Substrate Probes for the Mechanism of Aromatic Hydroxylation Catalyzed by Cytochrome P-450: Selectively Deuterated Analogues of Warfarin

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Optically pure analogues of (*R*)- and (*S*)-warfarin selectively deuterated in either the 6-, 7-, or 8-position were prepared and incubated with microsomal preparations from either nontreated, phenobarbital-pretreated, or β-naphthoflavone-pretreated male Sprague–Dawley rats. The amount of deuterium retained and the relative amount of hydroxylated product formed (6-, 7-, 8-, or 4′-hydroxywarfarin) from each of the six substrates for each of the treatments were determined by capillary gas chromatography–mass spectrometry. The degree of deuterium retention in all products from all substrates was largely independent of both absolute configuration and induction state. Conversely, the relative amounts of product formed were highly dependent upon both absolute configuration and induction state. These results suggest that all the hydroxylation reactions proceed through an addition rearrangement step prior to or in the absence of epoxide formation, which appears to be dictated by the nature of the heme–Fe^{3+}–oxene complex. In contrast, the position of hydroxylation or regioselectivity appears to be primarily dependent upon the nature of the apoprotein.

Numerous studies have linked the metabolic activity of the cytochrome P-450 isozymes to such pathological conditions as kidney and liver dysfunction, teratogenesis, and carcinogenesis.[1,2] The ability of these enzymes to bioactive various substrates to reactive intermediates that can then bind covalently to critical biomacromolecules appears to be the basis for the observed toxicities. Thus, an understanding of their mechanisms of action and the factors that modulate them is crucial.

Cytochrome P-450 isozymes are mixed-function monooxygenases; i.e., after activation of molecular oxygen, they catalyze the reduction of one atom of a molecule of oxygen to water and the transfer of the second to substrate. In addition to O_2 the enzymes require NADPH, lipid, and cytochrome P-450 reductase for full activity. They follow the stoichiometry expressed by eq 1, and all appear to involve a ferric bound oxygen.[3]

$$\text{Cyt P-450} + \text{R–H} + O_2 + 2e^- + 2H^+ \rightarrow \text{Cyt P-450} + \text{R–OH} + H_2O \quad (1)$$

Theoretically, the oxygen atom can either be singlet-like or triplet-like in nature. Which species it is has profound implications with respect to the formation of potential toxic intermediates and is especially applicable in the case of aromatic hydroxylation. Of the four reaction mechanisms proposed by Tomazewski et al.[4] (Figure 1) for aromatic hydroxylation, two can be classified as singlet-like (ii and iii) and two as triplet-like (i and iv). Both singlet mechanisms are expected to be synchronous, i.e., to occur in a single step. Mechanism ii involves the direct insertion of oxygen across the carbon–hydrogen bond and is, therefore, expected to be nontoxic in nature since it leads directly to product. Mechanism iii on the other hand would lead directly to the formation of a highly reactive electrophilic arene oxide in a single step by attack on the π system. This mechanism has been generally held by many workers in the field to be the primary mechanism for aromatic hydroxylation. Epoxides have in fact been demonstrated in microsomal incubations. In addition, their reality has also been implicitly inferred by the isolation of secondary reaction products such as dihydrodiols and glutathione conjugates.[5,6]

By contrast, both triplet mechanisms must be stepwise and involve either hydrogen abstraction followed by radical recombination or addition to the π system followed by rearrangement. Evidence in the literature suggests that aliphatic hydroxylation occurs by the first of these mechanisms,[7] while we have obtained evidence that indicates that the 7-hydroxylation of warfarin possibly occurs by the second.[8]

The suggestion that cytochrome P-450 can hydroxylate aromatic systems by more than a single mechanism has been advanced by Selander et al.,[9] who found that *m*-chlorophenol was a substantial metabolite of chlorobenzene. Model studies have demonstrated that ring opening of the arene 2,3- or 3,4-oxide leads exclusively to either the ortho or para chlorophenol, respectively. As a consequence, formation of a meta phenol is unexpected.[10] On the basis of these results, these workers proposed an arene oxide pathway for ortho and para hydroxylation of chlorobenzene but a direct insertion pathway for meta hydroxylation. The findings of Tomazewski et al.,[4] that the in vivo meta hydroxylation of both nitrobenzene and methyl phenyl sulfone is seemingly accompanied by significant positive isotope effects while the ortho and para hydroxylations of the same substrates are not, are consistent with this hypothesis.

