Biochemical Pharmacology, Vol. 26, pp. 109–114. Pergamon Press, 1977. Printed in Great Britain.

STEREOCHEMICAL BIOTRANSFORMATION OF WARFARIN AS A PROBE OF THE HOMOGENEITY AND MECHANISM OF MICROSOMAL HYDROXYLASES

LANCE R. POHL,* WILLIAM R. PORTER and WILLIAM F. TRAGER†
Departments of Pharmaceutical Chemistry, University of Washington, Seattle, Wash. 98195 and University of California, San Francisco, Calif. 94143

and

MICHAEL J. FASCO‡ and JOHN W. FENTON, II
Division of Laboratories and Research, New York State Department of Health, Albany, N.Y. 12201, U.S.A.

(*Received* 8 *March* 1976; *accepted* 11 *June* 1976)

Abstract—The hepatic microsomal metabolism of *R* and *S* warfarin by normal Wistar and Sprague–Dawley rats was compared with that of phenobarbital (PB) and 3-methylcholanthrene (3-MC)-pretreated animals. In all the microsomal systems examined, *R* warfarin was metabolized faster than the *S* enantiomer. Induction of microsomal mixed-function oxidase activity with PB, and especially 3-MC, caused significant alterations in the normal stereochemical pattern of *R* and *S* warfarin hydroxylation which were independent of the method of microsomal preparation and the technique employed in the quantitation of hydroxylated products. PB pretreatment of Sprague–Dawley rats resulted in an increase in all hydroxylated products but to differing extents. Similar results were obtained from Wistar rats except for the processes of 4′ and benzylic hydroxylation of (*S*) warfarin. 3-MC pretreatment resulted in the selective induction of 6- and 8-hydroxylation in both species. These results suggest that liver microsomes from normal animals contain at least two major, A and B, and two minor, C and D, mono-oxygenases which differ in their stereoselectivity (as measured by the rates for the formation of two enantiomeric products), regioselectivity (as measured by the ratio of any two isomeric products from the same substrate), and inducibility. In this model, normal animals have hydroxylase activity enzyme A which is not inducible by PB or 3-MC and which is stereoselective for the *R* enantiomer of warfarin in 7- and benzylic hydroxylation and for the *S* enantiomer in 4′-hydroxylation. Microsomes from normal and PB-induced animals contain additional hydroxylase activity, enzyme B, which catalyzes both the 6- and 8-hydroxylation of warfarin and which has low stereoselectivity but is regioselective for 6-hydroxylation. Enzyme B may also be responsible for some 4′-hydroxylation. PB-induced animals have additional mono-oxygenase activity, enzyme C, which displays the opposite stereoselectivity compared to enzyme A for benzylic and less stereoselectivity for 7-hydroxylation. 3-MC-induced animals have greatly enhanced levels of 6- and 8-hydroxylase activity, enzyme D, which is stereoselective for the *R* enantiomer and regioselective for 8-hydroxylation of *R* warfarin and 6-hydroxylation of *S* warfarin.

The existence of multiple forms of cytochrome P-450 associated with liver microsomes resulting from pretreatment with inducing agents is well established [1–8]. Both qualitative and quantitative differences in the spectral properties of microsomal preparations and their ability to hydroxylate various substrates after pretreatment with either 3-methylcholanthrene (3-MC) or phenobarbital (PB) have been documented [1, 2, 9–12]. Recent biochemical investigations have focused on the isolation, purification and characterization of the various inducible forms. Lu *et al.* [13–17], utilizing chromatographic, electrophoretic and immunologic techniques, have succeeded in separating and purifying the microsomal forms of cytochrome P-450 obtained from immature Long Evans rats after induction with either 3-MC or PB. The enzymes thus obtained not only have different spectral, catalytic and immunologic properties but also have different molecular weights. Similarly, Van der Hoeven *et al.* [18–20] have separated and demonstrated multiple forms of cytochrome P-450 from rabbit liver. The question of whether or not the inducible forms of the enzymes are present in normal liver is unsettled, but evidence exists which suggests they are, although in much lower concentration. Comai and Gaylor [21], using Sprague–Dawley rats, were successful in chromatographically separating three distinct forms (I, II and III) of cytochrome P-450 from untreated animals. These results have been confirmed by Welton and Aust [22], Alvares and Siekevitz [23] and more recently by Muna [24], who used Sprague–Dawley rats obtained from the same breeding colony employed in this study. Thus, normal microsomes

*Present address: Laboratory of Chemical Pharmacology, National Heart and Lung Institute, National Institute of Health, Bethesda, Md. 20014.

