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WARFARIN—STEREOCHEMICAL ASPECTS OF ITS METABOLISM BY RAT LIVER MICROSOMES

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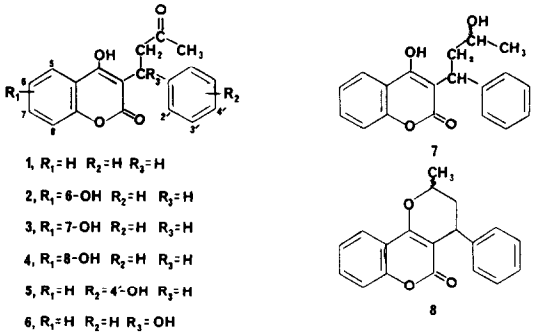
Abstract—The *R*- and *S*-enantiomers of warfarin were differentially metabolized by hepatic microsomes prepared from male Wistar and Sprague–Dawley rats. These studies were not only carried out with two strains of rats but were conducted independently in two laboratories employing different techniques. Although minor differences were observed, the same stereoselectivity was found for the microsomal transformations produced by both strains. The formation of 7- and 8-hydroxywarfarin was stereoselective for the *R*-enantiomer and in addition this enantiomer was metabolized more rapidly than the *S*-enantiomer. The converse stereoselectivity was found for the process of 4′-hydroxylation in Sprague–Dawley rats but it could not be conclusively shown for Wistar rats. A Michaelis–Menten analysis of the metabolic products of *R*- and *S*-warfarin formed by liver microsomes from male Sprague–Dawley rats is reported. The K_m for the processes of 6-, 7-, and 4′-hydroxylation for both isomers and the K_m for 8-hydroxylation of the *R*-isomer were all of the order of 0.03 to 0.11 mM and were not statistically different. The K_m for 8-hydroxylation of the *S*-isomer, 0.20 mM, was significantly greater. The K_m for benzylic hydroxylation of both isomers appeared to be still greater but was less precisely determined. The V_{max} for each of the enantiomeric pairs of products was statistically different. The kinetic data are interpreted as being inconsistent with the supposition that an arene oxide (6–7 and/or 7–8) may serve as the intermediate in the formation of 7-hydroxywarfarin from either isomer. Further, if product formation is assumed to be rate limiting, the data provide evidence for at least three distinct enzymatic processes which may or may not be distinct hemoproteins. Reduction of the side-chain ketonic function of warfarin to the corresponding diastereomeric warfarin alcohols by the 105,000 *g* supernatant fraction displayed both a high degree of stereoselectivity (*R*-isomer) and stereospecificity (*S*-reduction). This reduction was best catalyzed by NADPH rather than by NADH. Determination and quantification of the metabolic products obtained after incubation of *R*- and *S*-warfarin with the 10,000 *g* supernatant were consistent with the summation of those independently produced by the microsomal pellet and the 105,000 *g* supernatant.

The discovery that dicoumarol is the agent responsible for the hemorrhagic disorder produced in cattle [1, 2] by the ingestion of spoiled sweet clover led to the synthesis of a variety of structurally related compounds possessing potential anticoagulant activity. Of these, warfarin, *1*, has found extensive use both as a rodenticide and as a clinically effective oral anticoagulant in man. In recent years, increasing concern has developed over the appearance of warfarin-resistant strains of rats in Scotland, Northern Europe [3–6] and the United States [7]. At the clinical level, maintenance of an appropriate anticoagulant state with this drug has sometimes proved difficult due to variations in its hypoprothrombenemic activity caused by the concomitant administration of other medications [8].



Scheme 1.

Warfarin exists in two enantiomeric forms. The *S*-isomer is approximately five to six times more potent than the *R*-isomer in both the rat [9–11] and man [12], although the clinically available form of the drug is the racemate. Recent evidence has demonstrated that in man, the two enantiomeric forms are

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metabolized differently and that prior administration of other drugs, e.g. phenylbutazone, will quantitatively affect these metabolic pathways to different degrees [13]. This observation offers, at least in part, an explanation at the molecular level for the effects observed at the clinical level.

The biotransformation *in vivo* of racemic warfarin has been studied in the rat and most of the metabolic products have been determined [14]. Ikeda *et al.* [15] have demonstrated that phenobarbital pretreatment induces the metabolism of warfarin by the 9000 *g* microsomal homogenate obtained from rat liver and that a general increase in the levels of 6-, 7- and 8-hydroxywarfarin, *2*, *3* and *4*, respectively, is observed. Similar results have recently been reported by Townsend *et al.*, [16] except that their system induced the formation of 7-hydroxywarfarin to a much greater degree than the other oxidative products. To our knowledge, the metabolic fate of the individual warfarin isomers either *in vivo* or *in vitro* has not been reported, although certain of their pharmacokinetic parameters have been studied. Breckenridge and Orme [10] reported that the half-life of the *R*-isomer (8.6 hr) is significantly shorter than the half-life of the *S*-isomer (15.4 hr). This finding agrees with those of Hewick [11] and Yacobi and Levy [17].

