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METABOLISM OF 4-NITROANILINE BY RAT LIVER MICROSOMES

MARTIN M. ANDERSON[1], JOANN B. MAYS, RONALD K. MITCHUM, and JACK A. HINSON

National Center for Toxicological Research, Food and Drug Administration

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ABSTRACT:

The principal rat liver microsomal metabolite of 4-nitroaniline was isolated by high pressure liquid chromatography and was characterized as 2-amino-5-nitrophenol (2-hydroxy-4-nitroaniline) by comparison of its mass, nuclear magnetic resonance, and ultraviolet spectra and HPLC retention time to the synthetic compound. A metabolite with the chromatographic retention time of authentic *N*-hydroxy-4-nitroaniline was not detected. Pretreatment of rats with phenobarbital and 3-methylcholanthrene increased the rate of conversion of 4-nitroaniline to 2-hydroxy-4-nitroaniline by 2-fold and 4-fold, respectively; the reaction required NADPH and was inhibited by heat treatment of microsomes, by argon and carbon monoxide:oxygen atmospheres and by the cytochrome P-450 inhibitor, 2-[(2,4-dichloro-6-phenyl)phenoxy]ethylamine. In the presence of a molecular oxygen ($^{18}O_2$) atmosphere, ^{18}O was quantitatively incorporated into the metabolite. Microsomes did not catalyze the isomerization of *N*-hydroxy-4-nitroaniline to 2-hydroxy-4-nitroaniline. A primary isotope effect was not observed upon comparison of the rate of conversion of 2,6-dideutero-4-nitroaniline to 2-hydroxy-4-nitroaniline with that of the nondeuterated compound. The 2-hydroxy-4-nitroaniline derived from microsomal incubation mixtures of 2,6-dideutero-4-nitroaniline contained about 20%, 3,6-dideutero-2-hydroxy-4-nitroaniline.

4-Nitroaniline is an industrial intermediate used in the synthesis of a variety of pharmaceutical, veterinary, agricultural, and other consumer products. For example, the reduction product *p*-phenylenediamine is widely used in the manufacture of antioxidants. A number of dyes including Direct Yellow 44 and Acid Black 1 are produced from 4-nitroaniline. The rodenticide Vacor, (1-(3-pyridylmethyl)-3-(4-nitrophenyl)urea) is an *N*-substituted derivative of 4-nitroaniline.

4-Nitroaniline induces a number of toxicological effects. Human exposure has been associated with headache, nausea, stupor, cyanosis, and methemoglobinemia leading to anoxia. Chronic exposure is believed to lead to jaundice and anemia (1, 2). In rats, 4-nitroaniline produces methemoglobinemia (3) and it is also weakly mutagenic in the *Salmonella* reversion assay (4, 5).

Reports on the metabolism of 4-nitroaniline in rats have appeared from two different laboratories. Mate *et al.* (6) found 2-hydroxy-4-nitroaniline as a major oxidative metabolite and *p*-phenylenediamine as a major reductive metabolite in acid-hydrolyzed urine. Subsequently, Smith and Gorrod (7) reported that 4-nitroaniline was *N*-hydroxylated *in vitro* by liver microsomes from rats as well as four other species.

In the present work, we have studied in greater detail the *in vitro* metabolism of 4-nitroaniline using rat liver microsomes. The potential role of *N*-hydroxylation in the metabolism of this compound has been reexamined since *N*-hydroxy derivatives of other aromatic amines may be proximate carcinogens (8). We have also conclusively shown 2-hydroxy-4-nitroaniline to be a microsomal metabolite, and examined the mechanism of its formation.

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[1] Present address, Department of Chemistry, Clatsop Community College, Astoria, OR 97103.

Send reprint requests to: Dr. Jack A. Hinson, National Center for Toxicological Research, Jefferson, AR 72079.

Materials and Methods

Chemicals. [*Ring*-U-^{14}C]4-nitroaniline (specific activity, 1.88 mCi/mmol) (greater than 99% pure) was obtained from Midwest Research Institute (Kansas City, MO).

2,6-Dideutero-4-nitroaniline was synthesized by a modification of the method of Best and Wilson (9). 4-Nitroaniline (96 mg), 170 μl of 20% DCl (in D_2O, 100.0 atom % D), and 2.0 ml of D_2O (99.8 atom % D) were sealed in a glass tube and heated overnight in a boiling water bath. The solution was then neutralized with 10 μl of 0.05 M Na_2CO_3. The product was extracted into 2 ml of ethyl acetate, the extract was dried with anhydrous Na_2SO_4, and the solvent was evaporated. The residue was then recrystallized from benzene. Mass and NMR[2] spectral analyses confirmed the synthesis. Electron impact mass spectrometry showed that the molecular ion and all major mass fragment ions were increased by 2 atomic mass units compared to 4-nitroaniline and no significant amount of signal intensity (<1% of base peak) occurred at *m/z* values characteristic of nondeuterated 4-nitroaniline. The 100 MHz ^{1}H NMR signal for H-2 and H-6 (δ 6.6) was not detectable in the product and the doublet for H-3 and H-5 (δ 7.8) was converted to a singlet.