† To whom correspondence should be addressed at the University of Washington, Seattle, Wash. 98195.

‡ To whom correspondence should be addressed at the New York State Department of Health, Albany, N.Y. 12201.

apparently contain at least three distinct forms of P-450 whose relative concentrations and therefore whose catalytic properties are altered by inducing agents.

The study of multiple products produced from a single substrate should increase our understanding of the individual catalytic properties of the various microsomal hydroxylases. Selander *et al.* [25] have studied the ratio of *o*-, *m*-, and *p*-chlorophenol obtained from the biotransformation of chlorobenzene and concluded that at least three cytochrome P-450s operating by two distinct mechanisms were involved in the hydroxylation reactions. PB induced the levels of all the enzymes without significantly altering the product ratio whereas 3-MC induction resulted in a large selective increase in the formation of the *ortho* isomer. Careful studies by Burke and Bridges [26] of the metabolism of biphenyl revealed that PB preferentially induced the formation of 4-hydroxybiphenyl, while 3-MC induced the formation of 4- and 2-hydroxybiphenyl almost equally. Other workers have demonstrated preferential hydroxylation at aliphatic sites as a function of the nature of the inducing agent [27, 28]; but to our knowledge, no study has appeared which involves both aliphatic and aromatic hydroxylation within the same substrate.

In a previous paper [29], we reported that the hepatic mixed-function oxidase system in normal rats was stereoselective in both the aromatic and benzylic hydroxylation of *R* and *S* warfarin (Fig. 1). A Michaelis–Menton analysis of the various products derived from this system allowed us to conclude that the normal microsomal preparation catalyzed at least three kinetically distinct groups of hydroxylations. As a result of this finding, we have studied the effects of the inducing agents, PB and 3-MC, on the metabolism of the warfarin enantiomers in order to further probe the possible multiplicity and stereoselectivities of the microsomal mixed-function oxidase system. Microsomes were prepared from both Wistar and Sprague–Dawley rats to examine strain-dependent factors and to gain some information as to the possible generality of the effects of induction on the metabolism of warfarin.

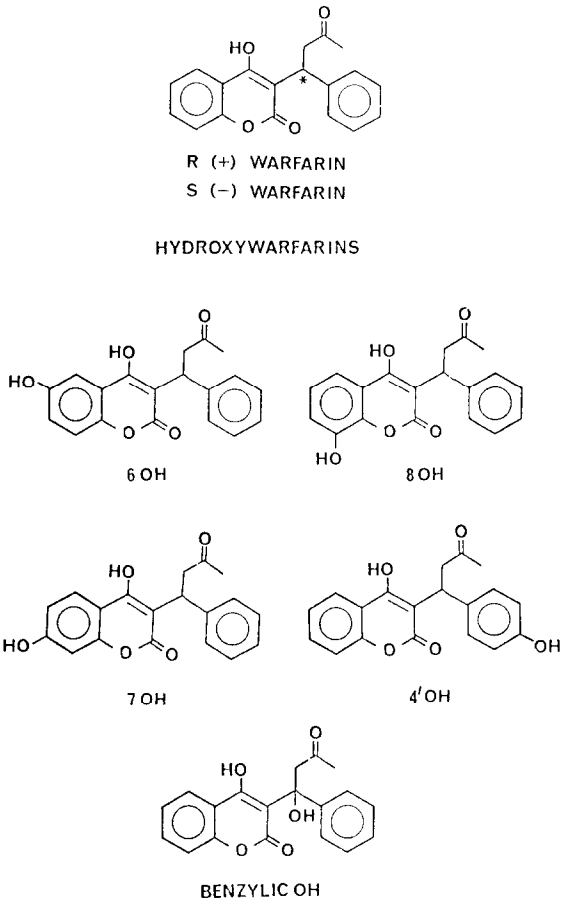

Fig. 1. Structure of warfarin and its hydroxylated metabolites.

