

STEREOCHEMICAL ASPECTS OF THE METABOLISM OF PHENPROCOUMON IN RAT LIVER MICROSOMES

CONRAD WHEELER and WILLIAM F. TRAGER*

Departments of Chemistry and Medicinal Chemistry, University of Washington, Seattle, WA 98195, U.S.A.

and

WILLIAM R. PORTER

School of Pharmacy, University of Wisconsin, Madison, WI 53706, U.S.A.

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Abstract—The oxidation of the enantiomers of phenprocoumon catalyzed by noninduced rat hepatic microsomes was determined, and the formation of products was found to be either nonstereoselective or selective for the (S)-enantiomer. Of the seven possible aromatic monohydroxylated metabolites, only the 4'-, 6-, 7- and 8-hydroxy derivatives were detected. These hydroxylation reactions were shown to be cytochrome P-450-dependent by decreased product formation in the presence of the inhibitors CO, metyrapone and SKF-525A (β -diethylaminoethyl 2,2-diphenylvalerate) and by decreased product formation when NADH was substituted for NADPH. Comparison of these results to those obtained earlier for warfarin reveals that major regioselective and stereoselective differences exist in the *in vitro* metabolism of these two similar coumarin anticoagulants.

Warfarin [4-hydroxy-3-(3-oxo-1-phenylbutyl)-2H-1-benzopyran-2-one], **1**, and phenprocoumon [4-hydroxy-3-(1-phenylpropyl)-2H-1-benzopyran-2-one], **2**, are potent anticoagulants that are currently in use in clinical medicine. Although the drugs are structurally quite similar and produce their effects by the same mechanism (i.e. competitive inhibition of the synthesis of specific vitamin K-dependent clotting factors) [1-4], major differences exist in their pharmacological properties. For example, **2** is significantly more potent than **1** [5], has a much longer biological half-life [5], is metabolized to a much lesser extent in the rat *in vivo* [6], and has a different oxidative metabolic profile [6]. The reasons for these differences are at present obscure.

In previous studies on the *in vitro* metabolism of **1** by noninduced rat liver microsomes, it was demonstrated that (1) the formation of four aromatic hydroxy metabolites (4'-, 6-, 7- and 8-hydroxy-warfarin) is mediated by cytochrome P-450 [7, 8]; (2) the oxidative metabolic pattern obtained *in vitro* [9] is similar to that obtained *in vivo* [10]; and (3) the patterns obtained are a function of the stereochemistry of the substrate, state of induction, and presence of inhibitors [9, 11-13].

Similar to **1**, **2** is metabolized *in vivo* to the 4'-, 6-, 7- and 8-hydroxy metabolites [10]; however, major differences exist in the relative amounts of the metabolites that are obtained [6]. For **1**, 7-hydroxylation is the major metabolic transformation observed, followed by 6-hydroxylation and to a much lesser extent 4'- and 8-hydroxylation [10]. For **2**,

7- and 8-hydroxylation are of minor importance only, whereas 4'- and 6-hydroxylation are of major importance [6]. To determine if the differences between the metabolic profiles of warfarin and phenprocoumon observed *in vivo* are mirrored *in vitro*, we have studied the metabolism of (R)- and (S)-**2** catalyzed by rat liver microsomes.

MATERIALS AND METHODS

The radiolabeled substrates of (-)-(S)-[2-¹⁴C]-**2** (1.23 mCi/mmol) and (+)-(R)-[2-¹⁴C]-**2** (1.29 mCi/mmol) were prepared as described [14] and were greater than 98 per cent optically pure. The metabolite standards, 2'-, 3'-, 4'-, 5-, 6-, 7- and 8-hydroxy-phenprocoumon were gifts from Dr. Lance Pohl [15]. Stock solutions of the radiolabeled compounds were prepared as follows: (-)-(S)-[2-¹⁴C]-**2** (50 mg, 0.018 mmol) was dissolved in a solution comprised of 0.1 N NaOH (20 ml) and 1.15% KCl in 0.01 M phosphate buffer (pH 7.5, 30 ml). This was repeated with the opposite enantiomer. These mixtures were frozen and stored until use. A standard solution of all the potential aromatic hydroxy metabolites was prepared by mixing 2 mg of each of the metabolites (2'-, 3'-, 4'-, 5-, 6-, 7- and 8-hydroxy-**2**) together in 1 ml of acetone. This solution was stored at 0° until use in preliminary microsomal experiments to check the spectrum of metabolites via autoradiography. The experimental results indicated that the potential metabolites 2'-, 3'- and 5-hydroxy-**2** were not present in measurable quantities. Therefore, all subsequent experiments were conducted using a standard mixing containing 4'-, 6-, 7- and 8-hydroxy-**2**.

