Isotopically Labeled Chlorobenzenes as Probes for the Mechanism of Cytochrome P-450 Catalyzed Aromatic Hydroxylation†

Kenneth R. Korzekwa,‡ David C. Swinney,§ and William F. Trager*,‖

Laboratory of Chemical Pharmacology, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892, Department of Drug Metabolism, A3/165, Syntex Corporation, Palo Alto, California 94303, and Department of Medicinal Chemistry, BG-20, University of Washington, Seattle, Washington 98195

Received April 24, 1989; Revised Manuscript Received June 30, 1989

ABSTRACT: Noncompetitive and competitive intermolecular deuterium isotope effects were measured for the cytochrome P-450 catalyzed hydroxylation of a series of selectively deuterated chlorobenzenes. An isotope effect of 1.27 accompanied the meta hydroxylation of chlorobenzene-$^2H_5$ as determined by two totally independent methods (EC–LC and GC–MS assays). All isotope effects associated with the meta hydroxylation of chlorobenzenes-3,5-$^2H_2$ and -2,4,6-$^2H_3$ were ~1.1. In contrast, competitive isotope studies on the ortho and para hydroxylation of chlorobenzenes-4-$^2H_1$, -3,5-$^2H_2$, and -2,4,6-$^2H_3$ resulted in significant inverse isotope effects (~0.95) when deuterium was substituted at the site of oxidation whereas no isotope effect was observed for the oxidation of protio sites. These results eliminate initial epoxide formation and initial electron abstraction (charge transfer) as viable mechanisms for the cytochrome P-450 catalyzed hydroxylation of chlorobenzene. The results, however, can be explained by a mechanism in which an active triplet-like oxygen atom adds to the π system in a manner analogous to that for olefin oxidation. The resulting tetrahedral intermediate can then rearrange to phenol directly or via epoxide or ketone intermediates.

Although arene oxides have been shown to be intermediates in the cytochrome P-450 mediated oxidation of aromatic substrates, a fundamental understanding of the mechanism of their formation is lacking. Of the four possible modes of initial attack of activated oxygen on a substrate first postulated by Tomaszewski et al. (1975), only oxygen addition, concerted or stepwise, remains a viable primary mechanism. A significant contribution by either direct insertion of oxygen across a carbon–hydrogen bond or initial hydrogen atom abstraction followed by recombination with hydroxy radical has been eliminated by the observation of the near universality of the N1H shift for all aromatic substrates that have been studied in this regard. Since the original proposal (Tomaszewski et al., 1975), Burka et al. (1983) have suggested that, rather than addition of an oxygen atom, abstraction of a π electron may be the initiating event for stepwise hydroxylation. Finally, the possibility of initial attack by oxygen with simultaneous migration of hydrogen to an adjacent site to form a ketone directly, as has been suggested for the oxidation of acetylenes (Komives & Oritz de Montellano, 1987), needs to be considered.

For mechanisms involving initial addition of oxygen, the transfer of the activated oxygen atom from enzyme to the π system of the substrate can occur in one of two ways; either it adds in a single concerted step to form an epoxide directly, or its adds stepwise to generate an initial radical containing tetrahedral intermediate which can subsequently collapse to epoxide. The first possibility implies the involvement of a "singlet-like" oxygen atom, while the second implies the involvement of a "triplet-like" oxygen atom.

As the evidence for epoxide formation in cytochrome P-450 catalyzed aromatic hydroxylation reactions mounted, its obligatory formation began to be tacitly assumed, and by inference so did the singlet pathway for its formation. However, in recent years additional evidence has accumulated which is inconsistent with direct obligatory formation and therefore suggests that non arene oxide pathways are possible. Such evidence includes the meta hydroxylation of chlorobenzene (Selander et al., 1975a,b) the hydroxylation of 2,2′,5,5′-tetrachlorobiphenyl (Preston et al., 1983), the meta hydroxylation of biphenyl (Swinney et al., 1984), the 7-hydroxylation of warfarin (Bush & Trager, 1982, 1985), and the aromatic hydroxylation of a series of selectively deuterated monosubstituted benzenes (Hanzlik et al., 1984). A non arene oxide mechanism which is consistent with all the data involves initial attack by a triplet-like oxygen atom to form a tetrahedral intermediate, which can then form the final product without passing through an epoxide, either directly or via a keto intermediate, Figure 1.

The purpose of this paper is to probe further the mechanism of aromatic hydroxylation by evaluating (1) the involvement, or partial involvement, of direct epoxide formation, (2) the potential involvement of the addition–rearrangement mechanism, and (3) the involvement of additional mechanisms such as initial electron abstraction or concerted ketone formation. In pursuit of these ends the deuterium isotope effects were determined for the hydroxylation of a series of selectively deuterated chlorobenzenes with either purified cytochrome P-450b or microsomes from phenobarbital-pretreated male Sprague-Dawley rats.

Experimental Procedures

Materials. Deuterated materials, $^2H_2$, CH_3O^2H, etc., were obtained from either Stohler Co. or Aldrich Chemical Co. Pentane and acetonitrile were obtained from Burdick and Jackson. Pentafluorobenzyl bromide was obtained from Pierce Chemical Co. All other organic chemicals were obtained from Aldrich Chemical Co., while all biochemicals were obtained from Sigma Chemical Co. All materials were used as received unless stated otherwise.

† This work was supported in part by the National Institutes of Health (Grant GM 36922) and in part by a PRAT fellowship (K.R.K.) from the Institutes of General Medical Sciences.

‡ National Institutes of Health.
§ Syntex Corp.
‖ University of Washington.

0006-2960/89/0428-9019$01.50/0

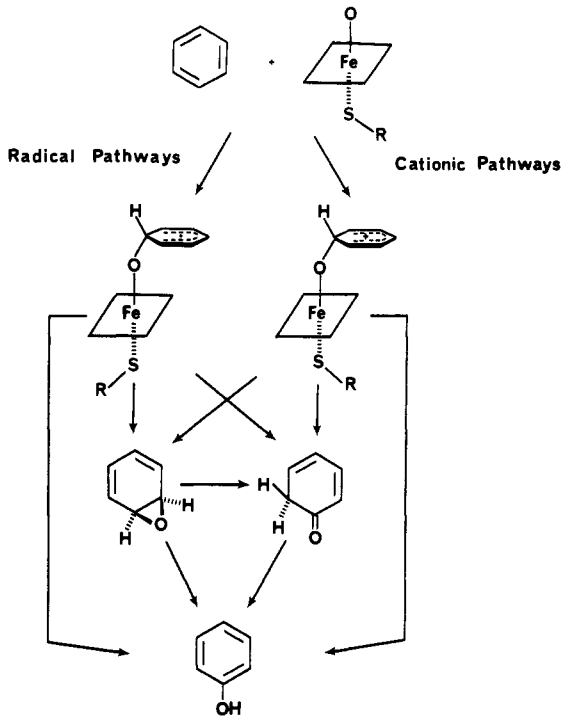


FIGURE 1: Possible addition–rearrangement to phenol.

Instrumentation. Gas chromatography was performed on an HP 5840 A gas chromatograph modified for use with a J & W DB-5 or DB-1 capillary column. Difference spectra were recorded on an HP 8451 A UV spectrophotometer. GC–MS analysis was performed on a VG SP7070 H mass spectrometer in the selected-ion recording mode, interfaced to an HP-5710 A GC fitted with a J & W DB-5 fused silica capillary column.

