Regioselective and Stereoselective Hydroxylation of R and S Warfarin by Different Forms of Purified Cytochrome P-450 from Rabbit Liver*

Michael J. Fasco, Kostas P. Vatsis, Laurence S. Kaminsky, and Minor J. Coon

The metabolism of the R and S enantiomers of warfarin by rabbit liver microsomes and by purified and partially purified forms of rabbit liver cytochrome P-450 (P-450<sub>LM</sub>) to yield multiple monohydroxylated products has been investigated to probe the substrate specificities and regio- and stereoselectivities of the cytochromes. The metabolism of warfarin by rabbit liver microsomes differed from that of rat microsomes with respect to the overall rates of warfarin metabolism, the relative rates of formation of the different metabolites, and the effects of inducing agents on the metabolism. These results imply that the rabbit hepatic cytochromes differ markedly from those of rat liver.

Based on their catalysis of warfarin metabolism, the purified and partially purified, reconstituted P-450<sub>LM</sub> enzymes were divided into three groups; P-450<sub>LM</sub>, and P-450<sub>LM</sub>, were inactive with warfarin; P-450<sub>LM</sub>, and P-450<sub>LM</sub>, had the same activities; and P-450<sub>LM</sub>, had a markedly different activity. The regio- and stereoselectivities of P-450<sub>LM</sub>, and P-450<sub>LM</sub>,-catalyzed metabolism of warfarin differed, and the results indicate that P-450<sub>LM</sub>, acts on warfarin only when it is oriented to the enzyme catalytic site at the coumarin side of the molecule, and preferentially when warfarin is in the R configuration. P-450<sub>LM</sub>, also acts on the coumarin side of warfarin, but preferentially when it is in the S configuration, and also on the phenyl ring, but preferentially when warfarin is in the R configuration. Comparison of warfarin metabolite patterns from microsomes and P-450<sub>LM</sub> enzymes indicates that regio- and stereoselectivities of the P-450<sub>LM</sub> enzymes were essentially maintained when the cytochromes were removed from the microsomal membrane and purified.

The broad substrate specificity of cytochrome P-450 has been partially attributed to the multiplicity of the cytochrome (e.g. Refs. 3 to 7). The concept of multiple forms of cytochrome P-450 has been supported by the results of numerous studies on the effects of inducing agents, together with immunological (6, 8, 9) and gel electrophoretic (3) evidence. However, the isolation and purification of a number of different forms of cytochrome P-450, most notably by groups in this country (10-14) and Japan (15, 16), have provided the most compelling evidence for the multiplicity of cytochrome P-450.

Highly purified and partially purified forms of cytochrome P-450 have been isolated, in one of the present laboratories, from uninduced and phenobarbital- or β-naphthoflavone-induced rabbit liver microsomes, and assigned a nomenclature based on their electrophoretic mobilities (10-12). The specificities of these purified enzymes have been investigated with a number of different substrates (11, 17), and the regio- and stereoselectivities of the purified forms of cytochrome P-450 are still not clearly understood, however, and further investigations are essential for elucidation of the multifaceted modes of action of the enzymes.

Rat P-450<sub>LM</sub> catalyzes the metabolism of warfarin (Scheme IA) to yield the following as shown in the scheme: B, 6-hydroxywarfarin; C, 7-hydroxywarfarin; D, 8-hydroxywarfarin; E, 4'-hydroxywarfarin; F, benzylic hydroxywarfarin (19, 20, 22); G, dehydrowarfarin. Warfarin contains an asymmetric carbon atom (21) and the enantiomeric forms, R and S warfarin, undergo stereoselective hydroxylation catalyzed by microsomal cytochrome P-450 (22). The relative formation rates of the warfarin metabolites are markedly altered by prior induction of the microsomes (23), indicating that a number of different forms of cytochrome P-450 are involved in warfarin metabolism.

Our recently developed HPLC assay for the simultaneous analysis of all the microsomal metabolites of the warfarin enantiomers (24) greatly facilitates the use of warfarin as a probe of cytochrome P-450-catalyzed metabolism.