N-Hydroxy-4-nitroaniline was synthesized from 1,4-dinitrobenzene by the method of Kuhn and Weygand (10). Mass and ^{1}H NMR spectral analyses of the product were consistent with *N*-hydroxy-4-nitroaniline (molecular ion at *m/z* 154, and major mass fragments at 138, 124, 91, 80, 76, and 63; 100 MHz NMR doublets at δ 6.8, 8.0, with two downfield singlets disappearing when D_2O was added to sample).

4-Nitrosonitrobenzene was synthesized from *N*-hydroxy-4-nitroaniline by a modification of a method described by Brill (11). To 10 ml of ethyl ether was added 96 mg of *N*-hydroxy-4-nitroaniline. While the solution was stirred, flushed with argon, and cooled in an ice bath, 106 mg of diethylazodicarboxylate in 10 ml of ether was added during 3 min. Stirring was continued for 2 hr, after which the ether was evaporated under a stream of argon. The solid residue was suspended in a small

[2] Abbreviations used are: NMR, nuclear magnetic resonance; DPEA, 2-[(2,4-dichloro-6-phenyl)phenoxy]ethylamine.

volume of benzene-hexane (1:7, v/v), applied to a Florisil column (60–100 mesh, 2.5 × 20 cm), and eluted with benzene-hexane (1:7). A green band was collected and the solvent was evaporated under a stream of argon. Mass spectral analysis revealed a molecular ion for 4-nitrosonitrobenzene (*m/z* 152) and a minor contaminant with a molecular ion corresponding to 1,4-dinitrobenzene (*m/z* 168). High pressure liquid chromatographic analysis indicated 4-nitrosonitrobenzene was contaminated with a minor amount of 1,4-dinitrobenzene.

4,4′-Dinitroazobenzene was synthesized from 1,4-dinitrobenzene by an adaptation of the method of Vogel (12). 1,4-Dinitrobenzene (2.1 g) was dissolved in a solution containing 55 ml of methanol and 55 ml of toluene. Magnesium turnings (0.75 g) and a small crystal of iodine were added and the solution was warmed until slow evolution of bubbles began. Another 0.75 g of Mg was added when most of the original Mg had reacted. The solution was refluxed until all Mg had dissolved (about 2 hr). The solvent was removed under reduced pressure and the residue was dissolved in 50 ml of methanol-acetone (1:1, v/v). The solution was poured into 200 ml of cold water, and acetic acid was added until the solution was acidic. The solution was cooled in an ice bath and the deep red precipitate which formed was collected and recrystallized from ethanol-acetone (1:1, v/v). Mass and 270 MHz NMR spectra corresponded to those of 4,4′-dinitroazobenzene (molecular ion, *m/z* 272; two doublets centered at δ 8.49 and 8.20 ppm, J = 9.2 Hz relative to trimethylsilane).

4,4′-Dinitroazoxybenzene was synthesized by a modification of the method of Manson (13). *N*-hydroxy-4-nitroaniline (25 mg) was dissolved in 10 ml of acetone and 10 ml of 0.1 M sodium phosphate, pH 7, was added. The solution was stirred at room temperature for 4 hr, with periodic addition of acetone to maintain a constant volume. After about 40 min, a precipitate formed, which did not immediately dissolve upon addition of acetone, but dissolved after an additional hour of stirring. The solution was cooled in an ice bath and held in the refrigerator overnight. The initial white precipitate, found to be sodium phosphate, was discarded. The remaining solution was evaporated and the residue in the flask was extracted with hot acetone. No solid appeared when the acetone extract was evaporated. The solid remaining in the flask was submitted to mass and NMR spectral analysis. Spectral characteristics were those of 4,4′-dinitroazoxybenzene (molecular ion, *m/z* 288; two aryl doublets containing four protons centered at δ 8.22 and 8.42, J = 9.2 Hz and an AB pattern containing four protons centered about 8.51, J = 9.9 Hz). A molecular ion for phenazine (*m/z* 270) was not observed nor were any singlets observed by NMR analysis.

Nicotinamide adenine dinucleotide phosphate was purchased from P-L Biochemicals. Disodium glucose 6-phosphate, glucose 6-phosphate dehydrogenase (type XXI), phenobarbital, 3-methylcholanthrene, and 4-nitroaniline were obtained from Sigma Chemical Co. 4,6-Diphenyl-1,10-phenanthroline (bathophenanthroline), D_2O (99.8 atom % D) and DCl (20% solution in D_2O, 100.0 atom % D) were products of Aldrich Chemical Co. DPEA was a gift from Eli Lilly Co. Oxygen ($^{18}O_2$) was purchased from Stohler Isotope Chemicals. 1,4-Dinitrobenzene was obtained from Tridom Chemicals, Inc. Florisil was ordered from Fisher Scientific Co. 2-Hydroxy-4-nitroaniline (2-amino-5-nitrophenol) was a product of Fluka (Switzerland).

