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DT-Diaphorase and Peroxidase Influence the Covalent Binding of the Metabolites of Phenol, the Major Metabolite of Benzene

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SUMMARY

The role of various enzymes and biological molecules on the activation and deactivation of the metabolites of phenol was investigated *in vitro*. Phenol, the major metabolite of benzene, is metabolized to hydroquinone and catechol. Activation of these metabolites and deactivation of their oxidized forms was assessed by the amount of covalent binding to microsomal protein. [^{14}C]Phenol and NADPH were incubated with hepatic microsomes isolated from phenobarbital-pretreated guinea pigs, and 2.33 nmoles of hydroquinone and 0.12 nmole of catechol were formed per minute per milligram of microsomal protein. Covalent binding of the metabolites to microsomal protein incubated with microsomes isolated from guinea pigs pretreated with phenobarbital was 252 pmoles bound/min/mg; with microsomes from untreated guinea pigs, covalent binding was 146 pmoles bound/min/mg. Covalent binding was inhibited greater than 90% with the addition of *N*-octylamine, ascorbate, or GSH. The addition of superoxide dismutase inhibited covalent binding with microsomes isolated from phenobarbital-pretreated guinea pigs 35% but did not inhibit it with microsomes isolated from untreated animals. Partially purified guinea pig hepatic DT-diaphorase [NAD(P)H (quinone acceptor) oxidoreductase, EC 1.6.99.2] inhibited covalent binding 70%. This effect was reversed in the presence of dicumarol, a specific inhibitor of DT-diaphorase. DT-diaphorase present in the $10^5 \times g$ supernatant fraction was also active in inhibiting covalent binding but only after the removal of endogenous reduced glutathione. This effect could also be reversed by dicumarol. The addition of diaphorase (NADH:lipoamide oxidoreductase, EC 1.6.4.3) partially purified from *Clostridium kluyveri* inhibited covalent binding 86%. The addition of hydrogen peroxide and horseradish peroxidase (peroxidase, EC 1.11.17) or myeloperoxidase(s) increased covalent binding 30-fold and 6-fold, respectively. Ascorbate decreased this binding greater than 95%. These results indicate that hydroquinone, catechol, and phenol as well as their oxidized forms can be activated or deactivated by several of the above model systems. These systems may play a role in the myelotoxicity of benzene by modulating covalent binding.

INTRODUCTION

Upon chronic exposure, benzene produces hemopoietic toxicity and in severe cases results in aplastic anemia (1, 2). Benzene has also been implicated as a human leukemogen (3). It is generally accepted that benzene requires metabolic activation to produce its toxicity. Several reports in the literature support this (4–8). Tunek *et al.* (9) found that a further metabolite of phenol, and not benzene oxide, was responsible for the majority of *in vitro* covalent binding to microsomal protein. Hydroquinone and/or catechol also accumulate or persist in the bone marrow when benzene, catechol, or hydroquinone is administered (10–12). Administration of hydroquinone and catechol resulted in a significant amount of covalent binding as measured in the bone marrow (12). Recently, the MFO[1] metabolism of phenol has provided additional information regarding the covalent binding of these metabolites (13). When *N*-acetylcysteine was included in the incubation mixture, covalent binding was completely inhibited with the corresponding formation of the *N*-acetylcysteine adducts of both hydroquinone and catechol (13). Greenlee *et al.* (12) proposed that covalent binding of hydroquinone and/or catechol in the bone marrow may be an important event in the expression of benzene's toxicity. They postulate that this binding is

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[1]The abbreviations used are: MFO, mixed-function oxygenase; HRP, horseradish peroxidase; PB, phenobarbital.

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due to a highly reactive semiquinone or quinone metabolites.

With regard to benzene or phenol metabolism, if the oxidized forms of hydroquinone or catechol are involved in covalent binding, then the effect of reducing these metabolites by DT-diaphorase (EC 1.6.99.2) warrants investigation. How the metabolites of phenol can be activated and deactivated by biological molecules and enzymes is important in determining the molecular mechanism of the selective toxicity of benzene. The present study is concerned with the effect of such enzymes as HRP, myeloperoxidase, and DT-diaphorase, and also the effect of such biological molecules as ascorbate and GSH, on the activation and deactivation of the metabolites of phenol. The delineation of the role of semiquinones versus quinones in covalent binding is also examined.