Other evidence exists in the literature that suggests that aromatic hydroxylation may proceed by a non arene oxide pathway. For example, Preston et al.[11] report that the 3-hydroxylation of 2,2′,5,5′-tetrachlorobiphenyl proceeds by a non arene oxide pathway although the mechanism is left undefined. Johansson and Ingelman-Sundberg[12] report

(1) Nelson, S. D.; Boyd, M. R.; Mitchell, J. R. "Drug Metabolism Concepts", ACS Symposium Series, no. 44, Jerina, D. M., Ed.; American Chemical Society, Washington, D.C., 1977; p 155.
(2) Hathway, D. E. "Foreign Compound Metabolism in Mammals"; Hathway, D. E., Senior Reporter; The Chemical Society: London, Vol. 5, p 190.
(3) White, R. E.; Coon, M. J. *Annu. Rev. Biochem.* **1980**, *49*, 315.
(4) Tomazewski, J. B.; Jerina, D. M.; Daly, J. W. *Biochemistry* **1975**, *14*, 2024.
(5) Low, L. K.; Castagnoli, N., Jr. "Burger's Medicinal Chemistry", 4th ed.; Wolff, M. E., Ed.; Wiley: New York, 1979; Part 1, p 171.
(6) Nelson, S. D. "Burger's Medicinal Chemistry", 4th ed.; Wolff, M. E. Ed.; Wiley: New York, 1979; Part 1, p 227.
(7) Groves, J. T.; McClusky, G. A.; White, R. E.; Coon, M. J. *Biochem. Biophys. Res. Commun.* **1978**, *81*, 154.
(8) Bush, E. D.; Trager, W. F. *Biochem. Biophys. Res. Commun.* **1982**, *104*, 626.
(9) Selander, H. G.; Jerina, D. M.; Daly, J. W. *Arch. Biochem. Biophys.* **1975**, *168*, 309.
(10) Bruice, P. Y.; Bruice, T. C. *J. Am. Chem. Soc.* **1976**, *98*, 2023.
(11) Preston, B. D.; Miller, J. A.; Miller, E. C. *J. Biol. Chem.* **1983**, *258*, 8304.
(12) Johansson, I.; Ingelman-Sundberg, M. *J. Biol. Chem.* **1983**, *258*, 7311.

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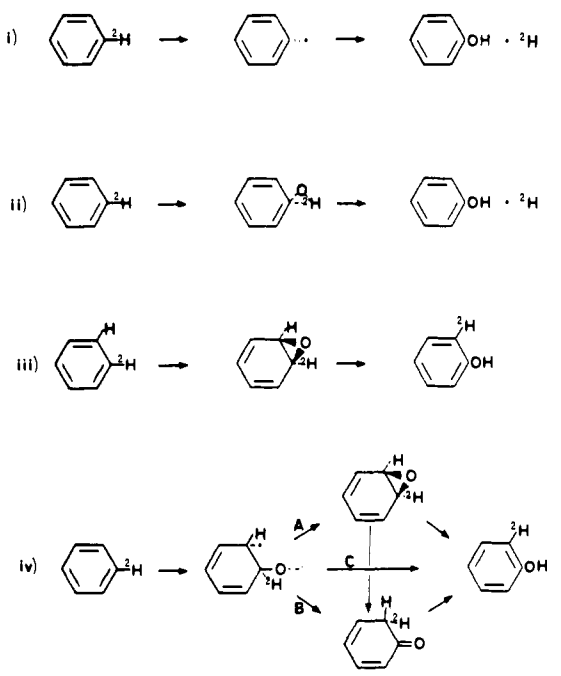
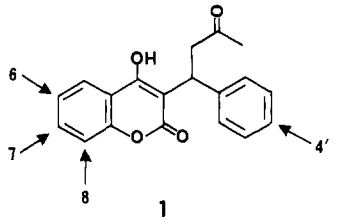


Figure 1. Possible reaction mechanisms for aromatic hydroxylation catalyzed by cytochrome P-450: (i) abstraction, (ii) insertion, (iii) addition, and (iv) addition rearrangement.

that the P-450-dependent hydroxylation of benzene is mediated primarily through hydroxy radicals. Burka et al.[13] have suggested that the ortho and para hydroxylation of the halobenzenes is initiated by removal of one electron from the aromatic ring to generate a radical cation followed by collapse to a tetrahedral intermediate similar to that shown in path iv, Figure 1. Finally, Hanzlik et al.[14] have demonstrated that direct aromatic hydroxylation, defined by total loss of a label at the position of hydroxylation, contributes significantly to the cytochrome P-450 catalyzed hydroxylation of simple substituted aromatic systems.