Instruments. Mass spectra were obtained with a solid probe using a Finnigan 4023 mass spectrometer operated in the electron impact mode at 70 eV and 250°C ionizer temperature. Proton NMR spectra were acquired with either a JEOL MH-100 or a Bruker WH-270 instrument. Samples for ^1H NMR analysis were dissolved in DMSO-d_6 and chemical shifts were referenced to tetramethylsilane. Ultraviolet spectra were obtained on a Gilford 2400-2 spectrophotometer.

Microsomal Preparations. Hepatic microsomes were prepared from fresh tissue of male Sprague-Dawley rats (200–400 g) from our breeding colony according to previously reported procedures (14). Protein concentrations were determined by use of the biuret reaction (15) and the microsomes were stored in liquid N_2 until use. For the induction experiments, rats received ip injections of 3-methylcholanthrene (20 mg/kg body weight) on each of 3 successive days, or phenobarbital (80 mg/kg body weight) on the day before sacrifice.

Microsomal Incubations and Assay of Hydroxylated Product. Standard incubation mixtures contained 0.5 mM EDTA, 2.5 mM glucose 6-phosphate, 1.0 mM $NADP^+$, 3 units of glucose 6-phosphate dehydrogenase, 0.1 M sodium phosphate, pH 7.4, 0.5 mM 4-nitroaniline (added in 10 μl 95% ethanol), and 2.0 mg of microsomal protein in a total volume of 1.0 ml. Incubations were conducted in 10-ml Erlenmeyer flasks at 37°C with shaking. At appropriate time intervals, 0.8-ml aliquots were removed and thoroughly mixed with 1.0 ml of water- and argon-saturated amyl acetate or ethyl acetate. The phases were then separated by centrifugation. Aliquots (0.5 ml) of the organic extracts were taken for assay by a modification (16) of the bathophenanthroline assay of Kadlubar *et al.* (17) or by measurement of 2-hydroxy-4-nitroaniline HPLC peak areas. When the rate of product formation was measured by the two methods, the rates were found to be the same and linear for at least 20 min.

To determine the role of molecular oxygen in 4-nitroaniline metabolism, incubation mixtures as described above, minus substrate, were shaken for 1 min with an argon or a CO/O_2 mixture (4:1). The flasks were then sealed, substrate was added through the seal with a syringe, and the incubations were carried out as above. For incubations with $^{18}O_2$, a sealed flask containing 0.5 mM EDTA, 2.5 mM glucose 6-phosphate, 1.0 mM $NADP^+$, 60 units of glucose 6-phosphate dehydrogenase, and 0.1 M sodium phosphate, pH 7.4 in a 20-ml volume, was repeatedly evacuated and flushed with N_2, then evacuated and filled with $^{18}O_2$. The other components of the incubation mixture, 4-nitroaniline and microsomes, were flushed with nitrogen and injected into the sealed flask. The incubation was conducted for 1 hr at 37°C with shaking. Immediately before and after the incubation, 1-ml samples were withdrawn from the vapor space and the $^{16}O_2$ and $^{18}O_2$ ratio determined by mass spectrometry. The metabolite was removed from the reaction mixture by two 20-ml extractions with ethyl acetate. The combined extracts were evaporated under a stream of argon and redissolved in methanol. The metabolite was isolated by HPLC on a μBondapak column (3.9 × 300 mm) eluted with 20% methanol using the system described below and was analyzed by mass spectrometry.

For the HPLC assay and metabolite isolation, the organic extracts were evaporated and redissolved in methanol and then injected into a Waters Associates HPLC system equipped with a U6K injector, a Tracor 970A detector, a model 660 solvent programmer, and two model 6000 pumps. Analytical separations were performed on a C_{18} μBondapak column (3.9 × 300 mm) and large scale separations on a C_{18} μBondapak column (7.8 × 300 mm). Elution was conducted with aqueous methanol (Fisher Scientific Co., HPLC grade). In experiments with radioactive substrate, 30-sec fractions were collected and the radioactivity was determined using Scintisol scintillation fluid (Isolabs, Akron, OH) and a Searle Mark III scintillation counter.

Results

Characterization of Major Microsomal Metabolites of 4-Nitroaniline. When 4-nitroaniline was incubated with rat liver microsomes and an NADPH-generating system, HPLC analysis of an ethyl acetate extract revealed a single major metabolite as shown in fig. 1. The same profile was obtained whether the microsomes used were from control, phenobarbital-induced, or 3-methylcholanthrene-induced animals. The initially eluting peak (I) (fig. 1) was not detected in an incubation mixture lacking a NADPH-generating system, and had a retention time and UV spectrum identical to those of authentic 2-hydroxy-4-nitroaniline (2-amino-5-nitrophenol). The peak which eluted second (II) (fig. 1) had a retention time on HPLC and UV spectrum identical to those of the substrate, 4-nitroaniline. To isolate enough metabolite to conclusively establish the identity of the metabolite as 2-hydroxy-4-nitroaniline, 4-nitroaniline was incubated for 1 hr in a 10-ml incubation mixture containing microsomes and a NADPH-generating system. The incubation mixture was extracted with ethyl acetate and the metabolite was isolated by multiple HPLC separations. The mass spectrum showed a frag-