MATERIALS AND METHODS

Experiments performed in Albany

Materials. 3-Methylcholanthrene was obtained from either K & K Laboratories (Albany, N.Y.) or CalBiochem (La Jolla, Calif.). Phenobarbital was obtained through the New York State Department of Health. Other materials utilized in the study were as previously described [29].

Tissue preparation, incubations and isolation procedures. Male Wistar rats (200–250 g) were used throughout this study and were allowed food and water *ad lib.* Induced mixed-function oxidase activity was achieved by pretreatment of animals with either PB (80 mg/kg, i.p., in physiological saline once daily for 3 days) or 3-MC (25 mg/kg, i.p., corn oil once daily for 3 days). Control animals received comparable doses of either corn oil or physiological saline. Twenty-four hr after the last injection, ten similarly treated animals were sacrificed by a blow to the head and their livers were excised and combined in cold 1.15% KCl. Microsomes were prepared, incubations conducted and products isolated and quantified as previously described [29].

Protein and cytochrome determinations. The concentration of microsomal protein was determined from the 105,000 *g* pellet by the biuret method [30] employing bovine serum albumin as standard.

Experiments performed in Seattle

Materials. 3-Methylcholanthrene was obtained from Sigma Biochemicals (St. Louis, Mo.). Phenobarbital (Luminal, Winthrop Laboratories) was obtained from Drug Services, School of Pharmacy, University of Washington. Other materials utilized in the study were as previously described.

Tissue preparation, incubations, isolation procedures and protein determinations. Six male Sprague–Dawley rats (130 ± 2 g) were employed for the control and each induction experiment. After 4 days of acclimation, the treated animals were given, i.p., either sodium phenobarbital (100 mg/kg in normal saline) or 3-methylcholanthrene (20 mg/kg in corn oil) for 3 days at 9:30 a.m. On the following day at 9:30 a.m., the animals were weighed (range 140–180 g), decapitated and exsanguinated. All other procedures were as previously described [29].

Table 1. Comparative oxidation *in vitro* of *R* and *S* warfarin by normal, and phenobarbital- and 3-methylcholanthrene-induced hepatic microsomes from Sprague–Dawley rats

	Product formed from rat microsomes* (nmoles/mg protein/10 min)					
	Normal microsomes		Phenobarbital-induced		3-Methylcholanthrene-induced	
Warfarin metabolites	*R* warfarin	*S* warfarin	*R* warfarin	*S* warfarin	*R* warfarin	*S* warfarin
6-Hydroxywarfarin	0.69 ± 0.05	0.64 ± 0.02	1.35 ± 0.04	1.23 ± 0.07	2.78 ± 0.17	1.70 ± 0.06
7-Hydroxywarfarin	1.92 ± 0.13	0.43 ± 0.01	4.36 ± 0.26	1.70 ± 0.05	0.94 ± 0.13	0.40 ± 0.02
8-Hydroxywarfarin	0.43 ± 0.02	0.13 ± 0.01	0.92 ± 0.04	0.42 ± 0.02	4.91 ± 0.09	0.54 ± 0.03
4′-Hydroxywarfarin	0.38 ± 0.02	0.64 ± 0.04	0.89 ± 0.03	1.12 ± 0.12	0.16 ± 0.02	0.27 ± 0.04
Benzylic hydroxywarfarin	0.26 ± 0.03	0.11 ± 0.01	0.40 ± 0.01	0.94 ± 0.04	0.09 ± 0.01	0.09 ± 0.01
Total	3.68	1.95	7.92	5.41	8.88	3.00
R/*S* (total)	1.89		1.46		2.96	

Data are expressed as the means ± standard deviations and represent three analyses. A warfarin concentration of 0.26 mM was employed in these studies.