NADPH (tetrasodium salt, type X), NADH (dipotassium salt) and bovine serum albumin were

* Author to whom correspondence should be addressed: William F. Trager, Department of Medicinal Chemistry, BG-20, School of Pharmacy, University of Washington, Seattle, WA 98195, U.S.A.

purchased from the Sigma Chemical Co (St. Louis, MO). SKF-525A (β -diethylaminoethyl 2,2-diphenylvalerate) was supplied by Smith Kline & French Laboratories (Philadelphia, PA), and metyrapone was provided by Dr. Mout Juchau (Department of Pharmacology, University of Washington). The animals used in this study were male Sprague-Dawley rats (140–160 g) from Tyler Laboratories (Bellevue, WA) and were given Purina Rat Chow and water *ad lib*. Homogenization was performed in a Potter-Elvehjem glass tube with a Teflon pestle having a 0.10 to 0.15 mm clearance. Centrifugation was carried out in polycarbonate tubes (in a 42.1 rotor head) in a Beckman L2-65B refrigerated ultracentrifuge. Protein concentrations were determined by the modified Lowry method as described previously [9] on a Gilford Stasar II spectrophotometer. P-450 determinations were performed using an Aminco (American Instrument Co., Silver Springs, MD), DW-2 spectrophotometer according to the method of Omura and Sato [16]. Incubations were carried out in 20-ml scintillation vials or (for CO inhibition studies) in 20-ml vials equipped with rubber septa in an American Optical model 2156 shaker with a water bath held constant at $37 \pm 0.5^\circ$. Reagent gases (oxygen, nitrogen and carbon monoxide) were purchased from Matheson (Cincinnati, OH). Thin-layer chromatographic separations were accomplished using silica gel 60 plates (E. Merck reagents, No. 5539, 20 cm \times 20 cm), and metabolite loci were visualized by u.v. light. Radioactivity determinations were made with a Beckman LS-230 liquid scintillation counter in 10 ml of Aquasol (New England Nuclear Corp., Boston, MA). An external ^{14}C counting standard was used to determine counting efficiency. For autoradiography Kodak No-Screen X-ray film was used. All solvents and common chemicals were reagent grade and were purchased from commercial suppliers.

Microsomes were prepared from groups of three rats for each experiment reported in Tables 2, 3 and 4 as described [9], except for the following modifications. A single 60-min centrifugation at 105,000 g was carried out and cold 0.05 M phosphate buffer was used to suspend the pellet. Protein determinations were made as described previously [9].

Incubations—general procedure. Incubations were carried out in a total volume of 4 ml of phosphate buffer (50 mM, pH 7.4), and contained (R)- or (S)-[2- ^{14}C]-2 (1.2 μ moles) and microsomes (4 mg protein). Inhibitors were added as described below. The mixtures were incubated for 1 min at 37° with shaking (90–100 oscillations/min), followed by the addition of NADPH (4 μ moles) to initiate reaction. Incubations on the shaker continued for 10 min before the reactions were terminated by the addition of ethyl acetate–diethyl ether (1:1, 10 ml). A standard metabolite mixture (15 μ l) was added, and the incubations were then stored at -20° . In each set of experiments, background values for the metabolite loci were obtained from incubations lacking NADPH or incubations containing boiled microsomes.

Cofactor requirement and inhibition studies. All cofactor and inhibition studies were performed in an air atmosphere. An equimolar amount of NADH was used to replace NADPH in a set of incubations

conducted to probe the dependence of the enzyme system on the source of reducing equivalents. The inhibitors SKF-525A and metyrapone were each dissolved in 2.0 ml of 1% HCl and an aliquot of these solutions (100 μ l) was added to the incubation mixture. Both inhibitors were used at a final concentration of 500 μ M, and the reaction was initiated by either NADPH or NADH.

