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# Characterization and Mechanism of Formation of Reactive Products Formed during Peroxidase-Catalyzed Oxidation of *p*-Phenetidine

## Trapping of Reactive Species by Reduced Glutathione and Butylated Hydroxyanisole

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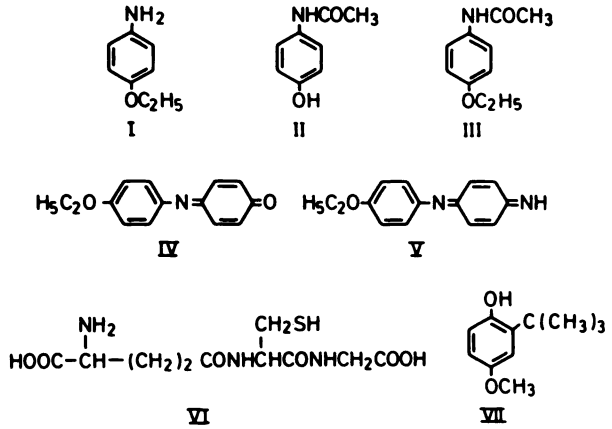
SUMMARY

The products of horseradish peroxidase (EC 1.11.1.7)-catalyzed oxidation of *p*-phenetidine were isolated and reactive species were trapped with reduced glutathione (GSH) and butylated hydroxyanisole (BHA). When BHA was added to a reaction mixture after 5 min, subsequent TLC and mass spectrometric analysis revealed the formation of an adduct of BHA and 4-(ethoxyphenyl)-*p*-benzoquinone diimine (A). The diimine derivative (A) was unstable and its expected degradation products, 4-(ethoxyphenyl)-*p*-benzoquinone imine (B) and ammonia, were recovered from the reaction in stoichiometric amounts. Ethanol was an early product of the reaction presumably resulting from radical coupling reactions and its formation agreed with the combined production of A and B, suggesting that this was its sole route of formation. The addition of GSH to a reaction at various times and subsequent TLC and high performance liquid chromatographic analysis revealed the presence of at least seven conjugates. Two conjugates were identified by fast atom bombardment mass spectrometry, one as a mono-GSH conjugate of A and another as a mono-GSH conjugate of B. When purified [$^{14}$C]B was mixed with [$^{3}$H]GSH, three conjugates were isolated by high performance liquid chromatography, two of which were tentatively identified as di-GSH conjugates. The conjugates isolated existed in both oxidized and reduced forms which could be easily interconverted by redox processes. The production of such reactive species and their conjugates *in vivo* may be a useful indicator of peroxidase-catalyzed metabolism.

## INTRODUCTION

Peroxidases such as PGS[1] (EC 1.14.99.1) and HRP (EC 1.11.1.7) can catalyze the oxidation of xenobiotics to reactive species which can bind to both protein and DNA (1, 2). Classically cytochrome P-450-catalyzed oxidations have been considered to be most relevant for the production of toxic derivatives of xenobiotics during metabolism but in certain tissues where peroxidase levels are high peroxidatic oxidation may play a significant role in induction of toxicity (1).

An organ which contains high levels of PGS is the kidney (3) and we have been investigating the role of peroxidase-catalyzed metabolism in the nephrotoxicity (4–7) elicited by chronic use of the analgesics phenacetin (Scheme 1, III) and acetaminophen (II). Phenacetin un-




SCHEME 1

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[1] The abbreviations used are: PGS, prostaglandin synthase; HRP, horseradish peroxidase; GSH, reduced glutathione; MS, mass spectrometry; FAB, fast atom bombardment; CI, chemical ionization; BHA, butylated hydroxyanisole; HPLC, high performance liquid chromatography; $t_r$, retention time.

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dergoes metabolism in the liver to produce both acetaminophen and *p*-phenetidine (I) (8), and we have shown that peroxidase-catalyzed activation of both of these compounds result in the production of species which are able to bind covalently to protein (9, 10). Peroxidase-catalyzed metabolism of *p*-phenetidine however, unlike that of acetaminophen, results in the production of species which bind to DNA and induce DNA single strand breaks in human fibroblasts (11, 12). The peroxidatic oxidation of *p*-phenetidine occurs via the generation of substrate-derived free radicals (13), whose production may be relevant for the genotoxic effects observed during *p*-phenetidine oxidation but has little importance for the protein binding associated with peroxidase-catalyzed metabolism of *p*-phenetidine (14). Thus, it seems that secondary reaction products are responsible for the protein binding observed during *p*-phenetidine oxidation.

Peroxidase-catalyzed metabolism of *p*-phenetidine yields many such products and so far we have characterized three of these: a *p*-phenetidine trimer, 4,4′-(diethoxy)azobenzene, and 4-(ethoxyphenyl)-*p*-benzoquinoneimine (IV) (13, 14). The latter compound is a reactive species which will bind to protein and which, in preliminary experiments, has been shown to react with glutathione to form conjugates (14).

It was the purpose of this study to characterize the chemical mechanism of formation of the metabolites generated during the peroxidase-catalyzed oxidation of *p*-phenetidine and to determine if any reactive species other than IV was produced. It was of particular interest to determine if the reactive quinoneimine dimer of *p*-phenetidine (IV) arose via the production of an ephemeral quinone diimine derivative (V); as a related compound, the diimine derivative of benzidine is a reactive species which forms thioether conjugates with glutathione (15) and has also been proposed to bind to DNA (16). This study describes the use of the nucleophilic trapping agents glutathione (VI) and BHA (VII) to isolate reactive species produced during the peroxidase-catalyzed oxidation of *p*-phenetidine and the characterization of the resultant adducts by mass spectrometry. The isolation and quantitation of these species and other products of the peroxidase-catalyzed oxidation of *p*-phenetidine have allowed us to describe the route of biotransformation of *p*-phenetidine during peroxidase-catalyzed oxidation.

## MATERIALS AND METHODS

GSH, BHA, HRP type VI (250 units/mg), catalase (3400 units/mg), and ammonia color reagent were purchased from Sigma Chemical Co., St Louis, MO. Ninhydrin spray (6758)-precoated thin layer chromatographic plates, cellulose (0.1 mm, 5552) and silica gel 60 (0.25 mm, 5721; 2 mm, 5745) were obtained from Merck, Darmstadt, West Germany. Porapak Q (100–120 mesh) gas chromatography packing material was purchased from Waters Associates, Milford, MA. Ring-labeled [$^{14}$C]*p*-phenetidine hydrochloride was synthesized as before (10). All other solvents and chemicals were of analytical grade and purchased from local commercial suppliers.