Synthesis and Incorporation of Substrates. (A) Chlorobenzene-4-^2H. This substrate was prepared by the catalytic reduction of 4-chlorobromobenzene in methanol-$^2H_1$, with $^2H_2$ gas and palladium on charcoal catalyst. The chlorobenzene was isolated by extraction into pentane, washed with water, and dried over sodium sulfate. The pentane solution was concentrated in vacuo to ~15 mL. The remainder of the pentane was removed by passing a stream of nitrogen over the sample in an evaporator tube. The final product (35% yield) was >99.9% pure by GC and had a deuterium incorporation of 97.2% $^2H_1$.

(B) Chlorobenzene-2,4,6-$^2H_3$. Successive exchanges of aniline hydrochloride in refluxing ^2H_2O were used to prepare aniline-2,4,6-$^2H_3$ deuteriochloride. Conversion to the diazonium salt with $NaNO_2$ in HCl/H_2O followed by chlorination with cuprous chloride at 70 °C (Hartman & Brethen, 1941) gave chlorobenzene-2,4,6-$^2H_3$. The chlorobenzene was isolated by extraction and purified by fractional distillation. The overall yield was 6.8% (>99.9% chlorobenzene by GC) with an isotopic composition of 98.04% chlorobenzene $^2H_3$, 1.94% chlorobenzene $^2H_2$, and 0.02% chlorobenzene-$^2H_1$.

(C) Chlorobenzene-3,5-$^2H_2$. Aniline-2,4,6-$^3H_2$ hydrochloride was converted to the acetanilide with acetic anhydride/triethylamine in chloroform. The acetanilide was monochlorinated in either the ortho or para position by the action of calcium hypochlorite in acetic acid (Chattaway & Orton, 1901). After base-catalyzed hydrolysis, the diazonium salts of the chloroanilines were generated with $NaNO_2$ and then reduced to chlorobenzene-3,5-$^2H_2$ with an excess of hypophosphorus acid (Swinney, 1985). Although the chlorination step gave both ortho and para isomers, reductive deamination of either results in chlorobenzene-3,5-$^2H_2$. Yields for the chlorination, hydrolysis, and reductive deamination steps were 91%, 87%, and 27% respectively. The isotopic composition of the product was 98.59% chlorobenzene-$^2H_2$, 1.36% chlorobenzene-$^2H_1$, and 0.05% chlorobenzene-$^2H_0$.

Chlorophenol-$^2H_4$ Internal Standards. The three isomeric chlorophenol internal standards were prepared by two successive acid-catalyzed exchange reactions of the parent chlorophenols in refluxing $^2H_2O_4/^2H_2O$ (50:50). The deuterated chlorophenols were extracted into dichloromethane; the organic phase was washed with water and then extracted into base. The aqueous phase was acidified and extracted into dichloromethane. Each of the dichloromethane solutions was concentrated, and the chlorophenols were used without further purification.

Enzyme Preparations. Liver microsomal fractions from phenobarbital-induced rats (male, Sprague-Dawley, 150–250 g) were isolated according to standard procedures (van der Haeven & Coon, 1974). The pellets were suspended in Tris buffer (100 mM, pH 8.2 at 25 °C unless otherwise specified) to obtain a protein concentration of 10 mg/mL. P-450b was purified to a specific content of 13 nmol mg^{-1} (Waxman & Walsh, 1982). Cytochrome P-450 reductase was prepared according to the procedure of Shepard et al. (1983).

Incubations. Microsomal incubation mixtures (1 or 2 mL) had final concentrations of 5 mg of protein/mL, 0.6 mM NADPH, and 4 mM (saturating conditions) appropriate substrate. Incubations were carried out at 25 °C unless otherwise specified. Incubation times were 1 h for the EC–LC studies and 30 min for the GC–MS study. Purified P-450b incubations were run under saturating conditions previously described (Jones et al., 1986) for 25 min with a substrate concentration of 4 mM.

EC–LC Analysis. Incubations were terminated by the addition of 20 μL of a solution of 18.4 M trichloroacetic acid containing 0.4 mM *o*-bromophenol. The metabolites were quantitated by liquid chromatography on a Du Pont 850 liquid chromatogaph fitted with an Altech C18 column (25 cm × 4.6 mm, spherical 5-μm support) and a Bioanalytical Systems Inc. Model LC-4A ampermeric detector. The mobile phase was 10% (v/v) 2-propanol in 0.05 M potassium phosphate buffer, pH 7.0. The flow was isocratic at 1.0 mL/min, and the column temperature was 55 °C. A freshly polished glassy carbon electrode was used in the ampermeric detector, and the oxidation potential applied to the electrode was +0.9 V. An aliquot (50 μL) of the supernatant from the trichloroacetic acid precipitated incubation was injected directly for analysis. Absolute amounts of chlorophenols were quantitated with *o*-bromophenol as the internal standard.

GC–MS Analysis. The assay used for GC–MS quantitation of the isomeric chlorophenols will be described elsewhere. In brief, the metabolites were isolated from the incubation mixtures by acid-base extraction and treated with pentafluorobenzyl bromide with K_2CO_3 and 18-crown-6 ether as catalysts (Davis, 1977) to form the pentafluorobenzyl ether derivatives. The mass spectra of the derivatives were recorded and the data analyzed with the least-squares method of Brauman (1966).

For the determination of the isotopic composition of the various substrates, 10% solutions (0.4 μL) of the labeled chlorobenzene(s) in acetonitrile were injected into the GC operating isothermally at 50 °C. In order to eliminate M − 1 contributions, the ionizing voltage of the mass spectrometer was maintained at 12 eV and the ion repeller was grounded to the source block.

Table I: Effect of Temperature and pH on the Percent Meta Hydroxylation of Separate Incubations of Saturating Concentrations of Chlorobenzenes-$^2H_0$ and -$^2H_5$ with Microsomes from Phenobarbital-Pretreated Rats As Determined by LC–EC

pH	temp (°C)	% $^2H_0$ meta	% $^2H_5$ meta	KIE[a]
7.9	37	4.0	3.6	1.11
8.2	37	4.9	3.6	1.23
8.1	30	4.5	3.5	1.29
8.4	30	3.7	3.1	1.19
8.2	25	3.5 (0.3)[b]	2.7 (0.3)	1.29 (0.18)
8.5	25	3.9	3.1	1.26

[a] Kinetic isotope effect. [b] Values in parentheses are the standard deviations. Since this pH and temperature gave the greatest turnover of substrate, the values were determined in quadruplicate.

Table II: Relative Percentages of Isomeric Chlorophenols Formed in Separate Incubations from the Hydroxylation of Saturating Concentrations of Selectively Deuterated Chlorobenzenes with Microsomes from Phenobarbital-Pretreated Rats As Determined by GC–MS

chloro-benzene	% total phenols			KIE
	ortho	meta	para	(meta)[a]
$^2H_0$	37.00 (0.54)[b]	6.60 (0.34)	56.42 (0.50)	
3,5-$^2H_2$	38.00 (0.26)	5.81 (0.08)	55.69 (0.21)	1.13 (0.06)
2,4,6-$^2H_3$	37.55 (0.29)	5.80 (0.08)	56.66 (0.38)	1.13 (0.06)
$^2H_5$	39.03 (0.40)	5.20 (0.15)	55.78 (0.49)	1.27 (0.07)

[a] Kinetic isotope effect for the meta position. [b] Values in parentheses are standard deviations.

Results

In preliminary studies, the intermolecular isotope effect associated with the meta hydroxylation of chlorobenzene-$^2H_5$ with microsomes from phenobarbital-pretreated rats was measured by a high-performance, liquid chromatography assay and electrochemical detection (EC–LC assay). The selectivity of the EC detector for phenols and its high sensitivity (picogram detection) allowed for the direct analysis of the incubation mixture after protein precipitation. Since it was found that the total amount of microsomal metabolism was very sensitive to both temperature and pH and that the normal conditions (37 °C, pH 7.4) did not give maximal metabolism, separate incubations for chlorobenzenes, -$^2H_5$ and -$^2H_0$, were run at six different pH–temperature combinations, with four additional sets of incubations run at the conditions showing the greatest metabolism (pH 8.2, 25 °C; Table I).