EXPERIMENTAL PROCEDURES

Materials. 1,2-Epoxy-3,3,3-trichloropropane, phenol, dicumarol, *p*-benzoquinone, hydroquinone, catechol, and 4-chlororesorcinol were purchased from Aldrich Chemical Company (Milwaukee, Wisc.). [^{14}C] Phenol (8.3 mCi/mmole) was purchased from New England Nuclear Corporation (Boston, Mass.). After adjustment of the specific activity to 0.16 mCi/mmole, [^{14}C]-phenol was extracted from a benzene solution by the addition of 2 ml of 0.15 N NaOH followed by extraction into 95% ethanol and neutralization with hydrochloric acid. The purity of [^{14}C]phenol was >99%. Radiochemical purity was >98% as determined by thin-layer chromatography (9). Sodium ascorbate, *S*-adenosyl-L-methionine, *N*-octylamine, diethyl maleate, superoxide dismutase, catechol *O*-methyltransferase, and diaphorase (lipoamide dehydrogenase) were purchased from Sigma Chemical Company (St. Louis, Mo.). Reduced glutathione and 3% hydrogen peroxide were purchased from Fisher Scientific Company (Fair Lawn, N. J.). Catalase was purchased from Boehringer Mannheim (Indianapolis, Ind.). HRP was purchased from Worthington Biochemical Corporation (Freehold, N. J.). ACS scintillation cocktail was purchased from Amersham (Arlington Heights, Ill.). The gas chromatography column and packing were purchased from Supleco, Inc. (Bellefonte, Pa.).

Guinea pig pretreatment and preparation of hepatic microsomes. Male Hartley guinea pigs (250–350 g) were treated with PB (1 mg/ml in the drinking water) for 5 days. Animals were decapitated on day 6, and their livers were removed, immediately placed on ice, and homogenized in 4 volumes of 100 mM sodium phosphate buffer (pH 7.4). The homogenates were centrifuged at 15,000 × *g* for 20 min, and the postmitochondrial supernatant was centrifuged at 100,000 × *g* for 1 hr to harvest the microsomes. The microsomes were made 4 times concentrated (16–20 mg of protein per milliliter) based on the initial supernatant volume. Frozen microsomes maintained activity for at least 3 months if stored at −20°. Microsomes prior to use were washed with 4 volumes of buffer, homogenized, and centrifuged at 100,000 × *g* for 30 min. The supernatant was decanted and the pellet was brought up to 6–7 mg of protein per milliliter. Protein was determined by the method of Lowry *et al.* (14), using bovine serum albumin as the standard.

Incubation conditions and assay for covalent binding. The standard incubation mixture consisted of washed microsomes (0.7–1 mg of protein per milliliter) which were added to 1 mM [^{14}C]phenol or phenol/1 mM NADPH/100 mM sodium phosphate buffer (pH 7.4). The total incubation volume was 1 ml. The incubation was carried out for 10 min at 37° in a Dubnoff shaker at 60 oscillations/min. This incubation mixture was used for the covalent binding assay and also for the quantitation of metabolites. Covalent binding was measured by the method of Tunek *et al.* (9). This method involves extraction of the incubation mixture three times with ethyl acetate to remove substrate and product, followed by extraction and precipitation of the protein with 50% ethanol. The protein was washed with an acetone:heptane mixture, centrifuged, and washed with methanol (9). The protein pellet was dissolved in 1 N NaOH and heated at 80° for 30 min. The clarified solution was neutralized with HCl, and an aliquiot was added to scintillation cocktail and counted. In the absence of NADPH, less than 15 pmoles of phenol equivalents were bound per minute per milligram of microsomal protein. This value was determined for each experiment and was subtracted from each experimental value reported. When either DT-diaphorase or diaphorase was added to the binding assay, 1 μM FAD was added to optimize enzyme activity (15). This concentration of FAD had no effect on covalent binding. GSH was depleted in the $10^5 \times g$ liver supernatant fraction (2.5 mg of protein per milliliter) by a final concentration of 10.8 mM diethyl maleate. The amount of GSH before and after (nondetectable) this treatment was measured by the method of Ellman (16). The final concentration of diethyl maleate in the incubation mixture used for covalent binding was between 0.25 and 1 mM, depending on the amount of supernatant used. Magnesium chloride (1 mM) was added to the incubation containing catechol *O*-methyltransferase or *S*-adenosyl-L-methionine.