### *Standard Incubation Mixture*

This consisted of 0.5 mM *p*-phenetidine hydrochloride, 0.2 μg/ml HRP (0.05 unit), and 1 mM EDTA in 100 mM phosphate buffer (pH 8). Reactions were initiated by the addition of $H_2O_2$ (1 mM final concentration) and were performed at room temperature.

### *Spectrophotometric Assay*

The spectra associated with a standard incubation mixture were recorded (250–700 nm) on a Shimadzu UV240 spectrophotometer at various times.

### *Synthesis and Purification of 4-(Ethoxyphenyl)-p-benzoquinoneimine (IV)*

This compound was synthesized using the standard incubation conditions as described above except that the incubation volume used was 100 ml. The reaction was started by the addition of peroxide and terminated by extraction with diethyl ether (200 ml) after 2 hr of incubation. The organic layer was evaporated and spotted on a TLC silica gel plate (2 mm) and run in $CHCl_3$:MeOH, 19:1. The orange band was scraped off, eluted from the plate using ether, concentrated, and subjected to further purification in the same solvent system using a plate (0.25 mm) which had been prewashed in this solvent system.

### *Quantification of Metabolites*

*BHA adduct and GSH conjugate(s) formation.* At various time points, standard incubation mixtures were divided into two halves to one of which BHA was added (1 mM final concentration) and to the other GSH (1 mM final concentration). Both BHA and GSH were allowed to react for 2 min. The incubations containing BHA were then extracted with 2 ml of ethyl acetate and the organic layers were subjected to TLC analysis on silica gel plates which were developed in *n*-hexane:ethyl acetate, 1:1. The blue spot corresponding to the BHA adduct was scraped off and radioactivity was determined by liquid scintillation counting. The recovery of the [$^{14}$C]BHA adduct from the plate was found to be 87 ± 3% ($n$ = 3) and all values presented are corrected for recovery.

Aliquots (50 μl) of the incubations containing GSH were spotted on cellulose TLC plates immediately after reaction with glutathione for 2 min and the plates were developed in 1-propanol:water, 60:40. After drying, the plates were sprayed with ninhydrin and heated at 80° for 5 min. Bands corresponding to ninhydrin-positive areas were scraped off and radioactivity was determined by liquid scintillation counting. The recovery of the [$^{14}$C]GSH conjugates referred to as ninhydrin-positive compounds 1 and 3 in the text was 85 and 89%, respectively. All values presented are corrected for recovery.

*4-(Ethoxyphenyl)-p-benzoquinoneimine (IV) formation.* Incubations contained 0.5 mM [$^{14}$C]*p*-phenetidine hydrochloride, 0.2 μg (0.05 unit) of HRP, and 1 mM EDTA in 100 mM phosphate buffer in a final volume of 1 ml. Reactions were initiated by the addition of 1 mM $H_2O_2$ (final concentration) and conducted at room temperature for different time periods. Reactions were terminated by extraction with 2 ml of ethyl acetate and the organic layer was spotted on a silica gel TLC plate and developed in $CHCl_3$:MeOH, 19:1. The orange spot corresponding to the quinoneimine dimer was scraped off and radioactivity was determined by liquid scintillation counting.

*Ammonia formation.* Ammonia was determined by the colorimetric method of Nessler (17) and the resultant absorption was determined at 420 nm using ammonium chloride as reference standard. Incubations (1 ml) contained 0.5 mM *p*-phenetidine hydrochloride, 0.2 μg (0.05 unit) HRP, 1 mM $H_2O_2$, and 1 mM EDTA in 100 mM phosphate buffer, pH 8. Reactions were started by the addition of peroxide, stopped with catalase (50 μl; 1500 units/ml), and extracted with 2 ml of ethyl acetate. To the aqueous phases (1 ml), Nessler's reagent (0.5 ml) was added and samples were left for 15 min before determination of $A_{420nm}$.

### *Ethanol Determination*

Incubations (5-ml volume, room temperature) contained 0.5 mM *p*-phenetidine hydrochloride, 1 μg (0.25 unit) of HRP, 1 mM $H_2O_2$, and 1 mM EDTA in 100 mM phosphate buffer, pH 8. Incubations were started by the addition of peroxide. Sample aliquots of 0.5-ml volume

were withdrawn at various times and stopped with 50 μl of 1 M HCl. Aliquots (5 μl) were injected into a Beckman GC 72-5 gas chromatograph equipped with a Porapak Q glass column (length, 3 m). Flow rates were: air, 270 ml/min; $H_2$, 45 ml/min; $N_2$, 30 ml/min; and carrier make-up, 65 ml/min. Temperature settings were: column, 100°; detector, 150°; injector, 125°.

*Analysis of GSH Conjugates*

*TLC analysis.* Incubations (1 ml) were conducted at room temperature and contained 0.5 mM [$^{14}$C]*p*-phenetidine hydrochloride, 0.2 μg (0.05 unit) of HRP, 1 mM GSH, and 1 mM EDTA in 100 mM phosphate buffer, pH 8. Reactions were initiated by the addition of 1 mM $H_2O_2$ (final concentration) and terminated at different time points with catalase (50 μl; 1500 units/ml). Glutathione was added 30 sec after the addition of catalase and allowed to react for 2 min. The mixtures were then extracted with ethyl acetate and 50 μl of the aqueous phase was spotted onto cellulose plates which were developed in 1-propanol:$H_2O$, 60:40. After drying, the plates were sprayed with ninhydrin and developed for 5 min at 80°. The ninhydrin-positive spots which were assumed to represent GSH conjugates were scraped off and radioactivity was determined by liquid scintillation counting.

*HPLC analysis.* A Spectra-Physics 3500 liquid chromatograph was used, equipped with a $C_{18}$ column (Nucleosil, 10 μ) and a UV detector (λ = 254 nm). Solvents used were: A, 90% ammonium formate, 0.05 M, pH 6.0/10% methanol; and B, 90% methanol/10% ammonium formate, 0.05 M, pH 6.0. Samples (100 μl) were injected using a WISP 710 B and eluted using a 0 to 100% B linear gradient (20 min) with a flow rate of 1 ml min$^{-1}$. In the case of radioactively labeled samples, fractions (1 ml) of the eluate were collected directly into scintillation vials and radioactivity was determined by liquid scintillation counting.

*Isolation, Purification, and HPLC Analysis of BHA Diimine Adduct*

The adduct was isolated from a standard incubation mixture (5 ml) using TLC as described previously, eluted from the plate with ether, concentrated, and subjected to further purification using the same TLC system. After further elution and concentration, the residue was dissolved in methanol and an aliquot (20 μl) was subjected to HPLC analysis using the following conditions: $C_{18}$ column (Nucleosil 10 μ); a linear gradient of 50% methanol/water to 100% methanol over 10 min; flow rate = 1 ml/min; λ = 605 nm.