Both $^2H_0$ and $^2H_5$ substrates were incubated with microsomes in separate experiments and quantitated relative to *o*-bromophenol as internal standard. Although there was considerable variation in total metabolism between duplicate incubations, useful data could be obtained by calculating the isotope effects associated with the percent meta hydroxylation relative to total for each set conditions. With this method the ortho and para metabolites serve as internal standards, and k_H/k_D is simply the ratio of the percent *m*-chlorophenol formed from the protiochlorobenzene relative to the percent formed from the deuteriochlorobenzene.[2] The observed isotope effects are given in Table I. A normal isotope effect was found for all incubation conditions, and the average isotope effect for the optimized conditions was 1.29 (Table I).

Table III: Substrate Composition, Metabolite Composition, and Isotope Effects for the Competitive Intermolecular Isotope Effect Experiment Using Purified Cytochrome P-450b and Saturating Concentrations of Substrate

chlorobenzene substrates	Substrate Composition: ratio of chlorobenzene-$^2H_0$ to chlorobenzene-^2H_x[a]
$^2H_0$/3,5-$^2H_2$	1.472 (0.009)
$^2H_0$/2,4,6-$^2H_3$	0.961 (0.006)
$^2H_0$/$^2H_5$	0.977 (0.004)

chlorobenzene substrates	Metabolite Composition: ratio of chlorobenzene metabolites, $^2H_0$ to ^2H_x[b]		
	ortho	meta	para
$^2H_0$/3,5-$^2H_2$	1.452 (0.016)	1.315 (0.011)	1.453 (0.005)
$^2H_0$/2,4,6-$^2H_3$	1.033 (0.005)	0.882 (0.006)	1.033 (0.015)
$^2H_0$/$^2H_5$	0.974 (0.017)	0.771 (0.022)	0.965 (0.018)

chlorobenzene substrates	Kinetic Isotope Effects: calculated isotope effects[c]		
	ortho	meta	para
$^2H_0$/3,5-$^2H_2$	1.014 (0.017)	1.119 (0.016)	1.013 (0.010)
$^2H_0$/2,4,6-$^2H_3$	0.939 (0.010)	1.090 (0.014)	0.930 (0.020)
$^2H_0$/$^2H_5$	1.003 (0.021)	1.267 (0.041)	1.013 (0.022)

[a] Each of three incubations was analyzed twice. Values in parentheses are standard deviations. [b] Average and standard deviation of four measurements. [c] Values in parentheses are standard deviations after propagation of errors.

Table IV: Deuterium Retention and Competitive Intermolecular Isotope Effects for the Hydroxylation of Saturating Concentrations of Chlorobenzene-4-^2H Using Purified Cytochrome P-450b

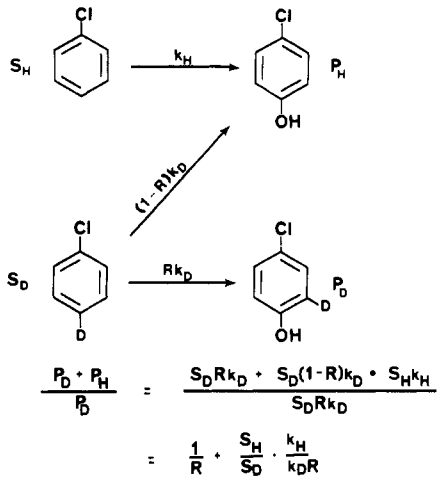
position of hydroxylation	% deuterium retention[a]	isotope effect[b]
ortho	99.8 (0.8)	1.012 (0.009)
meta	89.2 (0.8)	1.034 (0.010)
para	80.6 (0.6)	0.947 (0.007)

[a] Values in parentheses are standard deviations. [b] Values in parentheses are standard deviations after propagation of errors.

The second study used GC–MS and isotopic internal standards to correct for recoveries and to measure the intermolecular isotope effects for the meta hydroxylation of chlorobenzenes-$^2H_5$, -3,5-$^2H_2$, and -2,4,6-$^2H_3$, from the same enzyme source. Again the variation in the absolute amount of metabolism was much greater (~30%) than the variation in the percent meta hydroxylation. As a consequence the latter values were used to calculate kinetic isotope effects. The percent isomeric phenols and the isotope effect for meta hydroxylation for each substrate are given in Table II. The isotope effect for chlorobenzene-$^2H_5$ metabolism was 1.27, in good agreement with the value from the EC–LC study. In addition, both chlorobenzene-2,4,6-$^2H_3$ and chlorobenzene-

[1] When used for the mechanistic investigation of enzyme-catalyzed reactions, noncompetitive isotope effect experiments may provide erroneous conclusions, unless the absolute purity of the substrates is known. For example, if an isotopic substrate contains a small but significant amount of a potent inhibitor, the relative amounts of metabolism will differ. This potential problem is especially important when P-450 microsomes are used. Even when the amount of metabolite is compared to other regioisomeric metabolites (such as the percent meta hydroxylation), an inhibitor may affect one isozyme more than others. If the isozymes have different regiospecificity, an erroneous isotope effect may be observed.

[2] Because the substrates with more than one deuterium generate metabolites that contain at least one deuterium, they are always distinguishable (via mass spectrometry) from products resulting from hydroxylation of the protio substrate. This is no longer true with the monodeuterio substrate chlorobenzene-4-$^2H_1$, since para hydroxylation will lead to some *p*-hydroxy product that will not contain deuterium, and hence will be indistinguishable from that arising from the protio substrate. To circumvent the problem, one needs to know what percent of the nondeuterium-containing *p*-hydroxy metabolite arises from the monodeuterated substrate. This can be accomplished by accurately determining the percent deuterium in the substrate and then incubating this substrate alone with enzyme and precisely determining the percent deuterium retained in the *p*-hydroxy product. A better approach is outlined in the text.



$$\frac{P_D + P_H}{P_D} = \frac{S_D R k_D + S_D(1-R)k_D + S_H k_H}{S_D R k_D}$$

$$= \frac{1}{R} + \frac{S_H}{S_D} \cdot \frac{k_H}{k_D R}$$

FIGURE 2: Scheme and equations describing the linear relationships between substrate (S) and product (P) isotopic composition (H or D), deuterium retention (R), and isotope effects associated with the competitive para hydroxylation of chlorobenzene $^2H_0$ and chlorobenzene-4-$^2H_1$.

3,5-$^2H_2$ had isotope effects of 1.13 for meta hydroxylation.

To provide confirmatory results, to eliminate the effects of potential inhibitors,[2] and to avoid the greater experimental error inherent with noncompetitive (separate experiments) intermolecular isotope effect experiments, the competitive (a mixture composed of both protio and deuterio substrates) intermolecular isotope effects were determined for the same substrates with purified cytochrome P-450b. In order to calculate a competitive isotope effect, it is only necessary to know the isotopic ratios originally present in the substrate relative to those in the product. The measured isotopic compositions of the metabolites and substrates and the kinetic isotope effects are given in Table III. Again, the isotope effect for the meta hydroxylation of chlorobenzene-$^2H_5$ was 1.27. The meta isotope effects for the $^2H_2$ and $^2H_3$ substrates (1.12 and 1.09, respectively) are also in good agreement with the noncompetitive experiment. The metabolism of chlorobenzene-2,4,6-$^2H_3$ resulted in significant intermolecular inverse isotope effects, 0.94 and 0.93 for ortho and para hydroxylation, respectively (Table III). The isotope effects for the ortho and para hydroxylation of the 3,5-$^2H_2$ and $^2H_5$ substrates were not significantly different from 1.0.

