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Biotransformation of Phenol to Hydroquinone and Catechol by Rat Liver Microsomes

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SUMMARY

Hepatic microsomal biotransformation of phenol to hydroquinone and catechol has been investigated with special reference to the covalent binding to microsomal protein of reactive metabolites formed during microsomal metabolism of phenol. Incubation of [^{14}C]phenol with microsomes from phenobarbital-treated rat liver in the presence of an NADPH-generating system resulted in the formation of hydroquinone and catechol in the ratio of 20:1. No significant formation of 1,2,4-benzenetriol was observed. The biotransformation of phenol to both hydroquinone and catechol required NADPH and molecular oxygen. NADH was much less effective than NADPH as an electron donor and exhibited no significant synergistic effect when used together with NADPH. The biotransformation was inhibited by typical cytochrome P-450 inhibitors such as carbon monoxide, SKF 525-A, and metyrapone. These results indicated the involvement of cytochrome P-450 in the microsomal hydroxylation of phenol at both the *ortho*- and *para*-positions. Covalent binding of radioactivity to microsomal protein was observed when [^{14}C]phenol was incubated with rat liver microsomes in the presence of an NADPH-generating system. The covalent binding was also found to require NADPH and molecular oxygen. Inclusion of cytochrome P-450 inhibitors in the incubation mixture resulted in a decrease in the covalent binding. These results indicated that at least one step in the metabolic activation of phenol to the metabolites responsible for covalent binding to microsomal protein was mediated by cytochrome P-450. Inclusion of *N*-acetylcysteine in the incubation mixture resulted in the complete inhibition of the covalent binding of radioactivity derived from [^{14}C]phenol to microsomal protein, and there was a concomitant formation of *N*-acetylcysteine adducts of hydroquinone and catechol. These results indicated that hydroquinone and catechol were both precursors to reactive metabolites responsible for the covalent binding.

INTRODUCTION

Benzene is a well-known leukemogen and is extensively used in the chemical industry (1). Epoxidation by hepatic microsomal cytochrome P-450-containing monooxygenases has been well established as a major mechanism for the metabolic activation of a number of aromatic hydrocarbons (2). However, benzene oxide, the putative metabolite of benzene formed on epoxidation by cytochrome P-450, has been dismissed as the ultimate reactive metabolite responsible for covalent binding of benzene to protein in a reaction catalyzed by hepatic microsomes (3). Thus, recent studies have focused on further metabolism of phenol, the major metabolite of benzene both *in vitro* (3) and *in vivo* (4). Phenol is most likely derived from benzene oxide as the result of spontaneous or protein-catalyzed rearrangement (3, 5).

Hydroquinone and catechol have been identified as metabolites *in vivo* of benzene (4) and phenol (6). These metabolites are thought to be formed predominantly in the liver and subsequently accumulate in the bone marrow, a target tissue for benzene toxicity, when benzene is administered to rats (7–9). Furthermore, Tunek *et al* (10) have shown that the glutathione adduct, *S*-(2′,5′-dihydroxyphenyl)glutathione, is formed when benzene or phenol is incubated with rat liver microsomes in the presence of both an NADPH-generating system and glutathione. These findings suggest a role for hydroquinone and catechol in mediating the toxicity of benzene.

Although aromatic hydroxylation is one of the characteristic reactions catalyzed by the cytochrome P-450-containing monooxygenases, the involvement of the monooxygenase in the hydroxylation of phenol has not yet been confirmed. In the present report, the biotransformation of phenol to hydroquinone and catechol has been investigated. Also, the covalent binding of metabolites of phenol to microsomal protein has been examined. The results of these studies provide evidence for the involvement of cytochrome P-450 in the biotransformation of phenol to hydroquinone and catechol. In addition,

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these metabolites appear to be precursors to reactive molecules which covalently bind to microsomal protein.

EXPERIMENTAL PROCEDURES

Materials. [U-^{14}C]Phenol (specific activity 37.9 mCi/mmole) was obtained from Midwest Research Institute (Kansas City, Mo.) and diluted with unlabeled phenol to a specific activity of 0.17 mCi/mmole. The diluted [^{14}C]phenol was purified, prior to use, using preparative silica gel thin-layer chromatography as described by Tunek *et al.* (3). The purity of the [^{14}C]phenol used in this study was found to be >97.6% as judged by HPLC,[1] as described below.

Unlabeled phenol, hydroquinone, and catechol were the products of Aldrich Chemical Company (Milwaukee, Wisc.), Fisher Scientific Company (Pittsburgh, Pa.), and Sigma Chemical Company (St. Louis, Mo.), respectively. 5,5-Dimethyl-1-pyrroline-*N*-oxide and 1,2,4-benzenetriol were purchased from the Aldrich Chemical Company, and sodium azide and L-ascorbic acid were from the Fisher Scientific Company. SKF 525-A (2-diethylaminoethyl-2,2-diphenylvalerate, hydrochloride) and metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone) were purchased from Smith Kline & French Laboratories (Philadelphia, Pa.) and E. Merck (Darmstadt, Germany), respectively.