CO inhibition studies. Experiments utilizing a closed CO–O₂ atmosphere were conducted as follows. All components of the incubation mixture except NADPH were combined in open vials (20 ml) on ice. A rubber septum was added and the atmosphere of the vial was evacuated by means of a water aspirator. The vial was then filled with an atmosphere of N₂ and the process was repeated at least six more times. A premixed 4:1 CO:O₂ atmosphere was used in the final replacement. To ensure equilibration of the atmosphere with the solution the vials were left on ice for 5 min with occasional gently swirling, after which time they were transferred to the constant temperature shaker for another 2 min. The reactions were then initiated by the addition of an NADPH solution by syringe.

All reactions were allowed to continue for 10 min, shaking at a rate of 90–100 oscillations/min, and all were terminated by the addition of ethyl acetate–diethyl ether (1:1, 10 ml).

To allow a quantitative assessment of inhibition an equal number of incubations were conducted simultaneously in air for comparison.

Extraction and quantitation of metabolites. The frozen incubation vials were removed from the freezer and thawed, and 10% HCl (0.5 ml) was added. Following a 10-min shaking interval on the shaker the vials were immersed in a dry ice/acetone bath until the aqueous layer froze. The organic layer was decanted into a test tube (15 ml) and the solvent was removed under a stream of nitrogen. A fresh 10-ml aliquot of ethyl acetate–diethyl ether (1:1) was then added to the vials. The aqueous phase was allowed to thaw and the extraction process described above was repeated twice more. In each successive extraction the organic layer was added to the initial tube and evaporated. The residue in each of the test tubes was transferred to a vial (4 ml) by successive washings and evaporated under nitrogen. The samples were stored in a freezer until t.l.c. analysis.

For t.l.c. analysis the residues in the vials were dissolved in acetone–ether (1:1, 4 \times 200 μ l) and spotted in portions on a t.l.c. plate in a 1 by 2 cm square at the lower left-hand corner of the plate. One incubation extract was spotted per plate. The plates were eluted 2 cm with methanol (to concentrate the spots into a fine line), dried, re-eluted with methanol to the same point, dried, and developed in 1,2-dichloroethane containing 4% acetic acid. The plates were dried and redeveloped with the same solvent. After the second development in the first direction the plates were rotated 90° and the bands were concentrated into a line with methanol. A solution of phenprocoumon (5 mg/ml in acetone, 75 μ l) was then spotted on the plate below the development line. The phenprocoumon was moved up to the metabolite line with methanol. The plates were developed in the second dimension in a system

composed of *tert.*-butanol–benzene–NH₄OH–water (45:20:9:3). Parent compound and metabolite loci were located under u.v. light and circled in pencil.

For initial autoradiography experiments, the t.l.c. plates were placed in contact with X-ray film for 4–5 days and the film was developed according to the instructions of the manufacturer.

For experiments involving quantitation of metabolites, the loci were immediately cut out with scissors and added to scintillation vials containing Aquasol (10 ml). The spot containing unchanged **2** was also removed and the drug was extracted with methanol (5 ml). An aliquot (0.5 ml) was transferred to a scintillation vial, mixed with Aquasol (10 ml), and counted. The vials were counted for 10 min or until a 2 per cent counting error was reached.

To assess the degree of radioactivity recovered by these procedures, the extracted microsomal incubations, test tubes and vials were checked by scintillation counting.

Cold carrier experiments. A set of sixteen microsomal incubations of **2** was initiated with NADPH in the manner already described. At the end of the 10-min incubation period, the reactions were terminated and the flasks, were divided into four groups. To each flask in a given group was added one of the following amounts of cold standard metabolite solution: 0 μ l, 5 μ l, 10 μ l or 15 μ l. The

metabolites were extracted and applied to t.l.c. plates as described previously. Standard metabolite solution (5 μ l) was spotted on the four plates that received the microsomal extract from the flasks to which no standard had been added. After development the metabolites were quantitated by scintillation counting.

