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Characterization of the Oxidation of Amine Metabolites of Nitrotoluenes by Rat Hepatic Microsomes

N- and C-Hydroxylation

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SUMMARY

The rat hepatic microsomal oxidation of amine metabolites of mono- and dinitrotoluene isomers has been investigated. Microsomes catalyzed the NADPH-dependent oxidation of 2-amino-6-nitrobenzyl alcohol, 2-amino-4-nitrobenzyl alcohol, and the isomeric aminobenzyl alcohols to ethyl acetate-extractable compounds capable of reducing ferric iron. The microsomal metabolism of 2-amino-6-nitrobenzyl alcohol, a metabolite of the hepatocarcinogen 2,6-dinitrotoluene, was characterized in detail. High pressure liquid chromatographic analysis indicated the formation of two metabolites, both of which were reducing agents. One metabolite was identified as 2-hydroxylamino-6-nitrobenzyl alcohol by comparison of its chromatographic properties and mass spectrum with those of the authentic compound. Mass spectral, proton NMR, and UV-visible spectroscopic studies suggested that the other metabolite was 2-amino-5-hydroxy-6-nitrobenzyl alcohol. The microsomal oxidation of 2-aminobenzyl alcohol also resulted in the formation of two reducing agents, one of which was the corresponding hydroxylamine. The formation of 2-hydroxylamino-6-nitrobenzyl alcohol from the microsomal oxidation of 2-amino-6-nitrobenzyl alcohol was linear with respect to time for at least 20 min, while aminophenol formation was only linear for 3 min. The rate of the microsomal oxidation of 2-amino-6-nitrobenzyl alcohol was decreased by known inhibitors of cytochrome P-450, while heat inactivation of microsomal flavin-containing monooxygenase had no effect. The rate of formation of both metabolites was increased 1.5-fold by phenobarbital pretreatment. Pretreatment with β-naphthoflavone had no effect on the rate of N-hydroxylation, while a small but statistically significant increase in the rate of C-hydroxylation (117% of control) was observed. The rate of oxidation of 2-amino-6-nitrobenzyl alcohol was lower with microsomes from female rats than with those from males, yielding male/female ratios of 1.34 for aminophenol formation and 3.26 for hydroxylamine formation. These data indicate that 2-amino-6-nitrobenzyl alcohol, a metabolite of the hepatocarcinogen 2,6-dinitrotoluene, can be N-hydroxylated by hepatic microsomal cytochrome P-450. The results are consistent with the hypothesis that a hydroxylamine metabolite of 2,6-dinitrotoluene is sulfated *in vivo* to produce an electrophilic species.

INTRODUCTION

Technical grade dinitrotoluene (75.8% 2,4-isomer, 19.5% 2,6-isomer, 4.7% other isomers) produced a 100% incidence of hepatocellular carcinomas when fed to male Fischer-344 rats at a dose of 35 mg/kg/day for 1 year (reviewed in Ref. 1). The results of a subsequent 1-year feeding study indicated that the 2,6-isomer is primarily responsible for the carcinogenic activity of technical grade dinitrotoluene (reviewed in Ref. 1). Our laboratory has been investigating the metabolism of the nitrotoluenes in order to elucidate the bioactivation pathways of these compounds.

The results of detailed studies on the metabolism and genotoxicity of the dinitrotoluenes (reviewed in Ref. 1) suggested a bioactivation pathway involving initial hepatic biotransformation to dinitrobenzyl glucuronide, biliary excretion, deconjugation, and nitroreduction by intestinal microflora, and reabsorption of the resulting aminonitrobenzyl alcohol. Studies of the metabolism (2, 3) and genotoxicity (4) of the related mononitrotoluene isomers suggested that the genotoxicant 2-nitrotoluene

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is bioactivated in a manner similar to the hepatocarcinogen 2,6-dinitrotoluene. Recent *in vivo* studies have demonstrated that sulfotransferase inhibitors decrease the covalent binding of 2,6-dinitrotoluene and 2-nitrotoluene to hepatic DNA by greater than 95% (5, 6). Sulfation of hydroxylamine metabolites of these nitrotoluenes was postulated to result in the formation of an unstable *N*, *O*-sulfate which decomposes to an electrophilic nitrenium ion.

This paper describes the detailed characterization of the hepatic microsomal oxidation of amine metabolites of nitrotoluenes. The data indicate that 2-amino-6-nitrobenzyl alcohol, a metabolite of the hepatocarcinogen 2,6-dinitrotoluene, is both N-hydroxylated and C-hydroxylated by rat hepatic cytochrome P-450.

EXPERIMENTAL PROCEDURES

Chemicals. 2,4-Dinitrobenzyl alcohol and 2,6-dinitrobenzyl alcohol were prepared by dissolving 5 g (25.5 mmol) of 2,4-dinitrobenzaldehyde or 2,6-dinitrobenzaldehyde in 20 ml of ethanol. The ethanol solution was cooled in an ice bath, and 0.3 g (7.9 mmol) of sodium borohydride was added in portions with stirring. When addition was complete, the mixture was allowed to warm to room temperature and was stirred for an addition 4 hr. Fifty ml of water were added, and the mixture was extracted with 300 ml of diethyl ether. The ether layer was removed, dried over magnesium sulfate, and evaporated to dryness. The crude product was recrystallized once from ethanol/water and used in the synthesis of the aminonitrobenzyl alcohols without further purification.