Identification of hydroquinone and catechol. The products of phenol metabolism, hydroquinone, and catechol were extracted from the incubation mixtures and identified by gas chromatographic analyses. The reactions were stopped at various times with 0.5 ml of 10 N HCl and placed on ice for 10 min. 4-Chlororesorcinol (12.5 μg) was added to each tube as an internal standard. The incubation mixtures were centrifuged at 3000 × *g* for 5 min, and the acidified supernatant was removed. The supernatant was extracted four times with 1 ml of ethyl acetate. Ethyl acetate was evaporated to 0.2 ml at room temperature with a gentle stream of nitrogen. Twenty microliters of acetic anhydride, 20 μl of pyridine, and 2.5 ml of ethyl ether were added, and the samples were derivatized at 70° for 1 hr or until all of the ethyl ether had evaporated and only 0.2 ml of ethyl acetate remained. Samples were cooled to room temperature, and 0.2 ml of 0.5 N NaOH was added to remove excess reagent. The ethyl acetate phase was washed with 0.2 ml of pentane and 0.2 ml of distilled water and centrifuged at 3000 × *g* for 5 min; 2 μl were injected into a Varian 3700 gas chromatograph equipped with a flame ionization detector. The column (2 m × 2 mm inner diameter) was packed with 1% SP-1240-DA on 100/120 Supelcoport; the column and packing were purchased from Supelco. The injector temperature was 220°, column 157°, and detector 250°. The flow rate was 40 ml/min, and standard curves were obtained and plotted as the peak height ratios, catechol/4-chlororesorcinol and hydroquinone/4-chlororesorcinol. The ratio of catechol and hydroquinone formed from phenol to the standard, 4-chlororesorcinol, provided a direct index of the amount of product formed from the enzymatic incubation of phenol and NADPH. When NADPH was omitted from this incubation mixture, catechol and hydroquinone could not be detected.

Identification of phenol. Phenol was extracted from the incubation mixture and identified by gas chromatographic analyses using the same method as described above. However, the flow rate was decreased to 15 ml/min and the column temperature was decreased to 120°; after the phenol peak, the column temperature was increased to 180° to hasten the elution of the standard.

Assay of DT-diaphorase. Assays were performed using a modified method of Ernster *et al.* (17) and Benson *et al.* (15). The cuvettes contained 100 mM sodium phosphate buffer (pH 7.4), 250 μM NADH or NADPH, 5 μM FAD, bovine serum albumin (250 μg), and 50 μM *p*-benzoquinone or 100 μM *o*-benzoquinone, in a final volume of 1 ml at 25°. The supernatant fraction ($10^5 \times g$, 2.5 μg of protein) or partially purified DT-diaphorase (0.08 μg of protein) was added to the cuvette to start the reaction. The control cuvette contained all of the additions except enzyme; this resulted in the subtraction of the non-enzymatic reduction of *p*-benzoquinone by either NADH or NADPH. A final concentration of 10 μM dicumarol inhibited the reaction greater than 90%. The disappearance of NADH or NADPH was monitored at 340 nm using a molar absorption of 6.2×10^3 M^{-1} cm^{-1}. The apparent K_m

was determined by a double-reciprocal plot using the least-squares method.

Purification of DT-diaphorase. DT-Diaphorase was partially purified from male Hartley guinea pig livers by a revised method of Ernster *et al.* (17, 18). The livers were homogenized with 3 volumes of 10 mM sodium phosphate buffer (pH 6.4). The homogenate was centrifuged at 15,000 × *g* for 20 min and the postmitochondrial fraction was centrifuged at 100,000 × *g* for 1 hr. This supernatant was fractionated with ammonium sulfate. The 65–85% precipitate contained 30% of the initial activity and was further purified. The ammonium sulfate precipitate (5 ml) was dialyzed overnight against 4 liters of 10 mM sodium phosphate buffer (pH 6.4). The dialysate was applied to a DEAE-cellulose column. Ninety-eight per cent of the enzyme activity was found in the void volume. This void volume was put on another DEAE-cellulose column, and the subsequent void volume was collected and assayed. The specific activity using *p*-benzoquinone as the substrate was 33.3–58.3 μmoles of product formed per minute per milligram of protein, over 100-fold higher than the original homogenate. The activity could be inhibited with 10 μM dicumarol.

Preparation of a red blood cell-free homogenate of myeloperoxidase. Femurs were removed from five male Hartley guinea pigs weighing between 500 and 550 g. The marrow was removed from each femur and pooled in total volume of 50 ml of 0.07 M sodium phosphate buffer (pH 7.4). The preparation of the red cell-free homogenate was then prepared by the method of Himmelhoch *et al.* (19).

Statistical analyses. Data were subjected to statistical analyses, using Student's *t*-test.

RESULTS

Quantitation of the metabolites of phenol and their covalent binding to protein. The metabolism of phenol was studied using washed hepatic microsomes isolated from PB-treated guinea pigs. Under these assay conditions, 2.33 nmoles of hydroquinone and 0.12 nmoles of catechol per minute per milligram of microsomal protein were produced as measured by the gas chromatographic method described under Experimental Procedures. The rate of the reaction was linear for 15 min and proportional to the microsomal protein concentration. A protein concentration of 0.7–1 mg/ml and an incubation time of 5 or 10 min was used throughout all of the binding experiments. The generation of reactive metabolites of [^{14}C]phenol was assayed by the amount of covalent binding to microsomal protein. Incubation with NADH or NADPH with microsomes isolated from PB-pretreated or untreated guinea pigs resulted in covalent binding of phenol metabolites to microsomal protein (Table 1). In the presence of NADPH and microsomes from PB-pretreated guinea pigs, approximately 10% of the phenol metabolites were covalently bound. The amount of binding was linear with time and proportional to microsomal protein concentration.