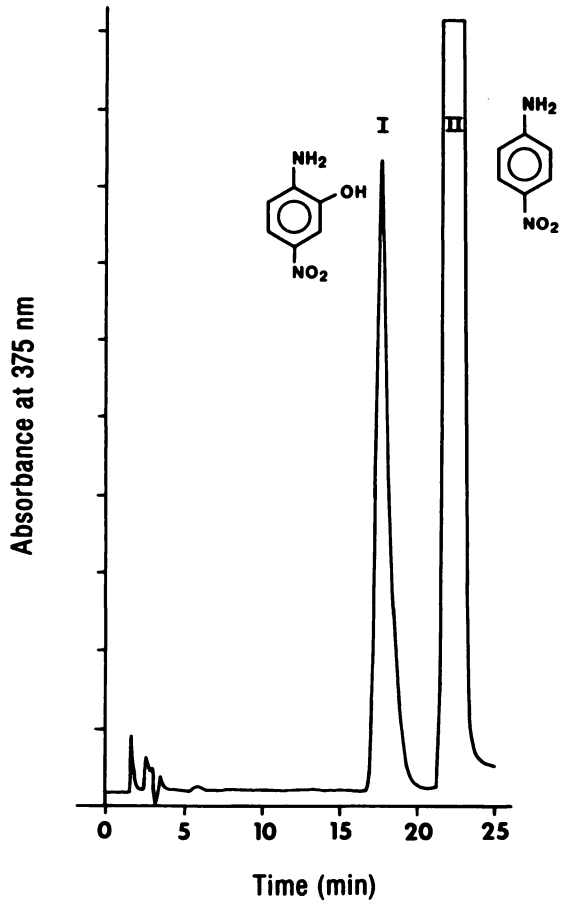

FIG. 1. *High pressure liquid chromatographic analysis of ethyl acetate extract from incubation of 4-nitroaniline with rat liver microsomes.*

4-Nitroaniline was incubated with rat liver microsomes as described in *Materials and Methods*. An ethyl acetate extract of the incubation mixture was analyzed on an analytical C_{18} μBondapak column eluting with 20% methanol at 1 ml/min.

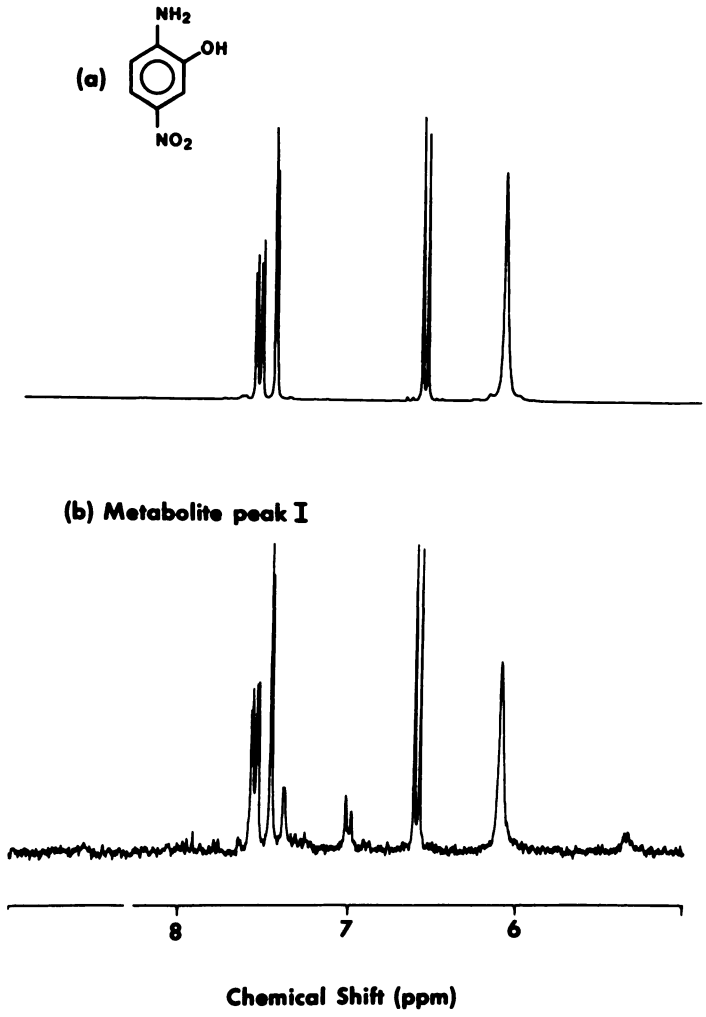

FIG. 2. *Aromatic region of 270 MHz ^1H NMR spectra of (a)* authentic 2-hydroxy-4-nitroaniline and *(b)* metabolite.

