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EVIDENCE AGAINST AN ABSTRACTION OR DIRECT INSERTION MECHANISM FOR CYTOCHROME P-450 CATALYSED META HYDROXYLATIONS

Ernie D. Bush and William F. Trager

Department of Medicinal Chemistry
School of Pharmacy, University of Washington
Seattle, Washington 98195

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SUMMARY: Warfarin, specifically labeled with deuterium in the 7 position, was incubated with liver microsomes from untreated rats or rats which were pretreated with either phenobarbital or β-napthoflavone. The four phenolic metabolites (6-, 7-, 8- and 4'-hydroxywarfarin) were isolated and quantitated by GC/MS and the percent deuterium retention calculated. In all induction states the 7-hydroxy metabolite of (7,^{2}H)warfarin retained greater than 77% of the deuterium. These results suggest that hydroxylation at the 7 position (meta hydroxylation) cannot proceed by either a direct insertion or abstraction mechanism.

Introduction: The family of enzymes collectively known as cytochrome P-450 is widely acknowledged to be of critical importance in drug metabolism (1). However, in spite of intensive investigation by a number of laboratories, the exact mechanism by which these enzymes catalyze aromatic hydroxylation is still not completely understood (2). Although direct epoxidation by a singlet oxygen atom is generally believed to be the mechanism for this oxidation Tomaszewski et al. (3) recognized that a total of four distinct reaction pathways could lead to product. These pathways are: 1) an initial abstraction of hydrogen either as a hydride ion or hydrogen atom followed by recombination with either hydroxide ion or hydroxyl radical to yield phenol; 2) the direct insertion of an oxygen atom across a carbon hydrogen bond to form a phenol in a single step; 3) the direct epoxidation of an aromatic ring by an oxygen atom to yield an arene oxide which subsequently rearranges to a phenol; and 4) the addition of an activated oxygen species to an aromatic ring to form a tetrahedral intermediate which subsequently rearranges to a phenol (either directly or through an epoxide intermediate). Since mechanisms 2 and 3 are concerted they are consistent with attack by a singlet oxygen

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atom, whereas mechanisms 1 and 4 are stepwise and are therefore consistent with, but not restricted, to attack by triplet oxygen.

There is a growing body of evidence which suggests that not all aromatic oxidations (or oxidations of π systems) proceed through a direct epoxidation mechanism (3-6). Indeed several investigators have postulated that meta hydroxylations of substituted benzenes proceed through an abstraction or direct insertion mechanism (3,4). This speculation is based largely on two sets of observations, 1) the existence of a measurable kinetic isotope effect for meta, but not for the ortho or para hydroxylation of several substrates (3,6), and 2) the formation of a significant amount of the meta hydroxy product from these substrates, in spite of the fact that this product is that least expected based on the normal chemical ring opening of an arene oxide intermediate (3,6,7).

The anticoagulant warfarin has been used by a number of investigators to probe the microsomal cytochrome P-450 system (7-9). Four phenolic products (6-, 7-, 8- and 4'-hydroxywarfarin) are obtained from incubations with purified P-450 enzymes and rat or rabbit liver microsomes, Fig. 1. The 6,7 and 8 positions in the aromatic ring correspond to the para, meta and ortho positions of substituted benzenes respectively (10).

Previous metabolic studies with warfarin have shown that, contrary to expectation, the 7-hydroxy metabolite which corresponds to meta hydroxylation is the major metabolite in man and the uninduced rat (11,12). Its formation appears to be distinct from pathways which lead to the 6- and 8- (ortho and para) hydroxywarfarins (7,13). This has led to speculation that the mechanism for 7-hydroxylation might not proceed through mechanism 3, direct epoxidation, as expected for 6- or 8-hydroxylation (14). To investigate the possibility of the involvement of a direct insertion or abstraction mechanism in the hydroxylation of warfarin, racemic (7,^{2}H)warfarin was synthesized (Fig. 2) and microsomal incubations conducted to determine if the deuterium was retained or lost after 7-hydroxylation. High deuterium retention in the 7-hydroxy metabolite would be strong evidence against a direct insertion or

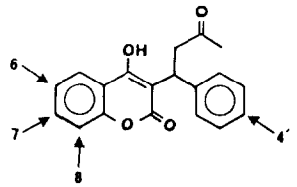

Fig. 1: Structure of warfarin

abstraction mechanism whereas loss of the deuterium would be strong evidence for such mechanisms.

Materials and Methods: Reagents and chemicals were of the highest commercially available grade. Racemic warfarin, NADP+ (monosodium salt), D-glucose-6-phosphate,D-glucose-6-phosphate dehydrogenase and bovine serum albumin were purchased from Sigma Biochemicals. β-Napthoflavone and Diazald were obtained from Aldrich Chemical Co., and sodium phenobarbital was obtained from Eli Lilly Company. BSTFA was obtained from Pierce Chemical Company. The deuterated hydroxywarfarin internal standards used for the mass spectral analysis were synthesized in our laboratories and will be reported elsewhere.†

Synthesis. (7,^{2}H)warfarin was synthesized as illustrated in Fig. 2, the details will be published elsewhere. Starting materials were commercially available and were used without further purification. The final product was extensively purified by preparative HPLC using a Whatman M-9 (2x50 cm, 10 um particle size) silica column on a Dupont 850 HPLC. The solvent system was 10% ethyl acetate and 90% dichloromethane (with 0.5% acetic acid), flow rate 2.5 mls/min. Deuterium content was shown to be 91% by mass spectrometry and the deuterium location was confirmed by NMR.