Since it appears that the warfarin isomers are differentially metabolized and that induced changes in metabolism may be the basis for at least some drug interactions, a model for the relatively rapid elucidation of such phenomena would be desirable. The present studies were undertaken in order to quantitate the biotransformation products *in vitro* of the warfarin enantiomers by rat liver microsomes. Initial experiments using lyophilized liver microsomes from male Wistar rats and employing u.v. absorption for the quantitation of *R*- and *S*-warfarin metabolites were performed in Albany. Subsequent investigations utilizing freshly prepared liver microsomes from Sprague–Dawley rats and employing isotopically labeled warfarin enantiomers to study the detailed microsomal metabolism of these isomers were performed in Seattle. Despite minor quantitative differences observed between the two rat strains, the same overall conclusions may be reached from the data of both laboratories. These data not only establish normal patterns for warfarin metabolism in the rat but provide the basis for further studies on drug interactions.

MATERIALS AND METHODS

Experiments performed in Albany

Materials. Racemic warfarin (CalBiochem, La Jolla, Calif.) was resolved [18] to yield the *R*- and *S*-warfarin isomers, which were determined to be 98 and 95.6 per cent optically pure respectively. 4′-Hydroxywarfarin was synthesized by the method of Hermodson *et al.* [19, 20], while 6, 7 and 8-hydroxywarfarin were supplied by Dr. W. F. Trager. The warfarin alcohols were prepared by reduction of the warfarin enantiomers followed by separation of the product diastereoisomers in each case, according to the method of Chan *et al.* [21]. NADP, glucose 6-phosphate, glucose 6-phosphate dehydrogenase and 0.4 M TES [(*N*-Tris hydroxymethyl)methyl-2-aminoethanesulfonic acid] buffer were purchased from CalBiochem. Homogenization was carried out in a Potter Elvehjem glass tube with a Teflon pestle. Centrifugations were done at 10,000 *g* in a Sorval RC-2 centrifuge and at 105,000 *g* in a Spinco model L ultracentrifuge. Incubations were performed in a New Brunswick rotary metabolic shaker at 37°. Thin-layer chromatography (t.l.c.) separations were carried out on 20 × 20 cm Silica gel plates, type DF-B (Camag, Inc.; New Berlin, Wisc.). Either a Carey model 14 double-beam spectrophotometer or a Gilford 2400 S spectrophotometer was utilized for quantitative u.v. spectral measurements. The extinction coefficient used for cytochrome P-450 determinations was 91,000 M^{-1} cm^{-1} ($\Delta A = A450 - A500$). Those for the hydroxywarfarins in 0.5 M NaOH were as follows: 6-hydroxywarfarin, 13,900 M^{-1} cm^{-1} (300 nm); 7-hydroxywarfarin, 27,400 M^{-1} cm^{-1} (327 nm); 8-hydroxywarfarin, 18,400 M^{-1} cm^{-1} (300 nm) and 4′-hydroxywarfarin 12,300 M^{-1} cm^{-1} (300 nm).

Tissue preparation. Male Wistar rats (200–250 g) were used throughout this study. The animals were killed by a blow to the head, and the livers were excised and placed in cold 1.15% KCl. The livers were then minced and homogenized with a Teflon pestle in 3 vol. of 1.15% KCl at 0–5°. The homogenates were centrifuged at 10,000 *g* for 20 min to remove cell debris and nuclei, and the supernatants recentrifuged at 105,000 *g* for 1 hr. The pellet was resuspended in 1.15% KCl to half its original volume, frozen in dry ice–acetone, and lyophilized. After drying, the microsomes were portioned into small aliquots (about 500 mg) and stored at −50°. Under these conditions, the enzymatic activity was found to remain stable for several months.