In the present study we have investigated the effects of PB and BNF induction on the rabbit hepatic microsomal metabolism of R and S warfarin by using the HPLC assay. Regio- and stereoselectivities of R and S warfarin metabolism catalyzed by purified rabbit P-450<sub>LM</sub>, P-450<sub>LM</sub>, P-450<sub>LM</sub>, and P-450<sub>LM</sub>, have been investigated and the results compared with those from the microsomal metabolism.

The abbreviations used are: P-450<sub>LM</sub>, liver microsomal cytochrome P-450; PB, phenobarbital; BNF, β-naphthoflavone; dilauroyl-GPC, dilauroylglycerol-3-phosphorylcholine; HPLC, high pressure liquid chromatography.

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The metabolic system containing P-450, from PB-induced rabbits catalyzed the metabolism of R warfarin to yield predominantly 4'-hydroxywarfarin with lesser quantities of 6- and 7-hydroxywarfarin. No metabolites were detected when NADPH was eliminated from the reaction mixture. In Fig. 1B the rates of formation of metabolites of R and S warfarin were determined by published spectral methods (12, 30). Protein concentrations were routinely determined by the method of Lowry et al. (31).

**Materials—**Racemic warfarin, purchased from Calbiochem (Los Angeles, Calif.), was resolved by a previously described method (21) into optically pure R (α = 149.7) and S warfarin (α = -149.7). The metabolites of warfarin; 6-, 7-, 4', and 8-hydroxywarfarin were synthesized by previously published methods (25, 27), where the treatment of animals is indicated in parentheses.

Preparation of Microsomes and Purification of Cytochromes—Microsomes were prepared from New Zealand male rabbits (2 to 2.5 kg), which were either uninduced or induced with PB or BNF as previously described (12). All other materials were obtained as described previously (18, 19). Water was deionized, glass-distilled, and filtered through a 0.22-μm membrane (Millipore Corp., Bedford, Mass.) prior to use in the HPLC.

Preparation of Microsomes from New Zealand Male Rabbits—New Zealand male rabbits were anesthetized with halothane (1.5% in air) and decapitated. The livers were removed, chilled in ice, and homogenized with a Potter-Elvehjem glass homogenizer in 50 mM Tris-HCl buffer, pH 7.4, and 0.9 μmol of NADPH were added. Components were preincubated for 3 min at 30°C with shaking, and the reaction was then initiated by the addition of 1.0 μmol of NADPH. Components were added and mixed in the manner previously described (17). Incubation was continued for 10 to 30 min, and the reaction was terminated by cooling in ice and filtering through a pad of filters as described above. A series of control experiments was performed with either one component being excluded in each case or boiled protein components being used. The precise concentrations of the components for each experiment are provided in the figure legends. All of the data are the averages of duplicate experiments.

The metabolic system containing P-450, from PB-induced rabbits catalyzed the formation of R and S warfarin, while 6-hydroxywarfarin from S warfarin was the major products with BNF-induced microsomes. Benzylic hydroxywarfarin and dehydrowarfarin were not detected as metabolites of R or S warfarin. No metabolites were detected when NADPH was eliminated from the reaction mixture. In Fig. 1B the rates of formation of metabolites of R and S warfarin were determined by published spectral methods (12, 30). Protein concentrations were routinely determined by the method of Lowry et al. (31).

**Metabolism of Warfarin by Microsomes—**R and S warfarin were metabolized by uninduced, PB-, or BNF-induced rabbit liver microsomes to yield 4'-, 6-, 7-, and 8-hydroxywarfarin. The rates of formation of the different metabolites are shown in Fig. 1A. 4'-Hydroxywarfarin from R warfarin and 6-hydroxywarfarin from S warfarin were the major products with uninduced and PB-induced microsomes, while 6-hydroxywarfarin from R warfarin and 7-hydroxywarfarin from S warfarin were the major products with BNF-induced microsomes. Benzylic hydroxywarfarin and dehydrowarfarin were not detected as metabolites of R or S warfarin. No metabolites were detected when NADPH was eliminated from the reaction mixture. In Fig. 1B the rates of formation of metabolites of R and S warfarin catalyzed by rat liver microsomes are shown for comparative purposes.

