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Mechanisms of hydroxylation by cytochrome P-450: Metabolism of monohalobenzenes by phenobarbital-induced microsomes

(kinetics/monohalophenols/Hammett constants/radical cation intermediate)

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ABSTRACT **The monohydroxylation of halobenzenes by phenobarbital-induced rat liver microsomes was studied. The *p*-halophenol was found to be the major metabolite from all four halobenzenes; *o*-halophenol formation decreased as the halogen atom size increased. V_{max} for total hydroxylation (*ortho* and *para* products) correlated well with the σ^+ Hammett constant with a negative ρ value. This implies a positively charged intermediate in the rate-determining step. V_{max} for either *ortho* or *para* hydroxylation alone did not correlate with a Hammett constant, implying that the product-determining step occurs after the rate-determining step. Rate-determining formation of a radical cation intermediate is postulated to explain this data.**

Metabolism of monohalobenzenes, especially chlorobenzene and bromobenzene, has been the subject of literally scores of studies. These compounds are of interest not only as primary environmental contaminants but also because they serve as models for more structurally complex haloaromatics such as polychloro- and polybromobiphenyls and polyhalogenated diphenyldioxins and diphenylfurans (1). Our recent interest in the biological oxidation of heteroatoms (2–5) led us to consider the possibility that oxidation of the halogen atom was the initial step in the cytochrome P-450-catalyzed metabolism of aromatic halides. Oxidation by removal of a hydrogen atom or an electron as an initial step in cytochrome P-450-catalyzed metabolism has been postulated for several substrates, including vinyl halides (6, 7), cyclopropyl amines (5, 8), sulfides (9), and norbornane (10). Evidence for a similar one-electron oxidation of aryl halides might be obtained from comparison of the kinetics of hydroxylation of the monohalobenzenes.

Although the aryl halides are a thoroughly studied group of compounds, consultation of the literature revealed an almost complete lack of kinetic data concerning their *in vitro* metabolism. An excellent product study of chlorobenzene metabolism progressing in complexity from reconstituted soluble hemoprotein systems to perfused rat livers was reported by Selander *et al.* (11). A Lineweaver–Burk plot for the metabolism of bromobenzene by a supernatant obtained at 9,000 × *g* from rat liver can be found in a study by Zampaglione *et al.* (12). However, it became obvious that, to make a valid comparison of the hydroxylation kinetics for the monohalobenzenes, a set of data generated in one laboratory with microsomes from a single isolation and with a consistent method of acquiring and presenting the kinetic data would be required.

MATERIALS AND METHODS

All chemicals were of the highest purity commercially available. Pentane was redistilled prior to use. Reduced pyridine nucleotide (NADPH) was obtained from Sigma; *o*- and *m*-iodoanisole were obtained by decomposing the corresponding diazonium salt with potassium iodide (13).

Microsomes. Male Sprague–Dawley rats (80–100 g each) were treated with phenobarbital (0.1% in drinking water) for 5 days. Rats were then killed, and liver microsomes were prepared as described (7). Microsomes were stored at −70°C in 10 mM Tris acetate buffer, pH 7.4/1 mM EDTA/20% (vol/vol) glycerol. Protein concentration was determined by the Lowry method (14) with bovine serum albumin as standard.

Incubations. All incubations were carried out at 37°C in a final volume of 2.0 ml per tube. Each tube contained potassium phosphate buffer (pH 7.4; 0.05 M), NADPH (2 mM for substrate concentrations <8.0 mM and 3 mM for substrate concentrations ≥8.0 mM), microsomes (4.68 mg of microsomal protein), and various concentrations of benzene, bromobenzene, chlorobenzene, fluorobenzene, or iodobenzene. Concentrated solutions of benzene or halobenzenes were prepared in acetonitrile. The maximum volume of acetonitrile added per tube was 2% or less of the total incubation volume. All incubations were started by the addition of NADPH.