These observations led to the following questions: Can a single isozyme operate by more than one mechanism (direct insertion vs. arene oxide formation) depending on substrate? Are different isozymes responsible for different mechanisms? Is arene oxide formation an obligatory first step in aromatic hydroxylation? Is arene oxide formation consistent with all of the pertinent experimental data present in the literature?

As an approach to answering at least some of these questions, we chose to investigate the mechanism of aromatic hydroxylation of selectively deuterated analogues of a substrate in which meta hydroxylation was an important process. Warfarin (1) insofar as we are aware is



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unique in that the major metabolite in both man[15] and rat[16] is 7-hydroxywarfarin, a phenolic product that corresponds to meta hydroxylation.[17] It is also hydroxylated in the adjacent 6- and 8-positions, which correspond to para and ortho hydroxylation, respectively. In addition, the relative ratios of the metabolites that are obtained are known to be a function of both the stereochemistry of the substrate and the source of P-450 isozymes.

To probe the system, both enantiomers of warfarin were selectively deuterated in the 6-, 7-, and 8-positions. Each labeled, optically pure substrate was then incubated with microsomes obtained from the livers of either normal, phenobarbital (PB) pretreated, or β-naphthoflavone (BNF) pretreated rats. We believed these substrates would serve as good models to probe the system for the following reasons. (1) If oxidation occurred by either direct insertion (path ii, Figure 1) or abstraction (path i, Figure 1), the deuterium label would be totally lost on conversion to the product. Conversely, if the label was retained in the product (by an NIH shift mechanism[18]), the most likely path would be direct epoxidation (path iii, Figure 1) or addition rearrangement (path iv, Figure 1). 2) If it is assumed that the initial transfer of oxygen to substrate in any of these four paths would be at least partially rate limiting and if an isotope effect for hydroxylation at the position of deuteration was observed, then the magnitude of the effect might indicate which path was operative. For example, the intrinsic isotope effect associated with path i is expected to be large because a C–D bond is broken in the transition state. The isotope effect associated with path ii would be moderate to small because of a probable three-centered nonlinear transition state. The isotope effect associated with path iii is expected to be zero because the breaking of a C–D bond is not involved in the rate-limiting step. Path iv might or might not yield an isotope effect depending on which of the three possible courses of rearrangement were followed. (3) If the pattern of the NIH shift or isotope effect changed as a function of induction state, then good evidence would be provided to indicate that different isozymes of cytochrome P-450 hydroxylate warfarin by different mechanisms. (4) The utilization of the optically pure enantiomers of warfarin allows the determination of the effect of stereochemistry on both the mechanism and pattern of hydroxylation.

Experimental Section

Materials. Racemic warfarin was obtained from Sigma Biochemicals and was resolved by the method of Preis.[19] Unlabeled phenolic warfarin metabolites (4′-, 6-, 7-, and 8-hydroxywarfarins) were synthesized according to the methods of Hermodson et al.[20] The 2′,3′,4′,5′,6′-pentadeuterio-6- and -7-hydroxywarfarins were a gift from Dr. L. K. Low, 2′,3′,4′,5′,6′-pentadeuterio-8-hydroxywarfarin was a gift from L. Heimark, and 5,6,7,8-tetadeuterio-4′-hydroxywarfarin was a gift from D. Swinney (all members of the Department of Medicinal Chemistry, University of Washington, Seattle, WA). The 6-, 7-, and 8-monodeuteriowarfarins