In order to verify the inverse isotope effect observed above for para hydroxylation, the competitive isotope effect was measured with chlorobenzene-4-$^2H_1$ as substrate (Table IV). Rather than a single mixture of protio and deuterio substrates being used to determine the isotope effects, four solutions, with different ratios of protio and deuterio substrate, were used.[3] The relationships between deuterium retention, isotope effect, and isotopic composition of the substrates and metabolites are shown in Figure 2. The equation in Figure 2 describes the linear relationship between the substrate isotopic ratio and the inverse of the percent deuterium in the product. Thus, a plot of the four substrate ratios versus the inverse of the fraction of $^2H_1$ present in the metabolites gives an intercept of 1/retention and a slope of (k_H/k_D)(1/retention) and allows rigorous determination of the statistics of the regression.

The isotopic ratios for the substrate solution were obtained from the average of three determinations. Each incubation mixture was analyzed one to three times, and the inverse of the fraction of $^2H_1$ present in the metabolite was calculated. The statistical program Mini-Tab was used to perform the regression analyses for each isomer. The intercept and slope coefficients and their standard deviations were used to calculate the deuterium retention and isotope effects. These values along with their standard deviations (after propagation of errors for the isotope effects) are given in Table IV.

The deuterium isotope effect for para hydroxylation is again inverse and agrees with the value observed in the previous experiment for the 2,4,6-$^2H_3$ substrate. Although the R^2 values for the regression were all >0.9995, perhaps a more meaningful assessment of the quality of the calculations can be seen from the ortho retention value. Since there is no deuterium in the ortho or meta positions, the degree of deuterium retention in the ortho phenolic product should be 100%. The calculated value of 99.8 ± 0.8% strongly supports the validity of the measurements.

Discussion

The proposed mechanisms for aromatic oxidation can be dividied into two general categories: singlet-like and triplet-like. The singlet mechanisms involve no intermediates and characterize the direct insertion pathways, i.e., insertion across a carbon–hydrogen bond or a carbon–carbon π bond, while the triplet mechanisms except for direct ketone formation are stepwise pathways involving the formation of an intial radical intermediate. These involve (1) initial hydrogen abstraction followed by recombination with hydroxy radical as indicated by the results of Groves et al. (1978) for aliphatic hydroxylation, (2) initial tetrahedral intermediate formation followed by rearrangement, and (3) initial electron abstraction followed by recombination with hydroxy radical and subsequent rearrangement as suggested by Burka et al. (1983).

For clarity, the discussion to follow will be divided into two main sections: (1) singlet mechanisms and (2) triplet mechanisms. The analysis assumes that the addition of oxygen to the substrate is irreversible (Harada et al., 1984).

Singlet Mechanisms

Direct insertion across a carbon–hydrogen bond can be eliminated as the primary pathway since deuterium at the site of oxidation is generally retained. Conversely, deuterium retention at the site of oxidation is entirely consistent with initial epoxide formation via singlet oxygen atom addition. The deuterium retention values for chlorobenzene are ~80% for para hydroxylation, 50–70% for meta hydroxylation, and ~50% for ortho hydroxylation when deuterium is substituted at the site of hydroxylation (data not shown). The lower ortho value can be rationalized by a shift of the deuterium to the chloro-substituted carbon, and subsequent loss.

If cytochrome P-450 catalyzed aromatic hydroxylation involves the initial formation of an epoxide in a single step, the presence of deuterium at either or both of the carbons being attacked should lead to an inverse secondary isotope effect because of sp^2 to sp^3 conversion on forming the three-membered ring. Once epoxide formation has occurred, then subsequent product formation can either be (i) determined, e.g., ratio of para to meta phenols independent of deuterium substitution, or (ii) undetermined, i.e., product ratios isotopically sensitive. If case i is operative, potential isotope effects associated with events beyond initial epoxide formation cannot be expressed, and a small inverse isotope effect should be observed when deuterium occurs at the site of hydroxylation. If case ii is operative, then again two cases must be considered depending upon whether epoxide ring opening is reversible or

[3] The isotope effect was modeled theoretically in a manner analogous to that of Shea et al. (1983). The equilibrium isotope effect was calcualted for charge transfer between benzene-$^2H_0$ and benzene-$^2H_5$, with the MNDO formalism, and was found to be 1.34.

irreversible. If it is reversible and one of the carbons forming the three-membered ring bears deuterium, then subsequent ring opening should increase at the carbon bearing the hydrogen relative to the carbon bearing the deuterium (sp^3 to sp^2 conversion). This effect would be more than offset (primary isotope effect versus secondary isotope effect) by the necessity of breaking the carbon–deuterium bond in the next step in generating the ketone, or progressing directly to phenol. The net result is that a normal isotope effect should accompany hydroxylation at the site of deuteration and the para to meta phenolic product ratio should change. If it is irreversible, then ring opening should also occur at the carbon bearing the hydrogen and thereby should generate the product in which the oxygen is bonded to the carbon bearing the deuterium atom. The net result is that the para to meta phenolic metabolic ratio should again change but an inverse isotope effect should be observed. On the basis of this analysis, if the mechanism of aromatic hydroxylation is initiated by epoxide formation in a single step, it can be accompanied by either a small inverse isotope effect (product determined and/or irreversible epoxide ring opening) or a normal isotope effect (reversible epoxide ring opening).

To our knowledge, a deuterium isotope effect for either ortho or para hydroxylation of any aromatic substrate has never been previously reported, suggesting that the magnitude of the isotope effect, if its exists, is small. However, problems arise when one considers meta hydroxylation. Selander et al. (1975) found that neither the 2,3 or 3,4 synthetically prepared chlorobenzene oxide opened to form *m*-chlorophenol. *o*- and *p*-chlorophenols were the sole products, respectively. These results contrasted dramatically with their enzymic results where they found the *m*-chlorophenol was formed from chlorobenzene by cytochrome P-450 but that the amount formed varied as a function of enzyme preparation (perfused liver, 20%; soluble enzymes, 0%) and induction state (different isozymes: phenobarbital, 10%; 3-methylcholanthrene, 2%). These data led the authors to conclude that the meta product probably is formed from a different isozyme of cytochrome P-450 than that (those) that form(s) the ortho and para products. They also suggested that meta hydroxylation may proceed by a direct insertion mechanism.

Normal isotope effects have been observed for the meta hydroxylation of several different substrates, e.g., nitrobenzene and methyl phenyl sulfone (Tomaszewski et al., 1975) and biphenyl (Swinney et al., 1984). This is also true in the present study. A normal isotope effect of 1.1–1.27 was found for all substrates that contained deuterium in the meta position of the substrate together with a high degree of deuterium retention. These data are only consistent with reversible epoxide ring opening. But, implicit in this model is that a corresponding inverse isotope effect must occur when the deuterium is present in the position adjacent to the meta position, i.e., ortho or para. This, however, is found not to be the case as the meta hydroxylation of chlorobenzene-2,4,6-$^2H_3$ is accompanied by a normal isotope effect (1.13; Table II; 1.09, Table III; 1.03, Table IV) irrespective of experimental design. Thus, one is forced to conclude that *m*-hydroxy products do not arise via an initial epoxide singlet mechanism. As a consequence it can be further concluded that either (a) meta hydroxylation proceeds by one of the triplet mechanisms and ortho and para hydroxylations proceed by initial epoxide mechanisms or (b) all regioisomeric hydroxylations proceed by one of the triplet mechanisms. Before any conclusion is reached as to which of these two possibilities is preferred, the addition–rearrangement mechanism (triplet) will be assumed for meta hydroxylation to assess how well the experimentally determined isotope effects correlate with those predicted by the model.