Microsomal Incubations. Liver microsomal preparations from either non-induced, phenobarbital induced or β-napthoflavone induced male Sprague-Dawley rats (140 to 160 grams from Tyler Labs, Bellevue, WA), were prepared according to the previously reported protocol (13). Protein concentrations were determined by a modified Lowry method (15), cytochrome P-450 concentrations were measured according to the method of Omura and Sato (16). Replicate one ml incubations were carried out in scintilation vials using the following protocol (all solutions are made up in 10 mM phosphate buffer, pH=7.4):

a) 400 ul substrate (1.62 mM warfarin)
b) 200 ul cofactors (2.80 mM NADP+, 23.4 mM G-6-P, 10.0 mM $MgCl_2$)
c) 200 ul dehydrogenase (5.00 U/ml G-6-P dehydrogenase)
d) preincubate for 5 min at 37°C
e) 200 ul microsomal suspension (10 mg/ml microsomal protein in isotonic KCl phosphate buffer)

The resulting mixture was exposed to the atmosphere and incubated for an additional 15 min. The incubations were terminated by addition of 600 ul acetone followed by the addition of the internal standard solution (20 ul containing 300 ng of each of the deuterated hydroxywarfarin standards).

†The deuterated metabolites were (2',3',4',5',6',$^2H_5$)6-hydroxy warfarin, (2',3',4',5',6',$^2H_5$)7-hydroxywarfarin, (2',3',4',5',6',$^2H_5$)8-hydroxywarfarin and (5,6,7,8,$^2H_4$)4'-hydroxywarfarin.

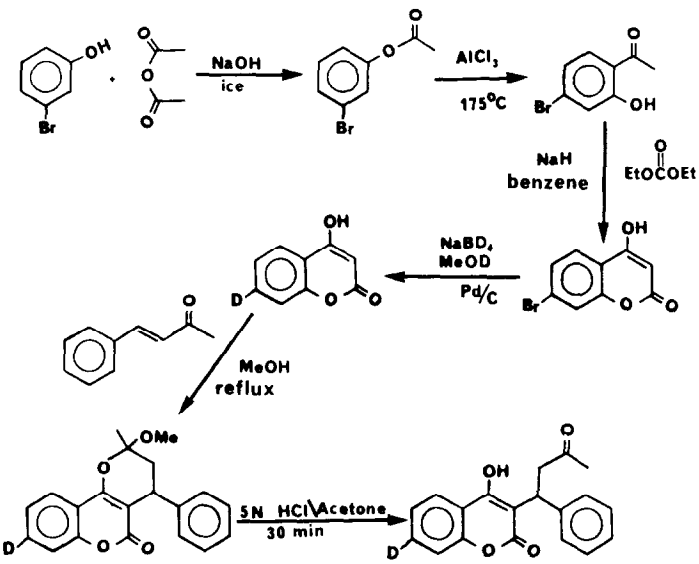

Fig. 2: Synthetic route followed for the preparation of (7,^{2}H)warfarin.

GC/MS Assay. After termination, the incubations were acidfied to pH 5.8-6.0 with NaH_2PO_4 (0.5 M, 200 ul). The slightly acidic solutions were extracted with cyclohexane (3 x 2 ml) to remove the unreacted starting material, followed by extractions with Et_2O/EtOAc, 1:1 (2 x 2 ml) to extract the metabolites. The solvent was removed from these extracts under a stream of N_2 and the dried samples were derivatized with etherial diazomethane. After 24 hrs the excess diazomethane and ether were removed and the samples taken up in 50 ul of BSTFA for injection on the GC. GC/MS was performed on a VG Analytical 7070H mass spectrometer fitted with a VG 2000 data system and a Hewlett Packard 5700 GC with a capillary injector. The assay utilized a wide bore DB-5 bonded phase, fused silica capillary column from J & W Scientific Company. Helium was used as carrier gas at a head pressure of 14 psi, with splitless injection. Oven temperature program: 1 to 2 minutes at 160°C, then to 250°C at 30°C/min, and held at 250°C for 12 min. The injection port and transfer line were held at 250°C. The mass spectrometer source was held at 200°C, all assays were run in EI mode at 70 eV. Absolute amounts of the deuterated and non-deuterated metabolites were calculated using previously generated standard curves.

