

Metabolic Activation of Phenol by Human Myeloperoxidase and Horseradish Peroxidase

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SUMMARY

The oxidation of phenol catalyzed by human myeloperoxidase and horseradish peroxidase resulted in extensive binding of phenol-derived metabolites to boiled rat liver protein. This binding paralleled closely the removal of phenol from the incubations and was inhibited from 83 to 99% by the addition of the antioxidants, ascorbate and glutathione, suggesting that metabolism and binding were occurring via a one-electron oxidation pathway. Metabolic studies employing both human myeloperoxidase and horseradish peroxidase resulted in the identification of 4,4'-biphenol and diphenoquinone as the principal identifiable metabolites. The addition of reduced glutathione to incubations containing horse-

radish peroxidase resulted in the formation of two conjugate species. These conjugate species were identified by fast atom bombardment mass spectrometry to be glutathione conjugates of diphenoquinone. The major glutathione conjugate was identified as 3-(glutathion-S-yl)-4,4'-biphenol by NMR spectroscopy. These results suggest that the formation of highly reactive species through the peroxidase-mediated metabolism of phenol and other phenolic compounds could play an important role in the hematopoietic toxicity observed during chronic benzene exposure.

Chronic exposure to benzene has been shown to lead to numerous blood and bone marrow disorders including pancytopenia, aplastic anemia, and leukemia (1, 2). Although the actual mechanism by which benzene exerts its hematopoietic effects appears to be complex, it is generally accepted that metabolic activation is required. Coadministration of toluene, a competitive substrate with benzene for cytochrome P-450 monooxygenase enzymes, and benzene reduced the levels of benzene metabolites in the bone marrow without affecting the benzene levels and resulted in protection against toxicity (3). Several studies have investigated cytochrome P-450 metabolism in the bone marrow and found the levels of cytochrome P-450 and the rates of benzene metabolism to be very low; such levels could not account for all of the accumulation and binding of radiolabeled compounds in the bone marrow (4-9). In addition, Sammett *et al.* (10) demonstrated that partial hepatectomy

in rodents also protected against benzene toxicity. These results suggested that metabolism within the liver was essential for toxicity but that toxicity was elicited distal from the liver in the bone marrow. A possible explanation for this is that stable benzene metabolites such as phenol, catechol, and hydroquinone travel from the liver to the bone marrow and there exert their toxic effects. Intravenous administration of the known benzene metabolites has failed to produce the same pattern of toxic effects observed after the administration of the parent compound (11, 12). Thus, it seems probable that it is not the hepatic metabolites of benzene which induce a myelotoxic response but the products of their further biotransformation within the bone marrow itself.

Bone marrow contains appreciable amounts of MPO which could be involved in the localized activation of benzene's metabolites (13). The target organ specificity demonstrated by benzene in recent animal carcinogenesis bioassays (Zymbal gland, Harderian gland, as well as others; Ref. 14) would also indicate a role for peroxidases in the metabolic activation of benzene since these target organs are known to contain significant peroxidase levels (15, 16).

Evidence for the involvement of peroxidases in the one-electron oxidation and metabolic activation of numerous xenobiotics has accumulated in recent years (17, 18). The perox-

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ABBREVIATIONS: MPO, myeloperoxidase; HRP, horseradish peroxidase; GSH, reduced glutathione; TCA, trichloroacetic acid; HPLC, high pressure liquid chromatography; FAB, fast atom bombardment; MS, mass spectrometry; GC, gas chromatography.

idase activity of prostaglandin synthase has been implicated in the nephrotoxicity induced by phenacetin (19) and acetaminophen (20) and in the induction of bladder cancer by nitrofurans (21) and aromatic amines (22). Peroxidases appear to be involved in the uterine toxicity and renal carcinogenicity induced by diethylstilbestrol (23) as well as in the induction of Harderian gland tumors by benzidine (24) and Zymbal gland tumors by *trans*-4-aminostilbene (15).

Phenol, the principal metabolite of benzene *in vivo* (1), has been shown to be a good reducing cofactor in the reduction of hydrogen peroxide by peroxidases. Early *in vitro* studies by Danner *et al.* (25) and Sawahata and Neal (26) have shown that phenol is metabolized to 2,2'-biphenol, 4,4'-biphenol, and diphenoquinone by HRP. Additional reports have indicated that phenol is converted to DNA- and protein-binding species during peroxidase-mediated metabolism and that the exogenous addition of antioxidants and sulfhydryl reagents had a protective effect (27, 28). Recent studies within our laboratory have demonstrated that protein binding of phenol occurs during the oxidative burst of human neutrophils and that this binding was due to peroxidase-mediated metabolism.¹ The purpose of this study was to investigate further the peroxidase-mediated metabolism of phenol by human MPO and HRP, to study the time course of protein-binding, and to identify the reactive protein-binding species.

