472 M^{-1} and at 296 nm was 6597 M^{-1}.

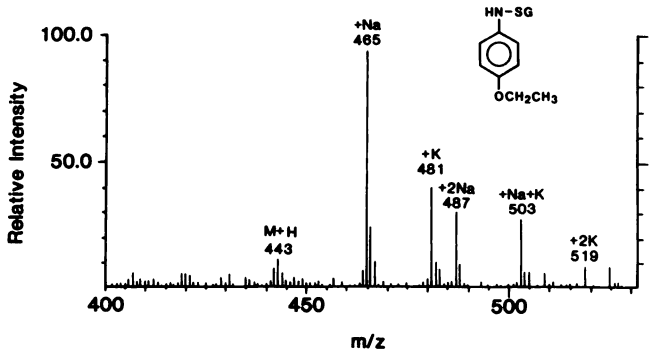

FIG. 3. *FAB mass spectrum of the p-nitrosophenetole-GSH conjugate*
The mass spectrum was obtained as described under Materials and Methods.

[^{3}H]GSH was incubated with *p*-nitrosophenetole, the 7-min fraction was coincident with eluted radioactivity. When this product was analyzed by FAB mass spectrometry (Fig. 3), it showed an M+H ion at *m/z* 443, which was consistent with a GSH conjugate of *p*-phenetidine. Other ions present at *m/z* 465, 481, 487, 503, and 519 were consistent with sodium and/or potassium mono-, di-, and mixed salts. Since the M+H ion of sulfinanilide *S*-oxides is a predominant ion in FAB mass spectrometry (16) and masses corresponding to the molecular weight of the sulfinanilide *S*-oxide were not present, the sulfur was not in the oxidized state.

A 500-MHz ^{1}H-NMR of the product showed clearly the resonances of GSH and *p*-phenetidine in a 1:1 ratio (Fig. 4). The presence of four aromatic protons, five ethoxy protons, and downfield shifts of the cysteine β protons (compared with GSH) were consistent only with a structure involving a covalent bond between the GSH sulfur atom and the nitrogen atom of *p*-phenetidine. Thus, the identity of the product was established as *N*-(glutathion-*S*-yl)-*p*-phenetidine. The oxidation state of this conjugate suggests that an intermediate *N*-hydroxysulfinanilide was formed which was subsequently reduced by GSH to the isolated conjugate.

GSH conjugate formation from N-hydroxy-p-phenetidine. When *N*-hydroxy-*p*-phenetidine was incubated with GSH in Tris buffer, a similar product was detected by reversed-phase HPLC analysis. A 500-MHz NMR of the purified product showed a spectrum identical with that in Fig. 4. Thus, the product obtained with *N*-hydroxy-*p*-phenetidine was also *N*-(glutathion-*S*-yl)-*p*-phenetidine.

Two mechanisms were envisioned by which the GSH conjugate could be formed from *N*-hydroxy-*p*-phenetidine. By the first mechanism, GSH reacts by direct nucleophilic attack on *N*-hydroxy-*p*-phenetidine to yield the conjugate; whereas, by the second mechanism, *N*-hydroxy-*p*-phenetidine oxidizes to *p*-nitrosophenetole, which subsequently undergoes a facile addition reaction with GSH. These possibilities were investigated by two different approaches. Initially, the rate of conjugation of *N*-hydroxy-*p*-phenetidine with GSH was investigated, since the rate would be concentration-dependent if the conjugate was formed by the first mechanism.

$$\text{Rate} = k[N\text{-OH-Pt}][\text{GSH}]$$

In Fig. 5, formation of the conjugate from *N*-hydroxy-*p*-phenetidine and GSH (1 mM) was shown to be relatively slow compared with the reaction of GSH with *p*-nitrosophenetole, which was formed maximally before the sample could be injected onto the HPLC (30 sec). Maximal conjugate formation from *N*-hydroxy-*p*-phenetidine was not observed before 20 min, at which time 75% of the added *N*-hydroxy arylamine was converted to the conjugate. Approximately 30% conversion was detected by 1 min, 60% at 5 min, and 70% at 10 min. Using 10 mM GSH, about 70% less conjugate was formed than at 1 mM GSH, and the rate of reaction was not increased. These data suggested that GSH did not react directly with *N*-hydroxy-*p*-phenetidine but with another species formed in the incubation mixture, presumably *p*-nitrosophenetole.