Incubation mixture. Lyophilized preparations were thawed in ice prior to use for approximately 1 hr. The powder was weighed, an appropriate amount of cold, double-distilled water was added, and the mixture gently homogenized with a Teflon pestle. For determination of cytochrome P-450, suspensions were diluted with 0.1 M phosphate buffer, pH 7.4, and the dithionite-reduced carbon monoxide difference spectrum was recorded in a Cary 14 double-beam spectrophotometer. Routinely, 50-ml Erlenmeyer flasks were placed in an ice bath to which were added: 1 ml of 0.4 M TES buffer, pH 7.5; 1 ml water containing 1 mg of the appropriate warfarin isomer (sodium salt); 1 ml of 20 mM magnesium chloride solution containing 2 μmoles NADP, 33 μmoles glucose 6-phosphate, and 5 units glucose 6-phosphate dehydrogenase (yeast). At zero time, duplicate flasks were placed in a rotary New Brunswick metabolic shaker equilibrated at 37° and allowed to preincubate for 7 min with gentle shaking to insure adequate starting concentrations of NADPH. At the end of this time, enough reconstituted microsomal preparation was added in 1 ml water to each flask to give a final cytochrome P-450 concentration of 4.1×10^{-6} M. The final reaction mixtures were incubated for an additional 20 min.

The activity of each preparation was monitored by its ability to *O*-demethylate *p*-nitroanisole by the slightly modified method employed by Ferris *et al.* [22]. In our procedure, 1.5 mg *p*-nitroanisole was added in 0.1 ml ethanol, and the reaction was terminated by the addition of 1.0 ml of 30% trichloroacetic acid. Quantitatively, no difference per mole of

cytochrome P-450 was observed between lyophilized and fresh microsomal preparations in their ability to either *O*-demethylate *p*-nitroanisole or hydroxylate the enantiomers of warfarin.

Isolation, identification and quantification of warfarin metabolites. At the end of the 20-min incubation period, the duplicate flasks were immediately poured into a separatory funnel containing 50 ml dichloromethane and 0.5 ml of concentrated HCl. The flasks were rinsed with 1 ml water and the washings combined with the original incubation mixture. After extraction, the organic phase was withdrawn and the aqueous phase re-extracted with 50 ml dichloromethane. The organic phases were combined and filtered through Whatman No. 40 paper. An 80-ml portion was removed, placed in a clean separatory funnel and extracted with 25 ml of 0.1 N NaOH. The organic phase was removed, and the aqueous phase was acidified with 1 ml of concentrated HCl and extracted two times with 50-ml portions of dichloromethane. The combined extracts were filtered through Whatman No. 40 paper, a 90-ml aliquot was removed, and the solution was evaporated to near dryness in a beaker. The residual solution was then quantitatively transferred to a small test tube and carefully evaporated to dryness with nitrogen.

For spotting, the residue was dissolved in exactly 0.2 ml acetone and 50 μg of this solution was applied to a 20 × 20 cm Silica gel plate (Camag, type DF-B) in a 1-cm streak running parallel to the bottom and 2 cm from it and the right-hand edge. The plate was developed with ethylene dichloride–acetone (7:3), dried, and redeveloped in the same system and direction. After drying, the plate was turned so that the original right-hand edge became the side immersed in the solvent and the spots were concentrated into a band by partial development with acetone. The plate was finally developed in ethyl acetate–methanol–trimethylamine (6:3:1) in the same direction as that used for the acetone development to separate the hydroxywarfarins, or alternatively, in toluene–ethyl formate–formic acid (10:5:1) to separate the warfarin alcohols.

The metabolites of warfarin were identified by their ability to co-chromatograph with authentic samples in three different solvent systems as well as by spectral comparison. For quantitation, the products were visualized with a u.v. light, scraped, and eluted from the Silica gel with 1.5 ml of 0.5 N NaOH. After centrifugation, the absorbance of the aqueous phase was read in a Gilford 2400 S spectrophotometer. Parent warfarin, added to boiled microsomes, was recovered by this procedure at 98 per cent of the theoretical yield.