Each dinitrobenzyl alcohol (2.5 g, 12.8 mmol) was dissolved in 20 ml of ethanol:HCl, 1:1, and cooled in an ice bath. A solution containing 6.2 g (32.8 mmol) of $SnCl_2$ in concentrated HCl (3 ml) was slowly added to the reaction mixture with stirring. When addition was complete, stirring was continued at room temperature for 2 hr. Fifty ml of water were added, and the reaction mixture was extracted twice with 300 ml of ethyl acetate. The ethyl acetate extracts were combined, dried over magnesium sulfate, and evaporated to dryness. The product was recrystallized once from ethyl acetate/hexane and then purified by silica gel chromatography (2 × 30-cm column, eluted with ethyl acetate:hexane, 1:1). 4-Aminobenzyl alcohol was prepared by stannous chloride reduction of 4-nitrobenzyl alcohol. All compounds were >99% pure as judged by HPLC.[2] All products gave mass spectra consistent with the expected structures.

2-Hydroxylamino-6-nitrobenzyl alcohol was synthesized in 40–50% yield by reduction of 2,6-dinitrobenzyl alcohol (1.0 mmol) with zinc dust (2.2 mmol) and ammonium chloride (1.0 mmol) in 3 ml of ethanol with stirring. After 3 hr at 60°, the ethanolic reaction mixture was cooled, filtered into 5 volumes of water, extracted twice with equal volumes of ethyl acetate, and the combined extracts dried over sodium sulfate. 2-Hydroxylaminobenzyl alcohol was synthesized in the same manner by reduction of 2-nitrobenzyl alcohol. Repeated attempts to purify 2-hydroxylamino-6-nitrobenzyl alcohol by various chromatographic procedures resulted in its decomposition. HPLC and colorimetric analyses (described below) demonstrated that only one reducing agent was present in the extract of the reaction mixture. The reducing agent was isolated by HPLC, extracted into ethyl acetate and allowed to react with BSTFA (Supelco, Inc., Bellefonte, PA), and subjected to gas chromatography-mass spectrometry as described below. The mass spectrum of the isolated reducing agent was consistent with that expected for the di(trimethylsilyl) ether of 2-hydroxylamino-6-nitrobenzyl alcohol (see Fig. 2 for mass spectrum). Therefore, an ethyl acetate extract of the synthetic reaction mixture was used as a standard. Hydroxylamine concentrations were determined using the colorimetric assay described below.

[2] The abbreviations used are: HPLC, high pressure liquid chromatography; BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide.

Bathophenanthroline, ferric nitrate, methimazole, NADPH, NADH, and glutathione were obtained from Sigma. Octylamine, nitrosobenzene, 2-aminobenzyl alcohol, 3-aminobenzyl alcohol, 2-nitrobenzyl alcohol, 4-nitrobenzyl alcohol, 2,4-dinitrobenzaldehyde, 2,6-dinitrobenzaldehyde, 2-amino-4-nitrophenol, 2-nitro-4-aminophenol, 2-nitro-3-methylphenol, 3-methyl-4-nitrophenol, 2-nitrophenol, and 4-nitrophenol were obtained from Aldrich. 2-Diethylaminoethyl-2,2-diphenylvalerate (SKF-525A) was a gift from Smith, Kline and French (Philadelphia, PA). Metyrapone and desferal mesylate were gifts from Ciba Pharmaceutical Co. Phenylhydroxylamine was synthesized by ascorbate reduction of nitrosobenzene in aqueous solution. The product was extracted with ether and recrystallized. The melting point and mass spectrum were consistent with the expected product. All other chemicals were of reagent grade and obtained from commercial sources.

Isolation of microsomes. Hepatic microsomes from 200–250-g male or 175–225-g female Fischer-344 rats (CDF (F-344)/CrIBR, Charles River Breeding Laboratories, Kingston, NY) were prepared by differential centrifugation (7, 8). The rats were housed in hanging wire cages in mass air displacement rooms (Bioclean, Hazleton Systems, Vienna, VA) and were given purified water (Nanopure System, Barnstead, Boston, MA) and NIH-07 rodent diet (Ziegler Brothers, Gardner, PA; analyzed for nutrients and contaminants by Lancaster Labs, Lancaster, PA) *ad libitum*. All animals were free of virus titers as determined using the standard rat viral antibody screen (Microbiological Associates, Bethesda, MD). Some male rats were given 0.1% phenobarbital in their drinking water for 7 days or a single i.p. injection of β-naphthoflavone (80 mg/kg) in corn oil 24 hr prior to sacrifice. Protein concentrations were determined by the biuret method (American Monitor Total Protein Kit, American Monitor Corp., Indianapolis, IN) using bovine serum albumin as the standard. Cytochrome P-450 concentrations were determined by the method of Omura and Sato (9) using an Aminco DW-2a spectrophotometer. The microsomal preparations from untreated, phenobarbital-pretreated, and β-naphthoflavone-pretreated male rats had specific contents of 0.35–0.48, 0.82–1.11, and 0.66 nmol of cytochrome P-450/mg of protein, respectively. The microsomal preparation from untreated female rats had a specific content of 0.35 nmol of cytochrome P-450/mg of protein.