To determine the extent of oxidation of the *N*-hydroxy derivative to *p*-nitrosophenetole by molecular oxygen, experiments were performed in air and in argon atmospheres. In the presence of argon, GSH conjugate formation was decreased by 70%. These data and the rate data described above suggest that the majority, if not all, of the conjugate formed in the *N*-hydroxy-*p*-phenetidine incubation with GSH is via reaction with *p*-nitrosophenetole. The extremely facile oxidation of *N*-hydroxy-*p*-phenetidine to its nitroso derivative was confirmed spectrophotometrically. Examination of the UV spectra of *N*-hydroxy-*p*-phenetidine indicated that absorptivity at 349 nm, which is characteristic of the nitroso derivative, increased rapidly when the solution was exposed to air.

Properties of N-(glutathion-S-yl)-p-phenetidine. The stability of the conjugate was studied under a variety of conditions and found to be relatively stable in water or a non-nucleophilic buffer such as bis-Tris-HCl, with a half-life of 6–7 hr (Table 1). In the presence of nucleophilic buffers, however, the half-life was much less. In phosphate buffer, for example, the half-life was 1 hr; in the presence of a strong nucleophile such as excess GSH (10 mM), the half-life was extremely short (7 min). In all cases, the *N*-(glutathion-*S*-yl)-*p*-phenetidine decomposed quantitatively to yield *p*-phenetidine.

In an acid buffer, the conjugate was also unstable

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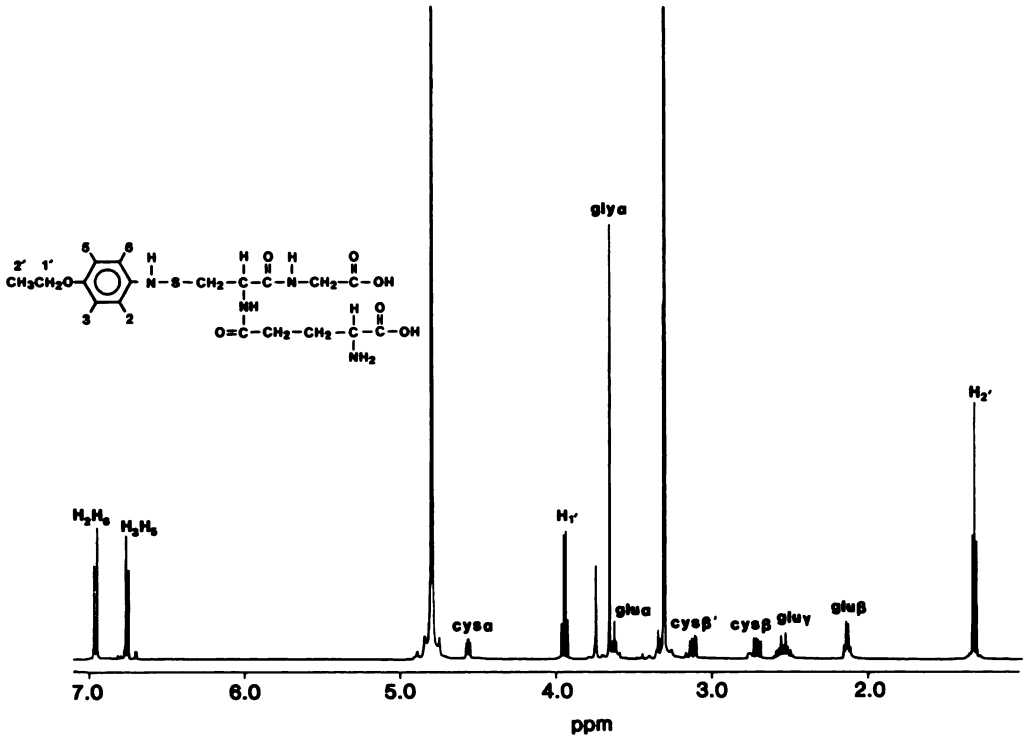