Factors affecting covalent binding. Various additions were made to the standard incubation mixture to study their effect on the amount of covalent binding to microsomal protein (Table 2). As shown in Table 2, GSH and ascorbate inhibited binding greater than 90%. Ascorbate has been shown to reduce benzoquinones to hydroquinones (20). GSH has been shown to form adducts with benzoquinones (21), and recently *N*-acetylcysteine adducts have been demonstrated for hydroquinone and catechol (13). *N*-Octylamine was added to the incubation mixture to inhibit the MFO system. *N*-Octylamine (5 mM) inhibited covalent binding 90%; this concentration inhibited the *O*-demethylation of *p*-nitroanisole greater than 90%. Trichloropropane oxide (1 mM), a known inhibitor of microsomal epoxide hydrolase, increased covalent binding only slightly; this concentration inhibited the formation of styrene glycol from styrene oxide greater than 90%.

DT-Diaphorase and reduction of o- and p-benzoquinones. The incubation of *p*-benzoquinone with the 10^5 × *g* hepatic supernatant in the presence of NADPH or NADH resulted in their disappearance at 340 nm. Both NADPH and NADH were capable of reducing *p*-benzo-

TABLE 1

Covalent binding of [^{14}C]phenol metabolites to hepatic microsomal protein in vitro

[^{14}C]Phenol (1 mM) was incubated with 0.7–1 mg of washed hepatic microsomal protein isolated from PB-pretreated guinea pigs or 0.7– 1 mg of washed hepatic microsomal protein isolated from untreated guinea pigs, NADPH (1 mM), and sodium phosphate buffer (100 mM, pH 7.4). The incubation was carried out for 10 min at 37°. The total volume was 1 ml.

Conditions	Covalent binding[a] (pmoles phenol equivalents bound/min/mg microsomal protein)
NADPH (1 mM), PB-induced	252.3 ± 20.1
NADPH (1 mM), non-induced	147.9 ± 6.4
NADH (1 mM), PB-induced	45.7 ± 9.9

[a] Each experiment was performed three times in duplicate. Values are means ± standard deviation.

TABLE 2

Effect of additions on covalent binding of [^{14}C]phenol metabolites to hepatic microsomal protein in vitro

[^{14}C]Phenol (1 mM) was incubated with 0.7–1 mg of washed hepatic microsomal protein isolated from PB-pretreated guinea pigs, NADPH (1 mM), and sodium phosphate buffer (100 mM, pH 7.4). The incubation was carried out for 10 min at 37°. The total volume was 1 ml.

Addition	Covalent binding[a] (pmoles phenol equivalents bound/min/mg microsomal protein)
None (control with NADPH)	252.7 ± 12.3 (4)
Glutathione (1 mM)	1.0 ± 1.9[b] (4)
Ascorbate (1 mM)	13.4 ± 1.7[b] (4)
S-Adenosyl-L-methionine (1.5 mM)	240.5 ± 7.7 (3)
S-Adenosyl-L-methionine (1.5 mM) + catechol *O*-methyltransferase (42 μg)	211.1 ± 7.9[b] (3)
N-Octylamine (5 mM)	19.6 ± 0.8[b] (3)
SKF 525-A (1 mM)	204.6 ± 17.5[b] (3)
Superoxide dismutase (55 μg)	161.4 ± 4.3[b] (6)
TCPO[c] (1 mM)	287.4 ± 8.7[b] (5)
NADPH (1 mM), non-induced[d]	146.6 ± 3.5 (3)
Superoxide dismutase (55 μg), non-induced	147.9 ± 6.4 (3)

[a] Numbers in parentheses are numbers of experiments performed in duplicate. Values are means ± standard deviation.

[b] Significantly different from control (with NADPH) ($p < 0.01$).

[c] TCPO, 1,2-epoxy-3,3,3-trichloropropane.

[d] Washed hepatic microsomal protein (0.7–1 mg) isolated from untreated guinea pigs.

quinone via an enzymatic reaction. The reaction rate was linear with time and proportional to protein concentration. There was no reaction with supernatant which had been boiled for 5 min. Dicumarol (10 μM), a specific and potent inhibitor of DT-diaphorase, inhibited the oxidation 90–95% of both NADPH or NADH with a corresponding inhibition in the reduction of *p*-benzoquinone. The addition of bovine serum albumin stimulated the activity of the enzyme. FAD was required for optimal activity of the enzyme. NADH and NADPH had approximately equal activities with this enzyme. The above are specific properties of DT-diaphorase (15, 17, 18). The apparent K_m of DT-diaphorase for *p*-benzoquinone was determined to be 8.4 μM, and the theoretical V_{max} was determined to be 624 nmoles/min/mg of supernatant protein. *o*-Benzoquinone is also a substrate for DT-diaphorase, but its apparent K_m and V_{max} could not be determined because of its instability.