**Results—**The reconstituted system containing P-450, from PB-induced rabbits catalyzed the metabolism of R warfarin to yield predominantly 4'-hydroxywarfarin with lesser quantities of 6- and 7-hydroxywarfarin. S warfarin was metabolized to 6-hydroxywarfarin with lesser quantities of 4'- and 7-hydroxywarfarin (Fig. 2). No 8-hydroxywarfarin was detected in the P-450, M.-catalyzed reactions. The overall rate of metabolism of S warfarin was greater than that of R warfarin. The metabolism rates for two different preparations of PB-induced P-450, M. are presented in Table I. Although there were dif-
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Fig. 1. A, rates of formation of 4'-hydroxywarfarin (4'-OH), 6-hydroxywarfarin (6-OH), 8-hydroxywarfarin (8-OH), and 7-hydroxywarfarin (7-OH) from R or S warfarin, catalyzed by control, PB-, or BNF-induced rabbit liver microsomes. Reactions were performed using 2 mg of microsomal protein/ml at 30°C, pH 7.4. All results are the averages of two experiments. B, rates of formation of warfarin metabolites from R and S warfarin catalyzed by control, PB-, or 3-methylcholanthrene-induced rat liver microsomes. Reaction conditions were as in A. Cytochrome P-450 concentrations were 1.0, 2.2, and 1.7 nmol/mg of microsomal protein for control, PB-, or 3-methylcholanthrene-induced microsomes, respectively.

TABLE I
Rates of formation of the metabolites of R and S warfarin catalyzed by the reconstituted system containing two different preparations of cytochrome P-450_{1M} from phenobarbital-treated rabbits

<table>
<thead>
<tr>
<th>Warfarin metabolite</th>
<th>Preparation 1</th>
<th>Preparation 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>R warfarin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4'-OH</td>
<td>50.4, 53.0*</td>
<td>36.0</td>
</tr>
<tr>
<td>6-OH</td>
<td>10.3, 9.7</td>
<td>7.9</td>
</tr>
<tr>
<td>7-OH</td>
<td>3.2, 3.2</td>
<td>2.5</td>
</tr>
<tr>
<td>S warfarin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4'-OH</td>
<td>23.9, 25.5</td>
<td>19.3</td>
</tr>
<tr>
<td>6-OH</td>
<td>99.9, 74.2</td>
<td>54.6</td>
</tr>
<tr>
<td>7-OH</td>
<td>2.8, 2.8</td>
<td>1.4</td>
</tr>
</tbody>
</table>

* Duplicate analyses.

The P-450_{1M}-catalyzed formation of all the metabolites from R warfarin was linear with time for 30 min (Fig. 3A), but with S warfarin metabolite formation was only linear for 20 min, with slight deviations being apparent after this time (Fig. 3B). In the absence of dilauroyl-GPC from the reaction mixture, rates of R warfarin metabolite formation were approximately one-fourth of the rates at the optimal dilauroyl-GPC concentration. The maximal rates of R 4', R 6-, and R 7-hydroxywarfarin formation all occurred at a ratio of approximately 30 to 45 mol of lipid/mol of P-450_{1M} (20 to 30 mg of lipid/nmol of P-450_{1M}) (Fig. 4A). When the ratio of dilauroyl-GPC to P-450_{1M} exceeded this ratio, the rates of all the metabolites were markedly decreased. The effects of dilauroyl-GPC on the P-450_{1M}-catalyzed metabolism of S warfarin were very similar (Fig. 4B) to the results with R warfarin.

In the absence of NADPH-cytochrome P-450 reductase, no metabolites were formed from R warfarin. As the reductase concentration was increased, the formation rates of all the metabolites increased until a molar ratio of reductase/P-450_{1M} of approximately 1.4 was reached. At higher ratios of reductase/P-450_{1M}, the rates were markedly decreased (Fig. 5A). Similar results were obtained with S war-
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Fig. 2. Rates of formation of 4'-hydroxywarfarin (4'-OH), 6-hydroxywarfarin (6-OH), 8-hydroxywarfarin (8-OH), and 7-hydroxywarfarin (7-OH) from R and S warfarin catalyzed by P-450<sub>1.M</sub> or P-450<sub>1.M</sub>, in the reconstituted enzyme system. Both forms of the cytochrome and the NADPH-cytochrome P-450 reductase were isolated from microsomes from PB-treated rabbits. Reaction mixtures contained in a total of 1.0 ml: P-450<sub>1.M</sub> (1.0 nmol), reductase (1.4 nmol, 4.2 units), dilauroyl-GPC (20 μg, 30 nmol), R or S warfarin (0.9 pmol), NADPH (1.9 μmol), and Tris-HCl buffer (50 mM). Reactions were run at 30°C and pH 7.4 for 20 min. Metabolites were analyzed by HPLC. All data points are the averages of two experiments.