FIG. 4. *A 500-mHz ^{1}H-NMR spectrum of the p-nitrosophenetole-GSH conjugate*

Proton chemical shifts are in parts per million downfield from trimethylsilane, and the coupling constants in Hertz for the conjugate are as follows: CH_3 (1.33, J = 7.0), Gluβ (2.13, multiplet), Gluγ (2.54, multiplet), Cysβ′ (2.70, J = 8.1, 14.3), Cysβ (3.11, J = 5.2, 14.3), methanol (3.30), Gluα (3.62, J = 5.9), Glyα (3.65), unknown (3.74), CH_2 (3.94, J = 7.0), Cysα (4.56, J = 5.5, 8.5), HOD (4.95), $H^{3,5}$ (6.75, J = 9.2), and $H^{2,6}$ (6.96, J = 9.2). Data acquisition conditions in CD_3OD were as follows for 500.13-MHz ^{1}H-NMR spectra: 32 K data size; sweep width 6024 Hz, flip angle 86° (90° pulse 11.5 μsec); pulse spacing 2.7 sec; and number of scans 74.

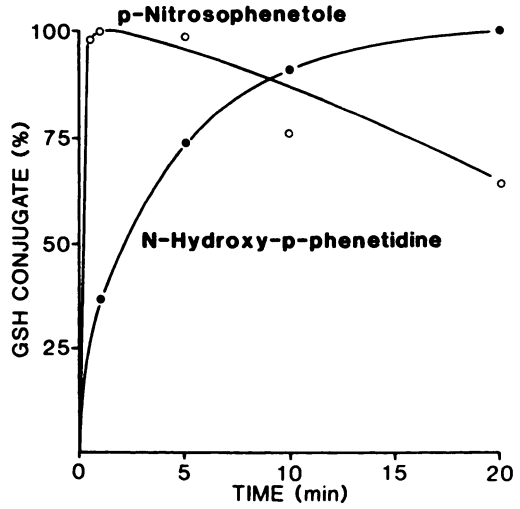

FIG. 5. *Time course for formation of the GSH conjugate from p-nitrosophenetole and N-hydroxy-p-phenetidine*

Percentage conjugate represents the maximal amounts detected at 1 min for *p*-nitrosophenetole and at 20 min for *N*-hydroxy-*p*-phenetidine. The 0.2-ml incubation contained 0.05 M Tris-HCl (pH 7.4) and 1 mM GSH, and were conducted in air. The incubation was started by addition of *p*-nitrosophenetole or *N*-hydroxy-*p*-phenetidine in 10 μl of Me_2SO. The amount of added *p*-nitrosophenetole was spectrophotometrically determined (32) to be 41.8 ± 3.4 nmol; at 1 min the amount of conjugate was 37.7 nmol. The *N*-hydroxy-*p*-phenetidine incubation contained 65 nmol of the *N*-hydroxy reactant, and 47.8 nmol of GSH conjugate was detected at 20 min.

TABLE 1

Effect of nucleophiles on stability of N-(glutathion-S-yl)-p-phenetidine

Incubations were performed at 37° using deionized distilled water and, when present, buffer concentrations of 50 mM (pH 7.4). Half-life determinations were made by repetitive HPLC analysis over a time period of at least one half-life. The data are presented as means of the incubation ± the standard deviation of the mean ($n \geq 3$).

Conditions	Half-life
Water	415 ± 95
bis-Tris-HCl	381 ± 65
Sodium phosphate	63 ± 12
Tris-HCl	73 ± 5
Tris-HCl + 1 mM GSH	40 ± 2
Tris-HCl + 10 mM GSH	7 ± 1

TABLE 2

Effect of pH on stability of N-(glutathion-S-yl)-p-phenetidine

Incubations were identical with those described in Table 1 except that pH values were varied using sodium citrate (0.05 M) as the buffer.