Materials and Methods

Chemicals and enzymes. Phenol, 2,2'-biphenol, 4,4'-biphenol, and ascorbic acid were purchased from Aldrich Chemical Co., Milwaukee, WI. GSH, HRP type VI (275 units/mg) (EC 1.11.1.7), H₂O₂ (30% solution), and guaiacol were obtained from Sigma Chemical Co., St. Louis, MO. Catalase (65,000 units/mg) was obtained from Boehringer Mannheim. [¹⁴C]Phenol (*ring*-UL; 30.5 mCi/mmol) was purchased from Pathfinders Laboratories, St. Louis, MO. [³H]GSH (glycine-2-³H; 1000 mCi/mmol) was obtained from New England Nuclear, Boston, MA. All other chemicals or solvents were generally the highest grade available and purchased through local commercial suppliers. Deionized water was purified using a Millipore Q system obtained from Millipore, Bedford, MA.

Preparation of human MPO. Human neutrophils were isolated as described by Markert *et al.* (29). After sonication by a Braunsonic cell sonicator (for 10 min at 70 W) and centrifugation (550 × *g* for 5 min), the supernatant was assayed for peroxidase activity as described by Klebanoff *et al.* (30). This MPO was stored at -20° and, after an initial drop in activity, was found to be relatively stable during the period of experimentation.

Standard incubation mixture. The standard incubation mixture consisted of 500 μM phenol, 10 μg/ml HRP or 1.5 units/ml MPO, and 1 mM H₂O₂ in 100 mM phosphate buffer (pH 7.4). Reactions were initiated by the addition of H₂O₂ and were performed at 37° in a shaking water bath. For the metabolic studies and fraction collections, the incubation was stopped at 2 min (for HRP) or 3 min (for MPO) by the addition of 650 units of catalase. In reactions to identify the GSH conjugate, this quenching with catalase was followed 15 sec later by the addition of 5 mM GSH.

Protein binding experiments. Rat liver 9000 × *g* postmitochondrial supernatant was prepared as described previously (31) and boiled for 30 min. An aliquot of this preparation containing 0.9 mg of protein was added to the standard incubation mixture and incubated for various time periods up to 30 min. All treatment additions were performed prior to H₂O₂ addition. The reaction mixture was quenched by the

addition of TCA (5% final concentration). Samples were maintained on ice and centrifuged prior to analysis by HPLC with electrochemical detection. Covalent binding to protein was determined by liquid scintillation counting following the procedure of Jollow *et al.* (32) with the following modifications. The precipitated protein was washed several times with ice-cold ethanol:ether (1:1) in addition to several washes with 5% TCA and methanol:H₂O (4:1). To help solubilize the protein, Tris buffer containing 0.25% sodium lauryl sulfate was used in combination with the NaOH treatment. The pH of the solubilized protein was adjusted to neutral pH before aliquots were removed for liquid scintillation counting and protein determination as described by Lowry *et al.* (33).

HPLC with electrochemical detection. The apparatus employed in these studies consisted of an isocratic reverse phase HPLC system (Beckman model 100A) with an amperometric detector (Bioanalytical Systems LC-4A) equipped with a glassy carbon working electrode (BAS TL-5) and an Ag/AgCl reference electrode. A working potential of +1.0 V was used. The system employed a 25 cm × 4.6 mm i.d. C-18 column (5 μm), from Supleco, and a Rheodyne injector (model 7125) with a 10-μl injection loop. The mobile phase consisted of 90% 0.1 M ammonium acetate buffer (pH 4.0) and 10% acetonitrile with a flow rate of 2 ml/min. Quantitation of phenol was performed by comparison to standard curves based on area counts as determined by a Varian Vista 401 chromatography data system.