Statistics. Nested factorial analysis of variance [17] was utilized after conversion of the data to logarithms and after determination that the variance was homogenous as determined by Bartlett's Test [18].

The hypothesis that two means for a single metabolite produced under two sets of conditions were significantly different at the 99 per cent confidence level was tested. The following comparisons between treatments means were made: (1) minus NADPH—versus boiled microsomes, versus plus NADPH, versus plus NADPH, and versus each inhibitor. (2) plus NADPH—versus plus NADH and versus each inhibitor. The results are presented in Tables 2, 3 and 4.

RESULTS

A two-dimensional t.l.c. system allowed complete resolution of all potential aromatic monohydroxylated metabolites of **2**, as shown in Table 1.

Table 1. *R_f* values of phenprocoumon (Phen.) and potential aromatic monohydroxylated metabolites after two-dimensional development on t.l.c. plates

	Phen.	<i>R_f</i> values						
		Position of hydroxylation						
		5	6	7	8	2'	3'	4'
First dimension*	0.72	0.36	0.28	0.30	0.56	0.60	0.47	0.39
Second dimension†	0.67	0.48	0.42	0.30	0.33	0.40	0.43	0.37

* The plates were developed twice in 1,2-dichloroethane containing 4% acetic acid.

† The plates were developed once in a solvent system composed of *tert.*-butanol–benzene–NH₄OH–water (45:20:9:3).

Table 2. Phenprocoumon metabolism: incubations conducted in air or in a CO:O₂ (4:1) atmosphere

Metabolite produced* [pmoles · (nmole cytochrome P-450) ⁻¹ · min ⁻¹]					
Treatment	Isomer	4'	Position of hydroxylation		
			6	7	8
+ NADPH (air)†	R	21.5 ± 2.9‡	10.7 ± 1.8‡	4.4 ± 0.9‡	2.3 ± 1.8
Normal control	S	28.7 ± 3.5‡	17.1 ± 2.3‡	4.1 ± 1.0‡	3.5 ± 1.1
+ NADPH	R	6.1 ± 1.3‡§	3.7 ± 1.0‡§	2.3 ± 0.7‡§	1.3 ± 1.1
(CO:O ₂ atm)	S	12.3 ± 2.0‡§	5.7 ± 1.1‡§	1.6 ± 0.8§	2.1 ± 1.0
– NADPH (air)	R	6.5 ± 0.4	6.2 ± 0.4	4.1 ± 0.2	9.0 ± 0.5
Background control	S	6.8 ± 0.4	4.8 ± 0.3	6.0 ± 0.4	7.6 ± 0.5

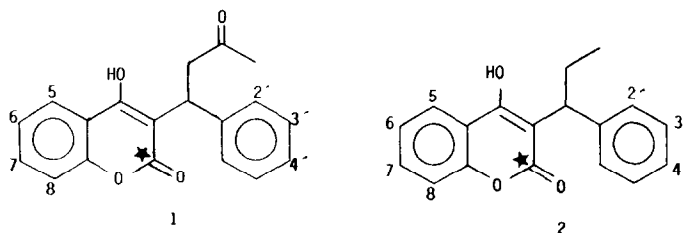
* Data are the means ± S.E. of three incubations. Statistical comparisons were made between two treatment means for a given metabolite of the same stereochemistry. Differences between two levels that are significant at the 99 per cent confidence level are denoted by the ‡ and § symbols (see footnotes below).

† Values for the first two treatments represent *net* metabolite levels (i.e. total amount minus background control).

‡ Significant difference from the background control data.

§ Significant difference from the normal control data.

|| Values for the last treatment are the background controls.



Qualitative results from autoradiography and quantitative results from scintillation counting established the identity of the metabolites produced from (R)- and (S)-**2** by noninduced rat liver microsomes as the 4'-, 6-, 7- and 8-hydroxy derivatives. None of the other possible aromatic monohydroxylated products could be detected, although several other minor (less than 10 per cent of total identified metabolites) unidentified radioactive spots were apparent. Maximum recovery of the metabolites from t.l.c. plates was obtained by using 15 μ l of a standard solution containing all the metabolites of **2** (2 mg/ml of each) as a cold carrier.