Triple Mechanisms

For clarity, the discussion will be divided into three sections: (a) evaluation of the isotope effects for meta hydroxylation with respect to the addition–rearrangement mechanisms, (b) evaluation of the ortho and para isotope effects, and (c) discussion of the potential involvement of the electron abstraction and direct ketone pathways.

(*a*) *Meta Hydroxylation.* Evaluation of the various pathways in the addition–rearrangement mechanism, Figure 1, whith respect to the observed isotope effects for meta hydroxylation is complicated not only by three independent routes of rearrangement emanating from a common intermediate leading to a final common product and three independent sites of intial attack (ortho, meta, and para) but also by the masking and unmasking of numerous potential isotope effects. Therefore, while a quantitative evaluation of the involvement of each pathway is impossible, a qualitative comparison can be made between the observed isotope effects and those expected for the addition–rearrangement mechanisms. For the sake of clarity, the following assumptions will be made: (1) Epoxides of chlorobenzene can only open to generate either ortho or para phenols [Selander et al. (1975a,b) and discussion above]. (2) Isotope effects associated with branched (alternate) pathways obey the kinetics described in the accompanying paper (Korzekwa et al., 1989).

A composite of multiple isotope effects would be expected to be associated with the individual discrete steps in the overall addition–rearrangement pathway. For example, an inverse secondary isotope effect would be expected for initial attack at the position of deuterium substitution to form the tetrahedral intermediate. From the tetrahedral intermediate, normal primary isotope effects would be expected for ketone and direct phenol formation. Additional inverse secondary isotope effects would accompany epoxide formation when closing to a position substituted with deuterium and ketone formation when the hydride is shifted to a deuterated carbon. Finally, a normal (>1.0) secondary isotope effect would be expected for epoxide ring opening (Figure 1).

Although a discussion on the interpretation of isotope effects associated with cytochrome P-450 oxidations is presented in the accompanying paper, equations for a simplified model will be presented to aid in the interpretation of the isotope effects for meta hydroxylation. For this discussion, it will be assumed that oxygen activation is rate limiting and irreversible, and therefore, the isotope effects are obserable because of branched reaction pathways in competition with the isotopically sensitive steps. Two different kinds of branching must be considered. First, branching can occur from the enzyme complex when more than one position on the aromatic ring is susceptible to initial oxygen attack or when substrate oxidation is in direct competition with the further reduction of the active oxygen to water (Gorsky et al., 1984; White et al., 1985; Atkins & Sligar, 1987). Second, branching can occur between epoxide, ketone, and direct phenol formation via a common tetrahedral intermediate, Figure 1. The reported experimental designs have allowed for the observation of isotope effects associated with either the percent meta hydroxylatin or, for the competitive experiments, the isotope effects on V/K. For branching from the tetrahedral intermediate, identical isotope effects will be seen on percent meta hydroxylation and V/K. For initial attack, water formation can unmask the isotope effect on V/K but will have no influence on the percent meta hydroxylation. This point will be further discussed later in the text.

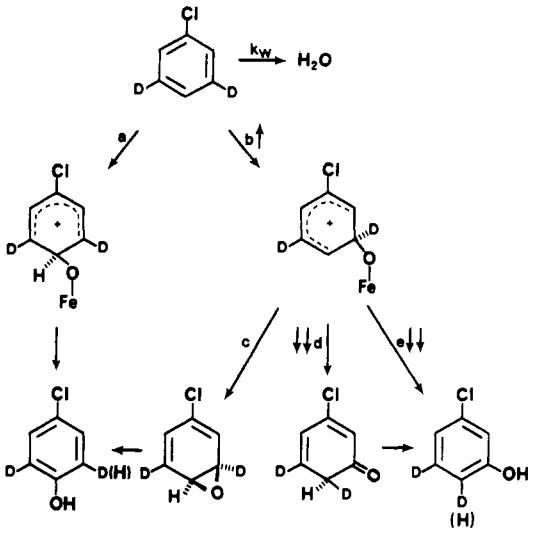


FIGURE 3: Addition–rearrangement pathways for the meta hydroxylation of chlorobenzene-3,5-$^2H_2$. Arrows (up, increase; down, decrease) represent effects of deuterium substitution on amount of meta phenol formed—single arrows represent secondary isotope effects, and double arrows represent primary isotope effects. The branched reaction pathway to water formation is represented by the rate constant k_w.

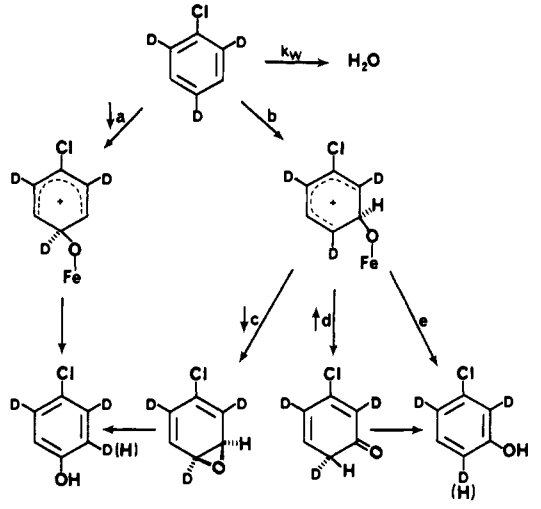


FIGURE 4: Addition–rearrangement pathways for the meta hydroxylation of chlorobenzene-2,4,6-$^2H_3$. Arrows (up, increase; down, decrease) represent effects of deuterium substitution on amount of meta phenol formed—single arrows represent secondary isotope effects, and double arrows represent primary isotope effects. The branched reaction pathway to water formation is indicated by the rate constant k_w.

The intermolecular noncompetitive and competitive isotope effects for the meta hydroxylation of chlorobenzenes-$^2H_5$, -4-$^2H_1$, -3,5-$^2H_2$, and -2,4,6-$^2H_3$ are given in Tables II–IV. Meta hydroxylation of the 3,5-$^2H_2$ and 2,4,6-$^2H_3$ substrates have similar isotope effects of ~1.1, while meta hydroxylation of chlorobenzene-$^2H_5$ has an isotope effect of 1.27.

Figures 3–5 show the addition–rearrangement schemes for the metabolism of these three substrates. In order to simplify these schemes and the following discussion, only the meta and para pathways are included. Although ortho hydroxylation is also involved for each of these substrates, the ortho and para positions have identical isotopic substitutions; therefore, these pathways can be combined.