Fig. 3. Effect of time on the quantity of 4'-hydroxywarfarin (■), 6-hydroxywarfarin (○), and 7-hydroxywarfarin (●) produced from (A) R warfarin and (B) S warfarin catalyzed by P-450<sub>1.M</sub> in the reconstituted enzyme system. Other conditions were as in Fig. 2.

Warfarin, but maximal rates were achieved at a somewhat higher reductase/P-450<sub>1.M</sub> ratio (Fig. 5B).

The effects of P-450<sub>1.M</sub> concentration on the rates of formation of metabolites of R and S warfarin are shown in Figs. 6 and 7. In Fig. 6, the concentrations of reductase were varied with the concentrations of P-450<sub>1.M</sub>, so as to maintain the optimal ratio of reductase/P-450<sub>1.M</sub>. Rates of formation of all the metabolites from R warfarin increased linearly with increasing P-450<sub>1.M</sub> concentration until a value approaching 1 nmol of P-450<sub>1.M</sub>/ml was reached, above which the increase in rates was much less rapid (Fig. 6A). With S warfarin as substrate (Fig. 6B), however, the rates of metabolite formation deviated slightly from linearity only at low P-450<sub>1.M</sub> concentrations. When the P-450<sub>1.M</sub> concentration was varied but the reductase concentration was maintained at a constant value (which was equivalent to that producing maximal rates at a P-450<sub>1.M</sub> concentration of 1 nmol/ml), the results shown in Fig. 7 were obtained. With R warfarin (Fig. 7A) and S warfarin (Fig. 7B), rates of metabolite formation increased linearly at low P-450<sub>1.M</sub> concentrations but deviated from linearity at higher enzyme concentrations.

Warfarin concentration (0.9 mM) was saturating but not inhibiting in all of these assays as was demonstrated by the

Fig. 4. Effect of dilauroyl-GPC concentration on the rates of formation of 4'-hydroxywarfarin (■), 6-hydroxywarfarin (○), and 7-hydroxywarfarin (●) from (A) R warfarin and (B) S warfarin catalyzed by P-450<sub>1.M</sub> in the reconstituted enzyme system. Dilauroyl-GPC was added in an ultrasonicated suspension. Other conditions were as in Fig. 2.

Fig. 5. Effect of NADPH-cytochrome P-450 reductase concentration on the rates of formation of 4'-hydroxywarfarin (■), 6-hydroxywarfarin (○), and 7-hydroxywarfarin (●) from (A) R warfarin and (B) S warfarin catalyzed by P-450<sub>1.M</sub> in the reconstituted enzyme system. Other conditions were as in Fig. 2.
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Equivalent results obtained when the warfarin concentration was altered to 0.45 mM. Addition of sodium cholate (0.2 mM) or sodium deoxycholate (0.2 mM) to the reconstituted system or the replacement of the Tris buffer by N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid buffer or phosphate buffer did not alter the rates of formation of the metabolites.

Metabolism of Warfarin by P-4501.M,-The reconstituted system containing P-4501.M, catalyzed the formation of R warfarin to yield predominantly 6-hydroxywarfarin with lesser quantities of 8- and 7-hydroxywarfarin. S warfarin was metabolized to a much lesser extent with 6-hydroxywarfarin being the predominant product and 8- and 7-hydroxywarfarin having lower rates of formation. No 4’-hydroxywarfarin was detected from either R or S warfarin with P-4501.M,. The metabolite formation rates catalyzed by P-4501.M, from uninduced, PB-, or BNF-induced rabbits are presented in Table II. In most cases, the P-4501.M, from uninduced rabbits produced the lowest formation rates for the metabolites. The relative contributions of the formation rates of each metabolite to the total formation rates of all products from the metabolism of R or S warfarin was essentially the same with P-4501.M, from control, PB-, or BNF-treated microsomes (Table II). This suggests that the P-4501.M, from these three sources is the same enzyme. Subsequent studies were performed using the P-4501.M, from PB-induced rabbits.

