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CHANGES IN THE METABOLIC PROFILES OF *R*- AND *S*-WARFARIN AND *R*- AND *S*-PHENPROCOUMON AS A PROBE TO CATEGORIZE THE EFFECT OF INDUCING AGENTS ON MICROSOMAL HYDROXYLASES

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Abstract—The biotransformation of *R*- and *S*-warfarin was examined using liver microsomes prepared from both noninduced rats and rats pretreated with Arochlor 1254, β-napthoflavone (BNF), pregnenolone-16α-carbonitrile (PCN), phenobarbital (PB), and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). For comparison, the metabolism of a closely related coumarin anticoagulant, *R*- and *S*-phenprocoumon, was determined using the same microsomal preparations. In noninduced microsomes the overall metabolism of warfarin was about five times that of phenprocoumon, with 7-hydroxywarfarin as the principle metabolite followed by the 6-, 4′- and 8-hydroxy derivatives (benzylic hydroxylation was not examined). For phenprocoumon a different regioselectivity was observed with 4′ hydroxylation being the greatest followed by 6-, and 7 and 8 hydroxylation. In the case of warfarin, hydroxylation with noninduced microsomes was either nonstereoselective (4′ hydroxylation) or selective for the *R*-enantiomer. The metabolic pattern observed for phenprocoumon showed hydroxylation to be either nonstereoselective (7 and 8 hydroxylation) or, in contrast to warfarin, selective for the *S*-enantiomer. Induction of cytochrome P-450 by Arochlor, BNF, and TCDD produced a similar metabolic pattern for both substrates in which 6 and 8 hydroxylation were greatly increased over control levels. In keeping with the pattern obtained from noninduced microsomes, a reversed stereoselectivity was again observed after induction with these three agents, i.e. warfarin metabolism was selective for the *R*-enantiomer and phenprocoumon metabolism was selective for the *S*-enantiomer. Based on cytochrome P-450 levels PCN decreased the metabolism of both substrates while PB had no effect. However, the induction with PB was readily apparent when calculations were performed on a per mg protein basis.

It is now generally accepted that microsomal cytochrome P-450 is not a single enzyme but rather a complex of an unknown number of closely related enzymes whose composition is both qualitatively and quantitatively a function of numerous variables, such as induction states, sex, species, age and nutrition [1–5]. As a consequence, present efforts are focussed on obtaining purified forms of the enzyme, delineating the numbers and metabolic profiles of the various species, and determining the nature of the changes produced by the numerous variables affecting the system.

One approach to examining this system is to take advantage of the various catalytic profiles exhibited by these enzymes in producing multiple products from a single substrate. A substrate that fulfills these conditions is the anticoagulant warfarin (**1**). Changes in the regioselective and stereoselective metabolism of the enantiomers of this drug have been utilized by a number of workers [6–11] to examine P-450 in various states of purity, from different sources and various states of induction.

Recently we examined the aromatic oxidative

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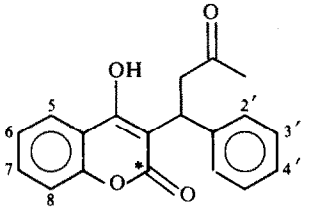

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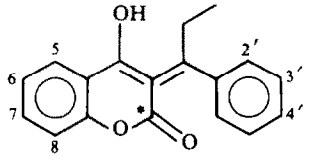

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Table 1. Rates of metabolite formation from the *R*- and *S*-enantiomers of warfarin and phenprocoumon with liver microsomes from noninduced, PB and PCN induced Sprague–Dawley rats

