NIH Shift in the Hydroxylation of Aromatic Compounds by the Ammonia-Oxidizing Bacterium *Nitrosomonas europaea.* Evidence against an Arene Oxide Intermediate†

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ABSTRACT: The migration of deuterium and hydrogen was observed in the aromatic hydroxylation of specifically deuterated, monosubstituted benzenes catalyzed by ammonia monooxygenase of *Nitrosomonas europaea.* The phenolic products of the hydroxylation of aromatics containing *ortho-/para*-directing substituents (F, Cl, Br, I, OH, NH_2, CH_3, CH_2CH_3, and OCH_3) were primarily *para*-phenols. In contrast, with aromatics containing *meta*-directing substituents (NO_2 and CN), the phenolic products were a more even mixture of *meta*- and *para*-phenols. *ortho*-Fluorophenol was the only *ortho*-phenolic product observed. The nature of the products suggested that the reaction involved an enzyme-specific, electrophilic addition to the aromatic ring so as to favor hydroxylation at either the *meta*- or *para*-positions. With the fluoro-, chloro-, and bromobenzene substrates, the values for the migration and retention of deuterium during hydroxylation (NIH shift) were nearly identical when the deuterium was either at the site of hydroxylation or at an adjacent site, indicating a possible common intermediate. The values of the NIH shift with the nitrobenzene substrate were significantly lower when the deuterium was at the site of hydroxylation than at an adjacent site, indicating the operation of a direct loss mechanism. The present results suggest that the aromatic hydroxylation involved a radical or carbocation intermediate which decayed, without the formation of an arene oxide, to form phenolic products with the accompanying direct loss of deuterium at the site of hydroxylation or the shift of the deuterium to an adjacent site.

The shift and retention of deuterium, tritium, methyl, or halogen substituents from the site of hydroxylation to an adjacent site during the oxidation of aromatic compounds (the "NIH shift") is a characteristic common to monooxygenase enzymes (Dalton et al., 1981; Daly et al., 1972; Green & Dalton, 1989; Guroff et al., 1967; Jerina & Daly, 1974) and has been demonstrated with chemical hydroxylations (Boyd et al., 1972; Jerina et al., 1971; Kasperek & Bruice, 1972). These reactions are thought to proceed through the slow formation of an arene oxide which spontaneously rearranges to a phenol (Jerina et al., 1970; Kasperek & Bruice, 1972). In the case of the hydroxylation of naphthalene by cytochrome P-450, 1,2-naphthalene oxide was identified as an intermediate metabolite (Jerina et al., 1968, 1970). It has been proposed that the arene oxide opens to form a cationic intermediate which breaks down by one of three distinct mechanisms to produce phenol products with differing levels of deuterium retention (Figure 1) (Daly et al., 1968). According to one mechanism, the cationic intermediate forms a *para*-ketone intermediate and shifts the *para*-deuterium or hydrogen as a hydride to the adjacent carbon (path A). The ketone then tautomerizes to a *para*-phenol with an expected isotope effect (k_H/k_D) of ~4 (Boyd et al., 1972; Hanzlik et al., 1984). Since both *para*- and *meta*-deuterated substrates pass through the same ketone intermediate, the deuterium retention values for *para*-phenol products are the same. By the second mechanism (path B), the epoxide opens to form the *meta*-ketone, with the shift of the *meta*-deuterium to either the *ortho*- or *para*-positions due to resonance of the cationic intermediate. Since both *para*- and *meta*-deuterated aromatic substrates could not pass through the same ketone intermediate, the deuterium retention values for *meta*-phenol products must be different. The carbocation could also form a cyclohexadieneoid intermediate by resonance stabilization of the positive charge by electron-donating substituents (path C). Subsequent collapse of this structure would form *para*-phenols with the complete loss of deuterium or hydrogen from the hydroxylation site. Since the Hammett constant is a measure of electron-withdrawing ability (Exner, 1978), one might predict that, as the value of the constant increases, the deuterium retention value will also increase as more of path A was followed (Daly et al., 1968; Jerina et al., 1971).

Mechanisms that do not involve arene oxide intermediates have also been proposed (Hanzlik et al., 1984; Tomaszewski et al., 1975). For example, cytochrome P-450 catalyzed the hydroxylation of chlorobenzene to *meta*-chlorophenol, and the latter was shown not to be produced by degradation of chemically synthesized arene oxides (Selander et al., 1975a,b). Similar results were observed in the hydroxylation of 2,2′,5,5′-tetrachlorobiphenyl to phenolic products by cytochrome P-450 (Preston et al., 1983). Several intermediates, other than an arene oxide, have been proposed, including a ketone, radical, or carbocation (Boyd et al., 1972; Daly et al., 1968; Hanzlik et al., 1984; Kasperek et al. 1972).