Incubation conditions. Unless otherwise specified, the reaction mixtures contained potassium phosphate buffer (0.1 M), pH 7.7, 2-amino-6-nitrobenzyl alcohol (0.5 mM), NADPH (1 mM), and 2.78 mg of microsomal protein in a final volume of 3 ml. The mixtures were preincubated in 10-ml Erlenmeyer flasks at 37° for 3 min. The reactions were initiated by the addition of NADPH, incubated with shaking for 3 min, and terminated by the addition of 3 ml of ethyl acetate. The quenched reaction mixtures were transferred to conical centrifuge tubes, vortexed, and centrifuged at 1500 rpm for 5 min. In some experiments, the microsomes were preincubated at 37° for 60 min to inactivate microsomal flavin-containing monooxygenase (EC 1.14.13.8) (10–12). This procedure has been shown to inactivate flavin-containing monooxygenase without affecting cytochrome P-450 activity (11). All incubations were carried out in duplicate or triplicate and appropriate controls were run with each incubation.

Colorimetric assay for reducing equivalents. The formation of ethyl acetate-extractable reducing equivalents was assayed by a modification of the method of Tsen (13). To 1 ml of the ethyl acetate extract, 0.8 ml of 2.5 mM bathophenanthroline in ethanol was added, followed by 0.04 ml of 10 mM ferric nitrate in 10 mM acetic acid. Thirty sec after the ferric nitrate was added, the absorbance of the resulting ferrous-bathophenanthroline complex was measured at 535 nm using a Bausch and Lomb Spectronic 710 spectrophotometer. The concentration of reducing equivalents was calculated using an extinction coefficient of 33.93 mM^{-1} cm^{-1} determined with phenylhydroxylamine.

HPLC assay. For HPLC analysis, 2-aminobenzyl alcohol (25.8 nmol) was added to the incubation mixtures as the internal standard prior to ethyl acetate extraction. The ethyl acetate extracts were dried over sodium sulfate, evaporated to dryness under a gentle stream of dry nitrogen, and reconstituted in 10 μl of ethyl acetate. The reconstituted

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extract was injected onto a Waters Associates HPLC (Milford, MA) equipped with two 30-cm Lichrosorb RP-18 columns (Brownlee Labs, Santa Clara, CA) connected in series. The microsomal metabolites of 2-amino-6-nitrobenzyl alcohol were separated using a 20-min linear gradient from 22–88% solvent B at a flow rate of 2 ml/min. The mobile phase consisted of water with 0.01% desferal mesylate (solvent A) and 90% acetonitrile/10% with water/0.01% desferal mesylate (solvent B). Desferal mesylate was added to the mobile phase to decrease chemisorption of the hydroxylamine (14). In the absence of desferal mesylate, 2-hydroxylamino-6-nitrobenzyl alcohol did not elute from the column, while 53% recovery was achieved in the presence of desferal mesylate. The internal standard 2-aminobenzyl alcohol had a elution volume of 17 ml. In some experiments, 0.5-min fractions were collected, diluted with 1.5 ml of water, and extracted with 1 ml of ethyl acetate. The ethyl acetate extracts were assayed colorimetrically as described above or processed for mass spectral or NMR analyses as described below.

The formation of 2-hydroxylamino-6-nitrobenzyl alcohol was estimated by the peak height ratio method using a standard curve generated by taking known amounts of the hydroxylamine through the procedure described above. The mixtures contained 2.8–51.7 nmol of 2-hydroxylamino-6-nitrobenzyl alcohol in potassium phosphate buffer (0.1 M), pH 7.7, containing 10 mM glutathione to prevent aerobic oxidation to the nitroso compound. Standard curves generated in the presence of microsomes yielded similar results but exhibited greater variability.

The formation of 2-amino-5-hydroxy-6-nitrobenzyl alcohol was estimated from the difference between total reducing equivalents and hydroxylamine formed. Six identical incubations were carried out for each determination. Three samples were assayed colorimetrically for total ethyl acetate-extractable reducing equivalents, while three samples were analyzed by HPLC for 2-hydroxylamino-6-nitrobenzyl alcohol. Since only two reducing agents were present in the ethyl acetate extracts of the incubation mixtures (see Fig. 1), the difference between the total and hydroxylamine was taken to represent the aminophenol metabolite.

Gas chromatography-mass spectrometry. The ethyl acetate extracts were dried over sodium sulfate, allowed to react with BSTFA, evaporated under nitrogen, and reconstituted in 20 μl of ethyl acetate. Aliquots (2 μl) were injected onto a Finnigan 3223 gas chromatograph/mass spectrometer equipped with a Durabond DB-5 fused silica capillary column (30 m, 0.32-mm inner diameter; J and W Scientific, Rancho Cordoba, CA). Splitless injections were made with the column held at 60°. After 1 min, the column was heated to 125° at 64°/min and from 125–260° at 10°/min. The carrier gas was helium at a flow rate of 3 ml/min. The compounds of interest had the following retention times: trimethylsilyl ether of 2-amino-6-nitrobenzyl alcohol, 14.8 min; di(trimethylsilyl) ether of 2-hydroxylamino-6-nitrobenzyl alcohol, 16.0 min; di(trimethylsilyl) ether of 2-amino-5-hydroxy-6-nitrobenzyl alcohol, 18.0 min. Some extracts were not reacted with BSTFA. Underivatized 2-amino-6-nitrobenzyl alcohol had a retention time of 12.8 min, while the aminophenol metabolite had a retention time of 13.9 min. 2-Hydroxylamino-6-nitrobenzyl alcohol could not be chromatographed underivatized. Ionization was by electron impact with the filament operated at 70 eV.

Spectroscopic measurements. Proton NMR spectra were obtained using a Varian 300 MHz superconducting spectrometer equipped with a Fourier transform accessory. Room temperature optical spectra were recorded on a Beckman DU-7 UV-visible spectrophotometer using cells with a pathlength of 1 cm.