Monitoring of parent compound recovery from early experiments showed a loss of 35–40 per cent. This loss was not due to either the extraction procedure or the transfer of the extract up to the point of t.l.c. analysis, since less than 1 per cent of the total radioactivity was left behind in the extracted mixture, test tube and vial. Phenprocoumon is known to be unstable on t.l.c. plates [19–21]; therefore, the stability of **2** and its four metabolites was determined in our t.l.c. systems. The recovery of [14 C]-**2** after two developments (\sim 3.5 hr on the plate) was 90–95 per cent. These results were determined via scintillation counting. With nonlabeled **2** the recovery determined via u.v. spectroscopy was found to be 68–72 per cent (acidic system) and 97–100 per cent (basic system). The recovery of either labeled or nonlabeled **2** from t.l.c. plates after development was essentially the same and indicated

that the loss occurred during the development in the acidic system. Similar results were obtained when **2** was recovered from incubation mixtures.

Since the above experiments with **2** showed close agreement between the two methods of quantitation, and since 14 C-labeled metabolites were not available, metabolite recoveries were determined spectrophotometrically using cold standards. The recovery of the metabolites from the acidic and basic solvent systems was: 4'-hydroxy-**2** 88 and 98 per cent, 6-hydroxy-**2** 67 and 90 per cent; 7-hydroxy-**2** 51 and 83 per cent; and 8-hydroxy-**2** 80 and 92 per cent respectively. Replicate determinations for any given metabolite did not vary by more than 2 per cent.

Incubations with boiled microsomes or incubations omitting NADPH served as background controls. Statistical comparisons between these two treatments for a given metabolite of the same stereochemistry showed no significant differences. Comparison of data obtained from incubations containing active microsomes plus NADPH (normal controls) with the background control data showed significant production of the 4'-, 6-, 7- and 8-hydroxy metabolites in all experiments except that reported in Table 2. In this experiment the amount of the 8-hydroxy metabolite formed from (R)-**2** was not significantly greater than the background at the 99 per cent level. In general, hydroxylation was regioselective for the 4' and 6 positions. The amount of 4'-hydroxy metabolite formed was approximately twice the amount of the 6-hydroxy metabolite formed. Formation of

Table 3. Phenprocoumon metabolism: experiments with the inhibitor metyrapone and the cofactor NADH

		Metabolite produced* [pmoles \cdot (nmole cytochrome P-450) $^{-1}$ \cdot min $^{-1}$]			
Treatment	Isomer	4'	6	7	8
+ NADPH†	R	16.2 \pm 2.0‡	4.7 \pm 0.9‡	2.0 \pm 0.4‡	1.7 \pm 0.7‡
	S	24.3 \pm 2.8‡	11.5 \pm 1.5‡	2.5 \pm 0.5‡	4.3 \pm 0.9‡
+ NADH	R	2.7 \pm 0.6‡§	4.1 \pm 0.8‡	0 \pm 0.2§	1.7 \pm 0.7‡
	S	2.6 \pm 0.6‡§	1.3 \pm 0.4‡§	0 \pm 0.2§	2.2 \pm 0.7‡§
+ NADPH + 500 μ M metyrapone	R	8.4 \pm 1.2‡§	3.5 \pm 0.7‡	1.0 \pm 0.3‡§	0.9 \pm 0.7§
	S	12.3 \pm 1.6‡§	4.8 \pm 0.8‡§	0.9 \pm 0.3‡§	1.6 \pm 0.6‡§
- NADPH Background control (no inhibitors)	R	3.0 \pm 0.2	3.7 \pm 0.2	2.0 \pm 0.1	5.5 \pm 0.3
	S	3.1 \pm 0.2	2.7 \pm 0.2	2.3 \pm 0.1	4.5 \pm 0.3

* Data are the means \pm S.E. of four incubations. Statistical comparisons were made between two treatment means for a given metabolite of the same stereochemistry. Differences between two levels that are significant at the 99 per cent confidence level are denoted by the ‡ and § symbols (see footnotes below).

† Values for the first three treatments represent *net* metabolite levels (i.e. total amounts minus background control).

‡ Significant difference from the background control data.