(*i*) *Chlorobenzene-3,5-$^2H_2$.* The kinetic scheme for the metabolism of chlorobenzene-3,5-$^2H_2$ is shown in Figure 3. The initial step for substrate oxidation is the addition of the

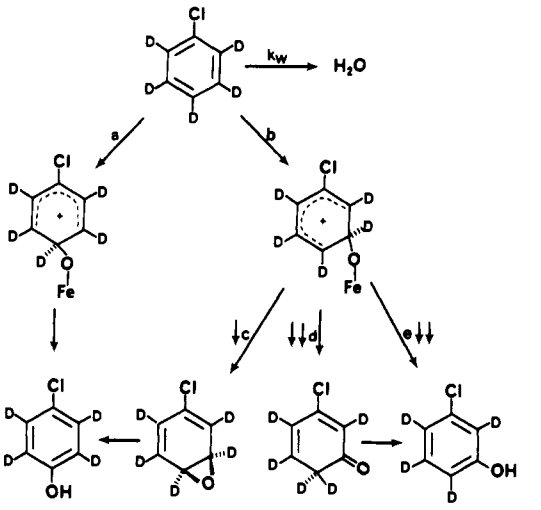


FIGURE 5: Addition–rearrangement pathways for the meta hydroxylation of chlorobenzene-$^2H_5$. Arrows (up, increase; down, decrease) represent effects of deuterium substitution on amount of meta phenol formed—single arrows represent secondary isotope effects, and double arrows represent primary isotope effects. The branched reaction pathway to water formation is indicated by the rate constant k_w.

oxygen to one of the carbon atoms. For carbons substituted with deuterium, an inverse isotope effect is expected. For this substrate, the isotope effect on the discrete step of C–O bond formation will result in an increase in meta attack (pathway b). This is depicted in Figure 3 by (↑), showing that meta tetrahedral intermediate formation will be increased by a secondary isotope effect. For this situation, the isotope effect for meta tetrahedral intermediate formation (for Figure 3) can be defined as eq 1, where k_b is the rate of addition to the

$$(k_H/k_D)_{obs} = \frac{k_{bH}/k_{bD} + k_{bH}/(k_a + k_w)}{1 + k_{bH}/(k_a + k_w)} \quad (1)$$

meta position, k_a is the total rate of addition to the ortho and para positions, and k_w is the rate of water formation (competitive reduction). This equation states that the magnitude of the observed isotope effect for meta addition is dependent on the intrinsic isotope effect for addition (k_{bH}/k_{bD}) and the ratio of meta to ortho plus para plus water pathways [$k_{bH}/(k_a + k_w)$]. The equation assumes that the rate of equilibration of the ortho, meta, and para positions of the substrate in the active site of the enzyme is much faster than the rate of substrate oxidation. Support for this assumption can be gained from the observations of Lindsay-Smith and Sleath (1983), who found that the para and methyl positions of anisole interchanged with sufficient rapidity to allow isotopically sensitive branching between the two sites. It should be noted that for the present meta isotope effects, the rate of water formation k_w is not included. However, if the rate of attack at the meta position is slow relative to those at the ortho and para positions, the isotope effect will already be unmasked and k_w will have a correspondingly smaller effect. Thus, if meta attack is not favored [$k_{bH}/(k_a + k_w)$ is small], the isotope effect for initial attack will be manifest in the final product distribution. It should also be noted that if initial attack of oxygen on the substrate is partially rate limiting, it can only serve to further unmask the intrinsic isotope effect (see accompanying paper).

Addition to the ortho and para positions will result only in ortho and para phenols (assumption 1). As a consequence, we need only consider pathways c–e. Both steps d and e in Figure 3 (ketone and phenol formation) would have primary isotope effects and are depicted by (↓↓) since they will decrease the amount of meta phenol formed. The ketone and phenol

pathways are also in competition with pathway c, which results in epoxides and, therefore, ortho and para phenols. Equation 2, derived from steady-state considerations, describes this relationship. In this equation, the ratio $(k_{dH} + k_{eH})/(k_{dD} + k_{eD})$ is the average of the ketone and phenol isotope effects, weighted by the relative contribution of the two deuterated pathways. This average isotope efect is modified by the relative contribution of pathway c (epoxide formation). As pathway c assumes importance in the oxidative scheme, $(k_{dH} + k_{eH})/k_c$ becomes smaller, and the isotope effects for ketone and phenol formation will begin to be unmasked and observed.

$$(k_H/k_D)_{obs} = \frac{(k_{dH} + k_{eH})/(k_{dD} + k_{eD}) + (k_{dH} + k_{eH})/k_c}{1 + (k_{dH} + k_{eH})/k_c} \quad (2)$$

Since the initial-attack isotope effect and the ketone/phenol isotope effect are associated with sequential steps in a reaction sequence, they are multiplicative. Therefore, provided that (1) the substrate can equilibrate in the active site prior to C–O bond formation and (2) the epoxide pathway from the meta tetrahedral intermediate is significant, the composite isotope effect for this substrate would be the product of an inverse secondary isotope effect and a normal primary isotope effect. The magnitude of the observed isotope effect would depend on the relative contribution of the epoxide pathway. If the epoxide is not formed from the meta tetrahedral intermediate, then only the inverse isotope effect for initial oxygen attack would be observed. If a large fraction of the meta tetrahedral intermediate ring closes to epoxide, then a significant normal isotope effect should be observed because of unmasking effects. The small observed isotope effect of ~1.1 is consistent with this model and suggests that while the epoxide pathway is significant, it is less than the sum of ketone plus phenol pathways.

(*ii*) *Chlorobenzene-2,4,6-$^2H_3$.* Figure 4 shows the addition–rearrangement pathways for chlorobenzene-2,4,6-$^2H_3$ metabolism. Although initial-attack isotope effects for this substrate will be observed only for ortho and para addition, these isotope effects can influence meta tetrahedral intermediate formation. Assuming again that the substrate can rapidly equilibrate in the active site, the result of the ortho and para isotope effects on meta addition is expressed by eq 3. This

$$(k_H/k_D)_{obs} = \frac{k_{aD}/k_{aH} + (k_b + k_w)/k_{aH}}{1 + (k_b + k_w)/k_{aH}} \quad (3)$$

equation states that, as the rate of ortho and para addition becomes favored, i.e., $(k_b + k_w)/k_{aH}$ becomes small, the observed effect on meta addition approaches the reciprocal of the isotope effect for ortho and para addtion (k_{aD}/k_{aH}). Since the isotope effect for addition is inverse, the net effect would be a decrease in the percentage of initial addition to the meta position. Thus, the ortho and para inverse isotope effects cause a switching from the meta positions to the ortho and para positions. In this case, however, any competing water pathway actually masks the observed isotope effect on the meta position.

Again, since the discussion is restricted to meta isotope effects, only pathways c–e need to be considered. For this substrate, since the carbon to which the oxygen is bonded is substituted with deuterium and undergoes an sp^2 to sp^3 conversion, pathway c (epoxide formation) would have an inverse isotope effect. For the same reason, pathway d (ketone formation) would also have an inverse isotope effect. The overall isotope effect for the pathways from the meta tetrahedral intermediate is expressed by eq 4. This equation contains three terms—$(k_{dH} + k_e)/(k_{dD} + k_e)$, k_{cD}/k_{cH}, and $(k_{dH} + k_e)/k_{cH}$. The last of these terms modifies the isotope effects in the usual manner. As epoxide formation is favored, the isotope effects are expressed. The term k_{cD}/k_{cH} is the reciprocal of the epoxide isotope effect and arises from the same situation described above (Figure 4) for initial attack—switching from the meta pathways to the epoxide (ortho and para pathways). Since the epoxide isotope effect is inverse, the result will again be a decrease in meta products. The remaining term, $(k_{dH} + k_e)/(k_{dD} + k_e)$, is a composite of the isotope effect for ketone formation and the rate constant for phenol formation. Thus, three inverse secondary isotope effects are associated with metabolism of this substrate. Two of these, initial attack and epoxide formation, will decrease meta phenol formation, and one, ketone formation, will increase meta phenol formation. As a consequence, each will give rise to a small normal isotope effect (reciprocal of an inverse isotope effect). The third, ketone formation, will increase meta phenol formation. Therefore, it will give rise to an inverse isotope effect. The overall observed isotope effect will be a composite of these three isotope effects and will be determined by the product of eq 3 and 4. If we first assume all three inverse isotope effects have a value of 0.9, an estimation of the observed isotope effect can be obtained as follows. If masking is minimal, i.e., $k_a \gg (k_b + k_w)$, then $(k_H/k_D)_{obs}$ from eq 3 with equal 1.11. We know from the observed isotope effect for the 3,5-dideuterio substrate that the epoxide pathway makes a significant contribution to product formation. In the case of the 2,4,6-trideuteriochlorobenzene this contribution, path c, leads to less *m*-chlorophenol formation (reciprocal of the inverse isotope effect), but it is offset by the inverse isotope effect associated with the ketone pathway, path d; thus, the observed isotope effect resulting from eq 4 would be expected to be either slightly positive or slightly inverse depending upon the relative contributions of paths c and d. Consequently, the product of eq 3 and 4 would be expected to be somewhere near 1.1. That the observed isotope effect is in fact 1.1 is not only consistent with the analysis but provides some evidence for the addition–rearrangement pathway.