*Mass Spectral Analysis*

Mass spectral analyses were performed with two LKB 2091 mass spectrometers. One of these was equipped with a FAB gas gun (FAB11NF; Ion Tech Ltd., Middlesex) which produced a xenon atom beam of 6 keV. The xenon beam was at a right angle to the sample ions produced. The dissolved sample was added to a thin layer of glycerol on the copper tip of the direct probe. Mass spectra of glycerol were recorded before the sample was added to the probe to be used for background subtraction. The sample holder and ion source were at room temperature.

The other mass spectrometer was equipped with chemical ionization units (18). Ammonia was used as reagent gas at an ion source pressure of about 0.3 torr. The dissolved sample was injected into a glass tube which was put into a temperature-programmed spiral on the direct inlet probe. The temperature of the ion source was kept at 200°.

FAB and CI mass spectra were obtained at a scanning rate of about 600 mass numbers in 20 responses/10 sec. Both LKB instruments were operated at an acceleration voltage of 3.5 keV. The instruments were connected to a PDP 11/34 computer system.

The ions at $m/z$ = 155 and 157 obtained in FAB mass spectra (Figs. 3, 10, and 11) are thought to result from copper/glycerol interactions due to the use of glycerol during the application of the sample to the copper tip of the probe.

## RESULTS

The spectral change associated with the HRP/$H_2O_2$-catalyzed oxidation of *p*-phenetidine is shown in Fig. 1.

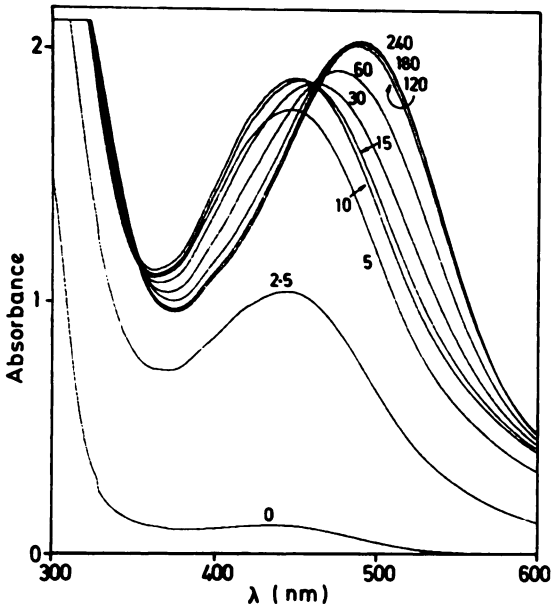


FIG. 1. *UV spectral change associated with the HRP/$H_2O_2$-catalyzed oxidation of p-phenetidine*

A standard incubation mixture was used (see Materials and Methods) and spectra were recorded at the times indicated (min).

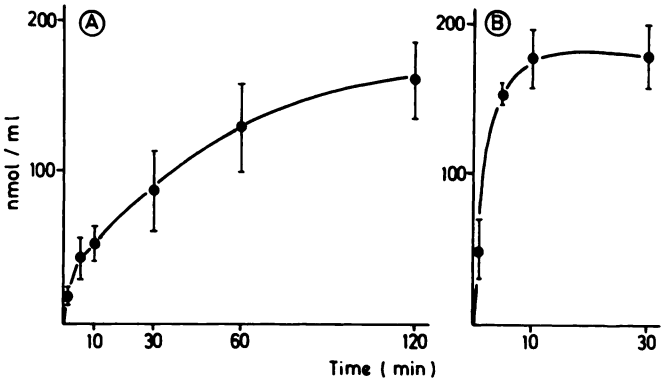


FIG. 2. *Time course of formation of IV: 4-(ethoxyphenyl)-p-benzoquinone-imine (A) and ethanol (B) during the HRP/$H_2O_2$-catalyzed oxidation of p-phenetidine*

A standard incubation mixture containing [$^{14}$C]*p*-phenetidine was used and reactions were stopped by the addition of 2 ml of ethyl acetate (A) or with 0.05 ml of 1 M HCl (B, see Materials and Methods). Data represent means ± standard deviations of at least three experiments.

The $\lambda_{max}$ of the reaction changes with time, suggesting the sequential production of a number of reaction products, but appears to be complete after 2 hr. The time course of formation of the quinoneimine dimer of *p*-phenetidine (IV), a compound which has been previously characterized by both chromatography and mass spectrometry (14), is shown in Fig. 2A. If the mechanism of formation of this dimer involves free radical coupling, then the ethoxy group of *p*-phenetidine should be lost as ethanol. That ethanol was a product of the HRP/$H_2O_2$-catalyzed oxidation of *p*-phenetidine was shown by gas chromatography and the time course of its formation is shown in Fig. 2B. It is clear from Fig. 2 that the time curves of the formation of ethanol and the quinoneimine dimer (IV) do not correlate. This suggested that the formation of a precursor of IV may also involve the

elimination of ethanol. It seemed probable that this precursor may be a quinonediimine derivative of *p*-phenetidine (V) presumably generated from the coupling of two *p*-phenetidine radicals.

In an attempt to trap the quinonediimine derivative, BHA was used in incubations as it has been shown previously that diimine derivatives react readily with BHA to form adducts (19) due to the electrophilic nature of the diimine nitrogen (20). The chemical structure of such adducts has not been fully described but it has been assumed that the reaction proceeds by a substitution reaction *ortho* to the hydroxy group of BHA upon the side of the aromatic ring away from the *tert*-butyl substitution [(21); see VII]. Addition of BHA (1 mM) to a standard incubation mixture after 5 min resulted in a change in the color of the reaction mixture from brandy to dark red. After extraction, concentration, and purification of this mixture by thin layer chromatography, a blue adduct typical of BHA diimine-coupling reactions was observed. Further purification by HPLC showed that this adduct consisted of at least two compounds, the major component representing 87% of the total product yield (assuming identical extinction coefficients of the two adducts). This is not surprising as BHA itself is a mixture of the 2- and 3-*tert*-butyl isomers. The major BHA adduct was subjected to FAB mass spectrometry and the results are shown in Fig. 3. By comparison with mass spectra of other quinones such as menadione and the quinoneimine derivative of *p*-phenetidine (IV), we have shown that quinone derivatives when subjected to FAB mass spectrometry produce base peaks at (M + 2) as well as a high intensity signal at $(M + 2)^+H$. Indeed the reduction of quinonoid compounds and the consequent generation of (M + 2) signals during electron impact mass spectrometry is well documented (22, 23). Hence, by analogy, the molecular weight of the BHA adduct formed in Fig. 3 is 374. If the reaction of BHA with diimine derivatives proceeds as suggested previously (21), then the resultant molecular weight of this adduct would be expected to be 405. It follows that formation of this particular adduct (Fig. 3) involves a substitution at the *para* position to the hydroxy group of BHA with elimination of the methoxy substituent (see VII). This has been confirmed recently by chemical studies of the interaction of BHA with the diimine derivative of benzidine (24) and by an investigation of the reaction of 2,6-dichloro-*p*-benzoquinone-4-chlorimine with various phenols (25). The formula of the proposed diimine-BHA adduct and assignments for fragmentation ions are shown in Fig. 3.