The metabolite sample was prepared by large scale (20 ml) incubation of 4-nitroaniline with microsomes from 3-methylcholanthrene-induced rats followed by ethyl acetate extraction and purification of the metabolite by HPLC on a μBondapak column (7.8 × 300 mm) eluted with 20% methanol. 270 MHz ^1H NMR spectra were obtained in DMSO-d_6. Chemical shifts are in ppm downfield from tetramethylsilane internal standard. The assigned resonances are: 6.59 (d, 1, H_6, $J_{5,6}$ = 9.0 Hz), 7.56 (d, 1, H_5, $J_{3,5}$ = 2.6 Hz), 7.47 (S, 1, H_3), and 6.10 (brS, 2, NH_2). Impurities are present at 7.0 and 7.4 ppm in the metabolite (6).

mentation pattern identical to that of an authentic sample of 2-hydroxy-4-nitroaniline (molecular ion, *m/z* 154; fragment ions, *m/z* 124, 108, 96, 80, and 63). The aromatic regions of the 270 MHz ^1H NMR spectra of the metabolite and authentic 2-hydroxy-4-nitroaniline are shown to be identical in fig. 2. Thus, the hydroxyl group was unequivocally placed on C-2.

Since other investigators have reported that 4-nitroaniline is converted to an *N*-hydroxy compound by rat hepatic microsomes, attempts were made to find evidence for metabolic *N*-hydroxylation. Careful examination was made of HPLC chromatograms of ethyl acetate extracts from standard and large scale microsomal incubations of 4-nitroaniline for radioactivity and UV absorbance at the retention time of *N*-hydroxy-4-nitroaniline (13 min in fig. 1). No evidence for the formation of this compound could be detected, even though the conversion of substrate to *N*-hydroxy product at a velocity of 20 pmol/min/mg protein would have been easily detectable. Also, when unlabeled *N*-hydroxy-4-nitroaniline (0.05 mM) was added to [^{14}C]4-nitroaniline incubation mixtures and extracts examined by HPLC analysis, only background radioactivity was detected in the recovered *N*-hydroxy-4-nitroaniline peak even though 92% of the *N*-hydroxy-4-nitroaniline was recovered and [^{14}C]2-hydroxy-4-nitroaniline was a major metabolite. Furthermore, HPLC chromatograms were inspected for peaks at retention times of further reaction products of *N*-hydroxy-4-nitroaniline. 4-Nitrosonitrobenzene, 1,4-dinitrobenzene, 4,4′-dinitroazobenzene, and 4,4′-dinitroazoxybenzene were used as markers, but formation of radioactive or UV-absorbing products at the corresponding retention times were not detected.

Role of Cytochrome P-450 in Formation of 2-Hydroxy-4-nitroaniline. The effects of pretreatment of rats with inducers of the microsomal cytochrome P-450 monooxygenases on the metabolism of 4-nitroaniline were examined. Phenobarbital pretreatment increased the rate of 2-hydroxylation from 0.48 nmol/min/mg microsomal protein to 1.00 nmol/min/mg microsomal protein whereas 3-methylcholanthrene induced the rate to 2.7 nmol/min/mg protein. Table 1 shows the effects of various incubation conditions on the rate of ring hydroxylation. The

TABLE 1

Rates of 4-nitroaniline metabolism under different conditions

Microsomes were prepared from nonpretreated rats. Incubations were performed and rates of metabolism were determined using the bathophenanthroline assay as described in *Materials and Methods*. Data are presented as mean and standard deviation of the mean, $n = 3$.

Conditions	Rate of 2-Hydroxy-4-nitroaniline Formation
	% control
Complete system	100 ± 3
Heat-treated microsomes	<3
−NADPH	<3
+Argon atmosphere	67 ± 4
+CO/O_2 (4:1) atmosphere	72 ± 7
+DPEA, 0.5 mM	28 ± 6

reaction was abolished by heat treatment of microsomes and required NADPH. Argon and an atmosphere containing carbon monoxide and oxygen inhibited the reaction, as did the cytochrome P-450 inhibitor, DPEA (18).

The role of molecular oxygen in the conversion of 4-nitroaniline to 2-hydroxy-4-nitroaniline was further examined by incubating 4-nitroaniline with microsomes and the NADPH-generating system in the presence of either air or an $^{18}O_2$ atmosphere. Large scale incubations (20 ml) were conducted for 1 hr and the metabolite was isolated by HPLC for mass spectral analysis. As shown in fig. 3, the molecular ion of the metabolite was increased from *m/z* 154 to 156 and the fragment ions at *m/z* 124, 108, and 96 were also increased by 2 μm when air was replaced by 98% $^{18}O_2$. In the spectrum for the $^{18}O_2$-derived metabolite, the *m/z* 156 peak is 98% of the total (154 + 156) M^+ signal intensity, suggesting that essentially all of the oxygen incorporated into the product was derived from molecular oxygen.

Mechanism of Formation of 2-Hydroxy-4-nitroaniline. Even though the above data suggested that 2-hydroxy-4-nitroaniline was not formed by isomerization of *N*-hydroxy-4-nitroaniline, it was still conceivable that 2-hydroxy-4-nitroaniline was formed by this mechanism within the microsomes. The possibility that microsomes may catalyze the isomerization of *N*-hydroxy-4-nitroaniline was thus investigated. *N*-hydroxy-4-nitroaniline was incubated in microsomal incubation mixtures with and without a NADPH-generating system. Mixtures incubated for 10 min and for 30 min were extracted with ethyl acetate, and the extracts were analyzed by HPLC. No evidence for the presence of 2-hydroxy-4-nitroaniline was detected in any of these microsomal incubation mixtures.