$$(k_H/k_D)_{obs} = \frac{[(k_{dH} + k_e)/(k_{dD} + k_e)](k_{cD}/k_{cH}) + (k_{dH} + k_e)/k_{cH}}{1 + (k_{dH} + k_e)/k_{cH}} \quad (4)$$

(*iii*) *Chlorobenzene-$^2H_5$.* The addition–rearrangement scheme for chlorobenzene-$^2H_5$ metabolism is shown in Figure 5. Since all positions in this substrate are deuterated, switching between the various positions of initial attack would not be significant, provided that the initial-attack isotope effects for each position are similar. However, branching via water formation can still be important, and can be described by eq 5. Thus, the isotope effect will be modified by the ratio of

$$(k_H/k_D)_{obs} = \frac{k_{bH}/k_{bD} + (k_{aH} + k_{bH})/k_w}{1 + (k_{aH} + k_{bH})/k_w} \quad (5)$$

total substrate oxidation to water formation.

For this substrate, four different isotope effects are associated with pathways c–e. The isotope effect for epoxide formation would be inverse, and a normal primary isotope effect would be expected for the phenol pathway. The ketone pathway would contain two isotope effects—a normal primary isotope effect for shifting the deuterium and an inverse isotope effect for the adjacent deuterium. The interactions of these pathways can be expressed by eq 6. Again, all isotope effects can be manifest provided that epoxide formation from the tetrahedral intermediate is signifiant; i.e., $(k_{dH} + k_{eH})/k_c$ is small. One might expect that the overall observed isotope effect for meta hydroxylation for the $^2H_5$ substrate should be

$$(k_H/k_D)_{obs} = \frac{[(k_{dH} + k_{eH})/(k_{dD} + k_{eD})](k_{cD}/k_{cH}) + (k_{dH} + k_{eH})/k_{cH}}{1 + (k_{dH} + k_{eH})/k_{cH}} \quad (6)$$

approximately equal to the product of 3,5-$^2H_5$ and 2,4,6-$^2H_3$ substrate isotope effects. The initial-attack isotope effects for chlorobenzene-2,4,6-$^2H_3$ and -3,5-$^2H_2$ metabolism should approximately cancel, and the $^2H_5$ substrate has the combined isotope effects of both substrates for pathways c–e. The measured isotope effect for chlorobenzene-$^2H_5$ metabolism is 1.27, in general agreement with the product of the other two isotope effects (1.22). The difference in these two values is in the expected direction. It can be shown that if significant masking occurs, e.g., if the rate of epoxide formation is slower or approximately equal to the rates of ketone and phenol formation, the observed isotope effect for the $^2H_5$ metabolism should be larger than the product of the observed 3,5-$^2H_2$ and 2,4,6-$^2H_3$ isotope effects. Therefore, the addition–rearrangement mechanism appears to provide a sound basis for the observed isotope effects associated with the meta hydroxylation of chlorobenzene.

(*b*) *Ortho and Para Hydroxylation.* In the analysis presented under singlet mechanisms it was concluded that either (a) meta hydroxylation proceeds by one of the triplet mechanisms and ortho and para hydroxylation proceed by an initial epoxide mechanism or (b) all regioisomeric hydroxylations proceed by one of the triplet mechanisms. Conclusion a appears untenable for a number of reasons. First, it seems highly unlikely that a purified enzyme, P450b, would transfer a triplet-like oxygen atom to the meta position of an aromatic substrate but a singlet-like oxygen atom to either of the adjacent positions (ortho or para). Second, both ortho and para hydroxylations of chlorobenzenes-2,4,6-$^2H_3$, -4-^2H, and -3,5-$^2H_2$ give significant inverse isotope effects (0.93–0.95) for phenol formation at a deuterium-substituted position, but not at the protio-substituted positions. These isotope effects are consistent with a stepwise addition mechanism, since the isotope effect data imply that only one carbon undergoes an sp^2 to sp^3 conversion, and are not consistent with symmetrical addition of oxygen across the double bond.

The similarity of these isotope effects to those reported for olefin epoxidation should also be considered. For the oxidation of styrene it was found (Hanzlik & Shearer, 1978) that substitution of deuterium at the position α to the phenyl ring resulted in an inverse isotope effect of 0.93, whereas substitution at the β position showed no isotope effect. Since the heme alkylation associated with olefin oxidation implies a stepwise radical addition (Ortiz de Montellano & Correia, 1983), the similar behavior for the chlorobenzene substrates provides strong support for a stepwise mechanism. Third, in an earlier study, we found that the 6-, 7-, and 8-hydroxylation (para, meta, and ortho, respectively) of warfarin selectively deuterated in the 6-, 7-, and 8-positions occurred with a remarkable consistency in the values of deuterium retention (Bush & Trager, 1985). That is, the degree of deuterium retention at the site of hydroxylation was independent of the isozyme of P-450 catalyzing the reaction and stereochemistry of the substrate. These data strongly suggested that all three regioisomeric products were arising from a common mechanism. Since meta hydroxylation cannot arise via a singlet mechanism, all three isomers (ortho, meta, and para) presumably arise from a common triplet mechanism. Of the two triplet mechanisms suggested by Tomaszewski et al. (1975), abstraction or addition–rearrangement, the abstraction mechanism can be eliminated because of deuterium retention in the product (N1H shift). Thus, the mechanism that fits all the data to this point in the analysis is the addition–rearrangement mechanism.

(*c*) *Charge Transfer and Direct Ketone Formation.* Evidence supporting the radical addition of an oxygen does not preclude the involvement of cationic intermediates. Theoretical calculations (Korzekwa et al., 1985) suggested that ketone and phenol formation from the tetrahedral intermediate are much more likely to occur from cationic intermediates. Moreover, 1,2-hydrogen shifts generally involve a hydride transfer to an adjacent cationic position (March, 1985) while direct phenol formation from a cationic tetrahedral intermediate is simply a deprotonation reaction. In addition, recent experimental evidence (Kurata et al., 1988) demonstrates that the N1H shift associated with Fenton and related oxidations of aromatic substrates involves electron transfer from initial tetrahedral radical species to generate cationic tetrahedral intermediates prior to ketone formation.

Given a triple-like active oxygenating species, a cationic tetrahedral intermediate can be generated in one of two ways. First, an initial charge transfer from the substrate ring to the heme would result in a substrate radical cation. Addition of the oxygen (as a hydroxy radical equivalent to the radical cation) would result in a cationic tetrahedral intermediate. Second, charge transfer could occur after formation of the tetrahedral intermediate; i.e., the radical in the substrate ring resulting from radical addition of oxygen is transferred to the porphyrin ring.

The first possibility, initial electron abstraction, would be expected to be accompanied by a normal isotope effect.[3] If this is the case, isotope effects for ortho and para hydroxylation should be normal. However, as seen, the observed ortho and para isotope effects are inverse, suggesting that electron abstraction is not the primary initiating event for the reaction. Conversely, electron transfer to porphyrin after formation of the tetrahedral intermediate would nicely account for the formation of meta chlorophenol and for the normal isotope effects associated with its formation.