RESULTS

The incubation of normal hepatic microsomes from Sprague–Dawley or Wistar rats with a single concentration of *R* or *S* warfarin yielded the same product distribution as reported previously [29] (Tables 1 and 2). When the values from the two laboratories were corrected for the differences in length of incubation, the stereochemical hydroxylation patterns obtained by the metabolism of the warfarin enantiomers were in good agreement irrespective of the species utilized, the method of microsomal preparation, the concentration of substrate, and the technique employed in the quantitation of hydroxylated products (Fig. 2). The influence of stereochemistry on product formation is demonstrated by the fact that 7- and 8-hydroxylation occurred preferentially with *R* warfarin [29] (see Tables 1 and 2 and Fig. 2).

Pretreatment of Sprague–Dawley rats with PB resulted in an increase in the rate of formation of all the *R* and *S* hydroxylation products/mg of microsomal protein, but the *R*/*S* ratio of total metabolism remained essentially normal (see Table 1). However, the increases in the rate of formation of the various metabolites were not equivalent. In addition to the fact that 7-hydroxylation became the major pathway for biotransformation of *S* warfarin, the most apparent difference was the 3- to 4-fold increase in the amount of 7- and 8-hydroxywarfarin obtained from this enantiomer. Further, a 9-fold increase in the amount of benzylic hydroxywarfarin was observed from the *S* isomer as compared to the approximate 2-fold increase in the amount of the remainder of the products produced from both *R* and *S* warfarin. The apparent preferential induction of 7- and 8-hydroxylation of *S* warfarin did not reverse the normal pattern of stereochemical preference, since larger quantities of 7- and 8-hydroxywarfarin were still obtained from the *R* enantiomer (see Table 1). However, induction did result in a reversal of the stereochemical preference for benzylic hydroxylation.

Hepatic microsomes from PB-pretreated Wistar rats displayed a very similar but not identical pattern of *R* and *S* warfarin hydroxylation to that of induced Sprague–Dawley microsomal preparations (see Table 2 and Fig. 1). For example, 7-hydroxylation was the major metabolic pathway for *S* warfarin and there was also an abnormal increase in the rate of 8-hydroxylation of this isomer. In contrast to Sprague–Dawley rat, however, phenobarbital induction of Wistar rat mixed-function oxidase activity resulted in an approximately 4-fold increase in the normal 7-hyd-

Table 2. Comparative oxidation *in vitro* of *R* and *S* warfarin by normal, and phenobarbital- and 3-methylcholanthrene-induced hepatic microsomes from Wistar rats

	Product formed from rat microsomes (nmoles/mg protein/20 min)					
	Normal microsomes		Phenobarbital-induced		3-Methylcholanthrene-induced	
Warfarin metabolites	*R* warfarin	*S* warfarin	*R* warfarin	*S* warfarin	*R* warfarin	*S* warfarin
6-Hydroxywarfarin	1.36 ± 0.12	1.30 ± 0.37	3.41 ± 0.61	1.83 ± 0.067	8.16 ± 0.23	3.2 ± 0.36
7-Hydroxywarfarin	3.31 ± 0.18	1.0 ± 0.12	13.41 ± 1.09	2.80 ± 0.56	1.65 ± 0.41	0.92 ± 0.15
8-Hydroxywarfarin	0.59 ± 0.12	0.10 ± 0.10	1.95 ± 0.28	0.97 ± 0.37	9.30 ± 1.46	0.64 ± 0.09
4′-Hydroxy + benzylic warfarin†	1.54 ± 0.02	2.01 ± 0.25	3.90 ± 0.95	1.46 ± 0.42	0.10 ± 0.10	1.28 ± 0.48
Total	6.80	4.31	22.67	7.06	19.11	6.04
R/*S* (total)	1.58		3.21		3.16	

* Data are expressed as the means ± standard deviations and represent three analyses. A warfarin concentration of 0.60 mM was employed in these studies.

† 4′-Hydroxy and benzylic warfarin were not separately quantitated.