Statistics. Statistical significance of the data was evaluated by Student's *t* test (15). The 0.05 level of probability was used as the criterion of significance.

RESULTS

Microsomal oxidation of amine metabolites of nitrotoluenes. Incubation of 2-amino-6-nitrobenzyl alcohol with hepatic microsomes and NADPH resulted in the formation of ethyl acetate-extractable compounds capable of reducing ferric iron (Table 1), consistent with the formation of hydroxylamine and/or aminophenol metabolites. Boiled microsomes did not support the reaction, indicating that it was enzyme catalyzed. The oxidation reaction required microsomes and NADPH. NADH could support the reaction at 20% of the NADPH-supported rate, while the rate was slightly greater than additive when both reduced pyridine nucleotides were present.

TABLE 1

Cofactor requirements for the microsomal oxidation of 2-amino-6-nitrobenzyl alcohol

The incubation conditions and colorimetric assay procedure were the same as described under "Experimental Procedures." The concentration of NADH was 1 mM when present.

Additions or deletions	Initial rate[a]
	nmol reducing equivalents formed/min/mg microsomal protein
None (complete system)	1.03 ± 0.04
Microsomes boiled 10 min	0
−Microsomes	0
−NADPH	0
−NADPH + NADH	0.21 ± 0.02
+NADH	1.35 ± 0.04

[a] Values are means ± S.E. of 3 determinations.

TABLE 2

Microsomal oxidation of isomeric aminonitrobenzyl alcohols and aminobenzyl alcohols

The reaction mixtures contained potassium phosphate buffer (0.1 M), pH 7.7, NADPH (1 mM), the indicated compound (0.5 mM), and 2.5 mg of microsomal protein. The reactions containing aminonitroenzyl alcohols were incubated for 3 min at 37°, while those containing aminobenzyl alcohols were incubated for 30 min. Ethyl acetate-extractable reducing equivalents were determined colorimetrically as described under "Experimental Procedures."

Compound	Nmol reducing equivalents formed/min/mg microsomal protein[a]
2-Amino-6-nitrobenzyl alcohol	1.65 ± 0.05
2-Amino-4-nitrobenzyl alcohol	1.70 ± 0.11
2-Aminobenzyl alcohol	0.29 ± 0.01
3-Aminobenzyl alcohol	0.18 ± 0.00
4-Aminobenzyl alcohol	0.04 ± 0.00[b]

[a] Values are means ± S.E. for 3 determinations.
[b] Value questionable as absorbance was not twice background.

Experiments were carried out to determine initial rate conditions for the microsomal formation of ethyl acetate-extractable reducing equivalents from 2-amino-6-nitrobenzyl alcohol. The reaction was linear with respect to time for 3 min and linear with respect to microsomal protein concentration through 2 mg/ml (data not shown). The oxidation reaction exhibited a broad pH optimum from pH 7.4–7.7; the rate decreased at higher and lower pH values (data not shown).

Several amine metabolites of the dinitrotoluene and mononitrotoluene isomers were substrates for microsomal oxidation (Table 2). The rate of oxidation of the aminonitrobenzyl alcohols was at least five times greater than that of the aminobenzyl alcohols. The apparent rate

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of the oxidation of the aminobenzyl alcohol isomers varied as 2– > 3– > 4–. However, the data presented in Table 2 for the aminobenzyl alcohols may not represent true initial rates since the assay conditions were not optimized for these substrates.

Microsomal metabolism of 2-amino-6-nitrobenzyl alcohol and 2-aminobenzyl alcohol. In order to investigate the identities of the microsomal metabolites of 2-amino-6-nitrobenzyl alcohol, ethyl acetate extracts of microsomal incubations were analyzed by HPLC. The results of a representative experiment are shown in Fig. 1. Two peaks in the chromatogram gave a positive response in the colorimetric assay, indicating that they were reducing agents. Control experiments demonstrated that all other peaks in the chromatogram except the substrate (retention volume of 21 ml) were derived from either microsomes or NADPH. The metabolite with a retention volume of 19 ml had the same retention volume by HPLC, the same retention time by gas chromatography, and a mass spectrum identical to that of authentic 2-hydroxylamino-6-nitrobenzyl alcohol (Fig. 2).

The metabolite with a retention volume of 14 ml was isolated by HPLC, allowed to react with BSTFA, and subjected to gas chromatography-mass spectrometry. The mass spectrum of this metabolite was consistent with a di(trimethylsilyl) ether, exhibiting an apparent molecular ion at *m/z* 328 (data not shown). Because the amino group of 2-amino-6-nitrobenzyl alcohol did not react with BSTFA under the conditions used, this result indicates the presence of two hydroxyl groups in the metabolite. The mass spectrum of the underivatized metabolite exhibited a molecular ion at *m/z* 184 and fragment ions at *m/z* 166 ($-H_2O$), 149 ($-H_2O$; $-OH$), and 121 ($-H_2O$; $-OH$; $-CO$) (data not shown), consistent with a phenolic metabolite of 2-amino-6-nitrobenzyl alcohol.