Finally, the possibility of concerted ketone formation for aromatic hydroxylation needs to be addressed for the sake of completeness, and since it has been suggested as the mechanism responsible for the cytochrome P-450 catalyzed oxidation of the triple bond of phenylacetylene (Komives & Ortiz de Montellano, 1987). The trapping of chlorobenzene oxide in microsomal incubations rules out the primary involvement of such a mechanism for para oxidation of chlorobenzene but does not eliminate such a mechanism for meta hydroxylation. Concerted ketone formation should result in an inverse isotope effect for the 2,4,6-$^2H_3$ substrate, a combination of primary and inverse effects for the $^2H_5$ substrate and a primary isotope effect for the 3,5-$^2H_2$ substrate. The observed isotope effects for meta hyroxylation for these substrates suggest that this mechanism is not tenable. Again, the only mechanism which appears to be consistent with all the data is the addition–rearrangement pathway. However, it must be pointed out that a nagging inconsistency exists for ortho and para hydroxylation of the $^2H_5$ substrate. That the isotope effects on ortho and para hydroxylation can be observed with competitive intermolecular isotope effect experiments suggests that an alternate pathway from the active oxygenating species is significant. Pathways from the active oxygen include isotopically sensitive branching between initial-attack positions in the substrate ring, competitive water formation, and substrate debinding. For both chlorobenzene-4-^2H and the chlorobenzene-2,4,6-$^2H_3$ analogue, branching from the various possible ring protio

positions to a deuterated ring site cannot account for the observed inverse isotope effects. In the case of the 2,4,6-$^2H_3$ substrate the degree of meta hydroxylation is quantitatively insufficient while in the case of the 4-^2H substrate switching from the ortho position to generate an inverse isotope effect at the para position would require that hydroxylation at the ortho position occur with a normal isotope effect. These data suggest that substrate debinding and/or water formation is responsible. Such branching is perhaps to be expected, given that chlorobenzene is a rather "poor" substrate (K_m = 2 mM; Burka et al., 1983).

The above analysis implies that if the isotope effects were due to a low commitment to forward catalysis, similar inverse isotope effects would be expected for the $^2H_5$ substrate. The observed isotope effects for both ortho and para hydroxylations of this substrate were 1.0. One possible explanation is that the expected inverse isotope effects are counterbalanced by normal binding isotope effects which become important as the number of deuteriums on the substrate is increased. Also, additional normal secondary isotope effects for initial attack may be involved. It is a general rule that a decrease in electron density at carbons substituted with deuterium will result in a normal isotope effect. Despite the uncertainty of the explanation of the results for the $^2H_5$ substrate, the observed inverse isotope effects for ortho and para hydroxylation can be more rationally explained by the addition–rearrangement mechanism than by any of the alternate mechanisms considered above.

Conclusions

In summary, (1) of all the mechanisms evaluated, the addition–rearrangement mechanism characterized by initial attack of an activated triplet-like oxygen atom on the π system to generate a tetrahedral intermediate is the most consistent with the measured isotope effects; (2) direct epoxide formation by a singlet-like activated oxygen atom cannot be involved; and (3) while initial electron abstraction is probably not involved, charge transfer from the tetrahedral intermediate to the porphyrin ring may well be involved since it accounts for the isotope effects associated with meta hydroxylation.

Acknowledgments

We are grateful to Dr. Jeffrey Jones for helpful discussions and Dr. Allan Rettie for a generous gift of P-450b.

References

Atkins, W. M., & Sligar, S. G. (1987) *J. Am. Chem. Soc. 109*, 3754–3760.

Brauman, J. T. (1966) *Anal. Chem. 28*, 607–610.

Burka, L. T., Plucinski, R. M., & MacDonald, T. L. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6680–6684.

Bush, E. D., & Trager, W. F. (1982) *Biochem. Biophys. Res. Commun. 104*, 626–632.

Bush, E. D., & Trager, W. F. (1985) *J. Med. Chem. 28*, 992–996.

Chattaway, F. D., & Orton, K. J. (1901) *J. Chem. Soc. 79*, 469–470.

Davis, B. (1977) *Anal. Chem. 49*, 832–834.

Gorsky, L. D., Koop, D. R., & Coon, M. J. (1984) *J. Biol. Chem. 259*, 6812–6817.

Groves, J. T., McClusky, G. A., White, R. E., & Coon, M. J. (1978) *Biochem. Biophys. Res. Commun. 81*, 154–160.

Hanzlik, R. P., & Shearer, G. O. (1978) *Biochem. Pharmacol. 27*, 1441–1444.

Hanzlik, R. P., Hogberg, K., & Judson, C. M. (1984) *Biochemistry 23*, 3048–3055.

Harada, N., Miwa, G. T., Walsh, J. S., & Lu, A. Y. H. (1984) *J. Biol. Chem. 259*, 3005–3010.

Hartman, W. W., & Brethen, M. R. (1941) *Organic Synthesis* (Gilman, H., & Blatt, A. H., Eds.) Collective I, pp 162–164, Wiley, New York.

Jones, J. P., Korzekwa, K. R., Rettie, A. E., & Trager, W. F. (1986) *J. Am. Chem. Soc. 108*, 7074–7078.

Komives, E. A., & Ortiz de Montellano, P. R. (1987) *J. Biol. Chem. 262*, 9793–9802.

Korzekwa, K., Trager, W. F., Gouterman, M., Spangler, D., & Loew, G. H. (1985) *J. Am. Chem. Soc. 107*, 4273–4279.

Korzekwa, K. R., Trager, W. F., & Gillette, J. R. (1989) *Biochemistry* (preceding paper in this issue).

Kurata, T., Watanabe, Y., Katoh, M., & Sawaki, Y. (1988) *J. Am. Chem. Soc. 110*, 7472–7478.

Lindsay-Smith, J. R., & Sleath, P. R. (1983) *J. Chem. Soc., Perkin Trans. 2*, 621–627.

March, J. (1985) in *Advanced Organic Chemistry*, 3rd ed., pp 942–943, Wiley, New York.

Ortiz de Montellano, P. R., & Correia, M. A. (1983) *Annu. Rev. Pharmacol. Toxicol. 23*, 481–503.

Preston, B. D., Miller, J. A., & Miller, E. C. (1983) *J. Biol. Chem. 258*, 8304–8310.

Selander, H. G., Jerina, D. M., Piccolo, D. E., & Berchtold, G. A. (1975a) *J. Am. Chem. Soc. 97*, 4428–4430.

Selander, H. G., Jerina, D. M., & Daly, J. W. (1975b) *Arch. Biochem. Biophys. 168*, 309–321.

Shea, J. P., Nelson, S. D., & Ford, G. P. (1983) *J. Am. Chem. Soc. 105*, 5451–5454.

Shephard, E. A., Pike, S. F., Rabin, B. R., & Phillips, I. R. (1983) *Anal. Biochem. 129*, 430–433.

Swinney, D. C. (1985) Ph.D. Thesis, University of Washington.

Swinney, D. C., Howald, W. N., & Trager, W. F. (1984) *Biochem. Biophys. Res. Commun. 118*, 867–872.

Tomaszewski, J. E., Jerina, D. M., & Daly, J. W. (1975) *Biochemistry 14*, 2024–2031.

van der Haeven, T. A., & Coon, J. J. (1974) *J. Biol. Chem. 249*, 6302–6310.

Waxman, D. J., & Walsh, C. J. (1982) *J. Biol. Chem. 257*, 10446–10457.

White, R. E., Miller, J. P., Favreau, L. V., & Battacharyya, A. (1985) *J. Am. Chem. Soc. 108*, 6024–6031.