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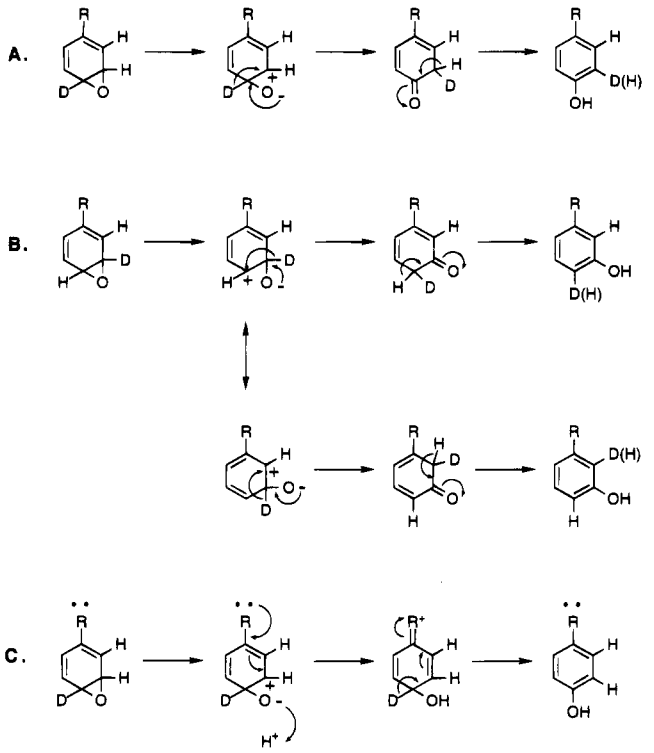

FIGURE 1: Possible mechanisms for the breakdown of an arene oxide by way of a cationic intermediate. (A) Formation of *para*-phenol with the migration of deuterium or hydrogen to the *meta*-position. (B) Formation of *meta*-phenol with the migration of deuterium or hydrogen from the *meta*-position to either the *ortho*- or *para*-positions. (C) Formation of *para*-phenol with the direct loss of deuterium or hydrogen from the hydroxylation site.

Although benzene, phenol (Hyman et al., 1985), and other aromatic substrates (Keener & Arp, 1994) have been shown to be degraded by ammonia monooxygenase (AMO)[1] of *Nitrosomonas europaea,* the mechanism of degradation is not known. In this paper, representative aromatic compounds have been tested as substrates and their products determined. With specifically deuterated aromatic compounds, the values of the retention of deuterium during hydroxylation to phenolic products suggest that an arene oxide intermediate was not required in the oxidation mechanism.

MATERIALS AND METHODS

Chemicals. Deuterium gas (99%) and [2,4,6-$^2H_3$]phenol (99%) were obtained from Cambridge Isotopes, Cambridge, MA. All other chemicals were obtained from Aldrich Chemical, Milwaukee, WI.

Bacterial Strains. N. europaea was grown and harvested as described previously (Logan, 1991).

Synthesis of Deuterated Compounds. [4-^{2}H]fluoro, [4-^{2}H]-chloro, [4-^{2}H]bromo-, and [4-^{2}H]ethylbenzenes were prepared by first reacting *para*-bromofluorobenzene, 1,4-dichlorobenzene, 1,4-dibromobenzene, or *para*-bromoethylbenzene with magnesium to make a Grignard reagent which was then quenched with deuterated acetic acid and D_2O. Deuterated [2,6-$^2H_2$]fluorobenzene, nitrobenzene, benzonitrile, and anisole were prepared by catalytic hydrogenation of the corresponding brominated precursors (Jerina et al., 1971). Deuterated aniline was prepared by repeatedly refluxing aniline hydrochloride in D_2O for 24 h to exchange *ortho* and *para* hydrogens with deuteriums (Swain et al., 1975). [3,5-$^2H_2$]-Chlorobenzene, [3,5-$^2H_2$]nitrobenzene, and [2,4,6-$^2H_3$]chlorobenzene were similarly prepared from their respective *para*- and *meta*-aniline hydrochlorides. The amino group was subsequently removed by diazotization (Starkey, 1943) and reduction (Korzeniowski et al., 1977) to give the deuterated substrate. The deuterated products were distilled and the deuterium content and position measured by mass spectroscopy and ^{1}H nuclear magnetic resonance (NMR), respectively.

Reaction Conditions. Incubations were in 125 mL serum vials sealed with Tuf-Bond Teflon laminated silicone discs (Pierce, Rockford, IL) and hypovial aluminum clasps. Ammonium, as a 1 M stock solution of ammonium sulfate, was added to a 100 mL solution of 100 mM, pH 7.8 phosphate buffer to a final concentration of 1 or 10 mM. Aromatic compounds were introduced either as 100 mM dimethyl sulfoxide stock solutions or as neat liquids to final concentrations of 10−1000 μM (Table 1). The reaction was initiated by the addition of 400 μL of a 200 mg/mL (wet weight) suspension of cells to a final protein concentration of 0.12 mg of protein/mL. The suspension was stirred at 23 °C for 60 min and was terminated by the addition of allylthiourea as a 1 M dimethyl sulfoxide stock solution to a final concentration of 100 μM.