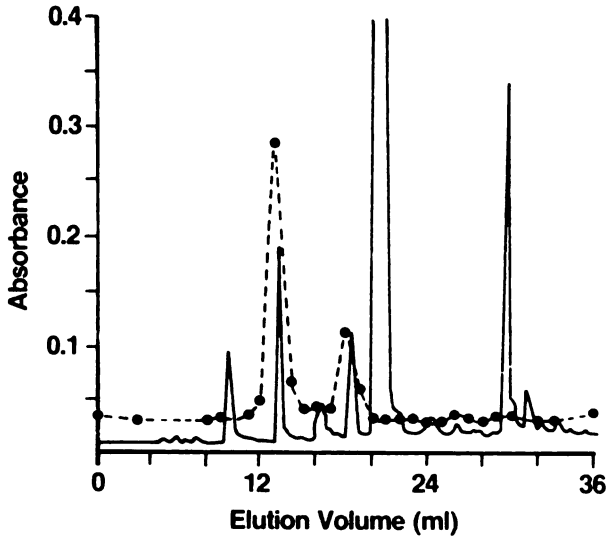

FIG. 1. *HPLC of the microsomal metabolites of 2-amino-6-nitrobenzyl alcohol*

The reaction mixtures were the same as described under "Experimental Procedures" except that microsomes from phenobarbital-pretreated rats (4.5 mg of protein) were used. The ethyl acetate extracts were processed and chromatographed as described under "Experimental Procedures." The *solid lines* show absorbance at 254 nm. Fractions (1 ml) were collected, extracted with ethyl acetate, and the extracts were assayed colorimetrically as described under "Experimental Procedures" (●– – –●, 535 nm).

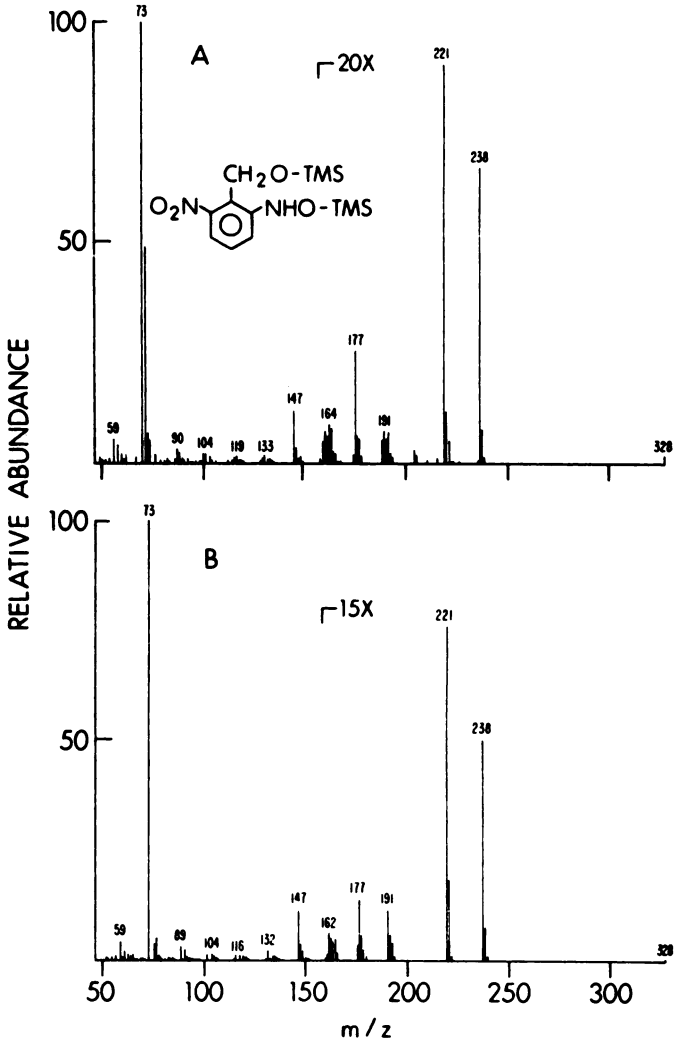

FIG. 2. *Mass spectrum of the di-(trimethylsilyl) ether f 2-hydroxylamino-6-nitrobenzyl alcohol*

The compounds were isolated by HPLC, allowed to react with BSTFA, and subjected to gas chromatography-mass spectroscopy as described under "Experimental Procedures." The abbreviation used is: TMS, trimethylsilyl. A. Authentic 2-hydroxylamino-6-nitrobenzyl alcohol. B. Microsomal metabolite.

Proton NMR experiments were carried out on the substrate and metabolite in an attempt to determine the position of the hydroxyl group. However, the chemical shifts of the aromatic ring protons of 2-amino-6-nitrobenzyl alcohol (multiplet from 6.85–6.95 ppm, triplet centered at 7.15 ppm) were not separated enough to allow positional assignment by decoupling experiments (data not shown). The NMR spectrum of the metabolite exhibited a singlet due to the phenolic proton at 6.55 ppm and a quartet (1:3:3:1) centered at 6.73 ppm (J = 8 Hz) ascribable to the aromatic ring protons (data not shown). The quartet is characteristic of *ortho*-proton coupling and suggests that the hydroxyl group is either adjacent to the amino group (C-3) or the nitro group (C-5).

Optical studies with model compounds demonstrated

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substantial differences in the absorbance spectra of *ortho-* and *para*-substituted nitrophenols in 1 N NaOH. For example, 2-amino-4-nitrophenol exhibited a large visible absorbance at 446 nm and a lesser UV peak at 275 nm (Fig. 3A), while 2-nitro-4-aminophenol exhibited an intense shoulder at 236 nm and relatively little visible absorbance (Fig. 3B). Similar results were obtained with the nitromethylphenol and nitrophenol isomer pairs. As shown in Fig. 3C, the phenolic metabolite of 2-amino-6-nitrobenzyl alcohol exhibited a shoulder at 241 nm and little visible absorbance in 1 N NaOH, suggesting that this metabolite is 2-amino-5-hydroxy-6-nitrobenzyl alcohol.