A mechanism of ring hydroxylation which involves cleavage of an aryl C-H bond (direct hydroxylation) in the rate-limiting step would be expected to produce a primary hydrogen isotope effect (19). To determine whether or not such an effect occurred in the formation of 2-hydroxy-4-nitroaniline, 2,6-dideutero-4-nitroaniline was used as the substrate. Standard incubations were conducted in parallel using either 4-nitroaniline or 2,6-dideutero-4-nitroaniline as substrates. Fig. 4 shows kinetic plots for both substrates. The rates of 2-hydroxylation were 3.26 ± 0.13 and 3.23 ± 0.15 nmol/min/mg protein for 4-nitroaniline and 2,6-dideutero-4-nitroaniline, respectively, which indicates no detectable change in rate caused by deuterium in the ring hydroxylation.

The incorporation of $^{18}O_2$ into 2-hydroxy-4-nitroaniline, the lack of formation of that compound from *N*-hydroxy-4-nitroaniline, and the lack of a kinetic isotope effect suggested that 2-

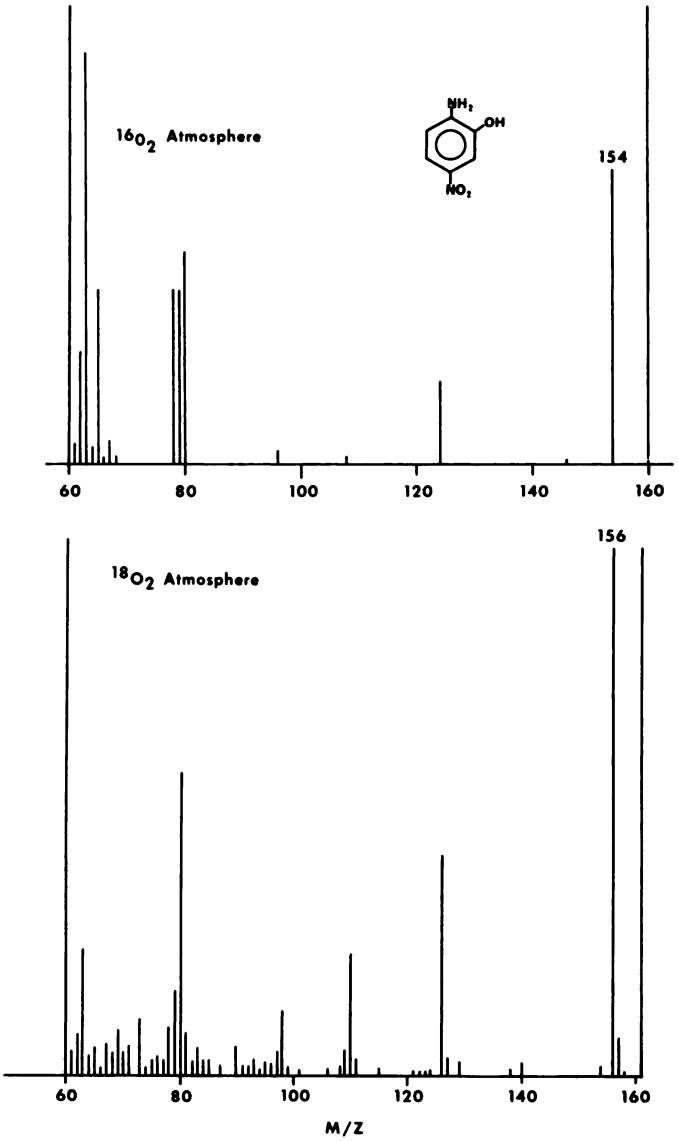

FIG. 3. *Mass spectra of microsomal metabolite derived from 4-nitroaniline incubation mixtures performed in $^{16}O_2$ and $^{18}O_2$ atmospheres.*

Metabolite samples were prepared from large scale (20 ml) incubations of 4-nitroaniline with microsomes from 3-methylcholanthrene-induced rats, gassed with $^{18}O_2$ as described in *Materials and Methods*, and isolated by ethyl acetate extraction and HPLC separation on a μBondapak column (7.8 × 300 mm) eluted with 20% methanol. Electron impact mass spectral analysis was conducted at 70 eV using a solid probe heated to 250°C.

hydroxy-4-nitroaniline may be formed by epoxidation of 4-nitroaniline followed by isomerization to the phenol. Many aromatic hydroxylations which take place by such a mechanism involve the migration of a substituent from the position being hydroxylated to an adjacent position (NIH shift) (20). To determine whether such a rearrangement occurs during the formation of 2-hydroxy-4-nitroaniline from 4-nitroaniline, 2,6-dideutero-4-nitroaniline was incubated with rat liver microsomes and the 2-hydroxy-4-nitroaniline was isolated and purified by extraction and HPLC, and subjected to mass spectral analysis. The mass spectra of the product and of 2,6-dideutero-4-nitroaniline substrate are shown in fig. 5. The signal at *m/z* 155 corresponds to