Gas Chromatography. For the determination of the phenolic products, *para*-cresol was added as an internal standard to the cell suspensions which were centrifuged to remove the cells. The solution was then extracted with either methylene chloride or ethylacetate. Due to *para*-cresol overlapping product peaks, biphenyl was added to the organic solvent as an internal standard for anisole, fluorobenzene, and toluene. For nitrobenzene, the aqueous layer was first acidified before extraction. For phenol and aniline, 250 mg of sodium dithionate was added to the aqueous layer previous to extraction. The organic layer was then concentrated down by evacuation to around 1 mL. Nitrobenzene, benzonitrile, phenol, and aniline products were then derivatized with acetic anhydride. The monohalogenated benzenes and anisole products were determined without derivatization. Iodo, bromo, and chlorophenols were separated on a 30 m, 0.25 mm internal diameter, 0.25 μm film thickness, DB-23 capillary column (J&W Scientific, Folson, CA). All other phenolic products were determined on a similar DB-5 capillary column (J&W Scientific). Chromatography was carried out on a Hewlett-Packard model 5890 gas chromatograph equipped with a flame ionization detector and a model 7673A autosampler.

Gas Chromatography−Mass Spectrometry. Deuterium content of the substrates and their phenolic products was measured with a Kratos model MS25, 70 eV electron impact gas chromatography−mass spectrometer (GCMS). The mass spectra of deuterated substrates and products were compared with authentic nondeuterated standards, and the deuterium content was determined by a least-squares calculation to remove the effects of naturally occurring isotopes (Brauman, 1966). The values of mole percent for the mixtures of deuterated and nondeuterated substrates and the products had an error of ±2%. The values of mole percent for the

[1] Abbreviations: AMO, ammonia monooxygenase; MMO, methane monooxygenase; sMMO, soluble methane monooxygenase; pMMO, particulate methane monooxygenase; NMR, nuclear magnetic resonance; GCMS, gas chromatography-mass spectrometer; TLC, thin layer liquid chromatography.

Table 1: Products of the Hydroxylation of Aromatic Substrates by AMO

substrates	[substrate] (mM)[a]	[NH_3] (mM)[a]	phenols (μmol)[b]			other hydroxylation products (μmol)[b]
			ortho	*meta*	*para*	
benzene	0.10	10				phenol (2.4)
naphthalene	0.10	10				1-naphthol (>0.1)
						2-naphthol (0.1)
aniline	1.0	1.0			(0.1)	
anisole	0.10	10			(0.5)	phenol (3.0)[c]
phenol	0.10	10			(0.9)	
toluene	1.0	1.0				benzylalcohol (5.8)
ethylbenzene	1.0	1.0			(0.4)	1-phenylethanol (3.4)
						2-phenylethanol (1.2)
fluorobenzene	0.10	1.0	(0.1)	(1.0)	(0.9)	
chlorobenzene	0.10	1.0		(0.2)	(2.8)	
bromobenzene	0.10	10		(0.1)	(1.7)	
iodobenzene	0.010	1.0		(>0.1)	(>0.1)	
benzonitrile	1.0	1.0		(2.1)	(3.1)	
nitrobenzene	1.0	1.0		(3.2)	(2.7)	

[a] Concentrations selected to produce the maximum amount of product in 1 h. [b] Amount of product produced in 1 h by 80 mg wet weight of cells (12 mg of protein). [c] Phenol was probably produced by hydroxylating the methyl of the methoxy group followed by an O-demethylation.

synthesized deuterated substrates are listed in Tables 2−5.

^1H Nuclear Magnetic Resonance. Compound were analyzed in deuterochloroform or [$^2H_6$]dimethyl sulfoxide solutions using a 300 MHz, Nicolet model NT-300 WB, superconducting NMR. Proton positions were assigned on the basis of the spectra of authentic standards and literature values (Sadtler Research Labs., Inc., 1966; Academic Press, Inc., 1985). Proton ratios for the deuterated products were determined by integration of the assigned peaks.

Thin Layer Liquid Chromatography. Nitrophenols were separated by TLC on 20 × 5 × 0.025 cm analytical silica gel plates (Whatman, Clifton, NJ) with benzene/ethylacetate (95:5) (Daly et al., 1968). Bands were identified by comparison with authentic standards, scraped off the plates, and extracted with sodium hydroxide (0.1 M). The aqueous solutions were acidified and extracted with methylene chloride. The solvent was then removed by evaporation, and the phenols were dissolved in deuterated methylene chloride for mass and ^1H NMR spectroscopy.