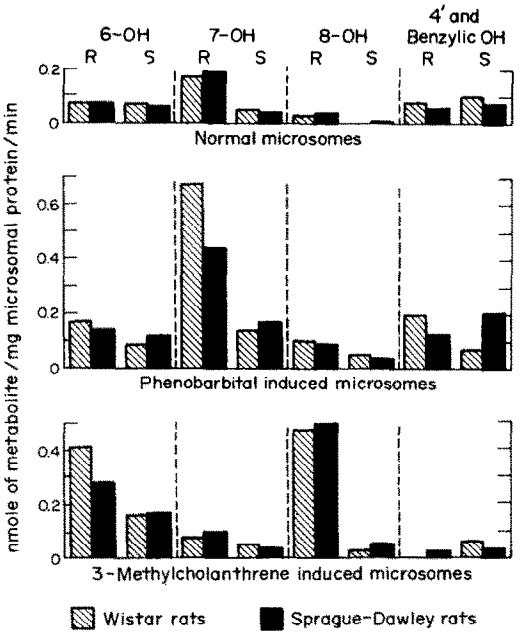

Fig. 2. Comparison of rate of metabolite formation by microsomes from Wistar and Sprague–Dawley rats. The data reported in Tables 1 and 2 were converted to the same units (nmoles metabolite formed/mg of microsomal protein/min of incubation time).

roxylation of *R* warfarin. This acceleration in 7-hydroxylation was primarily responsible for an *R*/*S* total metabolism ratio of 3.21; i.e. at least twice as large as that observed from PB-induced microsomes from Sprague–Dawley rats. Induced Wistar rat microsomes also possessed an apparent reduced capability to produce *S*-4′-hydroxy and/or benzylic warfarin. Due to the lack of an accurate extinction coefficient for benzylic warfarin, however, this observation requires further confirmation.

The mixed-function oxidases induced by 3-MC displayed less sensitivity to species variation and/or intrinsic factors that influence the synthesis of these hepatic enzymes than did those which were induced by PB. That is, the stereochemical hydroxylation patterns obtained from the incubation of *R* and *S* warfarin with 3-MC-induced microsomes from either species more closely resembled one another than did those obtained from PB induction (see Tables 1 and 2 and Fig. 2). The incubation of *R* and *S* warfarin with microsomes from 3-MC-treated rats resulted in a markedly different stereochemical pattern of hydroxylation than was obtained from normal microsomes. The most striking difference was that the *R* isomer was biotransformed primarily to 6- and 8-hydroxywarfarin at a rate of, respectively, four to six and eleven to sixteen times faster than normal. Conversely, the amount of 7-hydroxywarfarin was less than normal as were the concentrations of benzylic and 4′-hydroxywarfarin in Sprague–Dawley rats (see Table 1). Overall, *R* warfarin was metabolized approximately three times faster than the *S* enantiomer or nearly twice as fast as normal. The major biotransformation product obtained from *S* warfarin was 6-hydroxywarfarin, which was increased by a factor of two, while the concentration of 8-hydroxywarfarin was increased 3- to 4-fold. For the *R* enantiomer, the amount of 6-hydroxywarfarin was less than the amount of 8-hydroxywarfarin, whereas with the *S* isomer the amount of 6-hydroxywarfarin was greater than that of 8-hydroxywarfarin. The amount of 7-hydroxywarfarin produced from the *S* isomer was essentially normal. Microsomes from Sprague–Dawley rats also produced normal quantities of *S* benzylic warfarin, and less than the normal amount of 4′-hydroxywarfarin.

DISCUSSION

The data can most readily be analyzed in terms of the following regiospecific or kinetically distinguishable [29] hydroxylation pathways: *ortho* (8) and *para* (6) hydroxylation of the coumarin ring, *meta* (7) hydroxylation, *para* (4′) hydroxylation of the phenyl ring and benzylic hydroxylation.

Ortho, para *hydroxylation*

It has been suggested [21–24] that induction by 3-MC merely alters the relative proportions of multiple forms of cytochrome P-450. If the results from normal rats are considered as a control and are subtracted from the 3-MC results, the residual quantities must be due to the 3-MC-induced enzyme(s). When the data are viewed in this way, it is clear that the 3-MC-inducible enzyme(s) are stereoselective for the *R* enantiomer and regioselective for *ortho* hydroxylation of the *R* isomer and *para* hydroxylation of the *S* enantiomer. It is perhaps noteworthy that if the results for the *R* and *S* enantiomers are summed, the apparent regioselectivity is greatly reduced. These results illustrate the utility of enantiomers as probes of microsomal systems.