Using radioactively labeled *p*-phenetidine, the time course of formation of the diimine BHA adduct was determined (Fig. 4). Maximal formation of the adduct was apparent at early time points (5–10 min) and subsequently decreased due to either decomposition or further oxidation of the diimine prior to derivatization with BHA. If the diimine derivative of *p*-phenetidine (V) decomposed by hydrolysis to produce the quinoneimine derivative (IV), then ammonia should also have been produced. The evolution of ammonia was detected in a standard incubation mixture using a colorimetric method (see Materials and Methods) and its formation (Fig. 5) closely parallels the formation of the quinoneimine derivative of *p*-phenetidine (IV; see Fig. 2A).

In Fig. 6, the time course of ethanol production is compared with the combined production of the diimine BHA adduct plus the quinoneimine derivative of *p*-phenetidine (IV). The two curves correlate well and this suggests therefore that the mechanism of formation of compound IV, the quinoneimine derivative, involves the initial generation of compound V, the quinonediimine, with concomitant elimination of ethanol.

In order to determine whether the diimine derivative of *p*-phenetidine (V), in addition to the quinoneimine

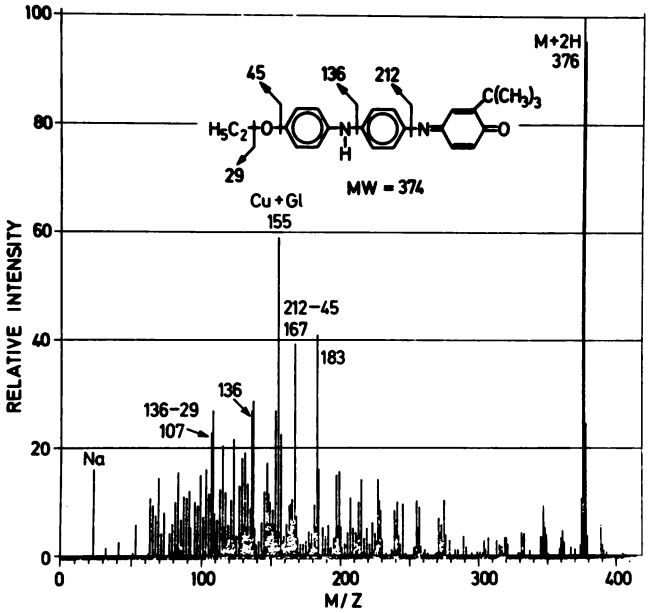


FIG. 3. *FAB mass spectrum of the proposed diimine-BHA adduct*
Analytical conditions are described in Materials and Methods.

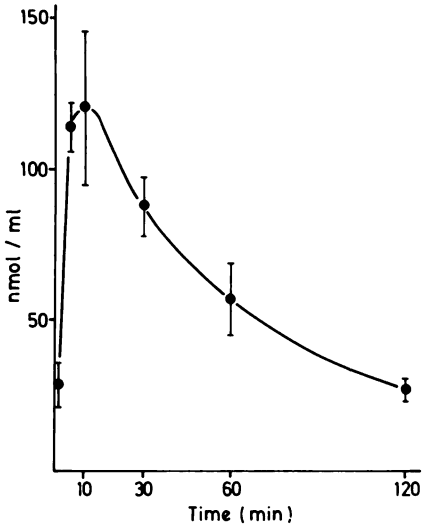


FIG. 4. *Time course of formation of the diimine-BHA adduct during the HRP/$H_2O_2$-catalyzed oxidation of p-phenetidine*
A standard incubation mixture containing [$^{14}$C]*p*-phenetidine was used and reactions were stopped with 2 ml of ethyl acetate (see Materials and Methods). Data represent means ± standard deviations of at least four experiments.

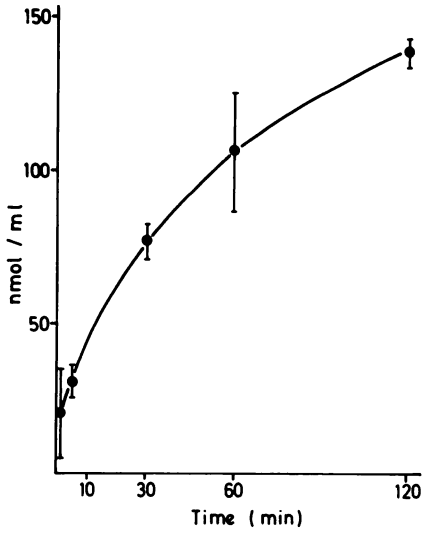


FIG. 5. *Time course of ammonia formation during the HRP/$H_2O_2$-catalyzed oxidation of p-phenetidine*

A standard mixture was used and reactions were stopped by the addition of catalase (0.05 ml, 1500 units/ml). Data represent means ± standard deviations of at least four experiments.

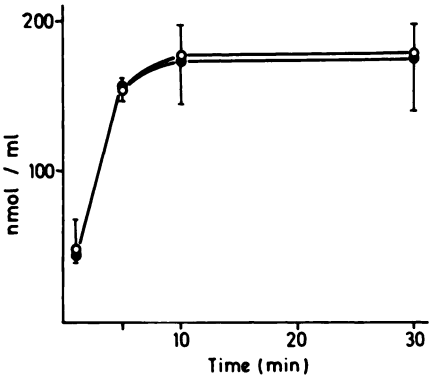


FIG. 6. *Time course of formation of ethanol (○) and (4-(ethoxyphenyl)-p-benzoquinone imine (IV) plus its diimine derivative (V) (●) during the HRP/$H_2O_2$-catalyzed oxidation of p-phenetidine*

Data are from Figs. 2 and 4.

dimer (IV), had the potential to react with biological nucleophiles, the reaction of the products of the HRP/$H_2O_2$-catalyzed oxidation of *p*-phenetidine with glutathione was investigated. When glutathione was added to a standard incubation mixture, after 1 hr the solution decolorized but on standing a red color returned. The colored products, however, could not be extracted from neutral, basic (pH 12), or acidic (pH 2) solution even though under the latter conditions the aqueous solution became violet. This suggested that the colored products were water-soluble conjugates.