Colorimetric Assay of Phenol. The total concentration of phenols was measured by the aminoantipyrine method (Ochynski, 1960). A 1 mL cell suspension was centrifuged to remove the cells. To the supernatant were added 200 μL of a 4% solution of sodium borate, 30 μL of a 2% solution of aminoantipyrine, and 20 μL of a 2% solution of ammonium persulfate. The red color that developed after 30 min was then quantitated spectrophotometrically at 500 nm using a standard curve developed with phenol solutions of 10−100 μM. Phenols with *para*- substituents other than a halogen will not develop the characteristic red color, so other methods were used. *para*-Aminophenol was determined spectrophotometrically by conversion to the indophenol dye (Fujita & Mannering, 1973). A 500 μL cell suspension was centrifuged and the supernatant placed in a test tube. Then 500 μL of a 1 N sodium carbonate solution and 500 μL of a 2% phenol, 0.5 N sodium hydroxide solution were added and allowed to react for 20−30 min. The blue color that developed was quantitated spectrophotometrically at 630 nm using a standard curve developed with aniline solutions of 10−120 μM. Ammonia at the concentration listed earlier did not interfere with this method. Nitrophenol was determined directly by quantitating spectrophotometrically at 400 nm using a standard curve developed with *para*-nitrophenol solutions of 10−120 μM.

Other Assays. Production of nitrite and rates of usage of oxygen were carried out as previously (Arciero et al., 1989; Hooper & Nason, 1965). Protein concentration was determined by the bicinchoninic acid method (Smith et al., 1985).

RESULTS

Incubations were carried out for 1 h with several concentrations of the aromatic compounds in the presence of either 1 or 10 mM ammonia. Concentrations of nitrite and phenolic products were determined. The initial concentration of substrate resulting in the greatest amount of phenolic product (Table 1) was used for subsequent experiments. As has been observed with other substrates of AMO (Hyman et al., 1988; Vannelli et al., 1990), the production of nitrite from ammonia was inhibited by the aromatic compounds, and their hydroxylation was stimulated by the presence of ammonia. In separate flasks and at the same time, deuterated and nondeuterated compounds were incubated with cells from the same harvest. Phenolic products were assayed by gas chromatography and were identified by comparison with authentic standards and by their mass and, in some cases, NMR spectra. The recovery of total substrate and product were reasonably high (e.g., 86% for anisole) but varied depending on the volatility and the initial concentration of the substrate. Products other than phenols may not have been detected. Acetanilide, pyridine, 1,3-dichloro-, 1,4-dichloro-, and trichlorobenzenes were not transformed. In contrast to most known substrates of AMO (Hyman et al., 1988; Vannelli et al., 1990), these compounds did not inhibit the production of nitrite from ammonia. Biphenyl was not soluble in the reaction buffer, and all conditions which solubilized biphenyl (e.g., use of detergents) inactivated ammonia oxidation in the cell suspension. The transformations of naphthalene, anisole, fluorobenzene, and benzonitrile by *N. europaea* were demonstrated here for the first time.

Identification of Products. Hydroxylation of aromatics containing *ortho*/*para*-directing substituents, except for fluorobenzene, resulted in *para*-phenols as the major product (Table 1). Hydroxylation of the monohalogenated benzenes all produced some *meta*-phenol. Hydroxylation of aromatics with *meta*-directing substituents resulted in both *meta*- and *para*-phenols. The major products of the hydroxylation of nitrobenzene or benzonitrile were the *meta*-phenol or the

Table 2: Deuterium Content and Values of the NIH Shift for the Hydroxylation of Benzene, Aniline, Phenol, Anisole, and Ethylbenzene to Phenols by AMO

substrates (mol %)[a]	phenol products (mol %)[a,b]	% ^2H_n retention	NIH shift value (%)[c]
[1,3,5-$^2H_3$]benzene			
$^2H_3$ (97)	(75)	77	77
$^2H_2$ (3)			
[2,4,6-$^2H_3$]aniline			
$^2H_3$ (91)	(0)	0	0
$^2H_2$ (7)	(97)		
$^2H_1$ (2)	(3)		
[2,4,6-$^2H_3$]phenol			
$^2H_3$ (96)	(17)	18	18
$^2H_2$ (4)	(81)		
$^2H_1$ (0)	(2)		
[4-^{2}H]anisole			
$^2H_1$ (96)	(56)	59	59
[4-^{2}H]ethylbenzene			
$^2H_1$ (83)	(32)	39	39

[a] The term ^2H_n represents the mol % of material containing n number of deuterium atoms. [b] Only *para*-phenol products detected. [c] See text.

para-phenol, respectively. In contrast to the report by Keener and Arp (1994), we observed that *para*-nitrophenol was a major product of the hydroxylation of nitrobenzene by AMO. *ortho*-Phenol was produced only in the hydroxylation of fluorobenzene.