HPLC with UV detection. A gradient HPLC system consisting of Beckman (model 100A) and Altex (model 100) pumps controlled by a Beckman microprocessor (model 420), an Altex sample injector (model 210), a Supelco 25 cm × 4.6 mm C-18 column (5 μm), a Perkin-Elmer LC-75 spectrophotometric detector (254 nm), and a Perkin-Elmer computing integrator (model M-1) was employed for all metabolic studies and fraction collection. A 100-μl loop was used for all studies except those employing fraction collection for FAB-MS, NMR, and peak collection with tritiated glutathione which employed a 1000-μl loop. The mobile phase used was 90% acetonitrile (A) and 0.1 M ammonium acetate buffer (B; pH 4.0), and the flow rate was 1 ml/min. Conditions were 93% buffer B for the first 10 min, after which a linear gradient was employed over the next 20 min to reach a final concentration of 10% buffer B.

Gas chromatography/mass spectrometry. Analysis was performed on ethyl acetate extracts of the standard incubations taken to near-dryness under N₂ and derivatized with diazomethane. The MS experiments utilized a Hewlett-Packard 5985B instrument equipped with an HP1000 computer and a model 5840A gas chromatograph. Separations were accomplished on a high performance methyl silicone column (10 m) with temperature programming (80–240°, 20°/min) with helium carrier gas (1 ml/min). Analysis was by selected ion monitoring using chemical ionization with methane (0.8 torr) as ionizing gas. FAB was carried out with xenon at 8 kV on a ZAB-VG instrument with the samples dissolved in a glycerol matrix.

Nuclear magnetic resonance. NMR was accomplished on a Banker 300 MHz instrument equipped with an Aspect 3000 computer. Samples were dissolved in D₂O and chemical shifts (δ) were determined relative to acetonitrile.

Statistical analyses. Following one-way analysis of variance on the log transformed data, statistical significance was determined using a one-tailed Dunnett's *t* test for multiple comparisons. Critical values were calculated using a 0.05 probability of type 1 error.

Results

The extent of HRP- and MPO-catalyzed oxidation of phenol to reactive species which bind to boiled rat liver protein is shown in Fig. 1. Extensive binding occurred very rapidly and paralleled very closely the removal of phenol from the incubation, indicating that an early reaction product was responsible for the binding. This binding was demonstrated to be H₂O₂ and peroxidase dependent and was 83–99% inhibited by the addi-

¹ D. A. Eastmond, R. C. French, D. Ross, and M. T. Smith, submitted for publication.

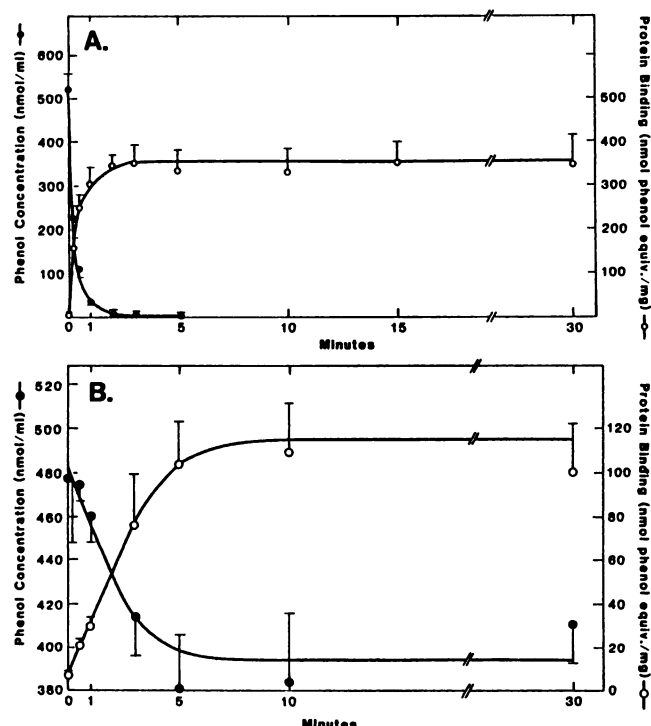


Fig. 1. Protein binding (○) and substrate removal (●) during the HRP (A)- and human MPO (B)-catalyzed oxidation of phenol. Incubation conditions are described in Materials and Methods. The data represent the means and standard deviations of three experiments.

tion of ascorbate or GSH to the incubation (Table 1). This binding was also shown to be protein dependent. When boiled protein was omitted from the incubations, the recovered radioactivity after TCA precipitation and solvent washes was less than 5% of that recovered in incubations containing protein. Phenol removal from the incubations was not observed with ascorbate and glutathione treatments, suggesting that these compounds were acting as antioxidants by reducing the phenoxyl radical back to phenol.