Liver microsomes were prepared from male Sprague-Dawley rats weighing 130–170 g as previously described (11). The rats were pretreated with phenobarbital (0.1% in the drinking water) for 5 days and killed 24 hr after discontinuance of the administration of phenobarbital. Superoxide dismutase (2500 units/mg of protein), catalase (4650 units/mg of protein), glucose 6-phosphate dehydrogenase, NADPH, NADP$^+$, NADH, NAD$^+$, glucose 6-phosphate, *N*-acetylcysteine, and lysine were obtained from the Sigma Chemical Company.

N-Acetylcysteine adducts of hydroquinone and catechol were synthesized by mixing a 10 mM ethanolic solution of *N*-acetylcysteine (1 ml) with a 10 mM ethanolic solution of *p*-benzoquinone (1 ml) or with a 10 mM etheral solution of *o*-benzoquinone (1 ml). An aliquot (20 μl) of each reaction mixture was analyzed by HPLC as described below. HPLC analysis indicated that the reaction was completed within 5 min or less. Additional HPLC analysis revealed that these compounds were slowly oxidized by air to other compounds. Another aliquot (0.1 ml) of each reaction mixture was added to 1 ml of 0.1 M diazomethane solution in ether. The mixture was allowed to stand at room temperature overnight, reduced in volume to approximately 0.1 ml, and then subjected to gas chromatography-mass spectrometry. The mass spectra were consistent with the methylated *N*-acetylcysteine adducts of hydroquinone and catechol (Fig. 4).

Methods. A typical incubation mixture contained [^{14}C]phenol (1 μmole); rat liver microsomes (1.3 mg of protein); EDTA (0.1 μmole); potassium phosphate buffer (pH 7.4, 0.1 mmole); and an NADPH-generating system consisting of NADP$^+$ (5 μmoles), glucose 6-phosphate (10 μmoles), $MgCl_2$ (5 μmoles), and glucose 6-phosphate dehydrogenase (1 unit). The final volume was 1 ml. For studies on the cofactor requirement, the NADPH-generating system was replaced by NADPH, NADP$^+$, NADH, or NAD$^+$ (1 μmole each). For the studies on molecular oxygen requirement and carbon monoxide inhibition, the reaction mixture was placed in a Thunberg tube and equilibrated with the gas mixture by 10 alternate cycles of gassing with the appropriate gas mixture followed by evacuating the tube under vacuum. The reactions were initiated by the addition of the NADPH-generating system or the pyridine nucleotides. The incubations were carried out in sealed vials at 37° for 10 min, unless otherwise specified. Following the incubation, 0.1 ml of 50 mM HCl-KCl buffer (pH 2.0) containing unlabeled hydroquinone and catechol (0.1 mmole/ml each) was added and the mixture was extracted with 1 ml of ethyl acetate. The addition of these unlabeled standards was necessary for monitoring the recoveries of the metabolites from the individual incubations as described below. No air-oxidation of hydroquinone or catechol to the corresponding benzoquinone was detectable spectrophotometrically (9) when these compounds were dissolved in HCl-KCl buffer (pH 2.0) and stored at 5° in a refrigerator for as long as 3 days. The mixture containing ethyl acetate was centrifuged at 1800 × *g* for 10 min to facilitate separation of the organic and aqueous layers. The ethyl acetate layer was removed and the aqueous layer was extracted twice more with ethyl acetate. The combined ethyl acetate extracts were reduced in volume under a gentle stream of nitrogen to near dryness, taking care to avoid completely drying the sample since catechol readily evaporates under these conditions. The residue obtained was dissolved in methanol and subjected to HPLC for the purpose of quantitation of the metabolites formed. The aqueous layer remaining from the ethyl acetate extractions was processed as described by Tunek *et al.* (3) for the determination of radioactivity covalently bound to microsomal protein.

In the studies examining the formation of *N*-acetylcysteine adducts of hydroquinone and catechol, [^{14}C]phenol (1 mM) was incubated at 37° for 30 min with rat liver microsomes (1.3 mg of protein per milliliter) in the presence of both the NADPH-generating system and *N*-acetylcysteine (2 mM) in a final volume of 1 ml. At the end of incubation, the reaction mixture was extracted three times with 2 ml of diethyl ether. The resulting aqueous layer was further extracted with 1 ml of ethyl acetate following addition of 0.1 ml of 5 N $HClO_4$ and 1 g of sodium chloride. The ethyl acetate layer was separated by centrifugation at 1800 × *g* for 15 min. The ethyl acetate extraction was repeated twice more. The combined ethyl acetate extracts were evaporated to dryness under a gentle nitrogen stream. The residue obtained was dissolved in 0.1 ml of methanol and subjected HPLC as described below.