Similar HPLC and colorimetric analyses were carried out on ethyl acetate extracts of microsomal incubations containing 2-aminobenzyl alcohol (data not shown). Colorimetric analysis of the HPLC eluate indicated the presence of two reducing agents. One of the metabolites had the same retention volume as authentic 2-hydroxylaminobenzyl alcohol (15.6 ml). The other reducing agent co-eluted with the substrate peak (18.0 ml) under the HPLC conditions described under "Experimental Procedures." Control experiments demonstrated that this reducing agent was not present in the substrate alone. Chromatographic separation and identification of this metabolite was not attempted.

The time course for the formation of the individual microsomal metabolites of 2-amino-6-nitrobenzyl alcohol is shown in Fig. 4. The formation of 2-hydroxylam-

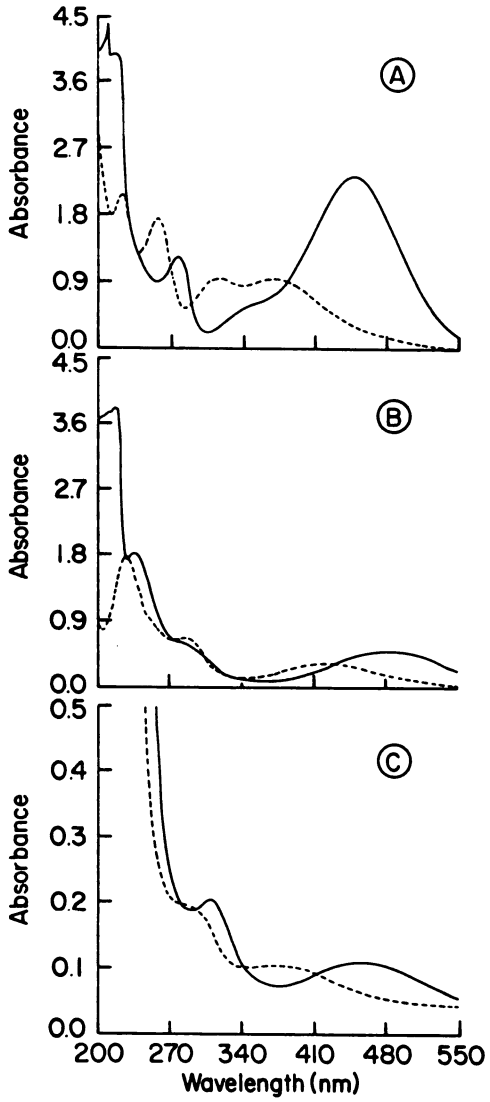

FIG. 3. *UV-visible spectra of isomeric amino nitrophenols and the phenolic metabolite of 2-amino-6-nitrobenzyl alcohol*

The *dashed lines* show the spectra in water, while the *solid lines* show the spectra in 1 N NaOH. A. 2-Amino-4-nitrophenol (0.20 mM). B. 2-Nitro-4-aminophenol (0.13 mM). C. Phenolic metabolite of 2-amino-6-nitrobenzylalcohol.

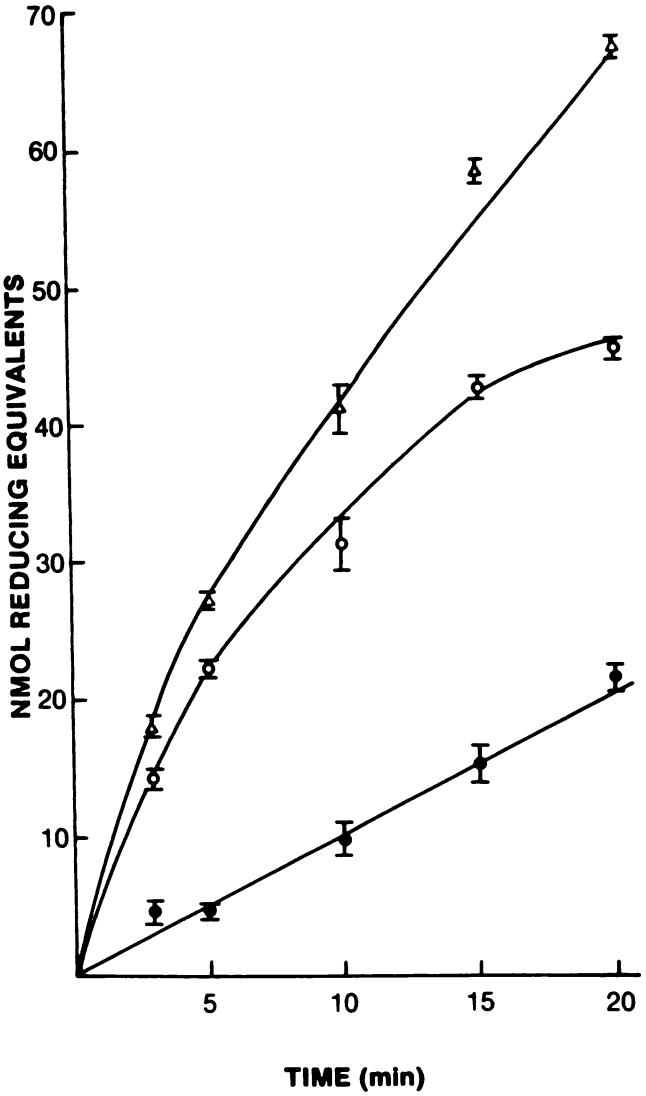

FIG. 4. *Time course for hydroxylamine and aminophenol formation from 2-amino-6-nitrobenzyl alcohol*