If the PB induction data are considered in the same manner, the PB-induced enzyme(s) are found to be both less stereoselective and less regiospecific. The metabolism of the *R* enantiomer still predominates, while *para* hydroxylation occurs preferentially with both the *R* and *S* enantiomers.

The stereoselectivity and regiospecificity observed for normal enzymes are intermediate to those observed for 3-MC- and PB-induced microsomes and are, therefore, on first analysis, consistent with the postulate that normal microsomes are comprised of a mixture of 3-MC- and PB-inducible enzymes (Fig. 3). However, the contribution of the 3-MC-inducible enzyme(s) must be minor on the following grounds: the stereoselectivity of the normal enzymes for 8-hydroxylation is close to that displayed by the PB-induced system and vastly different than the 3-MC system. Moreover, the regio-selectivity of the 3-MC system for *ortho* hydroxylation of the *R* isomer is inconsistent with the preferential formation of the *para* hydroxy metabolite in the normal system. Thus, the results are consistent with the 6- and 8-hydroxylase activity of normal microsomes being comprised primarily of PB-inducible enzyme(s). The possibility of a minor contribution from 3-MC- and/or non-inducible enzyme(s) cannot be excluded.

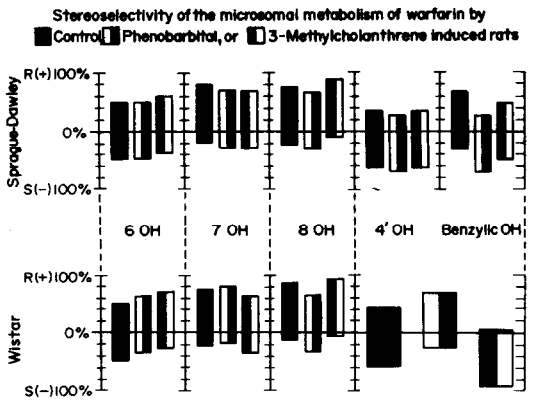

Fig. 3. Effect of inducing agents on the stereoselectivity of warfarin metabolism by microsomes from Sprague–Dawley and Wistar rats. The data in Tables 1 and 2 for the metabolites of both enantiomers were summed. The vertical bars indicate the percentage of each hydroxylated product derived from *R*-(+) or *S*-(−) warfarin.

Meta *hydroxylation*

The enzymatic processes responsible for the formation of 7-hydroxywarfarin can be distinguished from the coumarin *ortho, para* hydroxylases on the grounds of the differences of stereoselectivity in both strains of rats produced by induction with 3-MC and PB. This behavior is in marked contrast to the similarity in stereoselectivity between the strains for *orth–para* hydroxylation. These results clearly suggest that *meta* hydroxylase activity is dissociated from *ortho–para* hydroxylase activity and represents a distinct enzymatic process, confirming our prior conclusions based on kinetic evidence [29].

The alterations in stereoselectivity after induction suggest that normal microsomes contain a *meta* hydroxylase system which is not inducible and which is distinct from the PB-inducible *meta* hydroxylase. Changes in regioselectivity of the hydroxylation of the coumarin ring of warfarin caused by induction parallels those reported for simpler aromatic systems [25, 26], in that 3-MC induction favors *ortho* hydroxylation while PB induction favors *para* hydroxylation. The existence of arene oxides as intermediates in the formation of *ortho-* and *para*-hydroxylated products has been firmly established [31]. Moreover, Selander *et al.* [25, 32] have demonstrated that in at least several systems the 3,4 or 2,3 epoxides open, respectively, to yield either the *para* or *ortho* phenol, exclusively. *Meta* hydroxy products are apparently derived from an independent enzymatic process involving a direct insertion mechanism [25, 33] or perhaps via a nucleophilically catalyzed epoxide ring opening, as has been recently demonstrated by Johnson and Bruice [34] for a nonenzymatic reaction. In the case of warfarin, the latter possibility would seem less probable, since it would require, for example, the formation of the same 6,7 epoxide by two discrete enzymes, one of which leads to the production of the 6-hydroxy product in a normal fashion and the other of which leads to preferential generation of the 7-hydroxy product via catalysis by some critical nucleophile present at the enzymatic site.