After the addition of glutathione to the purified quinoneimine dimer (IV), two ninhydrin-positive compounds could be isolated using TLC (compounds 1 and 2, Table 1) which were not present in control incubations without glutathione or without IV. When glutathione was added to a standard incubation mixture containing HRP, $H_2O_2$, and *p*-phenetidine after 10 min, however, a different ninhydrin-positive compound (3, Table 1) could also be observed in addition to ninhydrin-positive bands

TABLE 1

*Ninhydrin-positive compounds formed upon addition of glutathione to the purified quinoneimine dimer of p-phenetidine (IV) and to an incubation mixture containing p-phenetidine, HRP, and $H_2O_2$*

Results are from one experiment typical of five.

| $R_F$ of ninhydrin-positive compound[a] | Formation on addition of GSH[b] to | |
|---|---|---|
| | Purified quinone imine dimer[c] | Incubation[d] mixture containing *p*-phenetidine/HRP/$H_2O_2$ |
| 1. 0.86 | + | + |
| 2. 0.58 | + | + |
| 3. 0.71 | − | + |

[a] TLC analysis was performed on cellulose plates developed in 1-propanol:$H_2O$, 60:40.

[b] Final concentration, 1 mM.

[c] Estimated concentration, 100 μM.

[d] Standard incubation mixture as described in Materials and Methods.

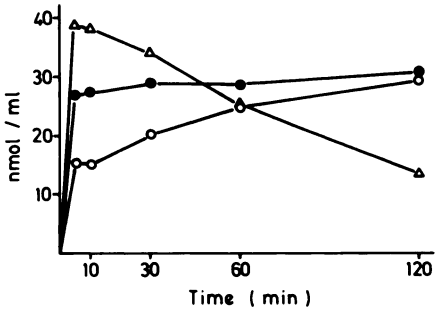


FIG. 7. *Time course of formation of ninhydrin-positive products formed after addition of glutathione to a standard incubation mixture*

Ninhydrin-positive compound 1 (○), $R_F$ = 0.86, 2 (●), $R_F$ = 0.58, and 3 (△), $R_F$ = 0.71. Data represent results of one experiment typical of two.

with $R_F$ values similar to compounds 1 and 2. By the use of [$^{14}$C]*p*-phenetidine and subsequent isolation of the ninhydrin-positive products by TLC after the addition of glutathione to a standard incubation mixture at various times, the time course of formation of these products was determined (Fig. 7). The time course of formation of ninhydrin-positive compound 3 (TLC $R_F$ = 0.71) was similar to that of the diimine-BHA adduct isolated during peroxidase-catalyzed oxidation of *p*-phenetidine, which suggested that compound 3 may be a glutathione adduct of the *p*-phenetidine diimine derivative (V).

When glutathione was added to a standard incubation mixture at various times and the products were analyzed by HPLC after extraction (see Materials and Methods), different products were observed (Fig. 8, peaks *a–h*). The time course of formation of peaks corresponding to ninhydrin-positive compounds 1 (TLC $R_F$ = 0.86; HPLC peak *g*) and 3 (TLC $R_F$ = 0.71; HPLC peak *f*) was confirmed but it was clear that a number of more polar derivatives were produced depending on the time of addition of glutathione. This mixture of compounds presumably represented the polar ninhydrin-positive material isolated using TLC ($R_F$ = 0.58). The HPLC peak corresponding to ninhydrin-positive species 2 (TLC $R_F$ = 0.58; HPLC peak *e*), which was formed by reaction of

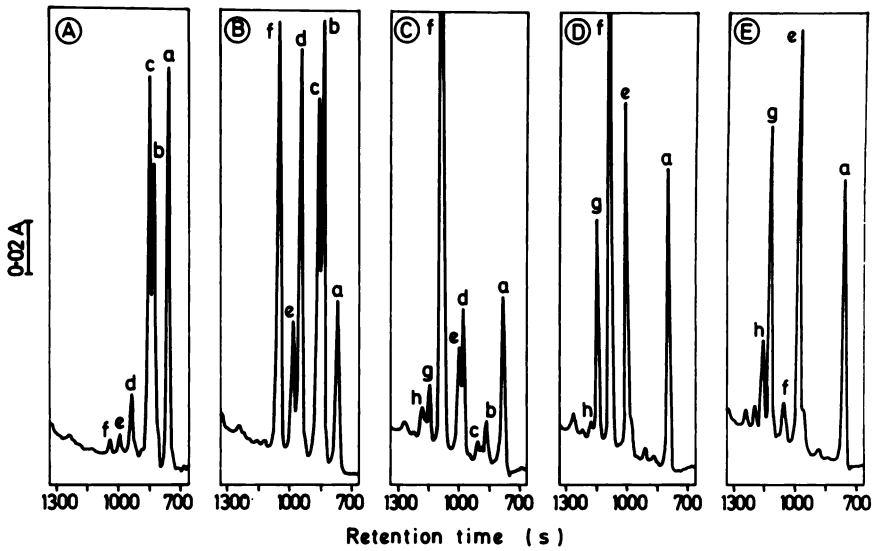


FIG. 8. *HPLC elution profiles obtained after the addition of glutathione to a standard mixture at various times*
Glutathione (1 mM) was added to a standard incubation mixture after 1 min (A), 5 min (B), 30 min (C), 60 min (D), and 240 min (E). Peaks *a–h* are shown. Mixtures without phenetidine and without glutathione and without $H_2O_2$ or HRP did not produce any peaks in this region of the chromatogram. Sample extraction and analytical conditions are described in Materials and Methods.