The incubation mixtures were the same as described under "Experimental Procedures" except that they contained 5.82 mg of microsomal protein from untreated rats. The mixtures were incubated at 37° for the times indicated. The formation of total reducing equivalents (△) was determined using the colorimetric assay. The formation of 2-hydroxylamino-6-nitrotoluene (●) was determined using the HPLC assay. Aminophenol formation (○) was determined from the difference between total reducing equivalents and hydroxylamine formed. The data are presented as the mean ± S.E. of 3 determinations.

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ino-6-nitrobenzyl alcohol was linear with respect to time for at least 20 min. In contrast, the formation of the aminophenol metabolite was only linear for 3 min, increasing nonlinearly thereafter.

Identification of the microsomal monooxygenase system catalyzing the oxidation reaction. As shown in Table 3, the cytochrome P-450 inhibitors SKF-525A, metyrapone, and octylamine significantly inhibited the formation of total reducing equivalents from 2-amino-6-nitrobenzyl alcohol. Methimazole, a high affinity substrate for microsomal flavin-containing monooxygenase (16), also significantly inhibited the reaction (control = 1.08 ± 0.02 nmol of reducing equivalents formed/min/mg of microsomal protein; 0.5 mM methimazole added = 0.74 ± 0.03). However, heat inactivation of microsomal flavin-containing monooxygenase had no effect on the rate of formation of reducing equivalents and abolished the inhibition by methimazole (control = 1.08 ± 0.02; heat inactivated = 1.05 ± 0.04 nmol of reducing equivalents formed/min/mg of microsomal protein). HPLC analysis demonstrated that heat inactivation of flavin-containing monooxygenase had no effect on the rates of formation of either the hydroxylamine or aminophenol metabolites.

Pretreatment of rats with phenobarbital significantly increased the rate of 2-hydroxylamino-6-nitrobenzyl alcohol formation from 2-amino-6-nitrobenzyl alcohol from 0.75 ± 0.11 to 1.27 ± 0.16 nmol/min/nmol of cytochrome P-450 and significantly increased the rate of aminophenol formation from 2.35 ± 0.05 to 3.49 ± 0.15 nmol/min/nmol of cytochrome P-450. Pretreatment with β-naphthoflavone had no effect on hydroxylamine formation (0.63 ± 0.09 nmol/min/nmol of cytochrome P-450) but produced a statistically significant increase in the rate of formation of the aminophenol metabolite (2.76 ± 0.16 nmol/min/nmol of cytochrome P-450).

Sex difference in the microsomal oxidation reaction. Preliminary experiments utilizing the colorimetric assay for total ethyl acetate-extractable reducing equivalents demonstrated that the rate of oxidation of 2-amino-6-nitrobenzyl alcohol by microsomes from female rats was lower than that of male rats, yielding a male/female ratio of 1.5 (data not shown). Quantitation of the individual metabolites by HPLC and colorimetric analyses revealed that the rates of formation of both the hydroxylamine and aminophenol were significantly lower with microsomes from female rats (0.23 ± 0.03 and 1.75 ± 0.07 nmol/min/nmol of cytochrome P-450, respectively) than with those from males (0.75 ± 0.11 and 2.35 ± 0.05 nmol/min/nmol of cytochrome P-450, respectively). The male/female ratio for the rate of formation of the aminophenol was 1.34 while that for the hydroxylamine was 3.26.

TABLE 3

Effect of cytochrome P-450 inhibitors on the rate of the microsomal oxidation of 2-amino-6-nitrobenzyl alcohol

The incubation conditions and colorimetric assay procedure were the same as described under "Experimental Procedures." The inhibitor concentrations were 0.5 mM when present.

Addition	Initial rate[a]
	nmol reducing equivalents formed/min/mg microsomal protein
None	1.22 ± 0.02 (100%)
SKF-525A	0.48 ± 0.01 (39%)[b]
Metyrapone	0.38 ± 0.02 (31%)[b]
Octylamine	0.62 ± 0.01 (51%)[b]

[a] Values are means ± S.E. for 3 determinations. The numbers in parentheses are the per cent of control value.

[b] Significantly different from control value, $p < 0.05$.

DISCUSSION

We have suggested previously that sulfation of hydroxylamine metabolites of 2,6-dinitrotoluene and 2-nitrotoluene results in the formation of electrophilic species which react with hepatic DNA (5, 6). The results of the present study demonstrate that 2-amino-6-nitrobenzyl alcohol and 2-aminobenzyl alcohol, amine metabolites of 2,6-dinitrotoluene and 2-nitrotoluene, can be N-hydroxylated by rat hepatic microsomes *in vitro*. Although red blood cells have been reported to be capable of N-hydroxylating aromatic amines (17), we observed that freshly isolated rat red blood cells did not N-hydroxylate 2-amino-6-nitrobenzyl alcohol,[3] suggesting that further metabolic activation of this molecule takes place in the liver.