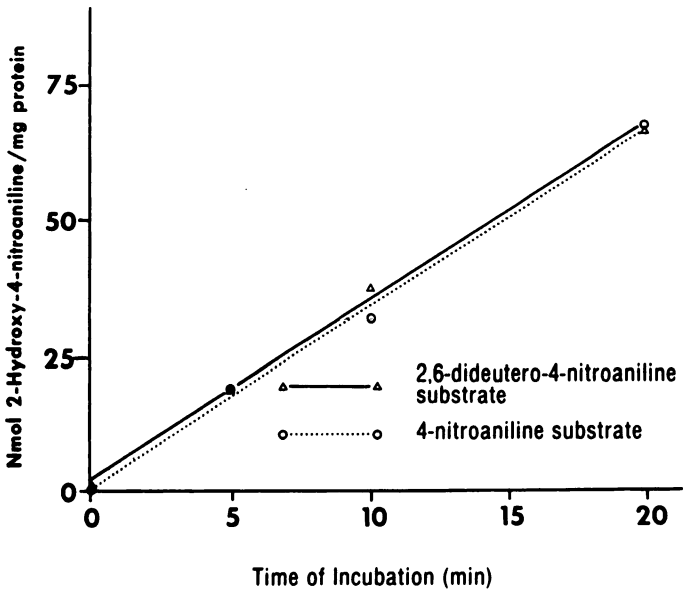

FIG. 4. *Formation of 2-hydroxy-4-nitroaniline from 4-nitroaniline and from 2,6-dideutero-4-nitroaniline.*

The 2,6-dideutero-4-nitroaniline was greater than 99% pure. The 2-hydroxy-4-nitroaniline was analyzed by the bathophenanthroline assay described in *Materials and Methods*. Microsomes from 3-methylcholanthrene-induced rats were used at 0.92 mg protein/ml.

the molecular ion of monodeuterated 2-hydroxy-4-nitroaniline and the signal at 156 to that of dideuterated 2-hydroxy-4-nitroaniline. From the intensities of these two signals, the relative amounts of mono- and dideuterated products were estimated. About 20% of the product molecules have retained both deuterium atoms, indicating significant retention of deuterium displaced from the ring position which was hydroxylated.

Discussion

Aniline and its derivatives are known to be metabolized by oxidations at either carbon or nitrogen (21). Whereas carbon oxidation usually serves as a detoxification mechanism for these compounds, nitrogen oxidation is believed to produce highly toxic intermediates. The *N*-hydroxy derivatives may produce methemoglobinemia, are frequently mutagenic, and may be proximate or ultimate carcinogens (23). Factors which predispose the aniline derivatives to be metabolized by one pathway or the other are not clearly understood (23). Moreover, *N*-hydroxy compounds in some cases are known to isomerize to the phenolic derivatives (24, 25). The importance of such isomerization in the metabolism of these derivatives has not been adequately defined.

In this study, we have examined the metabolism of 4-nitroaniline in microsomal incubation mixtures. Even though it has previously been reported to be *N*-hydroxylated in microsomal incubation mixtures (7), in this study, we have found no evidence for *N*-hydroxylation. Neither *N*-hydroxy-4-nitroaniline nor a number of its possible oxidation products were detected by HPLC analysis of microsomal mixtures. Moreover, when unlabeled *N*-hydroxy-4-nitroaniline was added to microsomal mixtures containing [^{14}C]-4-nitroaniline and the *N*-hydroxy-4-nitroaniline recovered by HPLC after incubation, no significant amount of radiolabel was found in the recovery of the *N*-hydroxy derivative. In these microsomal incubation mixtures, however,