(13) Burka, L. T.; Plucinski, T. M.; MacDonald, T. L. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 6680.
(14) Hanzlik, R. P.; Hogberg, K.; Judson, C. M. *Biochemistry* **1984**, *23*, 3048.
(15) Lewis, R. J.; Trager, W. F.; Chan, K. K.; Breckenridge, A.; L'Orme, M.; Rowland, M.; Schary, W. *J. Clin. Invest.* **1974**, *53*, 1607.
(16) Pohl, L. R.; Porter, W. R.; Trager, W. F.; Fasco, M. J.; Fenton, J. W., II *Biochem. Pharmacol.* **1977**, *26*, 109.
(17) Of the three adjacent aromatic sites, 6, 7, and 8, within the coumarin nucleus of warfarin, the 7-position can be considered as most meta-like. Calculations by Wald and Feuer [Wald, R. W.) Feuer, G. *J. Med. Chem.* **1971**, *14*, 1081] have demonstrated that the 6-position of 4-hydroxycoumarin bears a net charge of −0.113, the 8-position a net charge of −0.174, and the 7-position a net charge of only −0.061.
(18) Guroff, G.; Daly, J. W.; Jerina, D. M.; Renson, J.; Witkop, B.; Udenfriend, S. *Science* **1967**, *158*, 1524.
(19) Pries, S.; West, B. D.; Schroeder, C. H.; Link, K. P. *J. Am. Chem. Soc.* **1961**, *83*, 2676.
(20) Hermodson, M. A.; Barker, W. M.; Link, K. P. *J. Med. Chem.* **1971**, *14*, 167.

Table I. Percentage Deuterium Retention in the 4'-, 6-, 7-, and 8-Hydroxywarfarin Metabolites after (1) Normal Workup and (2) Reincubation Followed by Normal Workup

		4'-OH		6-OH		7-OH		8-OH	
substrate	microsomes	1	2	1	2	1	2	1	2
rac-6D	PB	84	85	66	65	76	76	82	83
rac-7D	BNF	86	83	84	84	52	50	76	76
rac-8D	PB	92	91	90	89	84	83	58	58

were synthesized and resolved by the authors and will be reported elsewhere. Proper placement of the deuterium label was confirmed by NMR. Diazomethane was generated from Diazald (Aldrich Chemical Co.) according to the directions supplied by the manufacturer. *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Pierce Chemical Co. Concentration tubes and caps were purchased from Lab Research Co., Los Angeles, CA. An Orion Research Model 701A digital pH meter was used for all pH measurements. $NADP^+$ (monosodium salt), D-glucose 6-phosphate, D-glucose-6-phosphate dehydrogenase, and bovine serum albumin were purchased from Sigma Biochemicals. β-Napthoflavone (BNF) was obtained from Aldrich Chemical Co., and sodium phenobarbital (PB) was obtained from Eli Lilly Co.

The animals used in this study were male sprague–Dawley rats (140–160 g) from Tyler Labs (Bellevue, WA) and were fed Purina Rat Chow and water ad libitum.

Tissue homogenization was performed in a Potter–Elevehjem glass tube with a Teflon pestle having a 0.10–0.15-mm clearance. Centrifugation at 9000*g* was performed in a 4 °C cold room, using an Angle Centrifuge (Model NSE, Ivan Sorval, Inc.). Ultracentrifugations were performed in polycarbonate tubes (in a 42.1 rotor head) in a Beckman 12-65B refrigerated ultracentrifuge. Protein concentrations were determined on a Gilford Stasar II spectrophotometer. Cytochrome P-450 determinations were performed with an Aminco (American Instrument Co.) DW-2 spectrophotometer. Incubations were performed in 20-mL scintillation vials in an American Optical Model 2156 shaker with the water held at a constant 37 ± 0.5 °C. Low-speed centrifugations were conducted on an IEC HN-SII benchtop centrifuge from Davon/IEC Division. The evaporator used was a Multivap (Model 113) from Organomation.

Capillary GC was performed on either wide or narrow bore DB-5 bonded phase (normal film thickness) fused silica columns (30 m) from J & W Scientific Co. GC/MS measurements were conducted on a VG Analytical 7070H mass spectrometer fitted with a VG 2000 data system and a Hewlett Packard 5700 GC with a capillary injector. GC/MS conditions were as previously reported.[21]