	Product formed from rat liver microsomes* [pmoles·(nmole cytochrome P-450)$^{-1}$·min·$^{-1}$]					
	Noninduced microsomes		PB induced		PCN induced	
	R	*S*	*R*	*S*	*R*	*S*
Warfarin metabolites						
4′-Hydroxywarfarin	49.0 ± 6.6	43.6 ± 6.2	45.9 ± 5.8	17.3 ± 3.6	31.5 ± 4.0	30.7 ± 4.0
6-Hydroxywarfarin	76.4 ± 9.4	39.9 ± 5.3	62.2 ± 7.4	27.2 ± 3.9	46.4 ± 5.2	49.9 ± 5.6
7-Hydroxywarfarin	162.5 ± 19.2	40.6 ± 5.4	191.9 ± 20.0	45.9 ± 5.4	73.0 ± 7.8	16.8 ± 2.2
8-Hydroxywarfarin	43.6 ± 5.7	13.2 ± 2.2	41.1 ± 5.1	8.4 ± 1.57	19.3 ± 2.5	7.3 ± 1.3
Total	331.5	137.3	341.1	98.8	170.2	104.7
R/S	2.4		3.5		1.63	
Phenprocoumon metabolites						
4′Hydroxyphenprocoumon	16.5 ± 2.4	29.8 ± 4.1	11.2 ± 1.5	8.9 ± 1.2	11.2 ± 1.8	10.1 ± 1.64
6-Hydroxyphenprocoumon	7.3 ± 1.2	19.9 ± 2.7	4.7 ± 0.9	14.9 ± 2.0	6.3 ± 1.0	5.2 ± 0.9
7-Hydroxyphenprocoumon	4.4 ± 1.0	4.1 ± 1.2	5.0 ± 0.8	5.1 ± 0.8	2.0 ± 0.5	2.1 ± 0.5
8-Hydroxyphenprocoumon	2.3 ± 1.3	3.5 ± 1.3	2.6 ± 0.6	3.4 ± 0.7	0.0 ± 0.6	0.0 ± 0.6
Total	30.5	57.3	23.5	32.3	19.5	17.4
R/S	0.53		0.73		1.12	

* Data are expressed as the means ± S.E. and represent three replicates.