Results and Discussion: Inspection of Fig. 3 reveals that the percent of deuterium retention in the formation of 7-hydroxywarfarin from (7,^{2}H) warfarin is at least 77% and is independent of the state of induction. These observations lead to two distinct conclusions. First, the high degree of retention is clearly inconsistent with either a direct insertion or abstraction mechanism for 7-hydroxylation. Second, the lack of dependence of the degree of retention on the state of induction implies that either a single cytochrome P-450 isozyme, present to varying degrees, is solely responsible

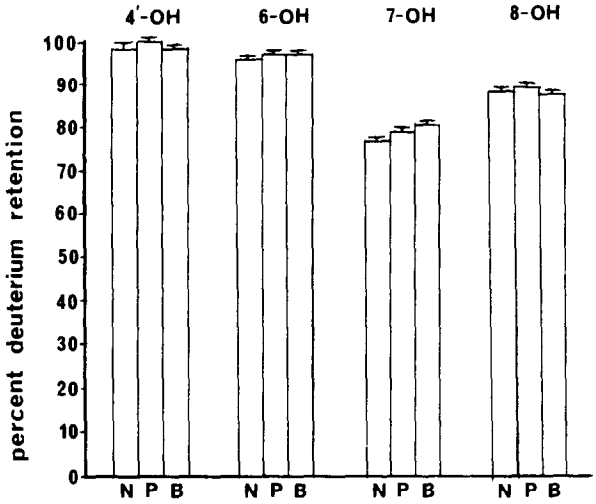

Fig. 3: Percent of deuterium retention data for the four phenolic metabolites (4'-OH, 6-OH, 7-OH and 8-OH) produced by microsomes from either noninduced (N), phenobarbital (P) induced or β-napthoflavone (B) induced rats. Error bars are standard deviation (N=4).

for the formation of 7-hydroxywarfarin or if more than one enzyme is involved the mechanism of hydroxylation is independent of the specific isozymes responsible. Inspection of Fig. 4 reveals that as a percent of total metabolism the relative amounts of 7-hydroxywarfarin vary dramatically from one

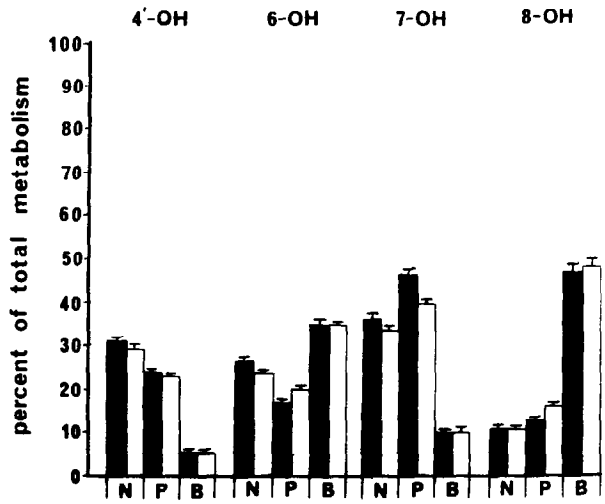

Fig. 4: Percent of total metabolism data derived from the same incubations (labeled substrates, unshaded bars) as given in Fig. 3. Individual values are calculated by dividing the amount of each metabolite by the sum of the amounts of all the metabolites in that incubation. The shaded bars represent incubations with unlabeled warfarin. N, P and B have the same meaning as in Fig. 3.

induction state to another. Again with regard to enzyme multiplicity these results can be interpreted in one of two possible ways; either the relative level of the enzyme responsible for the oxidation varies as a function of induction state or more than one enzyme is involved. If the later possibility is true, it, together with the constancy of the retention data, would suggest that the protein binding site of the enzyme is largely responsible for regioselectivity. The data in Fig. 4 also suggests that for 7-hydroxylation of (7,^{2}H)warfarin (with microsomes from phenobarbital induced rats) there is an isotope effect of approximately 1.2. This result is consistent with similar work in our laboratory on resolved deuteriowarfarin substrates (unpublished data) and with the meta-hydroxylation of deuterated nitrobenzene and methylphenylsulfone reported by Tomazewski et al. (3). This result is also inconsistent with the intermediacy of a warfarin epoxide, e.g. 6,7 and/or 7,8, in the formation of 7-hydroxywarfarin from these microsomes. Since direct insertion and abstraction can be eliminated by the deuterium retention data and since direct epoxidation, although not excluded, appears unlikely (due to electronic considerations in the ring opening of epoxides and the apparent isotope effect for 7-hydroxylation) the simplest mechanism for 7-hydroxylation that is compatable with all the data is mechanism 4, stepwise addition rearrangement.

In summary the picture that begins to emerge is that the meta hydroxylation of warfarin proceeds by an addition rearrangement pathway. Moreover, if more than one enzyme is involved in its formation then the retention data coupled to the induction state data suggests two significant conclusions. These are, the nature of the active oxygen-Fe-heme complex dictates the mechanism of the reaction independent of the specific isozyme and, the protein binding site of any specific isozyme dictates regioselectivity. Work is in progress to confirm or refute these hypothesis as well as to probe the generality of meta hydroxylation proceeding by an addition rearrangement mechanism.

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