The P-4501.M,-catalyzed formation of all the metabolites from R warfarin deviated slightly from linearity over 20 min (Fig. 8), but the relative quantities of the metabolites were unaltered and subsequent studies were performed with reactions run for 20 min. In the absence of dilauroyl-GPC from the reaction mixture, the rate of 6-hydroxywarfarin formation was approximately three-fifths of the rate at the optimal dilauroyl-GPC concentration (Fig. 9). The maximal rate of 6-hydroxywarfarin formation occurred at the ratio of approximately 30 mol of lipid/mol of P-4501.M, and when the lipid concentration was increased, the rate of formation decreased markedly. The rates of formation of 7- and 8-hydroxywarfarin

Table II

<table>
<thead>
<tr>
<th>Warfarin metabolite</th>
<th>Control</th>
<th>PB</th>
<th>BNF</th>
</tr>
</thead>
<tbody>
<tr>
<td>R warfarin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-OH</td>
<td>33.0, 40.9 (73) b</td>
<td>47.9, 48.2 (70)</td>
<td>39.9, 44.7 (72)</td>
</tr>
<tr>
<td>7-OH</td>
<td>4.2, 3.9 (7)</td>
<td>4.6, 4.9 (7)</td>
<td>4.3, 4.1 (7)</td>
</tr>
<tr>
<td>8-OH</td>
<td>8.7, 11.4 (20)</td>
<td>16.2, 16.5 (24)</td>
<td>10.9, 13.6 (21)</td>
</tr>
<tr>
<td>S warfarin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-OH</td>
<td>5.1, 6.1 (45)</td>
<td>12.5, 9.6 (48)</td>
<td>8.1, 7.6 (48)</td>
</tr>
<tr>
<td>7-OH</td>
<td>2.2, 2.9 (21)</td>
<td>4.6, 4.6 (20)</td>
<td>3.9, 3.9 (24)</td>
</tr>
<tr>
<td>8-OH</td>
<td>4.3, 4.3 (34)</td>
<td>7.9, 6.7 (32)</td>
<td>4.8, 4.5 (28)</td>
</tr>
</tbody>
</table>

* Duplicate analyses.  
* Percentage of total formation rates of all products from R or S warfarin.

Fig. 6. Effect of P-4501.M, concentration on the rates of formation of 4’-hydroxywarfarin (■), 6-hydroxywarfarin (○), and 7-hydroxywarfarin (●) from (A) R warfarin and (B) S warfarin catalyzed by P-4501.M, in the reconstituted enzyme system. The NADPH-cytochrome P-450 reductase concentration was varied to produce a constant P-4501.M,/reductase molar ratio of 1.4. Other conditions were as in Fig. 2.

Fig. 7. Effect of P-4501.M, concentration on the rates of formation of 4’-hydroxywarfarin (■), 6-hydroxywarfarin (○), and 7-hydroxywarfarin (●) from (A) R warfarin and (B) S warfarin catalyzed by P-4501.M, in the reconstituted enzyme system. The NADPH-cytochrome P-450 reductase concentration was kept constant at a level of 1.4 nmol/ml. Other conditions were as in Fig. 2.

Fig. 8. Effect of time on the quantity of 6-hydroxywarfarin (○), 7-hydroxywarfarin (●), and 8-hydroxywarfarin (□) produced from R warfarin catalyzed by P-4501.M, in the reconstituted enzyme system. Other conditions were as in Fig. 2.
were, however, maximal in the absence of dilauroyl-GPC and decreased with increases in the lipid concentration (Fig. 9).

In the absence of NADPH-cytochrome P-450 reductase, no metabolites were formed from R warfarin with P-450i,M. As the reductase concentration increased, the formation rates of the metabolites increased until maximal rates were achieved at a molar ratio of reductase/P-450i,M of approximately 1.4 (Fig. 10). At higher ratios, the rates of formation of the warfarin metabolites were decreased.

The effects of P-450i,M concentration on the rates of formation of R warfarin metabolites are shown in Fig. 11. In Fig. 11A, concentrations of the reductase were also varied with the P-450i,M concentration so as to maintain the optimal ratio of reductase/P-450i,M. Rates of metabolite formation were linear up to P-450i,M concentrations of 1 nmol/ml. When the reductase concentration was maintained at a constant level and only the P-450i,M concentration was varied (Fig. 11B), rates of formation of the metabolites still increased linearly with increases in enzyme concentration.