2-Amino-6-nitrobenzyl alcohol was also ring-hydroxylated by rat hepatic microsomes. Mass spectral, proton NMR, and UV-visible spectroscopic studies suggested that the metabolite was 2-amino-5-hydroxy-6-nitrobenzyl alcohol. Unequivocal assignment of the structure of this metabolite could be accomplished by ^{13}C NMR experiments or by chemical synthesis of the possible phenolic isomers followed by comparison of their chromatographic and spectral properties. Neither of these approaches were attempted in this study, since ^{13}C NMR would require isolation of mg quantities of metabolite and specific syntheses of the possible isomers would be extremely difficult.

Numerous studies of the microsomal N-hydroxylation of a variety of carcinogenic arylamines and arylamides have implicated cytochrome P-450 and/or flavin-containing monooxygenase as the catalyst(s) (18–21). The rate of N-hydroxylation of 2-amino-6-nitrobenzyl alcohol was decreased by known inhibitors of cytochrome P-450 (Table 3) and increased by phenobarbital induction of cytochromes P-450, indicating that cytochrome P-450 isozymes catalyze the reaction. Heat inactivation of microsomal flavin-containing monooxygenase had no effect on the rate of the oxidation reaction and abolished the inhibition produced by methimazole, a high-affinity substrate for flavin-containing monooxygenase (16). Previous studies have shown that the flavin-containing monooxygenase-dependent oxidation of methimazole produces a metabolite which inhibits the microsomal cytochrome P-450-catalyzed N-hydroxylation of 2-amino-6-nitrotoluene (22) and results in a loss of cytochrome P-450 (12). These results indicate that flavin-containing monooxygenase is not involved in the rat hepatic microsomal oxidation of 2-amino-6-nitrobenzyl alcohol.

The role of the aminophenol metabolite in the bioac-

[3] G. L. Kedderis, J. P. Chism, and D. E. Rickert, unpublished observations.

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tivation or detoxification of 2,6-dinitrotoluene is presently unclear. The N-hydroxylation of arylamines and arylamides is generally considered to represent an activation step (23, 24), while, at least in the case of the hepatocarcinogen 2-acetylaminofluorene, C-hydroxylation is frequently considered as a detoxification step (24). However, *in vitro* studies have shown that C-hydroxylated metabolites of 2-acetylaminofluorene can be oxidized by the cytochrome *c*-cytochrome oxidase system to reactive iminoquinones which covalently bind to protein (25, 26). An analogous oxidation of the aminophenol metabolite of 2-amino-6-nitrobenzyl alcohol by microsomal enzymes may account for its nonlinear time course of formation (Fig. 4). Alternatively, the differential time course for the formation of the hydroxylamine and aminophenol metabolites may suggest that the two metabolites are not formed from a common intermediate. We have observed that the aminophenol was not formed during incubations of 2-hydroxylamino-6-nitrobenzyl alcohol with or without hepatic microsomes in the presence or absence of NADPH.[4] These data suggest that an enzymic or chemical rearrangement of the hydroxylamine is not an operable mechanism of aminophenol formation. Additional *in vitro* studies are necessary to determine if the aminophenol metabolite of 2-amino-6-nitrobenzyl alcohol can be oxidized to a reactive iminoquinone species.

The rate of N-hydroxylation of 2-amino-6-nitrobenzyl alcohol was enhanced after induction of cytochrome P-450 isozymes by phenobarbital pretreatment but not after pretreatment with β-naphthoflavone. This result is in contrast to the results of studies of the rat hepatic microsomal cytochrome P-450-dependent N-hydroxylation of other arylamines and arylamides (20, 21), which have implicated the involvement of cytochrome P-450 isozymes induced by β-naphthoflavone, 3-methylcholanthrene, or Aroclor 1254 rather than by phenobarbital. Since multiple isozymes of cytochrome P-450 are induced by phenobarbital treatment (27), the present results cannot discern which isozymes are primarily responsible for the N-hydroxylation of 2-amino-6-nitrobenzyl alcohol by rat hepatic microsomes. Recent evidence has suggested that the N- and C-hydroxylations of 2-acetylaminofluorene are catalyzed by different isozymes of rat hepatic cytochrome P-450 (28). The same may also be true for the cytochrome P-450-catalyzed N- and C-hydroxylations of 2-amino-6-nitrobenzyl alcohol, since microsomes from β-naphthoflavone-pretreated rats formed the two metabolites in a different ratio than those from untreated or phenobarbital-pretreated rats. Future studies will address the isozyme specificity of the cytochrome P-450-dependent metabolism of 2-amino-6-nitrobenzyl alcohol.

The rate of metabolism of 2-amino-6-nitrobenzyl alcohol was significantly lower with microsomes from female rats compared with those from males, yielding male/female ratios of 1.34 for aminophenol formation and 3.26 for hydroxylamine formation. Marked sex differences have been observed for the microsomal metabolism of a variety of compounds and have been ascribed to the presence of unique forms of cytochrome P-450 in female rat liver (29). Previous studies on 2,6-dinitrotoluene metabolism have demonstrated a lower rate of biliary excretion of 2,6-dinitrobenzyl glucuronide in female rat liver, which has been suggested to be related to the lower incidence of hepatocellular carcinomas in female *versus* male rats fed technical grade dinitrotoluene (1). If the N-hydroxylation of 2-amino-6-nitrobenzyl alcohol represents a bioactivation step in the metabolism of 2,6-dinitrotoluene, then the sex difference in hydroxylamine formation may also be related to the sex difference in 2,6-dinitrotoluene-induced hepatocarcinogenesis.

[4] G. L. Kedderis and D. E. Rickert, unpublished observations.

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