To understand further the peroxidase-mediated metabolic pathways and to identify the binding species, various additional analytical approaches were employed. The HRP- or MPO-catalyzed oxidation of phenol was accompanied by the formation of a yellow chromophore ($\lambda_{\max} = 399 \text{ nm}$) and brown polymeric compounds. This yellow chromophore corresponds to the spectrum reported for diphenoquinone (26). Analyses of

either of these enzymatic oxidation mixtures employing HPLC with UV detection and monitoring at 399 nm failed to detect a peak corresponding to diphenoquinone. Monitoring at 254 nm, HRP-catalyzed oxidations showed one principal peak produced during the incubation (Fig. 2A). This peak co-chromatographed with a known standard for 4,4'-biphenol. Identical incubations performed with ^{14}C -phenol, in which 1-min fractions eluting from the HPLC were collected and analyzed by liquid scintillation counting, showed that the radioactivity co-eluted primarily with 4,4'-biphenol and phenol (Fig. 2A). In control experiments lacking HRP, virtually all of the radioactivity co-eluted with phenol (Fig. 2C). Confirmation of these results using HRP as an enzyme source was obtained by GC-MS. Analysis of the incubation products after extraction in ethyl acetate and derivatization by diazomethane showed one principal product which was identical in terms of chromatic retention time and mass spectrum to a derivatized standard of 4,4'-biphenol. Products corresponding to 2,2'-biphenol and diphenoquinone were also observed, but at levels only barely distinguishable from background. Similar results, but of a lesser magnitude, were obtained in incubations containing MPO. Since the metabolic profiles for HRP and human MPO appeared to be qualitatively identical, further analyses employed only HRP as the enzyme source.

In an attempt to trap the binding species, GSH was added to HRP incubations after 2 min of reaction. An immediate decrease in the yellow color of the incubation was observed. Analysis by HPLC with UV detection after the addition of glutathione resulted in a significant decrease in the 4,4'-biphenol peak with the formation of two additional peaks (1 and 2, Fig. 2B). Incubations using ^{14}C -phenol resulted in a decrease in radioactivity co-eluting with 4,4'-biphenol and increases in radioactivity co-eluting with the two new peaks 1 and 2 (Fig. 2B). Incubations using ^3H -glutathione, HRP, and unlabeled phenol showed major increases in radioactivity associated with the two new peaks. These data suggested that peaks 1 and 2 were glutathione conjugates but did not provide any evidence for the identity of the conjugating species. Authentic 4,4'-biphenol remained unchanged when mixed with glutathione and did not produce the putative glutathione conjugates represented by peaks 1 and 2. When 4,4'-biphenol, HRP, and glutathione were mixed, no removal of 4,4'-biphenol nor the appearance of peaks 1 and 2 was observed, whereas identical incubations including H_2O_2 in addition to the 4,4'-biphenol, HRP, and glutathione resulted in the formation of the putative

TABLE 1
Effect of various treatments on the HRP- and MPO-catalyzed metabolism of phenol

Additions	HRP/ H_2O_2		MPO/ H_2O_2	
	Phenol equivalent bound	Phenol remaining	Phenol equivalent bound	Phenol remaining
	nmol/mg	nmol/ml	nmol/mg	nmol/ml
None*	348 ± 66	1.4 ± 0.7 ^b	100 ± 19	410 ± 18
- H_2O_2	5 ± 2 ^c	523 ± 39	7 ± 1 ^c	478 ± 30
-Peroxidase	4 ± 1 ^c	ND ^d	7 ± 1 ^c	ND
-Phenol	1 ± 1 ^c	ND	1 ± 0 ^c	ND
+1 mM Ascorbate	60 ± 24 ^c	402 ± 112	12 ± 1 ^c	465 ± 24
+5 mM GSH	3 ± 1 ^c	478 ± 79	12 ± 4 ^c	447 ± 21

* Standard incubation conditions are described in Materials and Methods.

^b This value was determined after a 5-min incubation. All other values were determined after 30-min incubations.

^c These values differ significantly from the control (none) values, $p < 0.05$.

^d ND, not determined.