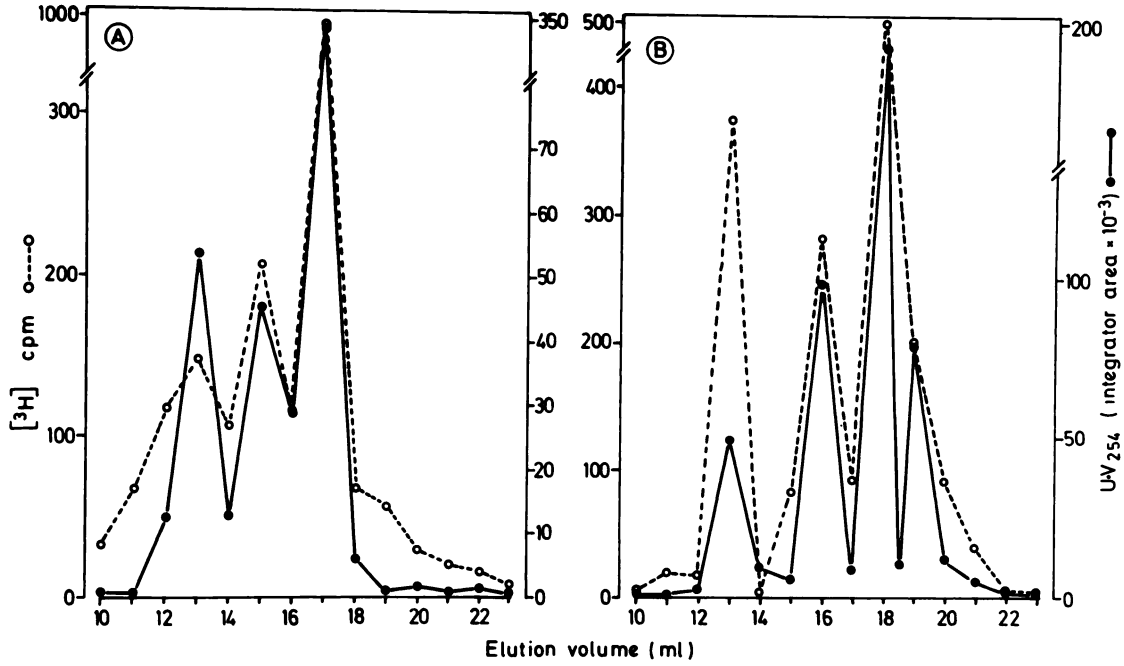


FIG. 9. *UV/$^3$H HPLC elution profiles obtained after the addition of [$^3$H]glutathione to a standard incubation mixture at various times*
[$^3$H]Glutathione (1 mM) was added to a standard incubation mixture after 5 min (A) and after 60 min (B). Sample extraction and analytical conditions are described in Materials and Methods.

the purified quinoneimine dimer (IV) with glutathione, was not observed in significant quantities until later time points.

To confirm the identity of these ninhydrin-positive species as glutathione conjugates, [$^3$H]glutathione was added to a standard incubation at two different time points (5 min and 1 hr), and the resultant mixtures were analyzed by HPLC. The HPLC eluate corresponding to the various peaks was collected (1-ml fractions), and the radioactivity contained in the various fractions was determined. The UV radioactive elution profiles obtained are shown in Fig. 9. Furthermore, after the addition of GSH to a standard incubation mixture after 1 min and subsequent extraction, the production of ammonia could be measured in the aqueous phase (data not shown), suggesting that conjugates themselves may decompose with resultant elimination of ammonia.

The identity of the conjugates was further investigated using mass spectrometry. The ninhydrin-positive compound isolated using TLC ($R_F$ = 0.86; HPLC peak *g*), which was formed during reaction of IV with GSH was subjected to mass spectral analysis before ninhydrin derivatization. Chemical ionization mass spectrometry produced fragmentation peaks ($m/z$ = 103,

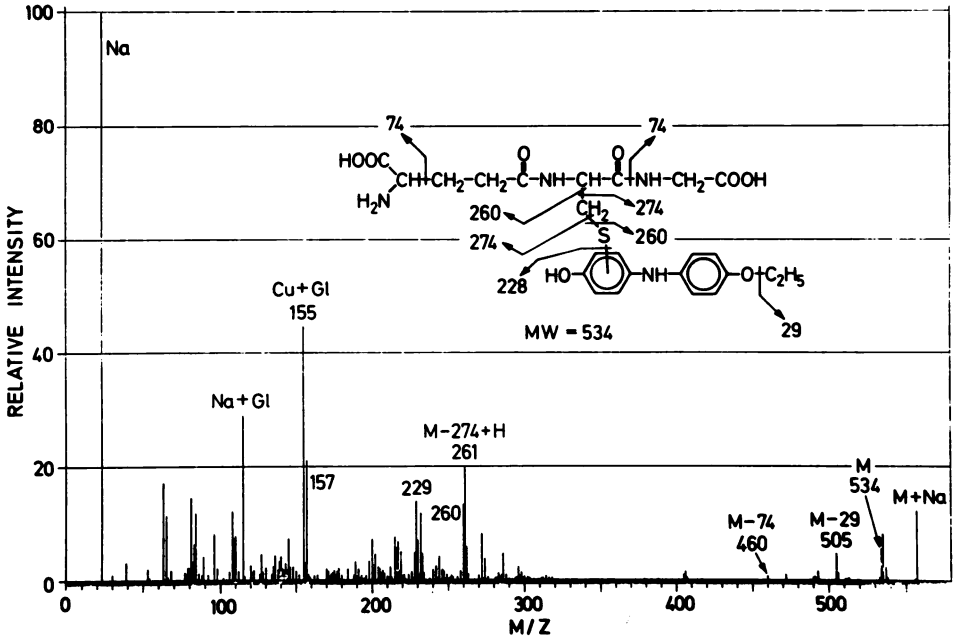


FIG. 10. *FAB mass spectrum of ninhydrin-positive compound 1 isolated using TLC ($R_F$ = 0.86)*
Analytical conditions are described in Materials and Methods. The proposed structure and assignments for fragmentation peaks are also shown. The mass spectrum does not allow unequivocal identification of the site of glutathione addition.

[$(OCNHCH_2COOH)H^+$]; $m/z$ = 130, [$(OCCH_2CH_2CH(COOH)NH_2)^+$]; $m/z$ = 262 [$(CH(CONHCH_2COOH)NHCOCH_2CH_2CH(COOH)NH_2)^+$] similar to octyl and hexyl glutathione conjugate standards but a molecular ion was not apparent (data not shown). FAB mass spectrometry (Fig. 10), however, revealed the presence of a molecular ion at $m/z$ = 534 and peaks at $m/z$ = 535 ($MH^+$) and $m/z$ = 557 (M + Na), the latter resulting from combination with sodium which is the base peak in the spectrum. This is consistent with previous analyses of glutathione conjugates using this method (26) and confirms that this material is a mono-GSH adduct of 4-(ethoxyphenyl)-*p*-benzoquinoneimine (IV). The proposed formula of this conjugate is shown in Fig. 10 together with assignments for various fragmentation ions. Analysis of the more polar conjugate formed upon reaction of GSH and IV (ninhydrin-positive compound 2; TLC $R_F$ = 0.58) by FAB mass spectrometry was unsuccessful. The remaining conjugate isolated using TLC (ninhydrin-positive compound 3 ($R_F$ = 0.71)), which