After 2 min, reactions were stopped by the addition of 200 μl of 10 M NaOH, and the tubes were placed on ice. A 50-μl volume of a standard solution of either *p*-chlorophenol (benzene and bromobenzene incubations) or *p*-bromophenol (chlorobenzene, fluorobenzene, and iodobenzene incubations) in acetonitrile was added to each tube as internal standard. The contents of the tubes were extracted once with 2 ml of pentane, and the supernatant was discarded. Dimethyl sulfate (200 μl) was added to each tube to convert phenols to anisoles. The tubes were heated at 40°C for 30 min with frequent vigorous shaking. The contents of the tubes were extracted twice with 2 ml of pentane. The upper phases were combined and transferred to 5-ml conical vials. The pentane solution was carefully concentrated by distillation at 38°C to a volume of about 50 μl. Control experiments established that selective methylation of one of the halophenols or evaporation of one of the derived anisoles under these conditions was not significant. Carbon tetrachloride (50 μl) was added to each vial. The vials were tightly capped and stored at −20°C until the contents were assayed.

Measurement of Phenol Production. Phenols were measured as methyl ethers (anisoles) by using a Shimadzu Scientific (Columbia, MD) model GC-Mini 2 gas chromatograph equipped with a flame ionization detector. Data were recorded, and peak areas were calculated with a Shimadzu C-RIB integrating recorder. A Supelco OV-1 glass capillary (30 m) was used to mea-

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Table 1. Michaelis–Menten parameters for hydroxylation of benzene and halobenzenes by phenobarbital-induced microsomes

Substituent	K_s, mM	K_m, mM	V_{max}, nmol·min^{-1}· (mg of protein)$^{-1}$	V_{max}/K_m	σ^+	π
H	2.9	3.0	3.3	1.1	0.0	
F	0.56	6.4	11	1.7	−0.07	0.14
Cl	1.8	1.9	6.7	3.5	0.11	0.17
Br	1.4	0.41	6.4	16	0.15	0.86
I	0.69	0.30	6.3	21	0.13	1.12

K_s, K_m, and V_{max} were determined as described. Values for σ^+ are taken from ref. 16. Values for π are taken from ref. 17.

sure phenols produced from benzene, chlorobenzene, fluorobenzene, and iodobenzene metabolism. A Supelco SE 54 glass capillary column (15 m) was used to measure phenols from bromobenzene metabolism. Column and detector temperatures were 150°C and 200°C, respectively, for chloroanisole, anisole, and iodoanisole determinations; 105°C and 150°C, respectively, for measuring fluoroanisoles; and 110°C and 200°C, respectively, for measuring bromoanisoles. Standard solutions of *o*-, *p*-, and *m*-anisoles for each compound were used to determine retention times, the detector-response ratios for the compounds, and the internal standard. The detector-response ratios were determined to be linear over the range used in the analysis. Each incubation was done in duplicate; each incubation mixture was analyzed at least twice. The minimum microsomal enzyme activity detectable by this procedure is about 0.1 nmol/min·mg of protein.

Determination of K_s. The interaction of halobenzenes and benzene with microsomal cytochrome P-450 was examined by determining K_s values for these compounds. The procedure used was based on that of Schenkman *et al.* (15). A Cary model 219 (Varian) spectrophotometer was used. Microsomes from the same batch used for K_m and V_{max} determinations were suspended in sample and reference cuvettes containing 0.1 M phosphate buffer (pH 7.4) to a final protein concentration of 4.68 mg per each 3-ml volume. Aliquots (1 μl) of substrate in acetonitrile were added to the sample cuvette. Equal volumes of acetonitrile were added to the reference cuvette. After addition of each aliquot, the spectrum was recorded from 350 to 450 nm. Substrate concentrations were within the same range used for measuring kinetic parameters. K_s for each substrate was determined from a regression equation calculated from a plot of the reciprocal absorbance change vs. the reciprocal of the substrate concentration. K_s equals the negative reciprocal of the *x*-axis intercept.

RESULTS

Hydroxylation of chlorobenzene was studied in some detail. Under our incubation conditions (i.e., NADPH added in one portion with no generating system), the rate of formation of chlorophenols was biphasic. A steep initial linear phase was observed for the first 2–3 min followed by another, more shallow, linear phase for up to 1 hr, the longest incubation time used. The linearity of the rate of hydroxylation with time was not investigated as thoroughly for the other substrates, but the rates were determined to be linear for at least 2 min.