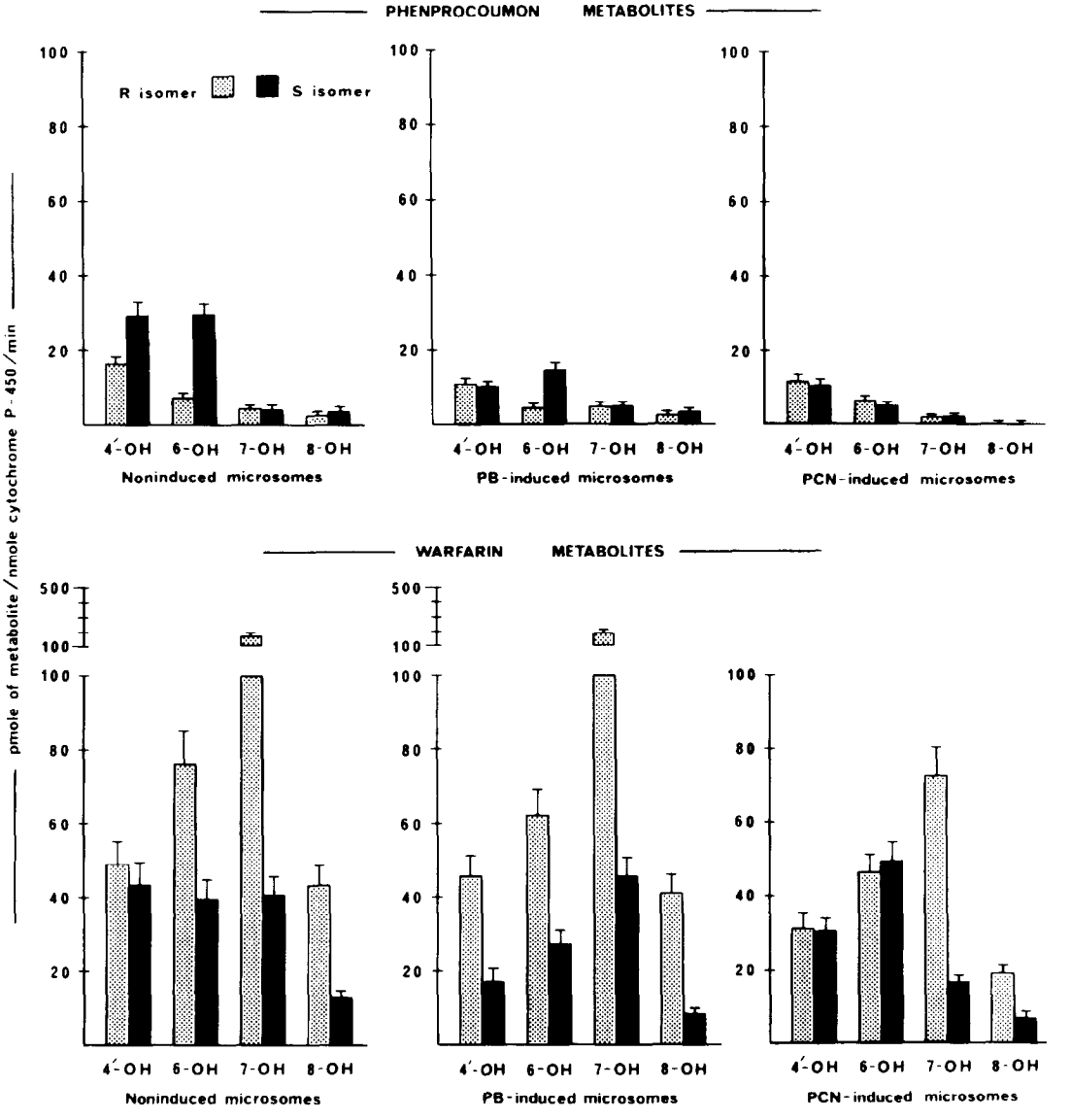

Fig. 1. Graphical presentation of the data in Table 1.

metabolism of a closely related coumarin anticoagulant, phenprocoumon (**2**), by noninduced rat liver microsomes [12]. Major differences were found not only in the general level of metabolism but also in the regio- and stereoselective patterns of the two drugs. Warfarin is metabolized primarily to the 7-hydroxy metabolite followed by the 6-, benzylic-, 4′-, and 8-hydroxy metabolites [6]. The formation of all the metabolites except the 4′-hydroxy derivative is selective for the *R*-enantiomer of warfarin. Conversely, phenprocoumon is metabolized under similar conditions to about one-fifth the level of warfarin and yields the 4′- and 6-hydroxy metabolites as the major metabolic products, while 7- and 8-hydroxyphenprocoumon are of minor importance [12]. Moreover, the formation of the 4′- and 6-hydroxy metabolites is selective for the *S*-enantiomer while the formation of the 7- and 8-hydroxy metabolites is apparently nonstereoselective. These major differences are suprising in view of the close structural similarity between the two substrates. In order to investigate the scope of the differences, we studied the metabolism of the two drugs using microsomes obtained from the Sprague–Dawley rats which had been treated with various inducing agents.

MATERIALS AND METHODS

Materials. β-Naphthoflavone (BNF) was obtained from the Aldrich Chemical Co. (Milwaukee, WI), and phenobarbital (PB) (sodium salt) was obtained from Eli Lilly & Co. (Indianapolis, IN). Pregnenolone-16α-carbonitrile (PCN) was a gift of Roger Bergstrom (Manager, Research Services, Searle Laboratories, Chicago, IL), Arochlor 1254 was a gift of Dr. Sid Nelson (Department of Medicinal Chemistry, University of Washington, Seattle, WA), and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was a gift of Dr. Richard Peterson (School of Pharmacy, University of Wisconsin, Madison, WI). *R*- and *S*-[2-^{14}C]Warfarin and the 4′-, 6-, 7- and 8-hydroxy standards were obtained as previously described [6]. The radiolabeled enantiomers of phenprocoumon and aromatic hydroxy metabolite standards were obtained or synthesized as described previously [12]. All other materials used for this study were obtained as described [12].

Tissue preparation. Male Sprague–Dawley rats (160–200 g, Tyler Labs, Bellevue, WA) were used throughout this study and were allowed food and water *ad lib*. Induction of microsomal cytochrome P-450 was achieved as follows: PB (80 mg/kg at a concentration of 80 mg/ml, i.p., in physiological saline, once daily for 3 days, animals killed on the following day), BNF (80 mg/kg at a concentration of 25 mg/ml, i.p., in corn oil, once daily for 3 days, animals killed on the following day), PCN (75 mg/kg, at a concentration of 30 mg/ml, i.p., suspended in distilled water with 1 drop of Tween-80/ml water, injections every 12 hr for 4 days, animals killed on the following day), Arochlor 1254 (500 mg/kg at a concentration of 200 mg/ml, i.p., in corn oil, one injection, animals killed 6 days later), TCDD (10 μg/kg, at a concentration of 10 μg/ml, i.p., in corn oil, one injection, animals killed 7 days later). Control animals received comparable doses of saline or corn oil. At the appropriate time, groups of three similarly treated animals were decapitated and microsomes were prepared [12].