Chromatographic conditions and scintillation counting. The HPLC conditions described by Greenlee *et al.* (12) for analysis of benzene metabolites were used for the analysis of hydroquinone and catechol as well as *N*-acetylcysteine adducts of these compounds. A Radial Pak-A octadecylsilane (ODS) column (Waters Associ-

[1] The abbreviation used is: HPLC, high-performance liquid chromatography.

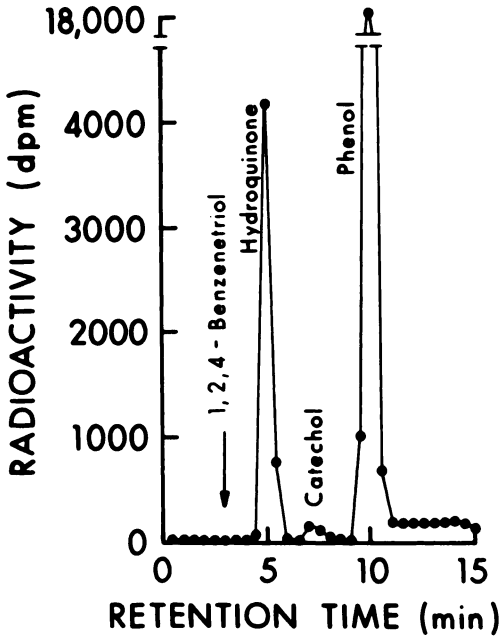

FIG. 1. *Typical chromatogram obtained by HPLC of ethyl acetate extract of the microsomal incubation of [^{14}C]phenol in the presence of an NADPH-generating system*

Liver microsomes from phenobarbital-pretreated rats (1.3 mg of protein per milliliter) were incubated with [^{14}C]phenol (1 mM) in the presence of an NADPH-generating system at 37° for 10 min, and the ethyl acetate extract of the incubation mixture was analyzed on an ODS HPLC column as described under Experimental Procedures.

ates, Milford, Mass.) and a linear gradient solvent system from 10% to 90% methanol in water over a period of 15 min were used. The solvents were acidified prior to use by the addition of 0.2 ml of 88% formic acid to 1000 ml of each solvent. Effluent fractions were collected directly into 7-ml scintillation vials at 0.5-min intervals, and 4 ml of ACS scintillation cocktail (Amersham Corporation, Arlington Heights, Ill.) were added to each. Radioactivity was determined using a Packard Model 460 scintillation counter fitted with an automatic external standard. Under the HPLC conditions, the retention times of authentic 1,2,4-benzenetriol, hydroquinone, catechol, phenol, *S*-(2′,5′-dihydroxyphenyl)-*N*-acetylcysteine, and the *N*-acetylcysteine adduct of catechol were 3.4, 4.8, 7.4, 10.6, 6.6, and 8.4 min, respectively. The recoveries of the metabolites, hydroquinone (73–91%) and catechol (68–94%), varied from one incubation to another, presumably due to differential air-oxidation of hydroquinone and different degrees of evaporation of catechol. Therefore, the recoveries from each incubation were calculated from the net UV-detector (280 nm) response due to the known amount of unlabeled metabolites which were added to the reaction mixtures prior to extraction with ethyl acetate. The recoveries were corrected by subtracting the responses due to the radioactive metabolites formed during the incubation from the apparent UV-detector responses. All data were corrected for the recoveries thus obtained.

Gas chromatography-mass spectrometry was carried out using a Finnegan Model 4000 gas chromatograph-mass spectrometer fitted with a 25-m (internal diameter; 0.035 inch) fused silica capillary column coated with OV-1 (Hewlett-Packard). The initial temperature of 35° was maintained for 30 sec after injection. The temperature of the column was then increased to 240° at a rate of 15°/min. The carrier gas was helium and was maintained at a flow rate of 5 ml/min. The elution of the compounds was detected by monitoring the total ion current. The mass spectrometer was operated at a filament current of 500 μamp and at electron energy of 70 eV.