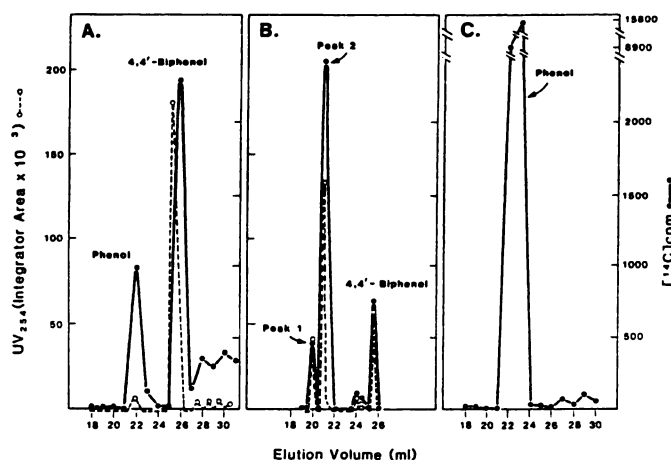


Fig. 2. UV/ ^{14}C HPLC elution profiles: A, HRP-catalyzed oxidation of phenol; B, a similar incubation with the addition of 5 mM glutathione after 2 min of reaction; and C, an incubation similar to that in A but lacking HRP. Incubation conditions are described in Materials and Methods.

TABLE 2

Profile of the metabolites identified during the HRP-catalyzed oxidation of phenol

	^{14}C -Phenol nmol	^3H -GSH nmol
Standard incubation ^a		
4,4'-Biphenol	23.4	
Standard incubation + GSH ^b		
4,4'-Biphenol	4.7	
Peak 1	4.1	4.0
Peak 2	11.8 ^c	11.9

^a Incubations contained 500 μM phenol, 10 $\mu\text{g}/\text{ml}$ HRP, and 1 mM H_2O_2 in 0.1 M phosphate buffer (pH 7.4). The reactions were stopped at 2 min by the addition of catalase.

^b Standard incubation conditions were followed but glutathione (5 mM) was added 15 sec after the catalase addition. Catalase (650 units/ml) was added at 2 min for the ^{14}C incubations, whereas 13,000 units/ml catalase were added at 2 min for the ^3H incubations. Under similar conditions containing protein, 96–98% of the phenol was removed from the incubations and 60% of the phenol equivalents were recovered as protein bound (see Fig. 1A).

^c This value was calculated after correction for unmetabolized phenol collected in the same fraction.

conjugates and removal of 4,4'-biphenol. These data show that the conjugating species was not 4,4'-biphenol itself but a common peroxidative oxidation product of both 4,4'-biphenol and phenol. This compound was presumably diphenoquinone, which could be easily reduced to 4,4'-biphenol during HPLC and GC-MS analyses. This quinone could then react directly with glutathione to form the two conjugates. A summary of the metabolic profile for the HRP/ H_2O_2 -catalyzed oxidation of phenol is shown in Table 2.

The HPLC eluate containing each of the conjugate peaks was frozen and lyophilized before being subjected to FAB-MS and NMR. The identities of peaks 1 and 2 were confirmed as glutathione conjugates of diphenoquinone by FAB-MS and NMR spectroscopy (Fig. 3). The mass spectrum of peak 2 (Fig. 3A) exhibits diagnostic masses for a glutathione addition product of 4,4'-biphenol as follows: M and M + 1 at m/z 491 and 492, corresponding sodium adducts on NaCl addition at m/z 513 and 514, and a glycerol adduct at m/z 575. In addition, the base peak (not shown) was at m/z 307 (glutathione). Peak 1 gave a similar FAB spectrum. Examination of the downfield region of the NMR spectrum of peak 2 confirms the presence of a substituted 4,4'-biphenol (Fig. 3B) indicating one unsub-