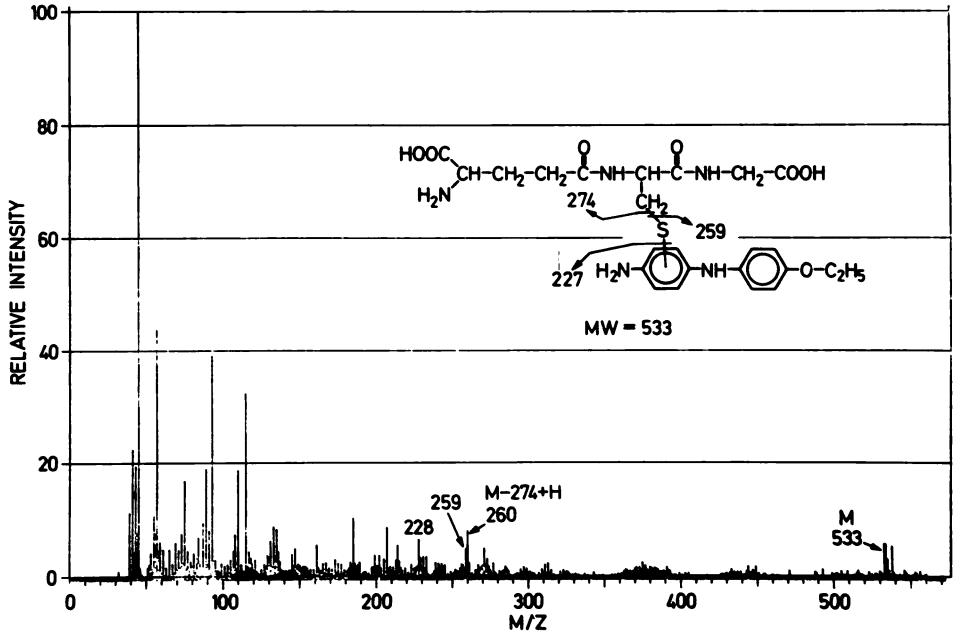


FIG. 11. *FAB mass spectrum of ninhydrin-positive compound 3 isolated using TLC ($R_F$ = 0.71)*
Analytical conditions are described in Materials and Methods. The proposed structure and assignments for fragmentation peaks are also shown. The mass spectrum does not allow unequivocal identification of the site of glutathione addition.

was proposed to be a conjugate of the diimine derivative (V) on the basis of its time course of formation (Fig. 7), produced the FAB mass spectrum shown in Fig. 11 (M = 533) which is consistent with its proposed structure as a mono-GSH adduct of V. Mass spectra from control samples show that the intense peaks below $m/z$ = 210 and 538 belong chiefly to background. A comparison of the two FAB mass spectra (Figs. 10 and 11) shows that sodium ($m/z$ = 23) is the base peak in Fig. 10 and a characteristic peak of M + Na is apparent ($m/z$ = 557), but in the spectrum shown in Fig. 11 both sodium and the M + Na peaks are absent. The mass spectrum in Fig. 10 (M = 534) shows major characteristic peaks at $m/z$ = 261, 260, and 229 whereas in Fig. 11 (M = 533) fragmentation peaks appear at $m/z$ = 260, 259, and 228, i.e., one mass unit lower than in Fig. 10. This one unit change in mass confirms that these ions contain the aromatic fragment. Other characteristic peaks of lower intensity in Fig. 11 also appear as one unit lower in mass as compared to those shown in Fig. 10.

When the quinoneimine derivative (IV) was isolated from an incubation mixture containing [$^{14}$C]*p*-phenetidine, purified, mixed with [$^{3}$H]glutathione, and analyzed using HPLC, two glutathione adducts (peaks *g* and *e*, Fig. 8) corresponding to the ninhydrin-positive compounds 1 and 2, respectively, were observed; in addition, a third more polar compound with a retention time similar to peak *a* (Fig. 8) was also formed. The radioactivity present in the eluate corresponding to each of the peaks was determined and the results are shown in Table 2. The conjugate peak *e* ($t_r$ = 1014 sec) corresponding to the ninhydrin-positive compound 2 and the additional polar compound ($t_r$ = 773 sec) had approximately twice the ratio of $^{3}$H/$^{14}$C as peak *g*, the mono-GSH conjugate ($t_r$ = 1169 sec) characterized by FAB mass spectrometry, which suggested that these compounds were di-GSH conjugates of compound IV. Interestingly, the HPLC peaks obtained upon analysis of a mixture of purified quinoneimine dimer and GSH existed as double peaks. The ratio of the components of such double peaks could be changed by the addition of dithionite to the reaction mixture immediately prior to HPLC analysis (results not shown), which demonstrates that these conjugates may exist in both oxidized and reduced forms.

TABLE 2

*HPLC analysis of GSH conjugates formed in reaction of the purified [$^{14}$C]quinoneimine derivative of p-phenetidine (IV) and [$^{3}$H]GSH*

Dimer concentration = 100 μM; GSH concentration = 1 mM.

| HPLC retention time | TLC $R_f$ of corresponding ninhydrin-positive compound | $^{3}$H/$^{14}$C in HPLC eluate appropriate to peak[a] |
|---|---|---|
| *sec* | | |
| 773 | Not observed | 2.0 |
| 1014 | 0.58 (ninhydrin-positive compound 2) | 1.8 |
| 1169 | 0.86 (ninhydrin-positive compound 1) | 1.0 |

[a] Values are the mean of two determinations. Eluates corresponding to the respective conjugate peaks were collected and radioactivity was measured as described in Materials and Methods.

## DISCUSSION

It has been shown previously that the HRP/$H_2O_2$-catalyzed oxidation of *p*-phenetidine is likely to proceed via the production of substrate-derived free radicals and that at least six oxidation products are subsequently formed in this reaction, presumably by radical coupling reactions (13, 14). One of these products, a quinoneimine dimer (IV) has been shown to bind covalently to protein (14), but the mechanism of its formation was unclear. The isolation of ethanol as an early product of the HRP/$H_2O_2$-catalyzed oxidation of *p*-phenetidine (Fig. 2B) suggested that this oxidation may proceed via the head to tail coupling of two *p*-phenetidine radicals with concomitant elimination of ethanol. If this was the case, then the initial product of radical coupling would be a quinonediimine derivative of *p*-phenetidine (V). This derivative has not been isolated previously during the HRP/$H_2O_2$-catalyzed oxidation of *p*-phenetidine but its pivotal role as an intermediate in this oxidation was confirmed by the use of BHA as a trapping agent and subsequent isolation and characterization of the adduct formed.