RESULTS

Involvement of cytochrome P-450 in the biotransformation of phenol to hydroquinone, catechol, and metabolites covalently bound to protein. Hydroquinone has been shown to be formed on incubation of phenol with rat liver microsomes in the presence of an NADPH-generating system. Tunek *et al.* (10) have reported that appreciable formation of hydroquinone is observed only in the presence of superoxide dismutase. However, as seen in Fig. 1, the formation of hydroquinone and catechol was easily detected even when the incubation was carried out in the absence of superoxide dismutase. Thus, aerobic incubation of [^{14}C]phenol (1 mM) with rat liver microsomes in the presence of an NADPH-generating system resulted in the formation of hydroquinone and catechol in the ratio of 20:1. Although 1,2,4-benzenetriol has been identified as a metabolite of phenol formed *in vivo* (6), no significant formation of the triol was detected in these microsomal incubations. As previously reported (3), covalent binding of radioactivity derived from [^{14}C]phenol to microsomal protein was also observed. Time course studies indicated that the formation of hydroquinone and catechol, as well as covalent binding to microsomal protein, were essentially linear for at least 10 min (Fig. 2). Therefore, 10 min was chosen as the

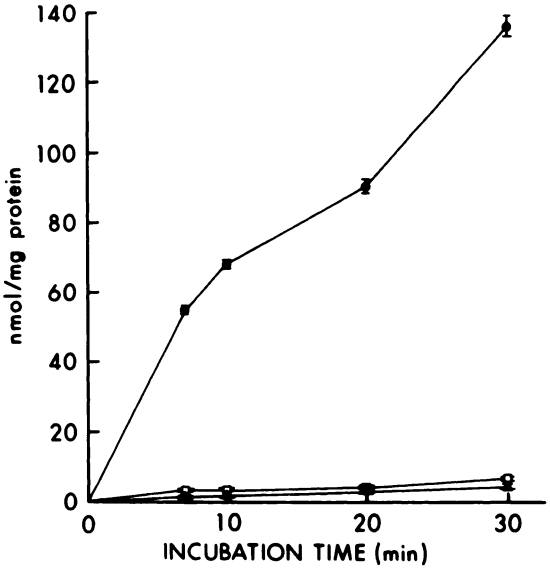

FIG. 2. *Time course of the formation of hydroquinone, catechol, and the covalent binding of radioactivity derived from [^{14}C]phenol to microsomal protein*

Incubations of [^{14}C]phenol with phenobarbital-treated rat liver microsomes in the presence of an NADPH-generating system were carried out as described in Fig. 1 for the periods of times indicated. Formation of hydroquinone (●) and catechol (□) and radioactivity covalently bound to microsomal protein (▲) were determined as described under Experimental Procedures. Each data point represents the mean ± standard deviation of three determinations.

TABLE 1

Cofactor requirement for rat liver microsomal hydroxylation of phenol and for the covalent binding of radioactivity derived from [^{14}C]phenol to microsomal protein

Incubations were carried out as described in Fig. 1 except that various pyridine nucleotides (1 mM each) were used in place of the NADPH-generating system. The results represent the mean ± standard deviation of triplicate determinations.

Cofactor	Products formed		
	Hydroquinone	Catechol	Covalent binding
	nmoles/mg protein/min		*nmoles bound/mg protein/min*[a]
NADPH	7.04 ± 0.16	0.37 ± 0.04	0.40 ± 0.06
NADH	0.67 ± 0.15	0.08 ± 0.06	0.09 ± 0.04
NADP	ND[b]	ND	ND
NAD	ND	ND	ND
NADPH + NADH	7.12 ± 0.21	0.36 ± 0.04	0.47 ± 0.07

[a] As estimated by the specific activity of the [^{14}C]phenol used in the incubations.

[b] ND, Not detectable (<0.01 nmole/mg of protein per minute).

incubation time for the additional studies described in this report.

As seen in Table 1, the biotransformation of phenol to hydroquinone and catechol by rat liver microsomes required NADPH. NADH was much less effective as an electron donor than NADPH and had no significant synergistic effect on the formation of the metabolites when used together with NADPH. The biotransformation of phenol by hepatic microsomes also required the presence of molecular oxygen and was inhibited by carbon monoxide (Table 2). Inclusion of other inhibitors of cytochrome P-450, SKF 525-A and metyrapone, in the incubation mixture resulted in significant inhibition of the microsomal metabolism of phenol to hydroquinone and catechol (Table 3).

The covalent binding of radioactivity derived from [^{14}C]phenol to microsomal protein also required NADPH (Table 1) and molecular oxygen (Table 2). Carbon monoxide (Table 2) and other cytochrome P-450 inhibitors (Table 3) significantly inhibited the covalent binding.

TABLE 2

Effect of carbon monoxide on the rat liver microsomal hydroxylation of phenol and the covalent binding of radioactivity derived from [^{14}C]phenol to microsomal protein

Incubations were carried out as described under Fig. 1 except that the components of incubation mixture were placed in a Thunberg tube and equilibrated with the gas mixture as described under Experimental Procedures. The results represent the mean ± standard deviation of triplicate determinations.

Gaseous phase	Products formed		
	Hydroquinone	Catechol	Covalent binding
	nmoles/mg protein/min		*nmoles bound/mg protein/min*[a]
100% Nitrogen	0.04 ± 0.03	0.02 ± 0.03	0.01 ± 0.01
Air/Nitrogen (1:1)	7.02 ± 0.09	0.44 ± 0.06	0.32 ± 0.05
Air/CO (1:1)	2.67 ± 0.06	0.26 ± 0.02	0.11 ± 0.07

[a] As estimated by the specific activity of the [^{14}C]phenol used in the incubations.