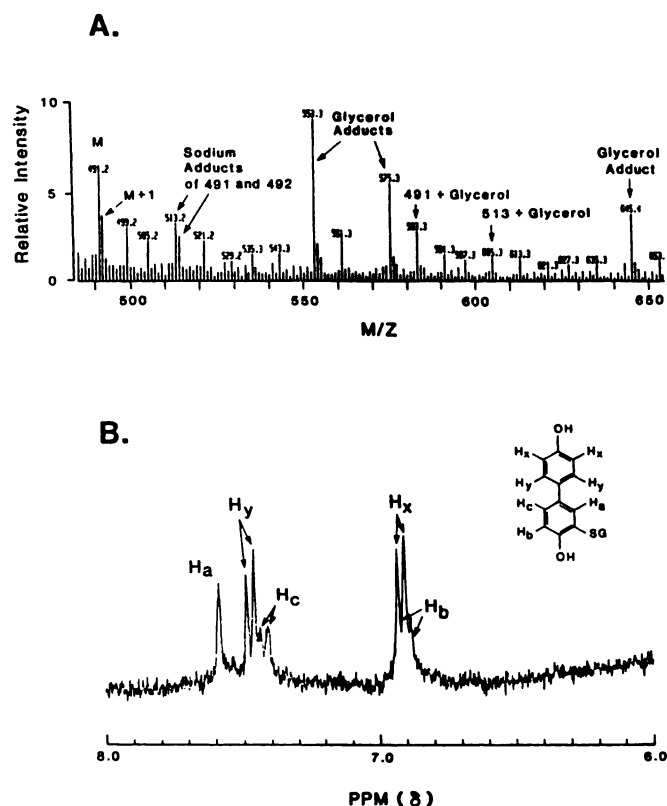


Fig. 3. FAB mass spectrum (A) and NMR spectrum of the aromatic region (B) of peak 2, the major glutathione conjugate isolated by HPLC. Analytical conditions are described in Materials and Methods. Coupling constants in Hz are as follows: $H_y = 8.4$; $H_x = 8.4$; $H_b = 9.2$; and $H_c = 9.2$. The upfield region of the NMR spectrum (2–5 ppm) exhibits resonances consistent with the presence of the glutathione moiety.

stituted ring (H_x and H_y doublets) and that the other ring contains a substituent at the 3-position since H_a appears as a singlet and H_b (partially obscured) and H_c exhibit the characteristic *ortho*-coupling for the structure shown (Fig. 3B). Although NMR is not available for peak 1 due to the paucity of material, it is proposed to be the corresponding glutathione adduct at the 2-position.

Discussion

The peroxidase-catalyzed binding of phenol to protein and DNA has been reported previously by several investigators (9, 26–28, 34, 35).² In these studies we have confirmed these reports and shown that extensive phenol binding occurs very rapidly with human MPO as well as with HRP and parallels very closely the removal of phenol from the incubation. The dependence of this binding on H_2O_2 , peroxidase, phenol, and protein demonstrates that the formation of these reactive metabolites was peroxidase mediated and indicates that these metabolites were actually protein bound. The inhibition of binding observed when ascorbate or glutathione was included in the incubation could be due to their antioxidant properties or to their ability to act as competitive substrates for the peroxidase enzymes. In recent experiments, Subrahmanyam and O'Brien (34) reported that little direct oxidation of ascorbate took place in H_2O_2 and HRP incubations in the absence

² D. A. Eastmond, R. C. French, D. Ross, and M. T. Smith, submitted for publication.

of phenol. However, in the presence of phenol, a rapid oxidation of ascorbate took place (34). In addition, other studies have failed to demonstrate an ESR signal from glutathione directly during HRP/H₂O₂ incubations but have shown a thiyl radical signal when the phenolic compound diethylstilbestrol was included in the incubation (36). These results indicate that both ascorbate and glutathione are functioning as antioxidants and are reducing the phenoxy radical back to phenol.

In these experiments, 4,4'-biphenol and diphenoquinone were the principal identifiable reaction products, whereas a trace amount of 2,2'-biphenol was detected by GC-MS. Other investigators have generally observed similar results, although 2,2'-biphenol was usually a more prominent metabolic product (25, 26, 34, 35). The reason for these differences in relative ratios of metabolic products is most likely the slight modifications in experimental conditions. In these experiments relatively high concentrations of HRP were used with a theoretical excess of H₂O₂ in order to effect a total removal of phenol. Due to the high affinity of 2,2'-biphenol for HRP and a reported increase in reaction rate in the presence of phenol (25), it is possible that the 2,2'-biphenol was formed rapidly during the incubation and subsequently converted to polymeric products which were not detected in our analyses.