Conditions	Half-life
Sodium citrate, pH 7.0	135 ± 45
Sodium citrate, pH 6.0	54 ± 12
Sodium citrate, pH 5.0	17 ± 1

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(Table 2). Decreasing the pH from 7.0 to 5.0 decreased the half-life from 135 min to 17 min. Thus, the decomposition was also acid-catalyzed. Quantitation of the breakdown of the conjugate indicated a stoichiometric quantitative (>95%) conversion to *p*-phenetidine.

Effect of GSH conjugation on DNA binding. Previous data have indicated that *N*-hydroxy arylamines can bind directly to DNA and result in genotoxicity (1–3, 8, 9). In addition, Vaught *et al.* (8) have reported that *p*-nitrosophenetole binds directly to tRNA. Thus, the effect of GSH on the binding of [^{14}C]*N*-hydroxy-*p*-phenetidine and [^{3}H]*p*-nitrosophenetole to calf thymus DNA was examined (Table 3). *N*-hydroxy-*p*-phenetidine, but not *p*-nitrosophenetole, was found to bind covalently to DNA, and the extent of the reaction at pH 5.0 was 4-fold greater than that at pH. 7.0. At pH 7.0, GSH decreased covalent binding by only a minor amount, whereas at pH 5.0 more binding was detected in the presence of GSH than in its absence. These data support the concept that the *N*-hydroxy-*p*-phenetidine is the electrophilic metabolite of phenacetin which binds to DNA and that GSH does not readily detoxify this species.

Since the GSH conjugate itself could be a reactive electrophile upon heterolytic cleavage, the possibility that the conjugate might lead to DNA binding was also investigated. However, as shown in Table 3, Experiment 2, [*ring*-^{14}C]*N*-hydroxy-*p*-phenetidine led to extensive DNA binding, whereas [*ring*-^{14}C]*N*-(glutathion-*S*-yl)-*p*-phenetidine did not result in an appreciable reaction.

DISCUSSION

GSH conjugation is believed to be one of the primary *in vivo* detoxification mechanisms and involves reaction of GSH with various electrophilic metabolites. For example, GSH protects the organism against liver toxicity produced by large doses of acetaminophen and bromobenzene. In both cases, GSH reacts with the electrophilic metabolite by conjugation to form stable metabolites that are excreted into the bile (25, 26). Under conditions where GSH is depleted, as following a large dose, the metabolite is not detoxified and interacts with cellular constituents, causing cell death.

TABLE 3

Effect of GSH on the covalent binding of [^{14}C]N-hydroxy-p-phenetidine to DNA

Incubations were carried out as described under Materials and Methods; results are expressed as nanomoles of phenetidine residues bound per milligram of DNA.

Incubation conditions	DNA binding	
	pH 7.0	pH 5.0
	nmol/mg	
Experiment 1		
N-Hydroxy-*p*-phenetidine	0.35	1.35
N-Hydroxy-*p*-phenetidine + GSH	0.29	1.88
p-Nitrosophenetole	<0.05[a]	<0.05[a]
p-Nitrosophenetole + GSH	<0.05[a]	<0.05[a]
Experiment 2		
N-Hydroxy-*p*-phenetidine	0.41	1.69
N-(Glutathion-*S*-yl)-*p*-phenetidine	<0.05[a]	<0.05[a]

[a] Judged to be the limit of detection.

With various chemical carcinogens the importance of GSH in modulating tumor induction is incompletely understood. Theoretically, if a proximate or ultimate carcinogen reacts with GSH, it is not available for interaction with macromolecules and this may be an important factor in preventing tumor initiation (27, 28). Reactions of GSH with various presumed ultimate carcinogens have been demonstrated. For example, metabolites of GSH have been reported for various polycyclic aromatic hydrocarbons (29, 30), aflatoxin (31), and *N*-methyl-4-aminoazobenzene (32). Also a number of GSH conjugates of 2-acetylaminofluorene are known. The sulfate ester of *N*-hydroxy-2-acetylaminofluorene reacts with GSH to yield conjugates substituted in positions 1, 3, 5, and 7 (33). In addition, two other GSH conjugates have been described which can be formed from the mutagenic metabolite, 2-nitrosofluorene; these were identified as *N*-(glutathion-*S*-yl)-2-aminofluorene and *N*-(glutathion-*S*-yl)-2-aminofluorene *S*-oxide (16).