Standard/Stock Solutions. Stock solutions of the substrates were made up as follows. The purified labeled or unlabeled warfarin (5.00 mg, 16.2 mM) was dissolved in 40% 0.1 N KOH (2 mL, from a solution prepared by adding 80 mL, 0.1 N KOH to 120 mL of 0.05 phosphate buffer, pH 7.4). After the warfarin had dissolved, 0.05 M phosphate buffer (pH 7.4, 6 mL) was added, and then 40% 0.1 N HCl (2 mL, from a solution prepared by adding 80 mL, 0.1 N HCl to 120 mL of 0.05 phosphate buffer, pH 7.4) was added to neutralize the base. This solution was kept frozen until use. In similar fashion standard solutions of the nonlabeled 4'-, 6-, 7-, and 8-hydroxywarfarins were prepared. A standard curve based on a fixed concentration of labeled metabolites vs. varying concentrations of nonlabeled metabolites was constructed as previously reported.[8,21]

Enzyme Cofactors. On the day of the incubations, this solution was prepared as follows. $MgCl_2 \cdot 6H_2O$ (101.65 mg, 0.5 mM), D-glucose 6-phosphate (304.1 mg, 1.17 mM), and $NADP^+$ monosodium salt (95.7 mg, 0.125 mM) were dissolved in 0.05 M phosphate buffer (pH 7.4, 50 mL) and the solution ket on ice until needed. Also on the day of the incubations, D-glucose-6-phosphate dehydrogenase (0.84 mg, 250 units) was dissolved in the 0.05 M phosphate buffer (pH 7.4, 50 mL) and the solution kept on ice until needed.

Microsome Preparation. Microsomes were prepared as previously described.[8] To determine the protein concentration of the microsomal suspensions, a 10-μL aliquot was added to 1 mL of water and assayed by the modified method of Lowry.[21] A series of four bovine serum albumin solutions (0.05, 0.1, 0.2, 0.3 mg/mL of water) were used as standards. Adsorption readings at a wavelength of 540 nm yielded concentration determinations following Beer's law. The protein concentration was adjusted to 10 mg/mL, using 0.01 M phosphate buffer.

Cytochrome P-450 concentrations were measured according to the method of Omura and Sato,[23] using an extinction coefficient of 91 000 M^{-1} cm^{-1} in the reduced, CO difference spectrum.

Incubations. All incubations for each group of pure enantiomers (*R* or *S*, OD (nonlabeled), 6D, 7D, and 8D) were performed simultaneously. The solutions were added in the following order: (a) 400 μL of substrate, (b) 200 mL of cofactors, (c) 200 μL of reductase, (d) 200 μL of microsomal suspension. The resulting 1-mL solutions were incubated for 10 min at 37 °C. All incubations were run in duplicate, and each experiment was repeated at least twice. The incubation products were analyzed by the previously published method.[21] Control experiments were performed by using the following modifications: (a) the incubation size was scaled up by a factor of 5; (b) on completion, the incubation was divided and both halves were extracted in the normal fashion; (c) the extract from one-half was analyzed by GC/MS according to the standard procedure, while the extract from the second half was resuspended in buffer and reincubated with microsomes in the absence of reducing equivalents; (d) the reincubate was extracted and analyzed in the normal fashion, and the results were compared to those obtained from the first half of the incubation mixture.

Data Analysis. As in the published procedure,[21] concentrations of unlabeled hydroxywarfarins were calculated by using eq 2, where A indicates unlabeled hydroxywarfarin, B indicates

$$\frac{\text{concn of A}}{\text{concn of B}} = m \times \frac{\text{area of A}}{\text{area of B}} + b \qquad (2)$$

5-*d*-labeled hydroxywarfarin, *m* and *b* = the slope and intercept coefficients, respectively, as obtained from the standard curve. The concentrations of 1-*d*-labeled hydroxywarfarin metabolites were estimated by using eq 3, where ^dA indicates 1-*d*-labeled

$$\frac{\text{concn of } {}^dA}{\text{concn of B}} = m \times \frac{\text{area of } {}^dA - (k \times \text{area of A})}{\text{area of B}} + b \qquad (3)$$

hydroxywarfarin, *k* = the fraction of unlabeled A, which, because of ^{13}C natural abundance, overlaps into the labeled channel. The percent deuterium incorporation was calculated by using eq 4.

$$\%\ \text{incorporation} = \frac{\text{concn of } {}^dA}{\text{concn of A} + \text{concn of } {}^dA} \times 100 \qquad (4)$$

The percent deuterium retention in the metabolite was calculated by using eq 5.

$$\%\ \text{retention} = \frac{\%\ \text{incorporation of product}}{\%\ \text{incorporation of substrate}} \times 100 \qquad (5)$$

Results and Discussion

The results from this study are listed in Table I and Figures 2 and 3. Table I lists the results obtained from control experiments in which potential deuterium loss on workup was determined. A large incubation mixture was divided into two equal portions following termination of the reactions. Both portions were extracted normally.