TABLE 3

Effects of some cytochrome P-450 monooxygenase inhibitors, superoxide dismutase, catalase, and some other compounds on hydroxylation of phenol and the covalent binding of radioactivity derived from [^{14}C]phenol to microsomal protein

The incubations were carried out as described in Fig. 1. All data represent the mean ± standard deviation of triplicate determinations. Student's *t*-test was used at a significance level of $p < 0.05$.

Addition	Products formed		
	Hydroquinone	Catechol	Covalent binding
	nmoles/mg protein/min		*nmoles bound/mg protein/min*[a]
None	7.55 ± 0.46	0.39 ± 0.04	0.38 ± 0.08
SKF 525-A (1 mM)	2.09 ± 0.63[b]	0.21 ± 0.05[b]	0.20 ± 0.04[b]
Metyrapone (1 mM)	3.78 ± 0.36[b]	0.27 ± 0.03[b]	0.13 ± 0.05[b]
Superoxide dismutase (50 μg/ml)	7.07 ± 0.17	0.42 ± 0.03	0.40 ± 0.03
Catalase (100 units/ml)	6.52 ± 0.21[b]	0.41 ± 0.04	0.43 ± 0.05
Sodium azide (1 mM)	3.22 ± 0.15[b]	0.31 ± 0.02[b]	0.27 ± 0.03
DMPO[c] (75 mM)	3.52 ± 0.47[b]	0.25 ± 0.04[b]	0.14 ± 0.06[b]
Ascorbic acid (1 mM)	5.69 ± 0.28[b]	0.39 ± 0.04	ND[d]
N-acetylcysteine (2 mM)	5.27 ± 0.03[b]	0.37 ± 0.01	ND[d]
Lysine (10 mM)	7.23 ± 0.42	0.41 ± 0.06	0.38 ± 0.06

[a] As estimated by the specific activity of the [^{14}C]phenol used in the incubations.

[b] Significantly different from the control ($p < 0.05$).

[c] DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide.

[d] ND, Not detectable (<0.01 nmole/mg of protein per minute).

Effects of superoxide dismutase, catalase, and various compounds on the formation of hydroquinone and catechol and on the covalent binding of radioactivity from [^{14}C]phenol to microsomal protein. As seen in Table 3, inclusion of superoxide dismutase (50 μg/ml) in the incubation did not significantly affect either the formation of the metabolites, hydroquinone and catechol, or the covalent binding of radioactivity derived from [^{14}C]phenol to microsomal protein. The formation of hydroquinone was significantly decreased when the incubations were carried out in the presence of catalase. However, catalase had no significant effect on the formation of catechol or the covalent binding of phenol metabolites to microsomal protein. Sodium azide, an inhibitor of catalase, significantly inhibited the microsomal hydroxylation of phenol but not covalent binding to microsomal protein.

The free radical trap, 5,5-dimethyl-1-pyrroline-*N*-oxide, markedly (63%) inhibited covalent binding of radioactivity to microsomal protein. The formation of hydroquinone and catechol (53 and 36%, respectively) was also inhibited by the *N*-oxide. Ascorbate, a free radical scavenger, slightly (25%) inhibited the formation of hydroquinone and completely inhibited the covalent binding. However, ascorbate did not significantly affect the formation of catechol.

An —SH group-containing amino acid, *N*-acetylcysteine, also completely inhibited covalent binding to

microsomal protein, while the primary amine-containing amino acid, lysine, did not significantly affect either microsomal hydroxylation of phenol or covalent binding to microsomal protein.

Formation of N-acetylcysteine adducts. In order to examine whether *N*-acetylcysteine, analogous to glutathione (3, 9), exerts its inhibitory effect on the covalent binding of radioactivity to microsomal protein by trapping reactive metabolites, microsomal incubations of [^{14}C]phenol were carried out in the presence and absence of *N*-acetylcysteine (2 mM). The reaction mixtures were extracted with diethyl ether to remove unmetabolized substrate and unconjugated metabolites. The aqueous layers were acidified with 5 N $HClO_4$ and further extracted with ethyl acetate. The ethyl acetate extracts were evaporated to dryness, dissolved in methanol, and subjected to HPLC.