Initial rates of formation of chlorophenols were determined for six concentrations of chlorobenzene ranging from 0.3 to 8.0 mM. The Michaelis–Menten parameters, K_m and V_{max}, for *ortho*, *para*, and total (*ortho* plus *para*) hydroxylation were determined from Lineweaver–Burk plots of the data with a least-squares fit. The correlation coefficient (r) was greater than 0.99 in each case.

Likewise, K_m and V_{max} were determined from initial rate data from three concentrations of fluorobenzene, 1.0–4.0 mM; four concentrations of bromobenzene, 0.3–4 mM; five concentrations of iodobenzene, 0.15–1.5 mM; and four concentrations of benzene, 1.0–8.0 mM. The correlation coefficient for the least-squares fit was 0.97 or greater for all determinations. Apparent inhibition was observed with concentrations of fluorobenzene and bromobenzene above 4.0 mM.

m-Halophenols were always minor products, detectable with certainty only at the highest concentrations of chloro- and fluorobenzene. For those incubations *meta*-substituted products comprised only 3–4% of the total monohydroxy compounds. *p*-Iodophenol was by far the principal product from iodobenzene. *o*-Iodophenol was determined with certainty only at the highest concentration and comprised about 8% of the total monohydroxylation products.

Table 1 gives the Michaelis–Menten parameters for total hydroxylation of the five substrates studied and the spectroscopic binding constant, K_s, for the substrates. The ratio of V_{max} to K_m was also calculated and varies over a 20-fold range for the four halobenzenes, increasing with increasing atomic weight of the halogen substituent.

Table 2 separates the Michaelis–Menten parameters for *ortho* and *para* hydroxylation. Not only does V_{max}/K_m for both

Table 2. Michaelis–Menten parameters for *ortho* and *para* hydroxylation of halobenzenes by phenobarbital-induced microsomes

	ortho			*para*			
Substituent	K_m, mM	V_{max}, nmol·min^{-1}· (mg of protein)$^{-1}$	V_{max}/K_m	K_m, mM	V_{max}, nmol·min^{-1}· (mg of protein)$^{-1}$	V_{max}/K_m	Hydroxylation ratio
F	5.2	3.7	0.71	7.5	7.5	1.0	1.4
Cl	2.5	3.0	1.2	1.7	3.8	2.2	1.8
Br	0.80	1.8	2.2	0.34	4.7	14	6.4
I	—	—	—	0.30	6.3	21	Large

K_m and V_{max} were determined as described. Hydroxylation ratio is V_{max}/K_m for *para* hydroxylation divided by V_{max}/K_m for *ortho* hydroxylation.

para and *ortho* hydroxylation increase with increasing atomic weight, but also the ratio of *para* hydroxylation to *ortho* hydroxylation follows this trend.

DISCUSSION

Two recent studies have suggested the intervention of halogen oxidation in the microsomal oxidation of aliphatic halides (2, 4). The microsomal oxidation of aromatic halides became of interest because these compounds would allow a comparison of the relative ease of halogen oxidation to oxidation of an aromatic system. This comparison might enable a qualitative assessment of the energy requirements for halogen oxidation. Halogen oxygenation in the cytochrome P-450-catalyzed oxidation of iodobenzene has been demonstrated (3, 18). Because the halogen oxides of the other halobenzenes are not expected to be stable, evidence for halogen oxidation would have to come from the other consequences (kinetics, product distribution) of this metabolic event.

One can envision a mechanism for the hydroxylation of aryl halides that involves an initial one-electron oxidation (see below). If oxidation of the halide is a rate-determining step, then a correlation of rate constants with ease of oxidation of the halides (i.e., F < Cl < Br < I) would be expected. The present studies were initiated to investigate this kinetic consequence of halogen oxidation.