4′-*Hydroxylation*

The enzymatic activity parallels that of the PB-inducible coumarin *ortho–para* hydroxylase system, but the reduced stereo-selectivity suggests that non-PB-inducible and non-3-MC-inducible 4′-hydroxylase activity is present in normal microsomes.

Benzylic hydroxylation

The preferential formation of *R*-benzylic hydroxywarfarin at high substrate concentrations employing microsomes obtained from either strain has been reported previously [35]. As noted above, the benzylic hydroxy metabolite was not studied separately with induced microsomes from Wistar rats. However, in the Sprague–Dawley rats, 3-MC induction failed to increase the synthesis of this metabolite from either enantiomer, whereas PB induction causes a greater increase in hydroxylation at this position than at any of the aromatic positions, except 7. Moreover, PB induction reverses the observed stereoselectivity (see Fig. 3), and a similar observation has been reported for another aliphatic hydroxylation [28]. This finding demonstrates that normal microsomes contain a non-inducible benzylic hydroxylase which is stereo-selective for the *R* enantiomer and which is kinetically different from normal coumarin hydroxylase [29].

Differences in the stereochemical preferences observed for the microsomal enzymes prepared from normal, PB-, or 3-MC-pretreated Sprague–Dawley and Wistar rats permit several classes of hemoproteins to be distinguished. The simplest but not unique hypothesis consistent with our findings is that liver microsomes from normal animals contain at least four hemoprotein mono-oxygenases, enzymes A–D, differing in their stereoselectivity and regiospecificity toward the enantiomers of warfarin.

These are classified as follows: (1) enzyme A, which is present in normal animals but is not inducible by PB or 3-MC, is stereoselective and regioselective for 7- and benzylic hydroxylation of *R* warfarin and 4′-hydroxylation of *S* warfarin; (2) enzyme B, which is present in normal animals, is inducible only by PB and is regioselective for 6-, 8-, and some 4′-hydroxylation; (3) enzyme C, which is present to a limited extent in normal animals, is inducible only by PB, and is regioselective for 7- and benzylic hydroxylation and more stereoselective for *S* warfarin than enzyme A; and (4) enzyme D, which is present in normal animals to a limited extent, is inducible only by 3-MC, and is stereoselective and regioselective for 6- and 8-hydroxylation of *R* warfarin.

The differences in regioselectivity displayed by the four enzymes are consistent with the hypothesis that two distinct hydroxylation mechanisms are operative. The first involves the formation of an arene oxide, which subsequently rearranges to the observed products (enzymes B and D, yielding primarily 6-, 8-, and 4′-hydroxylated metabolites). The second involves a direct insertion of oxygen between the carbon–hydrogen bond in either a stepwise or concerted fashion (enzymes A and C, yielding primarily 7, 4′, and benzylic hydroxylated metabolites).

These conclusions are supported in part by electrophoretic evidence [21, 24] and studies on simple aromatic systems [25, 26, 32, 33, 36]. The confirmation of

this hypothesis awaits the purification and characterization of all the catalytically active microsomal mono-oxygenases, and in this regard, the enantiomers of warfarin may serve as useful probes of the stereoselectivity and regiospecificity of such purified enzyme preparations.

Acknowledgements—This research was supported by NIH Training Grant 5-TO1-GM00728 (L. R. P.), the American Foundation for Pharmaceutical Education (L. R. P. and W. R. P.) and Grant HL 13160 (J. W. F.) from the National Heart and Lung Institute. The authors gratefully acknowledge the expert technical assistance of Mr. Robert Bales, Mrs. Wilhelmin B. Lawson and Mrs. Janis Bellantoni.