Experiments performed in Seattle

Materials. Warfarin specifically labeled with ^{14}C in the benzylic position, 4.27 μCi/mg, was prepared by Chan [23]. Briefly, labeled CO_2 generated from $Ba[^{14}C]O_3$ was reacted with phenylmagnesium bromide. The labeled benzoic acid thus obtained was reduced to benzyl alcohol with lithium aluminum hydride and then oxidized to labeled benzaldehyde with silver carbonate impregnated on Celite. The benzaldehyde was condensed with acetone to yield benzalacetone, which was condensed with 4-hydroxycoumarin to yield labeled warfarin in a 47 per cent yield based on starting $Ba[^{14}C]O_3$. Racemic warfarin was resolved by the method of West *et al.* [18] to yield *R*- and *S*-[^{14}C]warfarin, 0.18 μCi/mg, 3.9×10^5 dis./min/mg, 98 per cent enantiomerically pure. The 6-, 7-, 8- and 4′-hydroxywarfarin used as standards were either gifts from Dr. K. P. Link or were synthesized in this laboratory according to published procedures [19, 20]. NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Sigma Biochemicals (St. Louis, MO.). Homogenization was performed in a Potter–Elvehjem glass tube with a Teflon pestle with 0.10 to 0.15-mm clearance. Centrifugation was carried out in polycarbonate tubes (1 × 3.5 in. at 10,000 *g* and $\frac{5}{8}$ × 3 in. at 105,000 *g*) in a Beckman L2-65B refrigerated ultracentrifuge. Incubations were carried out on an AO model 2156 shaker in a water bath held constant at 37 ± 0.5°. T.l.c. separations were carried out on Eastman 6060 Silica gel plates. Radioactivity determinations were made with a Beckman LS-230 liquid scintillation counter in 10 ml Aquasol (New England Nuclear). Electron ionization mass spectra were obtained from an AEI MS-9 high resolution mass spectrometer at 70 eV. Samples were introduced via the direct insertion probe at a source temperature of 220–230°.

Tissue preparation. In each experiment, six male Sprague–Dawley rats (130 ± 2 g) were housed in a large stainless steel cage for 7 days with a sufficient food and water supply. The rats were programmed to a 12-hr on–off light cycle. On the morning of the last day they were weighed (range 140–180 g), decapitated and exsanguinated. All further preparations and transfers were conducted in a cold room (4 ± 4°). The livers were removed, weighed, pooled and minced. Homogenization was carried out in 3 vol. of cold 1.15% KCl–0.01 M sodium phosphate buffer, pH 7.4, using five upward and downward strokes of the pestle in 50–60 sec with the pestle driven by a stirring motor at 180–200 rev/min. After centrifugation at 10,000 *g* for 15 min at 4°, the supernatant was either utilized for 10,000 *g* supernatant studies or was recentrifuged (4°) at 105,000 *g* for 60 min. The microsomal pellet was resuspended manually with the homogenizer in a volume of cold 1.15% KCl buffer equal to that of the supernatant, which was saved for the 105,000 *g* supernatant studies and recentrifuged for 60 min at 105,000 *g*. The new supernatant was discarded and the final pellet was again suspended manually in a volume of cold 1.15% KCl buffer equal to that of the discarded supernatant.

Protein determinations. Prior to each incubation, the protein content of 1 ml of the 10,000 *g* supernatant, the 105,000 *g* supernatant, or the microsomal suspension, each diluted to 100 ml with distilled water, was determined by a modified Lowry procedure [24]. The undiluted microsomal suspension was then diluted with the appropriate amount of KCl buffer to obtain a concentration of 5.0 mg protein/ml of suspension. The undiluted 10,000 *g* (1 ml ~ 30 mg protein/ml) and 105,000 *g* (2 ml ~ 15 mg protein/ml) supernatants were utilized directly in the subsequent incubations without prior dilution.

Incubation mixture. K_m and V_{max} studies. All incubations were performed in triplicate in 25-ml Erlen-

meyer flasks. Substrate concentrations of 0.13, 0.26, 0.39, 0.52 and 0.78 mM of either *R*- or *S*-[^{14}C]-warfarin were employed. The reagents were added in the following order: substrate in 0.1 N KOH (0.040 ml, 0.65 μmole; 0.080 ml, 1.30 μmoles; 0.120 ml, 1.95 μmoles; 0.160 ml, 2.60 μmoles; and 0.240 ml, 3.90 μmoles); NADPH-generating system consisting of $MgCl_2$ (10 μmoles), glucose 6-phosphate (20 μmoles), and NADP (5 μmoles) in 1 ml of 0.1 M phosphate buffer, pH 7.4; 0.1 N HCl to neutralize the 0.1 N KOH (0.040, 0.080, 0.120, 0.160 and 0.240 ml, respectively); 0.1 N KCl to maintain equivalent ionic strengths in the incubation mixtures (0.40, 0.32, 0.24, 0.16 and 0 ml, respectively); 1.15% KCl–0.01 M phosphate buffer (1.52 ml); and a solution of glucose 6-phosphate dehydrogenase (1 ml, 5 units) prepared in 0.1 M phosphate buffer, pH 7.4. The resulting solutions (4.0 ml) were preincubated for 5 min at 37° to insure adequate starting concentrations of NADPH, and 1 ml of normal microsomal suspension was added to give a final volume of 5 ml/flask and initial concentrations of 1 mg protein/ml, 1.0 mM NADP, 2.0 mM $MgCl_2$, 4.0 mM glucose 6-phosphate, 1 unit glucose 6-phosphate dehydrogenase/ml, and 40 mM phosphate buffer. Incubations were conducted in open flasks at 37° and 120 oscillations/min for 10 min. Reactions were terminated by the addition of 1 N HCl (0.5 ml) and extracted twice with Et_2O (20 ml each). Control incubations were performed in duplicate using all reagents except the 1 ml (5 mg) of microsomal protein; an additional 1 ml of 1.15% KCl–0.01 M phosphate buffer was used in its place.