Effect of DT-diaphorase on covalent binding. The addition of 200 μg of $10^5 \times g$ hepatic supernatant to the standard incubation mixture inhibited covalent binding 66% (Fig. 1). If 20 μM dicumarol was included in the incubation mixture, very little effect was observed. When 10.8 mM diethyl maleate was incubated with the supernatant for 30 min, all of the GSH was depleted. If 200 μg of this supernatant were then added to the standard incubation mixture, there was a 26% inhibition in covalent binding (Fig. 1) as compared with 66% inhibition using the supernatant not depleted of GSH. If 20 μM dicumarol was added to an incubation mixture containing the GSH-depleted supernatant, a further decrease in the inhibition of covalent binding was observed from 26% to 5% inhibition (Fig. 1). This represents the contribution of DT-diaphorase. The concentration of diethyl maleate used in these studies had no effect on DT-diaphorase activity as determined by the spectrophotometric assay.

DT-Diaphorase was partially purified from guinea pig liver as described under Experimental Procedures. The addition of 2.0 units to the incubation mixture inhibited covalent binding 70% (Fig. 2). Addition of 20 μM dicumarol reversed this effect over 90% (Fig. 2). The purified preparation exhibited the specific properties of DT-diaphorase described previously. The addition of 2.0 units of partially purified diaphorase (lipoamide dehydrogenase) from *Clostridium kluyveri* inhibited binding 86% (Fig. 3). Both *p*-benzoquinone and *o*-benzoquinone are substrates for this enzyme as determined by the assay described under Experimental Procedures.

Effect of HRP, myeloperoxidase, and hydrogen peroxide on covalent binding. Phenol is oxidized by HRP and myeloperoxidase (22). The addition of HRP and hydrogen peroxide to microsomes in the absence of NADPH increased covalent binding 30-fold (Table 3) as compared with the standard binding assay in the presence of NADPH (Table 1). The addition of 1 mM ascorbate decreased this binding greater than 99% (Table 3). To determine whether ascorbate was preventing the oxidation of phenol by HRP and hydrogen peroxide, the concentration of phenol in the incubation mixture was measured in the presence and absence of ascorbate. In the absence of ascorbate, 57% of phenol was oxidized as measured by the gas chromatographic method described under Experimental Procedures. In contrast, in the presence of ascorbate, less than 3% of the phenol was oxidized. These experiments were carried out under the same conditions described in Table 3. The addition of

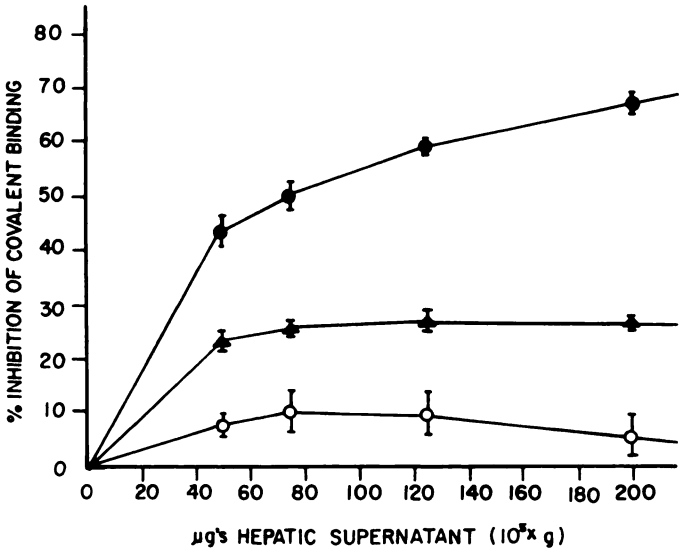


FIG. 1. *Effect of $10^5 \times g$ hepatic supernatant fraction, diethyl maleate, and dicumarol on covalent binding*

Assays were conducted as described under Experimental Procedures. ●——●, With $10^5 \times g$ supernatant; ▲——▲, with $10^5 \times g$ supernatant preincubated with 10.8 mM diethyl maleate; ○——○, with $10^5 \times g$ supernatant preincubated with 10.8 mM diethyl maleate added to the incubation containing 20 μM dicumarol. Each point represents the average of three experiments carried out in duplicate ± standard deviation.