Incubations and isolation procedures. Incubations with phenprocoumon were carried out as described [12] with NADPH as a source of reducing equivalents. Warfarin incubations were carried out in a similar manner in a total volume of 2 ml. Metabolite extraction and quantitation were as previously described [12] with the following exception. The t.l.c solvent used for the resolution of warfarin metabolites in the first dimension was $CHCl_3$ with 3% AcOH (three developments). Recovery of warfarin after t.l.c analysis was greater then 95 per cent.

Protein and cytochrome determinations. Protein determinations were performed with a Gilford Stasar II spectrophotometer according to the method previously described [6]. Cytochrome P-450 determinations were performed using an Aminco DW-2 spectrophotometer according to the method of Omura and Sato [13].

Statistical analysis. Treatments were applied to groups of three rats, with three incubations carried out for each inducer–isomer combination. Four aromatic hydroxy metabolites were measured for each replicate in a treatment. Benzylic-hydroxywarfarin, although separable by t.l.c., was not analyzed due to lack of a synthetic standard. Although the various experiments were carried out over a period of several months, similar experiments with warfarin and phenprocoumon were performed on the same day with aliquots from the same microsomal preparation. Complete incubations (minus NADPH, three replicates) served as background controls for each treatment–isomer combination. Since this study indicated that variations between the pooled enzyme activities of groups of three rats were not significant, the results presented here are based on a single set of studies.

The data were analyzed according to a nested factorial plan as described previously [12].

RESULTS

In preliminary studies it was determined that metabolite production from both warfarin and phenprocoumon was linear over a 10-min period at the microsomal protein concentration (1 mg/ml) and substrate concentrations used (warfarin, 750 μM; phenprocoumon, 300 μM). The *R*-isomer of warfarin was metabolized to a significantly greater extent than the *S*-enantiomer in almost all induction states (Figs. 1 and 2, Tables 1 and 2). With noninduced microsomes, 7-hydroxywarfarin was the major product followed by the 6-, 4′- and 8-hydroxy derivatives (Fig. 1, Table 1).

In comparison to the rate of warfarin metabolite formation by noninduced microsomes, PB induction caused little significant change except for the formation of *S*-4′-hydroxywarfarin when metabolite production was calculated on a per nmole cytochrome P-450 basis, (Fig. 1, Table 1). Induction with the steroidal inducer, PCN, actually resulted in a decrease in the rate of warfarin metabolite formation (when compared to noninduced microsomes). For example, the rate of 7-hydroxywarfarin formation was only about 50 percent of the rate observed with

noninduced microsomes (Fig. 1, Table 1). On the other hand, induction by Arochlor 1254, BNF and TCDD, while causing a 55 percent decrease in the formation of 7-hydroxywarfarin, resulted in a dramatic increase in the rate of 6- and 8-hydroxywarfarin formation [i.e. a 1.7- to 18-fold increase over the levels obtained with microsomes from noninduced rats for the (*R*)-isomer] (Fig. 2, Table 2).

Phenprocoumon incubations with noninduced microsomes show a regioselective pattern different from that obtained with warfarin (Fig. 1, Table 1). The 4′-hydroxy derivative is the major product, followed in decreasing order by the 6-, 7- and 8-hydroxy metabolites. In addition, with noninduced microsomes, phenprocoumon was metabolized to a smaller extent than warfarin. Induction by Arochlor, BNF and TCDD resulted in a huge increase in the production of 6- and 8-hydroxyphenprocoumon with both enantiomers to total levels which in several cases surpassed those seen with warfarin (Fig. 2, Table 2). Of these four phenprocoumon metabolites, *S*-6-hydroxyphenprocoumon was the principle metabolite. This was in contrast to warfarin metabolism in which the level of *R*-8-hydroxywarfarin was greater than the level of *R*-6-hydroxywarfarin. Similar to warfarin, little difference was seen when the metabolism of phenprocoumon by PB induced microsomes was compared to that obtained with noninduced microsomes (Fig. 1, Table1). In general, PCN induction also acted to decrease phenprocoumon metabolism.

In terms of stereoselectivity, the enzymes were either nonstereoselective or selective for the *R*-enantiomer of warfarin while the reverse occurred with phenprocoumon. For phenprocoumon the enzymes were either nonstereoselective or selective for the *S*-enantiomer.