The use of the nucleophilic tripeptide glutathione as an agent to trap binding species during metabolism has been reported previously (37). Our use of glutathione as a trapping agent resulted in the formation of two glutathione conjugates of diphenoquinone, 3-(glutathion-S-yl)-4,4'-biphenol, and another product, probably 2-(glutathion-S-yl)-4,4'-biphenol. The identification of 3-(glutathion-S-yl)-4,4'-biphenol as the principal glutathione conjugate was based upon MS, radioisotope, and NMR results, whereas the structure of the secondary conjugate, 2-(glutathion-S-yl)-4,4'-biphenol, was tentatively identified based upon MS and radioisotopic evidence. The conclusive identification of two species as glutathione conjugates of diphenoquinone represents the first definitive identification of a binding metabolite formed during the peroxidase-mediated metabolism of phenol. Although our HPLC results indicated that, in incubations containing HRP, 96–98% of the phenol was removed in 2 min, the subsequent trapping of diphenoquinone yielded only 6% of the phenol equivalents as glutathione conjugates. Under these conditions, 60% of the phenol equivalents were recovered as protein bound, indicating that other species formed during HRP/H₂O₂-catalyzed metabolism were responsible for most of the binding. These other species could possibly be the phenoxy or C-centered radicals, further oxidation products of 2,2'-biphenol or 4,4'-biphenol, or other polymerization products. Subrahmanyam and O'Brien (34, 35) have recently studied the HRP/H₂O₂-catalyzed oxidation of phenol and its binding to DNA. Their results indicate that a polymerization product of 2,2'-biphenol is a major DNA-binding species formed from phenol. A summary of the known metabolites formed during peroxidase-mediated metabolism of phenol and possible routes for the formation of binding metabolites is shown in Fig. 4.

The demonstration that highly reactive binding species are formed from phenol during peroxidase-mediated metabolism could be of importance in understanding the hematopoietic toxicity of benzene since phenol is the principal metabolic product formed from benzene *in vivo* (1) and the formation of DNA- and protein-binding products are often related to cyto-

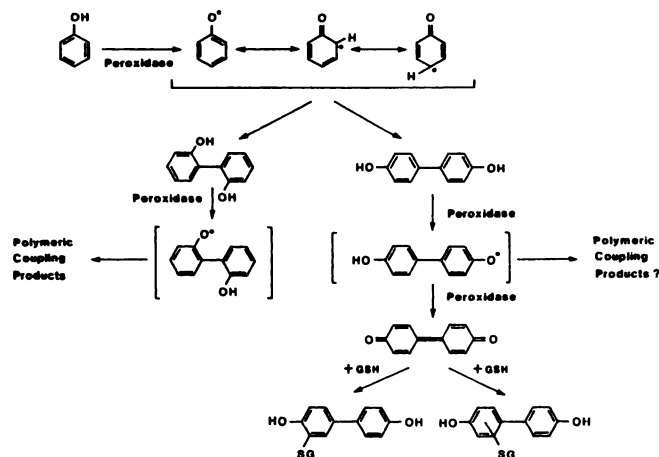


Fig. 4. Proposed scheme for the peroxidase-mediated metabolism of phenol and the reaction of diphenoquinone with glutathione to form the two identified glutathione conjugates.

toxic and genotoxic effects in cells (38, 39). There have been very few studies to examine the direct cytotoxic and genotoxic effects of the peroxidase-mediated metabolites of phenol. Nishioka and Ogasawara (40) reported that 2,2'-biphenol but not 4,4'-biphenol exhibited mutagenicity in *Escherichia coli* when tested in the DNA repair test. These investigators also reported that neither 2,2'-nor 4,4'-biphenol were mutagenic in strains TA98 and TA100 in the Ames *Salmonella* reversion assay. Recently, Erexson *et al.* (41) studied the effects of diphenoquinone, and 2,2'- and 4,4'-biphenol on the induction of sister chromatid exchange, changes in mitotic indices, and interference with cell cycle kinetics in human lymphocytes. The 2,2'- and 4,4'-biphenols induced slight but significant increases in sister chromatid exchange frequency, reductions in mitotic activity, and inhibition of cell cycle progression, whereas diphenoquinone caused only a reduction in mitotic activity. In addition, 4,4'-biphenol and diphenoquinone caused cytotoxicity to the cultured lymphocytes at fairly low concentrations, which suggests that these metabolites could be contributing to bone marrow cytotoxicity *in vivo*.

In summary, we present evidence to show that phenol is converted to highly reactive protein-binding species during metabolism catalyzed by human MPO and by HRP and that the mechanism probably involves the formation of a free radical intermediate. The peroxidase-mediated metabolism of phenol was shown to result in the formation of 4,4'-biphenol and diphenoquinone and that diphenoquinone is one of the binding species produced during metabolism. The identification of the other binding species and the relevance of these findings to the hematopoietic toxicity of benzene *in vivo* will require further investigation.

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