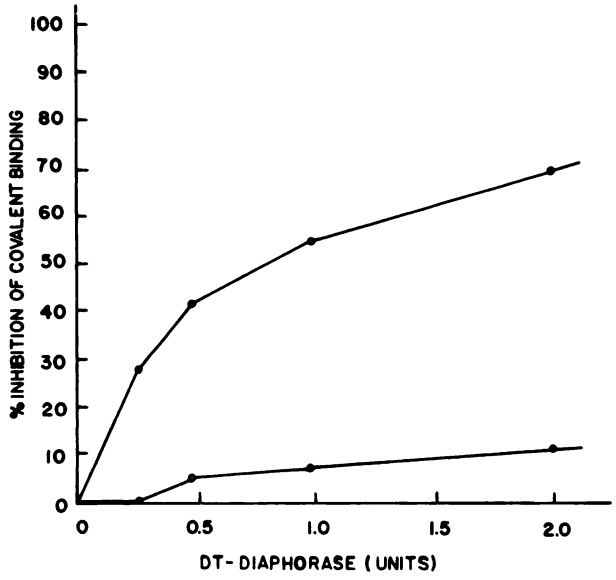


FIG. 2. *Effect of partially purified guinea pig hepatic DT-diaphorase on covalent binding*

All assays were conducted as described under Experimental Procedures. One unit of activity is defined as 1 μmole of *p*-benzoquinone reduced per minute. The specific activity of DT-diaphorase using *p*-benzoquinone as the substrate was 33.3–58.3 μmoles/min/mg. ●——●, With DT-diaphorase; ○——○, with DT-diaphorase and 20 μM dicumarol. Each point represents the average of two experiments carried out in duplicate; duplicates varied less than 5% about their mean.

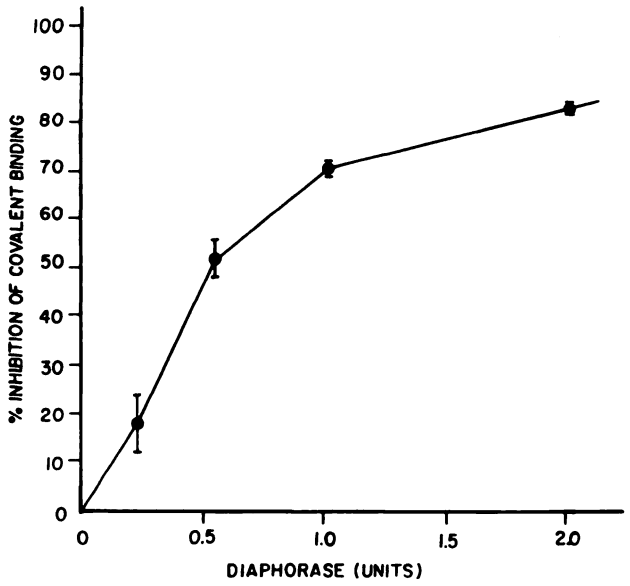


FIG. 3. *Effect of partially purified diaphorase (lipoamide dehydrogenase) from Clostridium kluyveri on covalent binding*

Assays were conducted as described under Experimental Procedures. One unit of activity is defined as 1 μmole of *p*-benzoquinone reduced per minute. The specific activity using *p*-benzoquinone as the substrate was 9.2 μmoles/min/mg of protein. Each point represents the average of three experiments carried out in duplicate ± standard deviation.

myeloperoxidase isolated from guinea pig marrow increased covalent binding 6-fold. This binding was also inhibited greater than 95% by ascorbate (Table 4). Three concentrations of hydrogen peroxide were used in Tables 3 and 4 to ensure that hydrogen peroxide was not limiting, since washed microsomes contain catalase activity.

DISCUSSION

Hydroquinone and catechol have been shown to accumulate in the bone marrow, the target tissue of benzene

TABLE 3

Effect of HRP on covalent binding of [^{14}C]phenol and metabolites to microsomal protein in vitro

[^{14}C]Phenol (1 mM) was incubated with 0.7 mg of washed hepatic microsomal protein isolated from PB-pretreated guinea pigs and sodium phosphate buffer (100 mM, pH 7.4). The incubation was carried out for 5 min at 37°. The total volume was 1 ml.

Condition	Covalent binding[a] (pmoles phenol equivalents bound/min/0.7 mg microsomal protein)
HRP (0.12 unit), H_2O_2 (2.2 mM)	5743.1 ± 887.7[b]
HRP (0.12 unit), H_2O_2 (4.4 mM)	7162.8 ± 1231.8
HRP[c] (0.12 unit), H_2O_2 (8.8 mM)	8287.8 ± 995.5
HRP (0.12 unit), H_2O_2 (8.8 mM), ascorbate (1 mM)	50.8 ± 23.3[b]
H_2O_2 (4.4 mM)	114.1 ± 32.1[b]

[a] Values are means ± standard deviation, $N = 4$; 1 unit = 1 μmole of guaiacol oxidized/min/mg; HRP = 126 units/mg.
[b] Significantly different from control ($p < 0.01$).
[c] Control.