In acquiring the kinetic data, only formation of monohydroxy compounds was considered. The justification for this simplification is: (*i*) dihydroxy aromatic compounds (catechols, resorcinols, hydroquinones) are the result of "two-pass" metabolism in cytochrome P-450 systems and are not kinetically important at short incubation times because the concentration of halophenol is very small with respect to halobenzene concentration, and (*ii*) dihydrodiols, if formed, are not present in large enough concentrations to appreciably alter the kinetic analysis. The amount of dihydrodiol found in bromobenzene microsomal metabolism was less than 2% of the phenol product (19). The dihydrodiol product from microsomal hydroxylation of chlorobenzene was found to be "insignificant" (11).

At low substrate concentration, the Michaelis–Menten expression simplifies to $v = V_{max}/K_m[S]$. Expressing the kinetic data for hydroxylation in terms of this ratio has obvious advantages. The low substrate concentration limit probably best reflects the *in vivo* situation. In addition, in order to determine correlation to a free energy relationship, such as the Hammett equation, one would like a rate expression of the form—a rate equals a rate constant times the concentration of reactant(s). Thus, in the simplified form, V_{max}/K_m becomes this rate constant, and it was of interest to determine the correlation of this ratio as well as the individual Michaelis–Menten parameters to a free energy relationship.

The results in Table 1 indicate that, at the low concentration limit, iodobenzene is hydroxylated at the fastest rate and bromobenzene is hydroxylated faster than chlorobenzene [i.e., V_{max}/K_m (iodobenzene) > V_{max}/K_m (bromobenzene) > V_{max}/K_m (chlorobenzene) > V_{max}/K_m (fluorobenzene)]. In addition, V_{max}/K_m for fluorobenzene is not very different from V_{max}/K_m for benzene itself, which has the smallest rate constant for hydroxylation at the low concentration limit. This is a surprising result because one usually assumes that halogens retard rather than promote metabolism of aromatic systems. This result is even more striking when one considers that the halobenzenes have essentially three sites of hydroxylation (two *ortho* and one *para*), whereas all six positions of benzene are equivalent.

A notable trend in Table 2 is that, as one proceeds from fluorobenzene to iodobenzene, the ratio of *para* to *ortho* hydroxylation increases. The effect of phenobarbital or 3-methylcholanthrene induction on the *ortho/para* ratio in bromobenzene metabolism has been investigated in some detail (19–21). Phenobarbital induction has been found to promote *para* hydroxylation, whereas 3-methylcholanthrene and β-naphthoflavone (βNF) pretreatment promotes *ortho* hydroxylation in mice and rats. The major cytochrome P-450 purified from 3-methylcholanthrene-pretreated rabbit liver gives almost totally *ortho* hydroxylation. Likewise, phenobarbital pretreatment has been found to promote *para* hydroxylation of chlorobenzene; 3-methylcholanthrene was found to promote *ortho* hydroxylation (11).

The free energy relationship initially developed by Hammett ($\log K/K_o$ or $\log k/k_o = \rho\sigma$) has found widespread use in probing mechanisms of organic reactions (16, 22). In this relationship σ is characteristic of a given substituent. The magnitude and sign of ρ gives some indication of the nature of the intermediate or transition state for the reaction. Several sets of σ parameters have been determined. The usual σ *meta* and σ *para* parameters are most suitable if a full electronic charge is not developed on the aromatic ring. The parameters σ^+ and σ^- are appropriate if the intermediate or transition state contains a positive or negative charge, respectively (16, 22). Although the original equation contains the logarithm of the ratio of an equilibrium or rate constant to some reference constant (K_o or k_o), normally the logarithm of the equilibrium or rate constant alone is used, as no change in the sign or the magnitude of the slope (ρ) is incurred by this simplification.