DISCUSSION

The results obtained for warfarin metabolism using normal and PB induced rat liver microsomes agree well with results obtained earlier in this laboratory, with the exception of the stereoselectivity of 4′ hydroxylation. Earlier results showed the reaction to be stereoselective for the *S*-enantiomer in noninduced (1.7/1), PB (1.3/1), and 3-methylcholanthrene (3-MC, 1.7/1) induced microsomes [7], whereas our current results along with those of others [10], showed the reaction to be either nonstereoselective or selective for the *R*-enantiomer (*R*/*S*, 2/1). The reason for the apparent discrepancy is not known.

Compared to warfarin the overall metabolism of phenprocoumon was about one-fifth as great, except in Arochlor, BNF or TCDD induced microsomes where it was equal to or greater than that seen with warfarin. With microsomes induced by these three agents phenprocoumon, like warfarin, is metabolized primarily to the 6- and 8-hydroxy derivatives. However, unlike warfarin, 6 and 8 hydroxylation were now highly selective for the *S*-enantiomer. A further difference was apparent when the relative amounts of the 6- and 8-hydroxy metabolite were compared. With warfarin 8 hydroxylation was greater than 6 hydroxylation while the opposite was true with phenprocoumon.

Warfarin and phenprocoumon appear to be useful in classifying at least several different inducing agents by virtue of the metabolic profiles that were produced. Clearly large changes in metabolite levels as a function of induction were the most useful. The large induction of 6 and 8 hydroxylation by Arochlor, BNF and TCDD resembles the pattern seen with 3-MC [7–10], and indeed these four compounds seem to behave similarly in their induction of AHH activity as measured by the hydroxylation of benzo[*a*]pyrene [14–17]. PCN and PB, on the other hand, produced smaller changes. PB induction did not produce a discernable effect other than in the stereoselectivity of 4′ hydroxylation when metabolite levels were calculated on a per nmole cytochrome P-450 basis. If, however, metabolite levels were calculated on a per mg protein basis, an increase following induction was statistically significant, particularly in the case of warfarin.

We conclude that PB induction more or less uni-

Table 2. Rates of metabolite formation from the *R*- and *S*-enantiomers of warfarin and phenprocoumon with liver microsomes from Arochlor 1254, BNF and TCDD induced Sprague–Dawley rats

	Product formed from rat liver microsomes* [pmoles · (nmole cytochrome P-450)$^{-1}$ · min^{-1}]					
	Arochlor induced		BNF induced		TCDD induced	
	R	*S*	*R*	*S*	*R*	*S*
Warfarin metabolites						
4′Hydroxywarfarin	22.5 ± 2.5	11.2 ± 1.5	19.8 ± 2.6	12.1 ± 1.8	13.4 ± 8.2	22.4 ± 13.0
6-Hydroxywarfarin	135.3 ± 13.8	43.5 ± 4.6	471.2 ± 47.6	192.0 ± 19.7	190.0 ± 26.1	82.6 ± 13.5
7-Hydroxywarfarin	45.1 ± 4.7	13.8 ± 4.1	100.7 ± 10.4	36.5 ± 4.1	53.2 ± 7.6	21.6 ± 4.2
8-Hydroxywarfarin	202.1 ± 20.4	14.8 ± 1.8	795.4 ± 80.0	64.9 ± 7.0	344.8 ± 43.5	35.6 ± 5.5
Total	405.0	83.3	1387.1	305.5	601.4	162.2
R/*S*	4.9		4.5		3.7	
Phenprocoumon metabolites						
4′-Hydroxyphenprocoumon	9.2 ± 1.3	22.4 ± 2.9	20.6 ± 2.9	53.7 ± 4.5	7.2 ± 1.1	5.9 ± 1.2
6-Hydroxyphenprocoumon	69.9 ± 8.2	367.2 ± 42.3	144.0 ± 16.9	904.6 ± 104.3	67.4 ± 7.1	416.5 ± 43.1
7-Hydroxyphenprocoumon	13.4 ± 1.6	57.9 ± 6.8	25.4 ± 3.2	117.2 ± 11.9	12.6 ± 1.4	63.5 ± 6.8
8-Hydroxyphenprocoumon	50.1 ± 6.1	94.0 ± 11.1	89.1 ± 11.1	220.2 ± 26.0	44.2 ± 5.3	98.6 ± 10.9
Total	142.6	541.5	279.1	1295.7	131.4	584.5
R/*S*	0.3		0.2		0.2	

* Data are expressed as the means ± S.D. and represent three replicates.