Quinonediimine derivatives are known to be unstable to hydrolysis and will decompose readily in aqueous solution to yield a quinoneimine derivative and ammonia (27). If this was the sole route of formation of the quinoneimine derivative of *p*-phenetidine (IV), then a stoichiometric elimination of ammonia would be expected. The production of ammonia during the HRP/$H_2O_2$-catalyzed oxidation of *p*-phenetidine did not differ significantly from the production of the quinoneimine derivative (IV) (Figs. 2A and 5), and the latter compound was quantitatively the major metabolite formed during the peroxidase-catalyzed oxidation of *p*-phenetidine. These results suggested that the major mechanism of formation of the quinoneimine derivative of *p*-phenetidine (IV) was via the hydrolysis of a quinonediimine derivative (V). This was confirmed when the formation of ethanol was compared with the combined production of the quinoneimine derivative (IV) and the diimine-BHA adduct. The two curves were in very good agreement, suggesting that this was the sole route of metabolism leading to ethanol production. The diimine derivative (V) is of quinonoid structure similar to the quinoneimine derivative (IV) and as such would be expected to react with biological nucleophiles in a similar manner to IV. Thus, the production of the diimine would be expected to be of toxicological relevance at least for protein binding. It is as yet unknown whether the production of the quinonediimine derivative (V) is of significance for the genotoxic effects observed during *p*-phenetidine oxidation (11, 12), but it has been proposed that a related compound, the diimine derivative of benzidine, may bind to DNA (16). Whether the quinonediimine derivative of *p*-phenetidine (V) and its quinoneimine analogue (IV) have genotoxic potential is currently under investigation in our laboratories but preliminary results indicate that the quinoneimine derivative (IV) does not bind to DNA.

The chemical nature of the reactive species formed during the HRP/$H_2O_2$-catalyzed oxidation of *p*-phenetidine was investigated by trapping the electrophilic inter-

mediates produced with the biological nucleophile glutathione. This approach offers useful information as to the type and number of reactive species produced in a reaction (28). We have shown previously that a large amount of water-soluble material, presumably representing conjugates, is formed when glutathione is added to a mixture containing HRP/$H_2O_2$ and *p*-phenetidine after 30 min (14). This was confirmed in this study by HPLC analysis of the GSH conjugates formed after the addition of [$^3$H]GSH and estimation of the radioactivity present in the eluate corresponding to the peaks. Initial experiments using TLC indicated the presence of three ninhydrin-positive bands, one of which (compound 3, $R_F$ = 0.71) had a time course of formation similar to the BHA diimine adduct, and subsequent FAB mass spectrometry (Fig. 11) confirmed that this material was indeed a mono-GSH conjugate of the diimine derivative (V). The remaining two ninhydrin-positive species isolated using TLC corresponded to the bands produced when glutathione was added to the purified quinoneimine dimer (IV). One of these (ninhydrin-positive compound 1) was confirmed as a mono-GSH adduct of IV (M = 534, Fig. 10) by CI and FAB mass spectrometry. The mass spectral data presented in Figs. 10 and 11 do not allow elucidation of the site of attachment of the glutathione moiety to derivatives IV and V but NMR studies to define the exact structure of these species are proceeding.

When a reaction mixture of quinoneimine (IV) and GSH was analyzed using HPLC, three conjugate peaks were apparent, two of which, peaks *g* and *e*, corresponded to ninhydrin-positive compounds 1 and 2, respectively, but another more polar compound was also present. Experiments using purified [$^{14}$C]quinoneimine dimer (IV) and [$^3$H]GSH indicated that these two unidentified conjugates (corresponding to peak *e*, Fig. 8, and the additional polar compound) were di-GSH conjugates. The identity of most of the conjugates isolated using HPLC analysis after addition of glutathione to a standard incubation mixture at various times is uncertain. The results do indicate, however, that products of the HRP/$H_2O_2$-catalyzed oxidation of *p*-phenetidine, other than the quinoneimine dimer (IV), are reactive species which are capable of forming conjugates with reduced GSH. On the basis of the mechanism proposed for the oxidation, it is tempting to speculate that these conjugates represent di-GSH conjugates of the quinonediimine dimer (V) or are products of the reaction of *p*-phenetidine radicals with GSH but definitive proof of such reactions is lacking.

It has recently been suggested that the formation of glutathione conjugates of certain compounds may be of toxicological relevance. For example, it has been shown that the glutathione conjugate of menadione can redox cycle and give rise to active oxygen species (29). Our observations in this study that the conjugates formed are easily interconvertible by redox processes may be relevant in this respect. Furthermore, Monks *et al.* (30) have shown that kidney-specific metabolism of glutathione conjugate(s) of bromohydroquinone may be responsible for the nephrotoxicity observed upon administration of the latter compound to rats. In addition, the cysteine

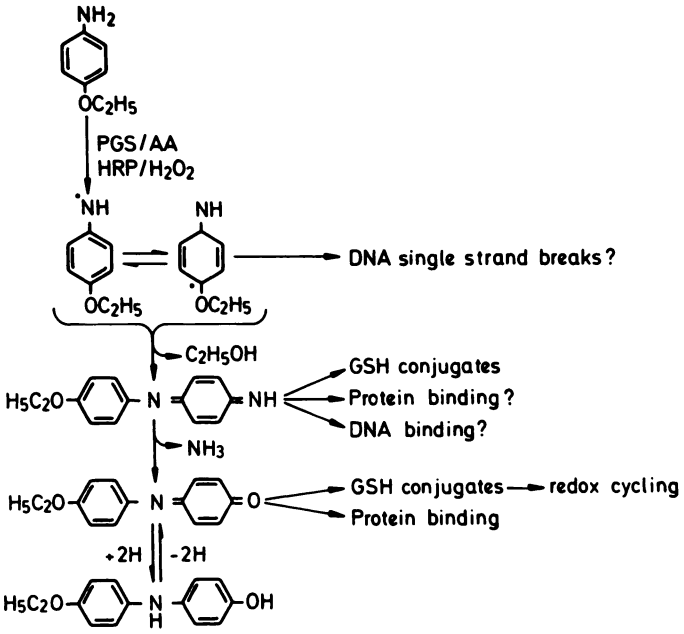


FIG. 12. *Proposed scheme for the HRP/$H_2O_2$-catalyzed oxidation of p-phenetidine*

and glutathione conjugates of hexachlorobutadiene have been implicated in the mechanism of nephrotoxicity of this hydrocarbon (31, 32). In this context, it is also pertinent to note that we have previously demonstrated a potentiation of the binding to DNA observed during the HRP/$H_2O_2$-catalyzed oxidation of *p*-phenetidine when glutathione was included in the reaction mixture (12). The importance of such binding as an index of toxicity, however, remains to be established.

In conclusion, we propose a mechanism for the HRP/$H_2O_2$ oxidation of *p*-phenetidine and the generation of reactive species which are capable of reacting with biological nucleophiles such as glutathione (Fig. 12). It is relevant that P-450-catalyzed activation of *p*-phenetidine results in the generation of reactive species different from that of peroxidase-catalyzed activation (33). Thus, the generation of the reactive species described (IV and V) and their glutathione conjugates *in vivo* after *p*-phenetidine administration may be a useful indicator of peroxidase-catalyzed metabolism. Whether such reactions occur *in vivo* in tissues rich in peroxidases and whether the formation of reactive species, or their glutathione conjugates during the peroxidatic oxidation of *p*-phenetidine has any relevance for phenacetin-induced nephrotoxicity are currently under investigation.

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