Nonaromatic hydroxylations occurred with anisole, toluene, and ethylbenzene. Anisole was converted to phenol and, to a lesser extent, 4-methoxyphenol. The phenol was probably produced by hydroxylation of the methyl of the methoxy group followed by an O-demethylation (Daly et al., 1968). Hydroxylation of ethylbenzene also occurred at both the ethyl substituent and on the aryl ring. The only hydroxylation products observed during the oxidation of ethylbenzene were 1- and 2-phenylethanol (separated and quantified for the first time here) and *para*-ethylphenol. The hydroxylation of toluene, which could, in theory, have produced cresols by aryl hydroxylation, produced only benzyl alcohol by methyl hydroxylation. Oxidation of aniline to nitrobenzene has been reported (Keener & Arp, 1994). However, we observed that *para*-aminophenol was the only product of the oxidation of aniline under conditions of analysis where production of nitrobenzene would have been observed.

Hydroxylation rates were highest when ring substituents were *meta*-directing deactivators and lowest when they were *ortho*-/*para*-directing activators. Halogenated substrates were oxidized at rates in the order of Cl > F > Br ≫ I (Table 1). This order was similar to that observed with the hydroxylation of halogenated benzenes by cytochrome P450 (Burka et al., 1983) or soluble methane monooxygenase (MMO) (Green & Dalton, 1989).

Values of the NIH Shift. Values of the retention of deuterium during the hydroxylation of specifically deuterated substrates by AMO were determined by mass spectroscopy of the phenolic products. Deuterium retention in the phenol product was determined by dividing the mole percent of the most deuterated product by the mole percent of the corresponding substrate and multiplying by 100% (Tables 2−5). The values for the shift of deuterium or hydrogen and resulting retention of the deuterium (values of the NIH shift) were calculated in a similar manner. Corrections were made in cases where only one of two equivalent sites were deuterated, i.e., [3-^{2}H]fluorobenzene with a mole percent of 91 was oxidized to [^{2}H]4-fluorophenol with a mole percent of 80 (89% retention of deuterium), giving a value of the NIH shift of 100%[80 − (91/2)]/(91/2) or 77%. Deuterium location in the product was determined by ^{1}H NMR for the *para*-phenol products of [4-^{2}H]chlorobenzene and [4-^{2}H]-bromobenzene and the *meta*-phenol product of [3,5-$^2H_2$]-nitrobenzene. The ^{1}H NMR spectra indicated that the location of the retained deuterium was at the equivalent 2- and 6-positions for 4-bromo- and 4-chlorophenol (data not shown) and at the 6-position for 3-nitrophenol (Figure 2). In cases where the deuterium or hydrogen could have shifted from the *meta*-position to either the *para*- or *ortho*-positions, it was assumed that migration occurred only to the *para*-position. This assumption was supported by the nearly identical values of deuterium retention in the *meta*-phenol products from the hydroxylation of the [4-^{2}H] and [2,4,6-$^2H_3$]chlorobenzene substrates (Table 4) and the shift of deuterium from the *meta*-position to only the *para*-position in the hydroxylation of [3,5-$^2H_2$]nitrobenzene to *meta*-nitrophenol (Figure 2).

In order to show that no spurious losses of deuterium occurred, [$^2H_5$]fluorobenzene, [$^2H_5$]bromobenzene, and [$^2H_5$]-nitrobenzene were incubated with cells, in the presence of ammonia. Each of the phenolic products were isolated and shown to contain four atoms of deuterium (data not shown). Further, hydroxylation of several of the aromatics occurred at a site that was not adjacent to a deuterium. In these cases, all of the deuterium was retained (Tables 3 and 5). Similarly, the phenol produced from anisole or the 1- or 2-phenyl-

Table 3: Deuterium Content and Values of the NIH Shift for the Hydroxylation of Fluorobenzenes to Phenols by AMO

substrates (mol %)[a]	phenol products (mol %)[a]			% ^2H_n retention			NIH shift value (%)[b,c]	
	ortho	*meta*	*para*	*ortho*	*meta*	*para*	*meta*	*para*
[2,6-$^2H_2$]fluorobenzene								
$^2H_2$ (24)	(8)	(21)	(25)	35	90	100[f]	*g*	*f*
$^2H_1$ (47)	(54)	(50)	(50)					
[3-^{2}H]fluorobenzene								
$^2H_1$ (91)	(78)	(80)	(81)	85	89	89	77[d]	78[d]
[4-^{2}H]fluorobenzene								
$^2H_1$ (95)	(93)	(69)	(77)	98[f]	72	81	72[e]	81

[a,c] See Table 2 for explanation. [b] Shift values for *ortho*-phenols were not determined. [d] Corrections were made for cases where only one of two equivalent sites was deuterated. [e] Shift value calculated as the retention of deuterium after migration of a hydrogen from the 3-position to only the 4-position with the subsequent loss of deuterium from the 4-position. [f] High deuterium retention values result from hydroxylation at a site that was not adjacent to deuterium(s); thus no shift was possible. [g] Not known if shift occurs.