TABLE 4

Effect of myeloperoxidase on covalent binding of [^{14}C]phenol and metabolites to microsomal protein in vitro

[^{14}C]Phenol (1 mM) was incubated with 0.7 mg of washed hepatic microsomal protein isolated from PB-pretreated guinea pigs and sodium phosphate buffer (100 mM, pH 7.4). The incubation was carried out for 5 min at 37°. The total volume was 1 ml.

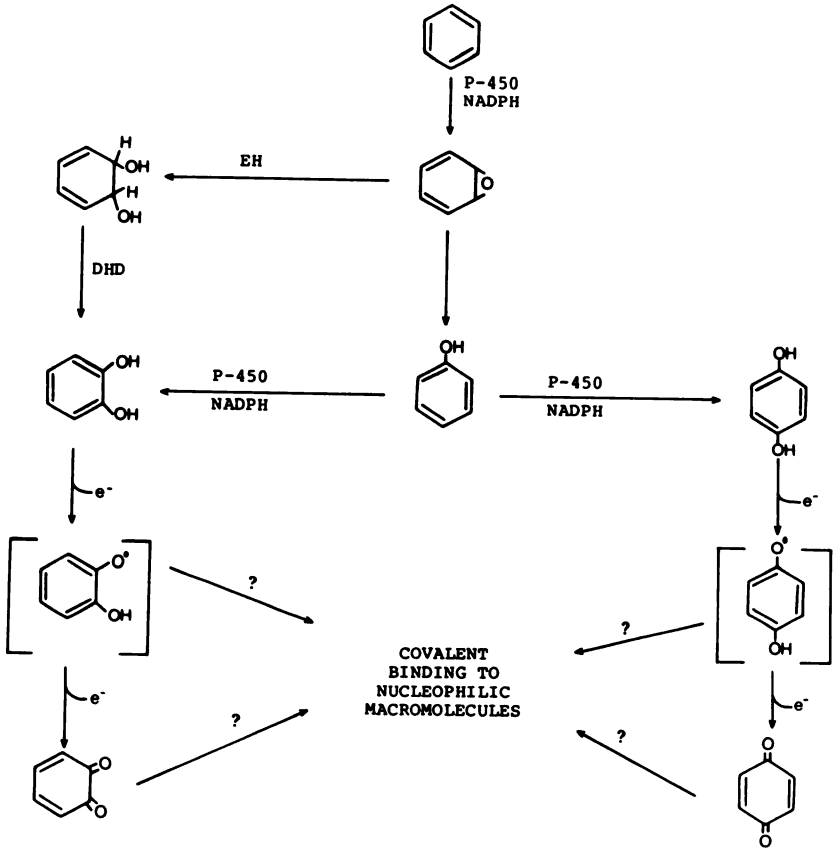
Condition	Covalent binding[a] (pmoles phenol equivalents bound/min/0.7 mg microsomal protein)
Myeloperoxidase (0.17 unit), H_2O_2 (2.2 mM)	1534.1 ± 128.1
Myeloperoxidase (0.17 unit), H_2O_2 (4.4 mM)	1474.4 ± 129.9
Myeloperoxidase[b] (0.17 unit), H_2O_2 (8.8 mM)	1458.6 ± 57.2
Myeloperoxidase (0.17 unit), H_2O_2 (8.8 mM), ascorbate (1 mM)	70.6 ± 20.1[c]
H_2O_2 (4.4 mM)[d]	114.1 ± 32.1[c]

[a] Values are means ± standard deviation, $N = 4$; 1 unit = 1 μmole of guaiacol oxidized/min/mg; guinea pig myeloperoxidase = 1.3 units/mg.
[b] Control.
[c] Significantly different from control ($p < 0.01$).
[d] Data from Table 3.

toxicity (10–12). It has been proposed that benzene-induced cytotoxicity may result from covalent binding of semiquinone and/or quinone metabolites to cellular macromolecules (12). We have shown that DT-diaphorase and diaphorase (lipoamide dehydrogenase) inhibit covalent binding. Both enzymes are capable of reducing *o*- and *p*-benzoquinones to catechol and hydroquinone. Iyanagi and Yamazaki (23) have demonstrated that DT-diaphorase reduces quinones through a direct two-electron transfer. This two-electron transfer results in the reduction of *p*-benzoquinone directly to hydroquinone. The covalent binding we observe, therefore, is due primarily to the electrophilic quinone species. If covalent binding were derived from the semiquinones, then one would expect the addition of DT-diaphorase either to increase covalent binding by reducing the quinones back to hydroquinone and catechol, making these more available for the oxidation to the semiquinones, or to have no effect, since the semiquinones would be covalently bound prior to their oxidation to the quinone forms (Scheme 1). The data presented therefore implicate *o*- and *p*-benzoquinone metabolites and not their semiquinones as the major species responsible for the observed covalent binding.