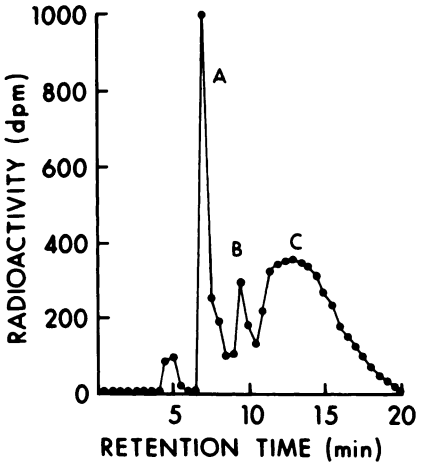

FIG. 3. *Formation of N-acetylcysteine adducts of hydroquinone and catechol*

Phenobarbital-treated rat liver microsomes (1.3 mg of protein per milliliter) were incubated at 37° for 10 min with [^{14}C]phenol (1 mM) in the presence of both an NADPH-generating system and *N*-acetylcysteine (2 mM) as described under Experimental Procedures. Extraction and HPLC analyses were carried out as described under Experimental Procedures.

Figure 3 shows a typical chromatogram of the ethyl acetate extract from the incubation carried out in the presence of *N*-acetylcysteine, revealing three peaks, *A*, *B*, and *C*. When the incubation was carried out in the absence of *N*-acetylcysteine, Peaks *A* and *B* were not observed but Peak *C* was still detectable. Therefore, Peak *C* appeared to be unrelated to *N*-acetylcysteine, and its identity was not pursued further. The ratio of the radioactivity corresponding to Peak *A* to that corresponding to Peak *B* was approximately 3:1. Peaks *A* and *B* were tentatively identified as *N*-acetylcysteine adducts of hydroquinone and catechol, respectively, by co-chromatography with authentic standards and by co-chromatography with these standard compounds on an ODS column after derivatization with diazomethane. Further identification of these adducts was carried out by gas chromatography-mass spectrometry following isolation by HPLC and derivatization with diazomethane. The methylated synthetic *N*-acetylcysteine adducts of hydroquinone and catechol were not separated under the gas chromatographic conditions used. Both compounds had a retention time of 15.6 min. The mass spectra of these authentic adducts were also similar to each other, the only major difference being in the relative intensity of the fragment ion peaks at *m/z* 168 and *m/z* 195 (Fig. 4). The methylated Peaks *A* and *B* had a retention time

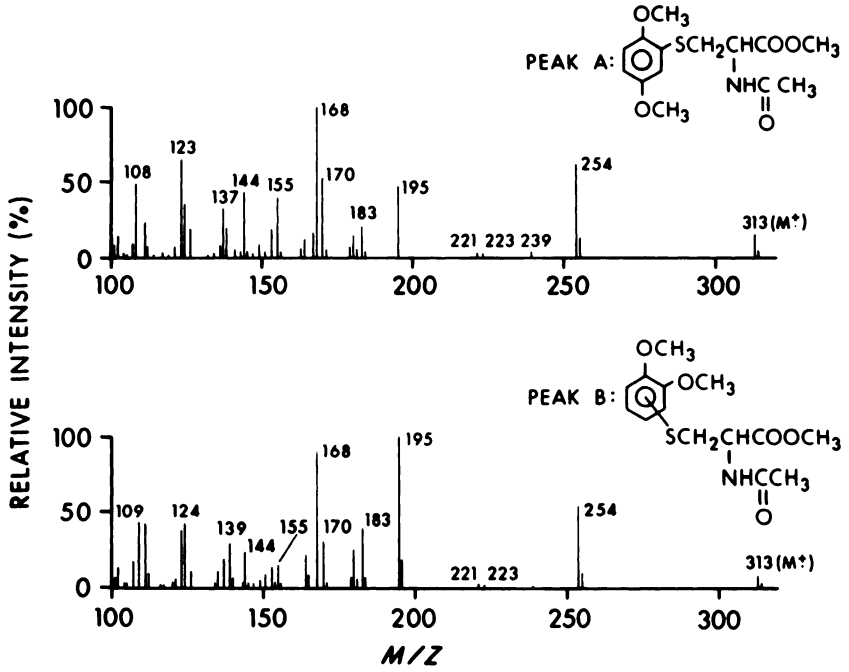

FIG. 4. *Mass spectra of methylated N-acetylcysteine adducts of hydroquinone and catechol*

The incubation was carried out as described in Fig. 3. The fractions corresponding to Peaks *A* and *B* in Fig. 3 were collected separately and 10 ml of 0.1 M diazomethane solution in ether were added. The residues obtained on evaporation of the solvent were dissolved in 0.1 ml of acetone and subjected to gas chromatography-mass spectrometry as described under Experimental Procedures.

identical with that of the standard methylated adducts and their mass spectra were identical with those of *N*-acetylcysteine adducts of hydroquinone and catechol, respectively.