§ Significant difference from the normal control data.

|| Values for the last treatment are the background controls.

Table 4. Phenprocoumon metabolism: inhibition studies with SKF-525A

Treatment	Isomer	Metabolite produced* [pmoles · (nmole cytochrome P-450) ⁻¹ · min ⁻¹]			
		4'	Position of hydroxylation		8
			6	7	
+ NADPH†	R	17.6 ± 2.5‡	9.1 ± 1.3‡	1.6 ± 0.3‡	2.9 ± 1.0‡
Normal control	S	29.4 ± 3.8‡	19.8 ± 2.6‡	6.0 ± 0.9‡	5.4 ± 1.3‡
+ NADPH	R	7.5 ± 1.3‡§	6.6 ± 1.0‡	2.7 ± 0.4‡	3.8 ± 1.1‡
+ 500 µM SKF-525A	S	8.8 ± 1.5‡§	6.0 ± 0.7‡§	2.3 ± 0.5‡§	4.0 ± 1.2‡
- NADPH	R	4.8 ± 0.2	2.9 ± 0.2	1.2 ± 0.1	6.0 ± 0.3
Background control	S	5.2 ± 0.3	3.9 ± 0.2	2.4 ± 0.1	6.9 ± 0.4
Boiled microsomes	R	5.4 ± 0.3	2.6 ± 0.1	1.2 ± 0.1	6.8 ± 0.4
+ NADPH (no inhibitor)	S	6.7 ± 0.3	3.6 ± 0.2	2.4 ± 0.1	8.0 ± 0.4

* Data are the means ± errors of six incubations. Comparison of boiled microsome data to background control data revealed no significant differences. Statistical comparisons were made between two treatment means for a given metabolite of the same stereochemistry. Differences between two levels that are significant at the 99 per cent confidence level are denoted by the ‡ and § symbols (see footnotes below).

† Values for the first two treatments represent *net* metabolite levels (i.e. total amount minus background control).

‡ Significant difference from the background control data.

§ Significant difference from the normal control data.

|| Values for the last two treatments represent the background controls.

the 7- and 8-hydroxy metabolites represented minor pathways. In addition, formation of the 4'- and 6-hydroxy metabolites was selective for the (S)-enantiomer, whereas formation of the 7- and 8-hydroxy metabolites was nonstereoselective.

Incubation under a 4:1 CO₂:O₂ atmosphere clearly inhibited production of the 4', 6- and 7-hydroxy metabolites. Inhibition of the 8-hydroxy metabolite was not clearly demonstrated. The incubations in which NADH was substituted for NADPH (Table 3) led to a significant diminution of metabolite production in all but two cases. Although the amount of the (R)-6- and (R)-8-hydroxy metabolites produced was significantly above background, the values did not differ from the respective normal control experiments. Metirapone (2-methyl-1,2-di-3-pyridyl-1-propanone) affected the production of six of the metabolites (Table 3). The production of the (R)- and (S)-4'-hydroxy, (S)-6-hydroxy, (R)- and (S)-7-hydroxy and the (S)-8-hydroxy metabolites was clearly inhibited even though the levels were still above background. The level of the (R)-8-hydroxy metabolite, on the other hand, while not significantly different from the level obtained without inhibitor, was reduced to a point where it was not significantly different from the background value of 5.48. The level of the (R)-6-hydroxy metabolite was not significantly different from the control value. Addition of SKF-525A to the incubations (Table 4) was less effective and did not reduce any metabolite production to background levels; however, the (R)- and (S)-4'-hydroxy, (S)-6-hydroxy, and (S)-7-hydroxy metabolites were formed at rates significantly below the rates obtained from incubations performed without the inhibitor.

DISCUSSION

In attempting to develop an assay for phenprocoumon metabolites, two major problems were

encountered. The first was due to the instability of metabolites on t.l.c. plates. The magnitude of this problem was determined by quantitating the recovery of metabolites from t.l.c. plates after development in either the acidic or basic solvent systems. These results showed that in each case the stability of the metabolite was greatest in the basic system. The overall recovery of each metabolite standard was as good as, or better than, the recovery of phenprocoumon with the exception of the 7-hydroxy derivative. With an overall recovery of approximately 40 per cent this compound appears to be unusually unstable. Studies have shown that the decomposition of the 7-hydroxy compound is highly dependent on the nature of the solvent and occurs only in the presence of oxygen [22].