(21) Bush, E. D.; Low, L. K.; Trager, W. F. *Biomed. Mass Spectrom.* **1983**, *10*, 395.

(22) Miller, G. L. *Anal. Chem.* **1959**, *31*, 964.

(23) Omura, J.; Sato, R. *J. Biol. Chem.* **1964**, *239*, 2370.

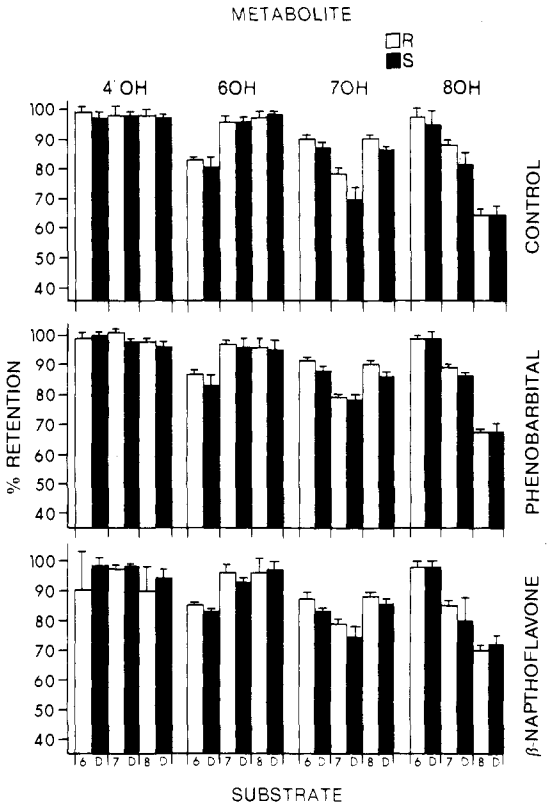

Figure 2. The percentage deuterium retention in the 4′, 6-, 7-, and 8-hydroxywarfarin metabolites from the (*R*)- and (*S*)-6-, 7-, and 8-deuteriowarfarin substrates after incubation with liver microsomes from either noninduced or induced (PB or BNF) male Sprague–Dawley rats. Products from the *R*-deuterated warfarins are indicated by the blank bars. Products from the *S*-deuterated warfarins are indicated by the filled bars. The error signs indicate the standard deviation for $n = 6$.

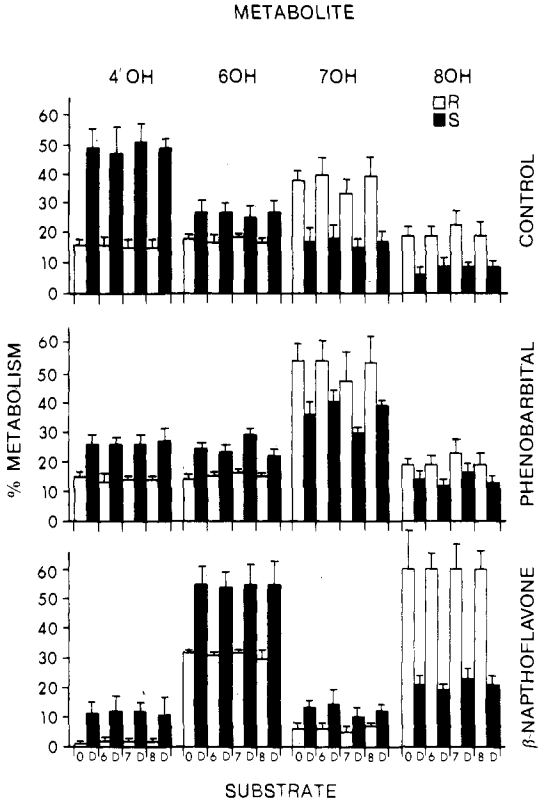

Figure 3. The percentage of each of the four metabolites (4′-, 6-, 7-, and 8-hydroxy) relative to total metabolism (the sum of 4′-, 6-, 7-, and 8-hydroxy) as a function of substrate (6′-, 7-, and 8-deuteriowarfarin) stereochemistry (*R* and *S* selectively deuterated warfarin) and induction state (noninduced and PB and BNF induced). The blank bars indicate percentages of metabolites from the *R*-deuterated warfarins and the filled bars indicate percentages of metabolites from the *S*-deuterated warfarins. The error signs indicate the standard deviation for $n = 6$.