Metabolism of Warfarin by P-450i,M, P-450i,M, and P-450i,M. With P-450i,M, P-450i,M, and P-450i,M, no metabolites of R or S warfarin were detected. With P-450i,M, metabolite patterns were detected which were identical to those of P-450i,M.

DISCUSSION

The rabbit hepatic microsomal metabolism of R and S warfarin is markedly different from that of the rat with respect to the overall rates of warfarin metabolism, the relative rates of formation of the different metabolites, and the effects of inducing agents on the metabolism. Comparisons of both R and S warfarin metabolism by rat' or rabbit microsomes indicate the following differences. In the rat R 7- and S 4'-hydroxywarfarin are the major uninduced microsomal metabolites from the warfarin enantiomers (22), while in the rabbit R 4'- and S 6-hydroxywarfarin are the major products. PB induction of rat microsomes results in the formation of R and S 7-hydroxywarfarin as the major metabolites (23) in contrast to rabbit microsomes where R 4'- and S 6-hydroxywarfarin predominate. BNF induction is similar to 3-methylcholanthrene or benzo[a]pyrene induction (22) in markedly enhancing the rates of formation of R 6- and R 8- and S 7-hydroxywarfarin as the major products. Furthermore, the metabolites dehydro-warfarin² and benzylic hydroxywarfarin, which are products of the rat hepatic microsomal metabolism of the aliphatic moiety of warfarin (22), are not detectable products of rabbit
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The overall rate of rat hepatic microsomal metabolism. The overall rate of rat hepatic microsomal cytochrome P-450-catalyzed metabolism of R or S warfarin is approximately twice that of the rabbit with uninduced and PB-induced microsomes, and six times that of the rabbit with BNIF-induced microsomes. These differences in function between rat and rabbit microsomes imply that the forms of cytochrome P-450 in the livers of the two species are different (cf Fig. 1, A and B).

The differences in the metabolic activities of the various purified and partially purified P-450 enzymes with warfarin, separate them into three groups: P-450M, P-450N, and P-450O, comprise a group which exhibits no activity with warfarin; P-450M and P-450N exhibit the same activity, which is markedly different from that of P-450O, which comprises the third group. From the results depicted in Fig. 2, it was determined that P-450O is stereoselective for R 4'- and S 6-hydroxywarfarin, while P-450M is stereoselective for R 6- and R 8-hydroxywarfarin. When the overall metabolism was considered, the two enzymes showed opposite stereoselectivities, with P-450M being stereoselective for S warfarin and P-450O for R warfarin. The regioselectivities of the two enzymes are also different, with P-450M being regioselective for R 4' and S 6-hydroxywarfarin, with no detectable formation of 8-hydroxywarfarin, and P-450O being regioselective for R 6- and S 6-hydroxywarfarin, with no detectable formation of 4'-hydroxywarfarin.

The regioselective and stereoselective properties of P-450M and P-450N enzymes must arise from differing activities of the enzyme catalytic sites with differently oriented warfarin molecules. Thus, P-450O, which only catalyzed the formation of metabolites which have the phenyl portion of the coumarin moiety hydroxylated (6-, 7-, and 8-hydroxywarfarin), has activity with warfarin only when it is oriented to approach the enzyme at the coumarin side of the molecule. Furthermore, the activity of P-450O, on warfarin in this orientation is greater with the R enantiomer than with the S enantiomer. In the case of P-450M, there is also potential activity with warfarin oriented with the coumarin moiety toward the catalytic site (6- and 7-hydroxywarfarin formed), but the S enantiomeric configuration is preferred. In addition, P-450O has the potential to interact with the phenyl substituent of warfarin, but for that interaction the R configuration of warfarin is favored.

It has been proposed that rat hepatic cytochrome P-450-catalyzed metabolism of warfarin to 8-hydroxywarfarin proceeds via formation of a 7,8-arene oxide, which preferentially opens to 8-hydroxywarfarin, and that 6-hydroxywarfarin similarly arises from a 6,7-arene oxide (22). If the same mechanism were applicable to rabbit P-450M, the metabolite patterns obtained (Fig. 2) indicate that P-450M only catalyzes the formation of the 6,7-arene oxide of warfarin, while P-450M catalyzes the formation of both the 6,7- and 7,8-arene oxides.