An excellent correlation of V_{max} for total hydroxylation (*o*- and *p*-halophenols) to the Hammett σ^+ parameter was obtained. Thus, a plot of $\log V_{max}$ vs. σ^+ gives a ρ of -1.1 with a correlation coefficient of 0.99. This excellent correlation implies a positively charged transition state or intermediate in the hydroxylation mechanism. The correlation of V_{max} for *ortho* hydroxylation or V_{max} for *para* hydroxylation with the Hammett σ^+ parameter is poor, $r = 0.83$ and 0.67, respectively. One explanation for the excellent correlation of the Hammett σ^+ parameter with V_{max} for total hydroxylation and poor correlation with each component V_{max} is that the hydroxylation involves rate-determining formation of one intermediate, which can yield both *ortho*- and *para*- hydroxylated products. The product-determining step may not be rate-limiting and, thus, not correlate with σ^+. The simplest Michaelis–Menten model, formation of an enzyme–substrate complex followed by one-step decomposition to products, may not hold. A more complicated reaction sequence of the enzyme–substrate complex involving several steps may occur, and V_{max} may contain several rate constants, one of which is common to both *para* and *ortho* hydroxylation and is rate-determining. [For related analyses of enzymatic and chemical oxidation processes, refer to Groves *et al.* (10), White and Coon (23), Ullrich (24), Walling (25), and Hamilton (26).]

A good correlation ($r > 0.95$) between K_m for total hydroxylation and parameters associated with the ease of oxidation or polarizability (or both) of the halobenzenes was found. These parameters include the Allred–Rochow electronegativity of the substituent atoms ($r = 0.97$) (27), the binding energy of the halogen nonbonding electrons in the halobenzenes as determined by photoelectron spectroscopy ($r = 0.97$) (28), and Hansch's π parameter for distribution between octanol and water ($r = 0.97$) (17). Only the Hansch parameters are given in Table 1. All of these parameters are probably interrelated, and correlation to one might infer correlation to all. Neither K_m nor V_{max}/K_m correlated well ($r < 0.9$) with σ^+.

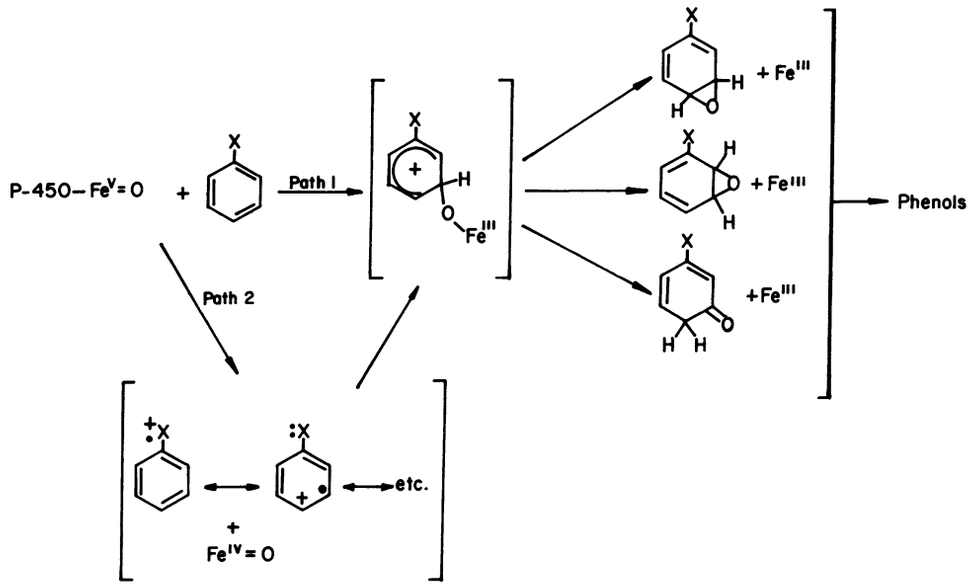
The substrate spectroscopic binding constant, K_s, was the same as K_m in two cases, benzene and chlorobenzene. Differ-

ences of up to a factor of 10 were observed for the other substrates. Microsomes are a mixture of cytochromes P-450, and perhaps only one or two of the isozymes are important in the metabolism of halobenzene. The K_s is for all cytochromes P-450 that bind substrate, whereas K_m reflects binding to those that mediate hydroxylation. Thus, one might not expect good correlation of K_s and K_m for microsomes. K_m also contains one or more rate constants for conversion of the enzyme substrate complex to products. If these rate constants are comparable to the rate constant for complex formation, then one would expect K_s and K_m to be different.