Diaphorase (lipoamide dehydrogenase) reduces quinones through a mixed mechanism involving one- and two-electron transfer (24). This enzyme can also inhibit covalent binding, as shown in Fig. 3. Although the mechanism of electron transfer is somewhat different from DT-diaphorase, the percentages of inhibition of covalent binding based on units of activity are similar (Figs. 2 and 3). This suggests that both enzymes are inhibiting covalent binding by reducing the quinone forms and not the semiquinones.

From our results it is clear that partially purified DT-



SCHEME 1. *Proposed metabolic pathway of benzene or phenol leading to intermediates capable of covalent binding*

diaphorase and diaphorase can substantially inhibit covalent binding from 70% and 86%, respectively. DT-Diaphorase present in the $10^5 \times g$ supernatant fraction is also capable of inhibiting covalent binding, but only after the removal of endogenous GSH. Depletion of GSH is known to occur *in vivo* through the action of a number of xenobiotics which are metabolized to electrophilic intermediates (25, 26). In the case of those xenobiotics which are metabolized to electrophilic quinones, DT-diaphorase may be important in the reduction of these, thus preventing the depletion of GSH, or it may be a secondary line of detoxication after GSH is depleted. We postulate that DT-diaphorase may function as a detoxication enzyme which can prevent the covalent binding of reactive electrophilic quinones to macromolecules. Previous studies by Lind *et al.* (27, 28) suggest that DT-diaphorase is also important in preventing semiquinone and superoxide anion generation and have demonstrated the necessity of quinone reduction by DT-diaphorase prior to the conjugation with glucuronic acid by UDP-glucuronsyltransferase.

Our results indicate that HRP can activate phenol to intermediates capable of covalent binding (29). We have demonstrated that HRP can increase covalent binding 30-fold (Table 3) as compared with the binding in the standard assay with NADPH. If benzene-induced toxicity is a result of the covalent binding of its metabolites, a peroxidase may be involved. Peroxidase activity has been demonstrated in bone marrow (19). In keeping with this, we have found peroxidase activity with hydroquinone as a substrate in bone marrow isolated from the dog.[2] Sawahata and Neal (22) have demonstrated that phenol can be oxidized to *o,o'*- and *p,p'*-biphenol by myeloperoxidase in the presence of hydrogen peroxide with subsequent covalent binding. We have concurred with their finding, in that myeloperoxidase isolated from guinea pig marrow also increases covalent binding. Importantly, we have found this effect to be reversed by ascorbate (Table 4). In the presence of ascorbate there was no significant oxidation of phenol by HRP, but in its absence 57% of the phenol was oxidized. Our studies indicate that ascorbate prevents the oxidation of phenol and thereby inhibits covalent binding and the formation of biphenol dimers. This effect may be due to the antioxidation property of ascorbate or it may be acting as a competitive inhibitor, since ascorbate is a substrate for HRP (30).

The addition of catechol *O*-methyltransferase with *S*-adenosyl-L-methionine inhibited covalent binding 12% (Table 2). This inhibition, although small, does implicate the involvement of catechol in covalent binding. Catechol *O*-methyltransferase does not methylate hydroquinone (31). Superoxide dismutase inhibited covalent binding 35% when incubated with microsomes isolated from PB-treated guinea pigs and had no effect with microsomes isolated from non-induced guinea pigs (Table 2). These results may be due to the induction of cytochrome P-450 reductase by PB treatment. Cytochrome P-450 reductase has been shown to reduce oxygen to superoxide anion

[2] R. C. Smart and V. G. Zannoni, unpublished results.

(32), and superoxide anion has been shown to activate a variety of catechols to reactive electrophiles capable of covalent binding (33). The slight inhibition of covalent binding by SKF 525-A as compared with *N*-octylamine (Table 2) could be due to selective inhibition of particular isozymes of cytochrome P-450 by SKF 525-A (34).

In summary, we have shown an effect of myeloperoxidase, DT-diaphorase, ascorbate, and glutathione on covalent binding of phenol and its metabolites. If benzene toxicity were a result of covalent binding of the oxidized forms of phenol, catechol, or hydroquinone within the bone marrow, then the ability of the bone marrow tissue to keep these metabolites in their reduced state could be related to the selective toxicity of benzene. The quantity or activity of the factors thus described may be of special significance in the myelotoxicity of benzene. This is presently under investigation.

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