Table 4: Deuterium Content and Values of the NIH Shift for the Hydroxylation of Chloro and Bromobenzenes to Phenols by AMO

	phenol products					
	(mol %)[a,b]		% ^2H_n retention		NIH shift value (%)[c]	
substrates (mol %)[a]	*meta*	*para*	*meta*	para	*meta*	*para*
[3-^{2}H]chlorobenzene						
$^2H_1$ (93)	(83)	(88)	89	95	79[d]	81[d]
[4-^{2}H]chlorobenzene						
$^2H_1$ (97)	(82)	(80)	83	81	83[e]	81
[3,5-$^2H_2$]chlorobenzene						
$^2H_2$ (94)	(73)	(78)	77	82	77	82
$^2H_1$ (5)	(25)	(21)				
[2,4,6-$^2H_2$]chlorobenzene						
$^2H_3$ (93)	(74)	(76)	79	81	79	81
$^2H_2$ (7)	(25)	(23)				
$^2H_1$ (0)	(1)	(1)				
[3-^{2}H]bromobenzene						
$^2H_1$ (96)	(77)	(90)	80	93	61[d]	87[d]
[4-^{2}H]bromobenzene						
$^2H_1$ (93)	(80)	(78)	86	83	86[e]	83

[a,c] See Table 2 for explanation. [b] No ortho-phenol products detected. [d,e] See Table 3 for explanation.

Table 5: Deuterium Content and Values of the NIH Shift for the Hydroxylation of Benzonitrile and Nitrobenzenes to Phenols by AMO

	phenol products					
	(mol %)[a,b]		% ^2H_n retention		NIH shift value (%)[c]	
substrates (mol %)[a]	*meta*	*para*	*meta*	*para*	*meta*	*para*
[4-^{2}H]benzonitrile						
$^2H_1$ (97)	(86)	(76)	89	78	89[d]	78
[2-^{2}H]nitrobenzene						
$^2H_1$ (97)	(93)	(96)	96	99[e]	*f*	*e*
[3,5-$^2H_2$]nitrobenzene						
$^2H_2$ (92)	(54)	(78)	59	85	59	85
$^2H_1$ (8)	(44)	(21)				
[4-^{2}H]nitrobenzene						
$^2H_1$ (98)	(93)	(63)	95	64	95[d]	64

[a,c] See Table 2 for explanation. [b] No ortho-phenol products detected. [d,e,f] See Table 3 for explanation.

ethanols produced from ethylbenzene had high values of deuterium retention (99%, 90%, or 91%, respectively) as expected if the hydroxylation occurred at a site other than the aryl ring.

Kinetic Isotope Effect. During the incubation of a 1:1 mixture of nitrobenzene and [$^2H_5$]nitrobenzene, a kinetic isotope effect (k_H/k_D) of 1.23 and 1.11 was observed for the production of *meta*-nitrophenol and *para*-nitrophenol, respectively. A similar isotope effect of 1.40 was seen with the oxidation of nitrobenzene to *meta*-nitrophenol by cytochrome P-450 (Tomaszewski et al., 1975). No kinetic isotope effect was observed with either fluoro- or bromobenzene (data not shown). Deuteration of the aryl ring of any of the substrates tested had no effect on the distribution of hydroxylation products. In each case, the hydroxylation product distribution for nondeuterated substrates was the same for partially or fully deuterated substrates (data not shown).

para-Deuterated Substrates. The *para*-phenol products of the hydroxylation of *para*-deuterated substrates had values of the NIH shift that ranged from 0 to 83% depending on the substituent group of the substrate (Tables 2−5). The

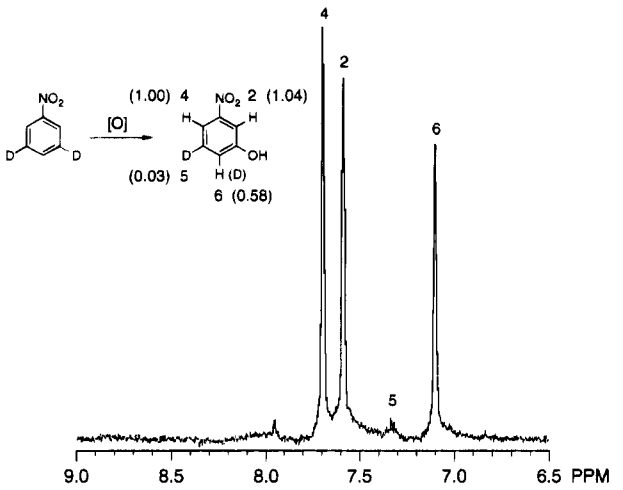

FIGURE 2: ^{1}H NMR spectrum of the *meta*-phenolic product from the oxidation of [3,5-$^2H_2$]nitrobenzene by AMO. The spectrum indicates that deuterium was shifted to the *para*-position, but not to the *ortho*-position. The mole number of protons at each position (x) was determined by dividing the area of each of the peaks in the spectrum by the area of the peak for the 4-position proton.