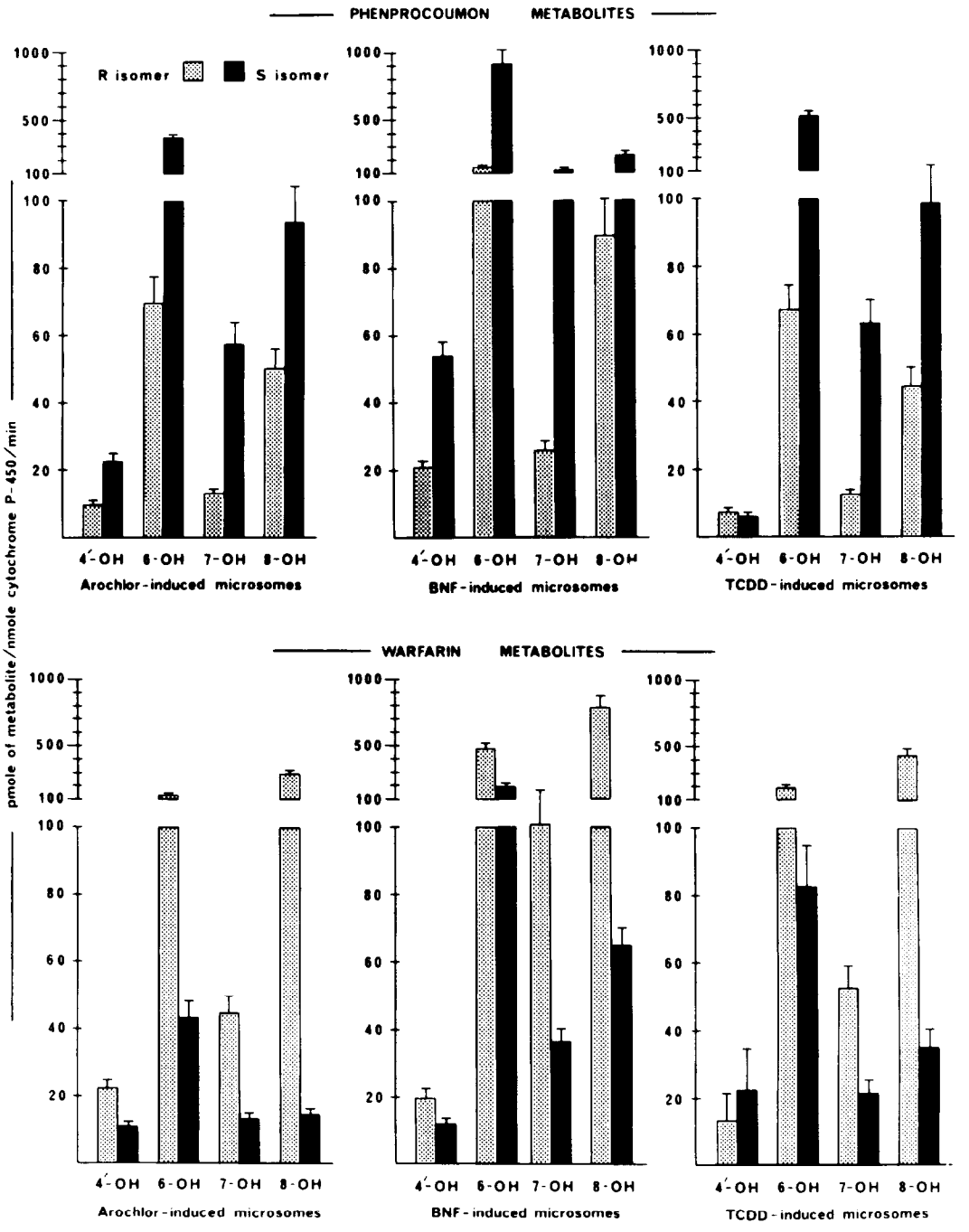

Fig. 2. Graphical presentation of the data in Table 2.

formly increased the levels of all the enzymes responsible for the aromatic hydroxylation of the two substrates. When the levels of the various metabolites (from the two states of induction; noninduced and PB induced) were normalized with respect to nmoles of cytochrome P-450, the two states were statistically indistinguishable. Arochlor 1254 is a mixture of various polychlorinated biphenyls, some of which appear to have a PB type of induction, as based on increases in ethylmorphine and aminopyrene *N*-demethylase activity [15, 18], but the component of PB induction arising from Arochlor pretreatment was not detectable with warfarin no matter how the calculations were made.