Warfarin undergoes a tautomeric-driven, ring closure in solution to form a hemiketal (Scheme 1H) (32). The extent of this reaction is dependent on the polarity of the environment of the warfarin molecule. The environment of the catalytic sites of the P-450 enzymes could thus influence the tautomerism to an unknown extent. The tautomeric form present could affect the metabolism of warfarin.

The fact that in the absence of added lipid, P-450M, catalyzed metabolism of R warfarin proceeds to a relatively much greater degree than P-450M, catalyzed metabolism, is consistent with results using other substrates (33). The inhibitory effect of excess lipid on the rates of P-450M, and P-450M, catalyzed R warfarin metabolism is a function of the lipid concentration, rather than the lipid concentration. This was determined from the studies of the effects of P-450M concentration on the rates of formation of the warfarin metabolites. At low P-450M concentrations, the ratio of lipid/P-450M was greatly in excess of the optimal ratio, but the metabolite formation rates were linearly related to P-450M concentrations (Figs. 6A and 11), indicating that a high ratio of lipid/P-450M was not, in itself, inhibitory. A similar argument may be applied to the effect of lipid concentration on S warfarin metabolism by P-450M, since the metabolism rates deviate slightly from linearity.

The variation in the effects of lipid concentration on the P-450M, catalyzed formation rates of the different metabolites (Fig. 9) can arise from lipid-imposed conformational restraints on the enzyme, such that its interactions with warfarin in different orientations are differentially affected. It has been previously suggested that lipids can affect microsomal cytochrome P-450 functions by inducing protein conformational changes (32, 34, 35). An alternative possibility is that there is more than one form of cytochrome P-450 in the P-450M, preparation. Although the P-450M preparations yield a single band on sodium dodecyl sulfate gel electrophoresis, and a single COOH-terminal residue (12), the NH2-terminal amino acid analysis of P-450M lends some support to the possibility of more than one enzyme component (36).

The requirements for NADPH-cytochrome P-450 reductase in the P-450M, and P-450M, reconstituted systems for the metabolism of R or S warfarin were similar (Figs. 5, A and B, and 10). In all cases, high levels of reductase apparently inhibited the reaction. We have eliminated the possibility that the NADP levels produced with high reductase concentrations inhibited warfarin metabolism, and thus it is possible that protein-protein interactions are the source of the inhibition. Either the reductase, when in excess, binds to the P-450M non-specifically and sterically hinders the interaction of the substrate with the P-450M, or the specific interaction causes conformational changes in the P-450M, which results in the inhibition of the reaction.

Although excess NADPH-cytochrome P-450 reductase in the reconstituted system clearly caused inhibition of warfarin metabolism at P-450M concentrations of 1 μM (Figs. 5 and 10), a similar effect did not occur at low P-450M concentrations. This was apparent from experiments where the reductase concentration was maintained at high levels and the P-
Results indicate that the selectivities of these two enzymes for similarities between the purified P-4501.~ and microsomal farin metabolism by BNF-induced microsomes, particularly microsomal metabolism with P-4501,M-catalyzed metabolism, the metabolite patterns derived from the P-4501,M, and the PB-induced metabolism of R and S warfarin with respect to 4' and 7-hydroxywarfarin formation. The induced microsome-catalyzed metabolism of R and S warfarin P-450/reductase ratios. However, the similarities between the purified P-4501.M enzymes have significant pharmacological implications in view of the following facts. The anticoagulation activity of S warfarin is five to eight times greater than that of R warfarin (37), and only 4'-hydroxywarfarin, of all the known metabolites, possesses significant anticoagulant activity (19). Thus, induction of P-4501,M leads to diminished rates of metabolism of the more active S enantiomer, which would enhance the pharmacological potency of clinically administered racemic warfarin. In contrast, induction of P-4501,M results in enhanced metabolism of S warfarin, and thus diminished pharmacological effects, but this would be offset to some extent by the formation of the active 4'-hydroxywarfarin metabolite.

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**REFERENCES**

Regioselective and stereoselective hydroxylation of R and S warfarin by different forms of purified cytochrome P-450 from rabbit liver.
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