In this study, we examined the importance of GSH conjugation with genotoxic phenacetin metabolites. Whereas reaction of GSH with *p*-nitrosophenetole occurred readily, reaction with *N*-hydroxy-*p*-phenetidine could not be detected. The latter conclusion is complicated by the facile oxidation of the *N*-hydroxy to the nitroso derivative, which rapidly yields the conjugate (Fig. 5). The finding that rate of formation of the conjugate as well as the total amount of conjugate formed from the *N*-hydroxy-*p*-phenetidine was not enhanced by increased GSH concentrations suggested that the conjugate was formed via another intermediate. Since an argon atmosphere decreased conjugate formation by 70%, it is likely that all of the conjugate formed from the *N*-hydroxy arylamine is mediated by its oxidative decomposition.

The FAB mass spectrum (Fig. 3) and the 500-MHz ^{1}H-nuclear magnetic spectrum (Fig. 4) indicated that the conjugate was *N*-(glutathion-*S*-yl)-*p*-phenetidine. Evidence for the formation of the corresponding *S*-oxide (sulfinanilide), as has been reported to be formed from 2-nitrosofluorene (16), *p*-nitrosotoluene (18), nitrosobenzene (17), and nitrosochloramphenicol (34), was not obtained with *p*-nitrosophenetole. Moreover, *p*-nitrosophenetole reacted quantitatively (>90%) with GSH within 0.5 min to yield the amine-GSH conjugate, *N*-(glutathion-*S*-yl)-*p*-phenetidine (Fig. 5) and not an *S*-oxide derivative. Why this GSH conjugation pathway predominates with *p*-nitrosophenetole but does not with other arylnitroso derivatives is at present unclear.

N-(Glutathion-*S*-yl)-*p*-phenetidine is an unstable conjugate. It slowly decomposed in water (half-life 6–7 hr) but was very unstable in the presence of excess GSH (Table 1). At 10 mM GSH, the conjugate decomposed rapidly to *p*-phenetidine, with a half-life of 7 min. Previous data suggest that the aminofluorene-GSH conjugate was likewise unstable in 10 mM GSH, whereas the corresponding *S*-oxide appeared to be stable under these conditions (16). In this regard, Dölle *et al.* (18) isolated both the amine and the amine-glutathione *S*-oxide conjugate from *p*-nitrosotoluene plus GSH incubations; however, the amine-GSH conjugate may also have been

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formed, yet was too labile, and only the amine could be isolated.

In this study, we also demonstrated that *N*-hydroxy-*p*-phenetidine binds covalently to DNA *in vitro* and that the reaction is enhanced under slightly acidic conditions. However, we could not confirm the nucleic acid-binding properties of *p*-nitrosophenetole as earlier reported (8). Even though *N*-hydroxy-*p*-phenetidine does not readily conjugate with GSH, it easily oxidizes *p*-nitrosophenetole, and subsequent conjugation of *p*-nitrosophenetole with GSH may represent a significant detoxification pathway *in vivo*. Alternatively, since other *N*-hydroxy derivatives have been reported recently to be enzymatically conjugated with GSH (35), glutathione transferase may catalyze conjugation of GSH with *N*-hydroxy-*p*-phenetidine. To test this hypothesis, as well as the role of *N*-hydroxy-*p*-phenetidine in phenacetin-induced tumors, additional studies are needed.

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Send reprint requests to: Dr. J. A. Hinson, Division of Carcinogenesis Research, National Center for Toxicological Research, Jefferson, Ark. 72079.

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