Despite the recovery problems, analysis of the various incubation experiments revealed that the *general* metabolic profiles obtained for 2 were independent of recovery values. Therefore, the data presented in Tables 2, 3 and 4 were not corrected for recoveries but were based on the raw counts obtained.

The second problem encountered was in the actual analysis of the various incubation mixtures. Since the substrate-to-product ratio was approximately 100:1, significant background counts were encountered throughout the plate due to trailing and/or decomposition of the substrate. This problem was satisfactorily diminished by sweeping the plate with cold phenprocoumon in the second dimension as described in Materials and Methods.

A number of statistical methods were considered for analyzing the data. Student's *t*-test was deemed not to be appropriate because of the multiple variances obtained. Factorial analysis in theory should be a better statistical method since comparisons of treatment means are made with respect to a pooled variance. Therefore, since each of the metabolites was produced within each treatment/isomer combination, a nested factorial analysis of variance [17]

was chosen as the best design for analysis of the data. Prior to evaluation by nested factorial analysis of variance, the data were converted to logarithms to ensure homogeneity of variance. This was necessary since initial inspection of numerous data sets indicated the variances of the means of metabolite formation were approximately proportional to the amount of metabolite formed. The variance of the transformed data was homogeneous as shown by Bartlett's Test [18] ($\alpha = 0.05$).

All comparisons of metabolite levels as indicated in the tables were made at the 99 per cent confidence level. The choice of this level of statistical significance was made to reduce the possibility that two metabolite levels would be judged significantly different when in reality they were not (a so-called Type One error). A low probability of making a Type One error was deemed necessary because of the high background levels and because of the multiple comparisons of mean metabolite levels needed to interpret the data.

In general, a number of criteria are utilized to demonstrate the involvement of cytochrome P-450 in metabolic transformations. These include: (1) decreased product formation in the presence of inhibitors such as SKF-525A, metyrapone, and CO; (2) either a lack or a substantial decrease in product formation upon substitution of NADH for NADPH as a source of reducing equivalents; (3) the lack of product formation with the use of heat-denatured enzyme; and (4) the demonstration that the source of the oxygen atom incorporated into the substrate is molecular oxygen and not water [23]. On the basis of the first three criteria, the results presented here clearly establish the involvement of cytochrome P-450 in the oxidative metabolism of **2**. The differential inhibition displayed by the inhibitors presumably reflects the fact that cytochrome P-450 is not a single enzymatic species but a family of closely related enzymes.

As discussed earlier, major differences exist in the regioselectivity observed for the metabolic profiles obtained *in vivo* from warfarin and phenprocoumon. Based on the present study a similar pattern is evident at the *in vitro* level when the data for both enantiomers is summed (the *in vivo* studies were performed with racemates), even if recoveries are taken into account. The lower level of overall phenprocoumon metabolism is also reflected at the *in vitro* level. A new and perhaps even more fundamental difference in the metabolic profile of these structurally similar drugs that has emerged from the present study is the change in substrate stereoselectivity. Whereas the cytochrome P-450-mediated formation of metabolites from warfarin is selective for the (R)-enantiomer, the formation of metabolites from phenprocoumon is either nonstereoselective or selective for the (S)-enantiomer. These differences cannot be accounted for by any differential binding to extraneous non-cytochrome P-450 protein in the incubations for the following reasons. The metabolic pattern of **1** was obtained under a saturating substrate concentration of 760 μ M. The metabolic pattern presented here for (R)- and (S)-**2**, although obtained at a lower concentration of 300 μ M, was qualitatively the same pattern as that obtained at higher concen-

trations up to and including 720 μ M [8]. The fact that the observed stereoselective patterns were obtained under saturating concentrations of substrate implies that the stereoselectivity manifested by both **1** and **2** is a function of the interaction between these substrates and the cytochromes P-450 and, as such, presumably reflects the binding characteristics of the catalytically active sites. Why these differences between the two substrates exist is not clear, but work is presently in progress to clarify the problem.

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