DISCUSSION

The results described in the present report indicate that the hepatic microsomal metabolism of phenol to both hydroquinone and catechol is mediated by the cytochrome P-450-containing monooxygenase system. Different susceptibility of hydroquinone and catechol formation from phenol to inhibition by various cytochrome P-450 inhibitors may reflect the possible involvement of different isozymes of cytochrome P-450 in the formation of hydroquinone and catechol, as is the case with hydroxylation of bromobenzene (13). Very recently Ingelman-Sundberg and Ekström (14) have reported that the cytochrome P-450-catalyzed *para*-hydroxylation of aniline is mediated by hydroxyl radicals generated by an iron-catalyzed Haber-Weiss reaction between superoxide anion radicals and hydrogen peroxide. In this system, hydrogen peroxide is produced by the dismutation of superoxide anion radical, which, in turn, is generated by the autoxidation of the oxy-cytochrome P-450 complex (15). As a consequence, the cytochrome P-450-dependent and hydroxyl radical-mediated hydroxylation of aniline is markedly inhibited by superoxide dismutase (14). In the present report, superoxide dismutase had no inhibitory effect on the microsomal hydroxylation of phenol (Table 3). Although catalase inhibited the formation of hydroquinone, the magnitude of the inhibition was relatively small, and sodium azide, an inhibitor of catalase, had a significant inhibitory effect rather than the expected stimulatory effect on the microsomal hydroxylation of phenol (Table 3). Furthermore, hydroxyl radical-mediated hydroxylation of phenol has been reported to result in the predominant formation of catechol, the *ortho*-hydroxylation product of phenol (16). The cytochrome P-450-mediated hydroxylation of phenol resulted in the predominant formation of hydroquinone, the *para*-hydroxylation product. Therefore, it appears the involvement of hydroxyl radicals in the hydroxylation of phenol catalyzed by hepatic microsomes occurs only to a small extent, if at all.

Covalent binding of radioactivity to protein seen on incubation of [^{14}C]phenol with hepatic microsomes required the presence of molecular oxygen and NADPH (Tables 1 and 2) and was inhibited by cytochrome P-450 inhibitors (Tables 2 and 3). These results indicate that at least one of the steps in the metabolic activation of phenol to the species responsible for the covalent binding to microsomal protein is mediated by cytochrome P-450.

According to the current hypothesis concerning its metabolic activation (1), benzene is converted to phenol via benzene oxide. Phenol is then metabolized to hydroquinone and catechol, which are further oxidized to the corresponding semiquinone radicals and quinones. Analogous to catecholamines and catechol-estrogens (17–20), semiquinones and quinones are believed to be the ultimate reactive metabolites responsible for the covalent binding of benzene to microsomal protein. In this connection, it is of interest that the spin-trapping reagent 5,5-dimethyl-1-pyrroline-*N*-oxide had significant inhibitory effects on both the microsomal hydroxylation of phenol and the covalent binding to protein (Table 3). Although the *N*-oxide is known to trap hydroxyl radicals and superoxide radicals (21), it is less likely that the inhibitory effect of the *N*-oxide was the result of trapping these free radicals since superoxide dismutase did not have any inhibitory effect on the microsomal hydroxylation of phenol or on the covalent binding to microsomal protein. Furthermore, ascorbate, which also reacts rapidly with hydroxyl radicals, had only a slight inhibitory effect on the formation of hydroquinone (Table 3). It is possible that the apparent inhibitory effect of the *N*-oxide is the result of trapping the semiquinone radicals, thus preventing the semiquinone radicals from disproportionating to form dihydroxybenzenes and quinones (see below; Eq. 2). However, it is also possible that the *N*-oxide itself is a substrate for cytochrome P-450, thus inhibiting the microsomal hydroxylation of phenol. Resolution of these possibilities must await the electron spin resonance studies and studies of the interaction of 5,5-dimethyl-1-pyrroline *N*-oxide with cytochrome P-450.

Tunek *et al.* (10) have proposed that superoxide anion is involved in the conversion of hydroquinone to semiquinone and quinone. This is based on observations that (a) the hydroquinone was formed in appreciable amounts only in the presence of superoxide dismutase, (b) superoxide dismutase inhibited the covalent binding of phenol metabolites to microsomal protein, and (c) the decrease in the covalent binding to protein seen in the presence of superoxide dismutase was compensated for by the increase in the formation of hydroquinone. These workers used microsomes from untreated rat livers, a relatively low concentration of phenol (3.6 μM), and silica gel thin-layer chromatography for the separation and quantitation of hydroquinone and catechol. In the present study, by using microsomes from phenobarbital-treated rat livers, 1 mM phenol, and the anaerobic HPLC method devised by Greenlee *et al.* (12) for quantitation of the metabolites of benzene, the formation of hydroquinone was easily detectable even when the incubation was carried out in the absence of superoxide dismutase (Fig. 1). In the presence of superoxide dismutase (50 μg/ml), the formation of hydroquinone and catechol was not significantly increased (Table 3). In addition, superoxide dismutase did not significantly affect the covalent binding of radioactivity to microsomal protein (Table 3). Tunek *et al.* (10) used a large amount (1 mg/3 ml) of superoxide dismutase in their experiments. However, even when the concentration of superoxide dismutase was increased up to 0.5 mg/ml (data not shown), we did not observe any significant effects of superoxide dismutase on the formation of the metabolites and covalent binding to microsomal protein.