REFERENCES

1. A. H. Conney, *Pharmac. Rev.* **19,** 317 (1967).
2. R. Kuntzman, *A. Rev. Pharmac.* **9,** 21 (1969).
3. W. Levin, A. Alvares, M. Jacobson and R. Kuntzman, *Biochem. Pharmac.* **18,** 883 (1969).
4. H. Glaumann, *Chem. Biol. Interact.* **2,** 869 (1970).
5. A. H. Conney, A. Y. H. Lu, W. Levin, A. Somogyi, S. West, M. Jacobson, D. Ryan and R. Kuntzman, *Drug Metab. Dispos.* **1,** 199 (1973).
6. G. E. R. Hook, T. C. Orton, J. A. Moore and G. W. Lucier, *Biochem. Pharmac.* **24,** 335 (1975).
7. V. Ullrich, P. Weber and P. Wollenberg, *Biochem. biophys. Res. Commun.* **64,** 808 (1975).
8. M. D. Stonard, *Biochem. Pharmac.* **24,** 1959 (1975).
9. T. C. Pederson and S. D. Aust, *Biochem. Pharmac.* **19,** 2221 (1970).
10. S. D. Aust and J. B. Stevens, *Biochem. Pharmac.* **20,** 1061 (1971).
11. A. Jori and R. Pescador, *Chem. Biol. Interact.* **8,** 297 (1974).
12. J. Werringloer and R. W. Estabrook, *Archs Biochem. Biophys.* **167,** 270 (1975).
13. A. Y. H. Lu and W. Levin, *Biochem. biophys. Res. Commun.* **46,** 1334 (1972).
14. A. Y. H. Lu, S. B. West, D. Ryan and W. Levin, *Drug Metab. Dispos.* **1,** 29 (1973).
15. W. Levin, D. Ryan, S. West and A. Y. H. Lu, *J. biol. Chem.* **249,** 1747 (1974).
16. D. Ryan, A. Y. H. Lu, S. West and W. Levin, *J. biol. Chem.* **250,** 2157 (1975).
17. D. Ryan, A. Y. H. Lu, J. Kawalek, S. B. West and W. Levin, *Biochem. biophys. Res. Commun.* **64,** 1134 (1975).
18. T. A. Van der Hoeven and M. J. Coon, *J. biol. Chem.* **249,** 6302 (1974).
19. T. A. Van der Hoeven, D. A. Haugen and M. J. Coon, *Biochem. biophys. Res. Commun.* **60,** 569 (1974).
20. D. A. Haugen, T. A. Van der Hoeven and M. J. Coon, *J. biol. Chem.* **250,** 3567 (1975).
21. K. Comai and J. L. Gaylor, *J. biol. Chem.* **248,** 2947 (1973).
22. A. F. Welton and S. D. Aust, *Biochem. biophys. Res. Commun.* **56,** 898 (1974).
23. A. P. Alvares and P. Siekevitz, *Biochem. biophys. Res. Commun.* **54,** 923 (1973).
24. W. J. Muna, *Ph.D. Thesis*, University of Washington (1974).
25. H. G. Selander, D. M. Jerina and J. W. Daly, *Archs Biochem. Biophys.* **168,** 309 (1975).
26. M. D. Burke and J. W. Bridges, *Xenobiotica* **5,** 357 (1975).
27. M. Kiese and W. Lenk, *Biochem. Pharmac.* **22,** 2575 (1973).
28. H. E. May, R. Boose and D. J. Reed, *Biochemistry* **14,** 4723 (1975).
29. L. R. Pohl, S. D. Nelson, W. R. Porter, W. F. Trager, M. J. Fasco, F. D. Baker and J. W. Fenton, II, *Biochem. Pharmac.* **25,** 2153 (1976).
30. R. Richterich, in *Clinical Chemistry—Theory and Practice*, p. 245. Academic Press, New York (1969).
31. D. M. Jerina and J. W. Daly, *Science, N.Y.* **185,** 573 (1974).
32. A. Y. H. Lu, W. Levin, H. Selander and D. M. Jerina, *Biochem. biophys. Res. Commun.* **61,** 1348 (1974).
33. J. E. Tomaszewski, D. M. Jerina and J. W. Daly, *Biochemistry* **14,** 2024 (1975).
34. D. M. Johnson and T. C. Bruice, *J. Am. chem. Soc.* **97,** 6901 (1975).
35. L. R. Pohl, S. D. Nelson, W. A. Garland and W. F. Trager, *Biomed. Mass Spectrosc.* **2,** 23 (1975).
36. N. Zampaglione, D. J. Jollow, J. R. Mitchell, B. Stripp, M. Hamrick and J. R. Gillette, *J. Pharmac. exp. Ther.* **187,** 218 (1973).