In summary, the hydroxylation of halobenzenes was found to be characterized (*i*) by a V_{max} that correlates to σ^+ and implies a positively charged transition state or intermediate and (*ii*) by a K_m that correlates well to polarization or ease of oxidation or both. A direct or concerted epoxidation of the aromatic ring without intermediates is not consistent with our data because little charge separation would be anticipated to occur in the direct epoxidation. A similar conclusion was reached by Hanzlik and Shearer (29) in a study of the microsomal oxidation of styrenes.

cies attacking an electron-rich aromatic ring to electrophilic aromatic substitution is a compelling one. However, in order to obtain the *meta*-substituted intermediate in mechanism 1 and the observed rates of hydroxylation (e.g., PhI > PhBr > PhCl > PhF > PhH), the halogens would have to be *meta*-directing and activating with respect to hydrogen. Halogens are *ortho–para* directors in electrophilic aromatic substitution and deactivating with respect to hydrogen, however.

Mechanism 2 in Scheme I inserts a rate-determining one-electron oxidation of the halobenzene to give a radical cation and the one-electron reduced oxygenated heme intermediate [Fe(IV)=O], which has radical character (31). These two radicals could collapse to give essentially the *meta*-substituted intermediate of mechanism 1. Mechanism 2 involves formation of a positively charged intermediate (radical cation), which is in accord with our data. Selective formation of a *meta*-substituted cationic intermediate may be a result of electronic distribution within the radical cation. That is, the Fe(IV)=O species may be expected to react at regions of high electron density (31). *Ab initio* calculations can be made to determine what the electronic distribution in the radical cation is likely to be. Preliminary Hückel-type calculations indicate that, in the halobenzene radical cation, positive charge resides in the *ortho* and *para* positions and radical character (regions of high electron density) lie on the halogen atom and *meta* carbons. Thus, radical–radical recombination of the [Fe(IV)=O]·radical cation pair should give *meta* or halogen oxygenation as the initial step.



Scheme I

Scheme I depicts the two mechanisms for hydroxylation that are to be considered in light of these kinetic results. Mechanism 1 is essentially electrophilic aromatic substitution of the P-450 perferryl oxide intermediate (23, 24) of the halobenzene *meta*-position to give a tetrahedral intermediate, which can close to give either of two epoxides. Presumably closure of the *meta*-tetrahedral intermediate to either the 2,3- or 3,4-oxide is dictated by physical factors such as substrate binding and orientation in the active site. Alternatively, a 1,2-hydride shift can occur in the tetrahedral intermediate to give a ketone that would become the *meta*-hydroxylated product. *Ortho*- and *para*-hydroxylated product would arise from rearrangement of the 2,3- and 3,4-epoxides. This mechanism is in essence one of four proposed by Tomaszewski *et al.* (30). Mechanism 1 involves a positively charged intermediate and would appear consistent with our data. It is especially attractive because one intermediate can give rise to all products, a requirement of our V_{max} vs. σ^+ data. The analogy of an electron-deficient oxenoid P-450 spe-

Alternate pathways, not strictly ruled out by the data, are attack of the Fe(IV)=O species at one of the *ortho*, *meta*, or *para* positions of the radical cation, followed by either epoxide formation or hydride transfer to give phenols without forming an epoxide but still involving an "NIH Shift." Although our data do not prove any mechanism, it is our opinion that mechanism 2 should be considered as likely as the more traditional mechanism 1.

Cytochrome P-450-mediated halogen oxidation may be an important process for alkyl halides; however, the correlation of V_{max} to σ^+ for the hydroxylation of halobenzenes seems to indicate that oxidation of the aromatic ring may be a lower-energy

process for the aryl halides. This may not be unexpected because the binding energy of the lone-pair electrons in halobenzenes, as determined by photoelectron spectroscopy, is higher than the first ionization potential for that compound (32). Only in iodobenzene is the lone-pair energy comparable to the first ionization potential of the compound.

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