One-half was then analyzed, while the other half was redissolved in buffer and further incubated with microsomes in the absence of reducing equivalents. It was then extracted without the addition of internal standard. With use of this technique, if exchange due to nonenzymatic sources occurred, the percentage deuterium found in samples that were reincubated should be less than that found in samples treated normally. Clearly, the results show that very little, if any, nonenzymatic exchange takes place on workup.

Figures 2 and 3 show the cumulative results from three sets of experiments. The percentage of deuterium retention for each phenolic metabolite obtained from either the *R* or *S* enantiomer from all treatments is plotted in Figure 2. The relative yield (percentage of total metabolism) for each phenolic metabolite is plotted in Figure 3.

Several observations based on the data in Figures 2 and 3 deserve comment.

1. The percent deuterium retention is always high irrespective of the site of hydroxylation even when hydroxylation occurs at the site of deuteration. For example, in the 6-hydroxy product obtained from 6-deuteriowarfarin 83% of the deuterium is retained, in the 7-hydroxy product obtained from 7-deuteriowarfarin 72% of the deuterium is retained, and in the 8-hydroxy product obtained from 8-deuteriowarfarin 65% of the deuterium is retained. These results are nearly independent of the stereochemistry of the substrate and the source of the microsomes (normal, PB-pretreated, or BNF-pretreated animals).

2. The overall pattern of deuterium retention is approximately the same for all induction states and all optical isomers. Thus, if there is a difference in mechanism between the three induction states, it is not expressed in the extent of the NIH shift.

3. χ^2 analysis of the data (Figure 3) for each optical isomer series does not indicate statistically significant differences between relative metabolite levels within an induction state. That is, the metabolic pattern for (*R*)-7-deuteriowarfarin for example is not statistically different from (*R*)-6-deuteriowarfarin using microsomes from Pb-pretreated animals. This is also visually apparent from the figure. However, there is a consistent trend worth noting. During metabolism of the 7-deuterio analogue by noninduced and PB-induced microsomes, the relative yields of the 7-hydroxy metabolite were consistently lower, while those of the 6- and 8-hydroxy metabolites were consistently higher, as compared to results with BNF microsomes. However, the differences were not statistically significant. Despite the lack of statistical significance, the data at least suggest that an isotope effect is associated with 7-hydroxylation as evidenced by an apparent metabolic switching. This effect is occasionally seen with the BNF-induced microsomes, but no consistent pattern could be established, presumably due to the low levels of the 7-hydroxy metabolite produced by this system.

4. The absolute amounts of total metabolism obtained from d_0 (nonlabeled) and 6-, 7-, and 8-deuteriowarfarin are approximately equal (data not shown).

The results of these studies suggest the following conclusions.

1. Since high retention of the deuterium label is observed in the products from all substrates and all induction states, little or no oxidation can occur by either the direct

insertion or abstraction pathways. This follows since pathways 1 and 2 require the total loss of deuterium in the product.

2. The percentage of deuterium retention in the phenolic metabolites is not a function of absolute configuration and appears to depend on the state of induction only to a minor degree. Thus, large differences in the composition of isozymes as noted previously[8,24-26] have little effect on percent deuterium retention. This result strongly suggests that hydroxylation of substrate irrespective of the position of hydroxylation or the specific microsomal preparation is occurring by a similar mechanism. Moreover, it provides additional evidence to support the notion that the nature of the heme–Fe^{3+}–oxene complex dictates the mechanism as previously suggested.[8,14,25]

In contrast to deuterium retention, the metabolic patterns (relative yields of metabolites) are very strongly dependent upon both induction state and stereochemistry. This result strongly suggests that the protein-binding site of the enzyme dictates both regioselectivity and stereoselectivity but has little influence on the actual mechanism of the oxidative process.