Greenlee *et al.* (9) have proposed a mechanism by which hydroquinone undergoes air-oxidation to semiquinone and quinone. Air-oxidation of hydroquinone (H_2Q) results in the formation of *p*-benzosemiquinone radical ($Q^{\cdot-}$) and superoxide anion radical:

$$H_2Q + O_2 \rightarrow Q^{\cdot-} + 2H^+ + O_2^{\cdot-} \quad (1)$$

Under aerobic conditions, the semiquinone sponta-

neously disproportionates to form *p*-benzoquinone (Q) and hydroquinone:

$$2Q^{\cdot-} + (2H^+) \underset{k_f}{\overset{k_d}{\rightleftarrows}} H_2Q + Q \qquad (2)$$

The rate constant k_d has been reported to be 7×10^7 (22) or 8×10^7 M^{-1} sec^{-1} (23), and the rate constant k_f to be 2.6×10^8 M^{-1} sec^{-1} (22). The semiquinone also reduces molecular oxygen to form superoxide anion radical:

$$Q^{\cdot-} + O_2 \underset{k'}{\overset{k}{\rightleftarrows}} Q + O_2^{\cdot-} \qquad (3)$$

The rate constant k has been reported to be 4.5×10^4 M^{-1} sec^{-1} (24), and the rate constant k' to be 9.8×10^8 (25) or 10^9 M^{-1} sec^{-1} (26). Therefore, in the presence of superoxide dismutase, air-oxidation of hydroquinone is accelerated by preventing the reaction of *p*-benzoquinone and superoxide anion radical to form the semiquinone radical and molecular oxygen (9, 24). As a theoretical consequence, superoxide dismutase would be expected to have an accelerating effect on covalent binding of radioactivity derived from [^{14}C]phenol to microsomal protein if air-oxidation of hydroquinone plays a major role in generating the reactive metabolites responsible for the covalent binding. However, superoxide dismutase had neither an accelerating nor an inhibitory effect on the covalent binding to microsomal protein. Furthermore, experiments using *N*-acetylcysteine (Figs. 3 and 4) clearly demonstrate that both hydroquinone and catechol are precursors of the reactive metabolites responsible for the covalent binding of radioactivity to microsomal protein. Formation of *N*-acetylcysteine adducts of hydroquinone and catechol in the ratio of 3:1 suggests that the rate of conversion of catechol to a reactive metabolite is faster than that of hydroquinone, since the ratio of the formation of hydroquinone and catechol from phenol is 20:1. Since hydroquinone is known to undergo air-oxidation more rapidly than does catechol (27), these data further suggest that the air-oxidation-mediated conversion of hydroquinone and catechol to the corresponding semiquinones and quinones does not play a significant role in generating the reactive metabolites responsible for the covalent binding of phenol to microsomal protein. If hydroquinone and catechol are converted to semiquinone radicals by air-oxidation or by reacting with superoxide anion free radicals in a microsomal incubation system, a general labeling of microsomal proteins would be expected. However, it has been shown that a microsomal protein with an apparent molecular weight of 72,000 becomes selectively radiolabeled when [^{14}C]phenol is incubated with rat liver microsomes in the presence of an NADPH-generating system (28). Therefore, it again appears unlikely that the conversion of hydroquinone and catechol to the corresponding semiquinone radicals and quinones mediated by air-oxidation or by free superoxide anion radicals plays a major role in generatng the reactive metabolites responsible for the covalent binding of phenol to microsomal protein. Assuming that the enzyme responsible for the generation of the reactive metabolite has the greatest probability of being labeled by the reactive metabolite, as has been shown in the case of inactivation of cytochrome P-450 by suicide substrates (29), we speculate that the final steps in the conversion of hydroquinone and catechol to the reactive metabolites responsible for the covalent binding to protein are mediated by an enzyme other than cytochrome P-450. This is suggested by the discrepancy between the molecular weights (48,000–54,000 daltons) of the various cytochrome P-450 isozymes (30) and the apparent molecular weight (72,000 daltons) of the protein which is selectively labeled by metabolites of phenol (28).

In conclusion, the results presented in this report demonstrate the involvement of cytochrome P-450 in the hepatic microsomal metabolism of phenol to hydroquinone and catechol. The results also provide evidence that both hydroquinone and catechol are obligatory intermediates in the hepatic microsomal metabolism of phenol to reactive metabolites which covalently bind to microsomal protein.

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