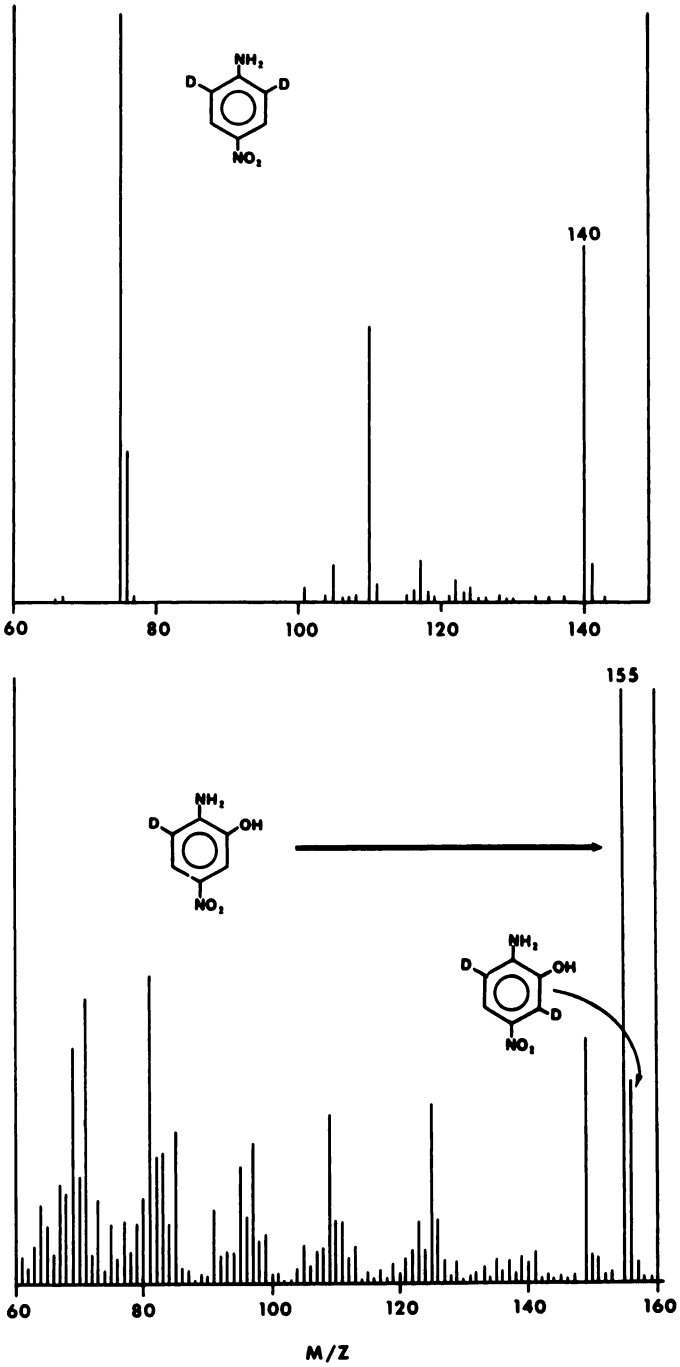

FIG. 5. *Mass spectra of 2,6-dideutero-4-nitroaniline substrate and deuterated 2-hydroxy-4-nitroaniline products.*

Metabolite samples were prepared from a large scale (20 ml) incubation of 2,6-dideutero-4-nitroaniline with microsomes from 3-methylcholanthrene-induced rats, followed by ethyl acetate extraction and HPLC separation on a μBondapak column (7.8 × 300 mm) eluted with 20% methanol. Electron impact mass spectral analysis was conducted at 70 eV using a solid probe heated to 250°C.

2-hydroxy-4-nitroaniline was a major metabolite of 4-nitroaniline (figs. 1 and 2). This finding confirms the previous urinary metabolite study that 2-hydroxylation is a major pathway of 4-nitroaniline metabolism.

The rate of formation of the 2-hydroxy metabolite was increased by pretreatment of rats with 3-methylcholanthrene and

phenobarbital. In the presence of an argon atmosphere, a carbon monoxide-oxygen atmosphere, or the cytochrome P-450 inhibitor DPEA, the formation of the metabolite was inhibited (table 1). Also $^{18}O_2$ experiments revealed the phenolic oxygen is derived from molecular oxygen (fig. 3). These data suggest that the metabolite was formed by action of a cytochrome P-450.

In other experiments, the mechanism of this ring hydroxylation of 4-nitroaniline was examined. The 2-hydroxy metabolite apparently was not formed by an initial *N*-hydroxylation followed by isomerization, since the incubation of *N*-hydroxy-4-nitroaniline with microsomes and NADPH yielded no 2-hydroxy-4-nitroaniline.

Tomaszewski *et al.* (18) have shown that *meta*-hydroxylation of deuterated nitrobenzene proceeds at a slower rate than that of the corresponding protonated derivative. They suggested an insertion of oxygen at the aryl C-H bond (direct hydroxylation) may occur. Since the hydroxylation of 4-nitroaniline occurs *meta* to the nitro group, the possibility was investigated that 4-nitroaniline is converted to the 2-hydroxy-4-nitroaniline derivative by a similar mechanism. The finding that 2,6-dideutero-4-nitroaniline was converted to the 2-hydroxy derivative at the same rate as nondeuterated 4-nitroaniline (fig. 4) suggests that oxygen insertion is not the mechanism of hydroxylation of 4-nitroaniline.

The deuterium isotope experiments provided further evidence regarding the hydroxylation mechanism. When the 2-hydroxy-4-nitroaniline metabolite from the microsomal incubation mixtures containing 2,6-dideutero-4-nitroaniline was analyzed by mass spectrometry, about 20% of the metabolite contained two deuterium atoms (fig. 5). Other investigators have shown that such isotope retention by migration (NIH shift) is characteristic of aromatic hydroxylations which occur by way of epoxide intermediates (20). Thus, 4-nitroaniline is apparently converted at least in part to 2-hydroxy-4-nitroaniline through an intermediate 2,3-epoxide.

The importance of oxidative metabolism in the toxicities of 4-nitroaniline is unclear. 4-Nitroaniline is mutagenic in the *Salmonella* reversion assay only in the presence of microsomal enzymes (4). Since 2-hydroxy-4-nitroaniline is mutagenic in this system in the absence of added enzymes (27, 28), the previously reported mutagenicity of 4-nitroaniline may be mediated by intermediacy of 2-hydroxy-4-nitroaniline. However, the importance of this pathway in the toxicities of 4-nitroaniline in higher organisms remains to be determined.

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