meta-phenol products exhibited values of the NIH shift (72−95%) that were similar to, but generally higher than, those observed with the *para*-phenol products (Tables 3−5).

meta-Deuterated Substrates. The values of the NIH shift were also high in the hydroxylation of *meta*-deuterated substrates to *meta*-phenols (59−79%) but were consistently lower than the values observed in the hydroxylation of *para*-deuterated substrates to *para*-phenols (78−87%) (Tables 3−5).

ortho-Deuterated Substrates. The value of deuterium retention for the hydroxylation of [2,6-$^2H_2$]fluorobenzene to *ortho*-fluorophenol, the only *ortho*-product detected, was 35% (Table 3). Although the value of deuterium retention indicates that an NIH shift did occur, the exact value of the shift cannot be determined. The *ortho*-deuterium could have been shifted to the position with the fluoride where it would not have been retained, resulting in a lower than expected value for the NIH shift. The values of deuterium retention for *meta*-phenols produced from *ortho*-deuterated nitrobenzene and fluorobenzene (96% and 90%, respectively) were extremely high and may indicate that the *meta*-hydrogen shifted only to the *para*-position and not the *ortho*-position.

DISCUSSION

The Hydroxylation of Aromatics by AMO May Occur by an Electrophilic Addition. The nature of the phenolic products of the hydroxylation of aromatics by AMO suggest that the reaction involved an electrophilic addition. Substrates with activating or deactivating *ortho-/para*-directing substituents were converted predominantly to *para*-phenols. Substrates with *meta*-directing deactivators were converted to both *meta*- and *para*-products, but the amount of the *meta*-product was larger with *meta*-directing than with *ortho-/para*-directing substituents. As is depicted in Figure 3, an enzyme-bound activated oxygen species could react with an aromatic substrate to form a radical or carbocation tetrahedral intermediate which could breakdown to form phenol. The radical or carbocation nature of the intermediate may depend upon the relative electron-withdrawing or -donating nature of the substituent on the aryl ring of the substrate and may

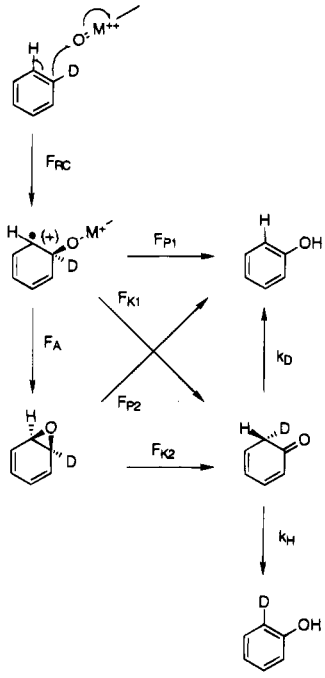

FIGURE 3: Proposed reaction pathways which involve a radical or cationic tetrahedral intermediate. (F_{RC}) Aromatic substrates react with an enzyme-bound, activated oxygen species to form a radical or carbocation tetrahedral intermediate. The radical or cationic intermediate can then breakdown to form the following: (F_{P1}) a phenol with the direct loss of a deuterium at the site of hydroxylation; (F_{K1}) a ketone which can spontaneously enolize to form phenol with the loss (k_D) or retention (k_H) of deuterium; (F_A) an arene oxide which can spontaneously tautomerization to form either a phenol with the loss of deuterium (F_{P2}) or a ketone intermediate (F_{K2}) which can spontaneously enolize to form phenol with the loss (k_D) or retention (k_H) of deuterium.

affect the NIH shift of deuterium on the substrate (Figure 3).

The type of aromatic hydroxylation products reported here are similar to those obtained with the cytochrome P-450 (Burka et al., 1983; Daly et al., 1968) and soluble methane monooxygenase (sMMO) (Burrows et al., 1984; Colby et al., 1977; Dalton et al., 1981; Higgins et al., 1979) but differ in important ways. With cytochrome P-450, the predominate products were *ortho*- and *para*-phenols; very little or no *meta*-phenol was observed. Although sMMO and AMO oxidize many of the same substrates, only sMMO will oxidize pyridine to pyridine-*N*-oxide, and toluene to benzyl alcohol and *para*-cresol. Interestingly, particulate methane monooxygenase (pMMO) and dopamine-β-hydroxylase do not oxidize aromatic rings (Burrows et al., 1984; May et al., 1981). Substrates with deactivating substituents were oxidized by AMO more readily than those with activating substituents (Table 1). A mechanistic interpretation of this effect is not possible since it may be due to differences in the K_m, solubility, and accessibility to the active site of the substrate.