In addition to these observations, consideration of all the data suggests a tentative conclusion as to which of the two remaining pathways (arene oxide formation or addition rearrangement) best fits the data.

(a) The large amount of the (*R*)-7-hydroxy metabolite relative to the 6- and 8-hydroxy metabolites formed by either noninduced or Pb-induced microsomes is inconsistent with the directional opening expected from either a 6,7- or 7,8-epoxide intermediate.

(b) There is considerable evidence summarized in recent reviews that suggests that in other chemical systems cytochrome P-450 oxidations proceed by a stepwise process involving a radical or triplet-like oxygen. A brief summary of some of this evidence is that racemization occurs at the site of hydroxylation in alkanes,[27] that epoxidation of double bonds is a stepwise process as shown by inverse isotope effects,[28] that theoretical studies favor a triplet oxygen as the active species in cytochrome P-450 oxidations,[29,30] and, most important to the present paper, that kinetic evidence suggests that formation of the ortho and para phenols from the halobenzenes does not involve initial epoxide formation.[13]

(c) The addition rearrangement pathway offers a logical explanation for the consistency of the deuterium retention data over all induction states and stereoisomers as described in conclusion 2.

(d) The addition rearrangement pathway is also consistent with the formation of epoxide metabolites, a process known to occur in cytochrome P-450 metabolism.

To summarize, the catalytic nature of the heme–Fe^{3+}–oxene complex appears to dictate the exact mechanism of the retentions catalyzed by cytochrome P-450, while the associated apoprotein dictates stereoselectivity and regioselectivity.

Of the four mechanisms considered herein, it appears that the addition rearrangement pathway offers the simplest explanation for the mechanism of aromatic hydroxylation of warfarin. Specifically, the mechanism for all oxidations would follow a similar first step; that is, the initial attack of oxygen on the ring to form a tetrahedral intermediate, which then may collapse to either form an epoxide, eliminate a proton (hydrogen atom) directly to give the phenol, or shift the hydride (hydrogen atom) to form a keto intermediate (see Figure 1).

Alternately an initial abstraction of an electron from the aromatic system by activated cytochrome P-450 to generate a radical cation followed by collapse to the tetrahedral intermediate as suggested by Burka et al.[13] is equally consistent with all the data and, therefore, the two mechanisms cannot be distinguished by the evidence presented herein. Howeve, if either pathway is the general mechanism by which aromatic hydroxylation proceeds, then an understanding of the factors that govern the relative rates of rearrangement to either the epoxide (k_1), phenol (k_2), or keto intermediate (k_3) is of crucial importance to understanding potential toxicity as a function of the structure of the substrate.

Acknowledgment. This work was supported by the National Institutes of Health (Grant GM 25136).

Registry No. Cytochrome P-450, 9035-51-2; (*R*)-6-monodeuteriowarfarin, 96455-18-4; (*R*)-7-monodeuteriowarfarin, 96455-19-5; (*R*)-8-monodeuteriowarfarin, 96455-20-8; (*S*)-6-monodeuteriowarfarin, 96455-21-9; (*S*)-7-monodeuteriowarfarin, 96455-22-0; (*S*)-8-monodeuteriowarfarin, 96455-23-1.

(24) Daly, J.; Jerina, D.; Farnsworth, J.; Guroff, G. *Arch. Biochem. Biophys.* **1969**, *131*, 238.
(25) Daly, J.; Jerina, D. *Arch. Biochem. Biophys.* **1969**, *134*, 266.
(26) Claeson, M.; Moustafa, M. A. A.; Aldine, J.; Vanderworst, D.; Poupaert, J. H. *Drug Metab. Dispos.* **1982**, *10*, 667.
(27) Groves, J. T.; McClusky, G. A.; White, R. E.; Coon, M. J. *Biochem. Biophys. Res. Commun.* **1978**, *81*, 154.
(28) Hanzlik, R. P.; Shearer, G. O. *Biochem. Pharmacol.* **1978**, *27*, 1441.
(29) Pudzianowski, A. T.; Loew, G. H. *J. Am. Chem. Soc.* **1980**, *102*, 5443.
(30) Loew, G. H.; Hjelmeland, L. M.; Kirchner, R. F. *Int. J. Quantum Chem.* **1977**, *4*, 225.