PCN treatment appears to have significantly and uniformly diminished the metabolism of both substrates. This result occurred despite the fact the cytochrome P-450 content per mg protein increased (0.95 vs 0.61 nmole cytochrome P-450/mgprotein in noninduced microsomes).

Previous studies with PCN have shown a large increase in ethylmorphine *N*-demethylase activity

after induction [19, 20]. This is an example of aliphatic hydroxylation, whereas the products of phenprocoumon and warfarin metabolism considered in this study result from aromatic hydroxylation. The decreased metabolism of both substrates can be explained either in terms of induction of a form(s) of cytochrome P-450 showing a preference for aliphatic hydroxylation or in terms of inhibition due to residual PCN. This latter possibility is unlikely in view of the levels of metabolism observed with other substrates after PCN induction [19, 20].

In an earlier paper, reversed stereoselectivity was observed for metabolite formation from phenprocoumon and warfarin with 3-MC microsomes, and a possible explanation, based on different preferred conformations, was advanced [21]. In this study, the results for the two substrates show that the same stereochemical trend was maintained after various inducing treatments and in some cases was very pronounced. For example, after induction with BNF, 6 hydroxylation of *S*-phenprocoumon was increased 45-fold and 8 hydroxylation was increased 62-fold. The formation of both metabolites was highly selective for the *S*-enantiomer with *S*/*R* ratios of ~6.0 and ~2.5 respectively. When the same microsomal preparation was used to catalyze the hydroxylation of *R*-warfarin, the 6 and 8 hydroxylations were again the major processes induced; 6 hydroxylation increased ~10-fold and 8 hydroxylation increased ~16-fold. The stereoselectivity for the 6 and 8 hydroxylation processes was opposite that observed for phenprocoumon, with *R*/*S* ratios of ~2.5 and ~12.0 being observed respectively. Although an explanation based on solution conformation may account for the reversed stereoselectivity, it is realized that other differences in the metabolism of warfarin and phenprocoumon cannot be accounted for solely by this explanation.

The most obvious difference in terms of regioselectivity was seen in 7 hydroxylation. In noninduced microsomes, 7 hydroxylation of warfarin was the major metabolic transformation of this substrate while 7 hydroxylation occurred to an almost insignificant extent with phenprocoumon. Earlier studies indicated that 7-hydroxywarfarin, produced from noninduced and PB induced microsomes, probably does not arise through an arene oxide intermediate but is formed by an enzyme different from those responsible for 6 and 8 hydroxylation [6, 7]. Kaminsky *et al.* [11] have recently reported immunological evidence which further supports this hypothesis. Thus, if true, phenprocoumon is a very poor substrate for this enzyme.

Hydroxylation at the 7 position did increase when microsomes obtained after Arochlor, BNF, and TCDD induction were used to catalyze the oxidation of phenprocoumon. Under the circumstances, however, the increase may have resulted from the induction of 6 and 8 hydroxylase activity. The 6 and 8 hydroxyphenprocoumon metabolites presumably arose from the 6-7 and 7-8 epoxides respectively. If a small percentage of these epoxides open to yield the 7-hydroxy metabolite then this pathway would be minor when 6 and 8 hydroxylation were minor but would increase when the latter were induced. Indeed, this is the pattern that was seen.

Another difference that must be accounted for is the 5-fold difference in rate of hydroxylation observed for the two drugs in noninduced microsomes. The reason for this difference is not apparent but may be related to differences in the physical–chemical properties of the two substrates, e.g. differences in pK_a, differences in binding resulting from the ketonic side chain of warfarin, etc. [21, 22].

In conclusion, it would appear that the changes in regioselectivity, and stereoselectivity, particularly when coupled with the use of structurally modified substrates, can provide a powerful tool for probing the nature and multiplicity of microsomal cytochrome P-450.

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