Evidence for the Specific Orientation of Substrates in the Hydroxylation of Aromatics by AMO. The present observations suggest that aromatic compounds bind in AMO so that the substituent group is placed either for hydroxylation, as with anisole, toluene, and ethylbenzene, or directly away from the hydroxylating site, as with all other substrates. Anisole and ethylbenzene can apparently bind in either orientation to allow hydroxylation on the substituent or at the 4-position of the ring. Hydroxylation at the 2-position generally does not occur, presumably because of steric hindrance by substituents with large van der Waals radii. Since the van der Waals radius of fluorine is similar to that of hydrogen, it may have allowed fluorobenzene to bind in more than one orientation in the active site, thus producing the small amount of *ortho*-fluorophenol observed. Because *ortho*-fluorophenol was further oxidized to an unknown compound, the true proportion of isomers is not known.

Evidence against an Arene Oxide Intermediate in the Hydroxylation of Aromatics by AMO. The absence of *ortho*-phenolic products indicates that 1,2-arene oxides were not involved in the oxidation of any of the substrates tested with the possible exception of fluorobenzene oxidation to *ortho*-fluorophenol. The production of *meta*-chlorophenol from the hydroxylation of chlorobenzene by AMO could not have arisen from an arene oxide intermediate, since the breakdown of chemically synthesized 2,3- or 3,4-chlorobenzene oxide has been shown to produce only *ortho*- and *para*-chlorophenols (Selander et al., 1975b). Nevertheless, the production of *meta*-chlorophenol by AMO proceeded with the NIH shift (Table 4). Further, the values of NIH shift were nearly identical in the hydroxylation of *para*- or *meta*-deuterated chlorobenzene to *meta*-chlorophenol, suggesting a common enzyme intermediate. One such possible intermediate is shown in Figure 3. The hypothetical activated oxygen species could have reacted with the aromatic substrate to form either a radical or a carbocation tetrahedral intermediate. This intermediate could then have decayed to phenol by several different pathways with different levels of deuterium retention. One such pathway involves the direct formation of a ketone intermediate with the shift of deuterium or hydrogen from the site of hydroxylation to an adjacent site. The ketone intermediate could enolize to phenol with the retention (Figure 3, k_H) or loss (Figure 3, k_D) of deuterium. Substrates deuterated at the site of hydroxylation or at an adjacent site would share the same intermediate in this model and thus would have the same values for the NIH shift. During the hydroxylation of chlorobenzene or nitrobenzene to *meta*-phenols, deuterium or hydrogen was shifted from the *meta*-position to the *para*-position, but not to the *ortho*-position. Since the decay of an arene oxide involves a cationic intermediate that can isomerize to allow the shift of deuterium or hydrogen to both the *ortho*- and *para*-positions (Figure 1B), an arene oxide intermediate is very unlikely to have been involved in the hydroxylation of nitrobenzene to *meta*-nitrophenol. Since an unusual enzyme-directed opening of an arene oxide could have, in theory, occurred, an arene oxide intermediate cannot be entirely ruled out for all of the substrates tested (Figure 3). However, it is certainly not supported by the present data.

Direct Loss of Deuterium or Hydrogen Occurred at the Site of Hydroxylation during Hydroxylation of Nitrobenzene by AMO. When nitrobenzene was oxidized to *para*- and *meta*-phenols, significantly lower deuterium retention values were observed when deuterium was at the site of hydroxylation than when deuterium was at an adjacent site (Table 5). The lower than expected values for the NIH shift may have been due to a direct loss mechanism (Figure 3). The radical or carbocation tetrahedral intermediate could form phenol directly (Figure 3, F_{P1}) with the complete loss of deuterium from the site of hydroxylation. The direct loss pathway would have no effect on the ratio of *para*- to *meta*-

phenolic products but would effect deuterium retention dependent upon which pathway (direct loss or ketone intermediate) was followed.

Other possible direct loss mechanisms involve the breaking of a carbon−hydrogen (deuterium) bond. One such possible mechanism would be the radical rebound mechanism proposed for the oxidation of alkanes. In this mechanism, the activated oxygen species abstracts a hydrogen radical from the aryl ring to form a phenyl radical intermediate which then recombines to form the phenol. Another possible mechanism would be the direct insertion of oxygen into the carbon hydrogen bond. Because a carbon−hydrogen (deuterium) bond would be broken in either of these mechanisms, large kinetic isotope effects or changes in product distribution (molecular switching) would be expected. Since small kinetic isotope effects and no changes in product distribution were observed, these mechanisms are inconsistent with our data.

Conclusion. The data suggest that AMO specifically binds aromatic substrates so that hydroxylation occurs at either the *meta-* or *para*-position on the aryl ring or at the substituent. The identity of the phenolic products is consistent with an electrophilic addition mechanism with a concurrent shift of hydrogen or deuterium. Taken together, the values of the NIH shift, the unlikely formation of an arene oxide intermediate, and the direct loss of deuterium during the hydroxylation of nitrobenzene indicate that the reaction involves a common intermediate, probably a ketone. Further, the enzyme influences not only the site of hydroxylation but also the degree of the NIH shift during the hydroxylation of aromatic substrates. This strongly suggests that an enzyme-bound intermediate, probably a cation or radical, was formed.

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