105,000 *g* Supernatant studies. All incubations were performed in 25-ml Erlenmeyer flasks, employing a substrate concentration of 0.65 mM and 2 ml of the 105,000 *g* supernatant fraction. Each substrate (*R*- and *S*-[^{14}C]warfarin) was incubated with the unfortified supernatant fraction, and with the supernatant fraction fortified with NADH or an NADPH-generating system. The reagents were added in the following order: substrate in 0.1 N KOH (0.20 ml, 3.25 μmoles); NADPH-generating system described above; 0.1 M phosphate buffer (2 ml, pH 7.4) for the NADH and nonfortified incubations; NADH solution (0.2 ml, 6.5 μmoles dissolved in H_2O); 0.1 N HCl to neutralize the 0.1 N KOH (0.2 ml); 1.15% KCl–0.01 M phosphate buffer (0.40 ml for the NADH incubations and 0.60 ml for the NADPH and nonfortified incubations); and a solution of glucose 6-phosphate dehydrogenase (1 ml, 5 units) prepared in 0.1 M phosphate buffer, pH 7.4, for the NADPH incubations. The resulting solutions (3.0 ml) were preincubated for 5 min at 37° and 2 ml of similarly preincubated 105,000 *g* supernatant was added to give a final volume of 5 ml/flask. Incubations with appropriate controls and the termination of the reactions were conducted as described above.

10,000 *g* Supernatant studies. All incubations were performed in 25-ml Erlenmayer flasks, employing a substrate concentration (*R*- or *S*-[^{14}C]warfarin) of 0.65 mM and 1 ml of the 10,000 *g* supernatant. The reagents were added in the following order: substrate in 0.1 N KOH (0.20 ml, 3.25 μmoles); the NADPH-generating system described above; NADH solution (0.2 ml, 6.5 μmoles dissolved in H_2O); 0.1 N HCl to neutralize the 0.1 N KOH (0.2 ml); 1.15% KCl–0.01 M phosphate buffer (1.4 ml); and a solution of glucose 6-phosphate dehydrogenase (1 ml, 5 units) prepared in 0.1 M phosphate buffer, pH 7.4. The resulting solution (4.0 ml) was preincubated for 5 min at 37°, and 1 ml of similarly preincubated 10,000 *g* supernatant was added to give a final volume of 5 ml/flask. Incubations with appropriate controls and the termination of the reactions were conducted as described above.

Isolation and quantification of metabolites. K_m and V_{max} studies. The ether extracts were filtered through anhydrous $MgSO_4$ and vacuum evaporated to give a small amount of oily solid residue. The residues were transferred into 300-μl conical screw-top vials (Reacti-Vials, Pierce Chemicals) with acetone, evaporated with N_2 to dryness, and then redissolved in 25 μl acetone. A standard solution containing 6-, 7-, 8-, and 4′-hydroxywarfarin, approximately 1 mg/ml of each, was then applied (5 μl, 1.5-cm streak) onto activated (15 min at 110°) Eastman 6060 t.l.c. plates. Portions (40 per cent) of the acetone solutions were then taken and applied with 5-μl disposable microcapillary tubes onto the t.l.c. plates over the regions containing the standards. The plates were eluted twice with chloroform–acetic acid (100:1, 15 cm, R_f, 6- and 7-hydroxy, 0.09; 4′-hydroxy, 5, 0.14; benzylic hydroxy [25], 6, 0.20; 8-hydroxy, 0.28; warfarin, 0.68), blown dry with an air gun (cold), turned 90°, eluted with MeOH in order to concentrate fluorescent bands, and then eluted with *t*-butanol–benzene–NH_4OH–H_2O [90:40:18:6, 6 cm, R_f (from concentrate line), 7-hydroxy, 0.35; 6-hydroxy, 0.5; 4′-hydroxy, 0.43; benzylic hydroxy, 0.58; 8-hydroxy, 0.37; warfarin, 0.65]. The loci corresponding to unchanged warfarin, 6-, 7-, 8-, 4′-, and benzylic hydroxywarfarin were then cut from the plates with scissors, placed into scintillation vials and eluted with MeOH (200 μl, 5 min), followed by the addition of Aquasol. The samples were counted three times (20 min/vial). The control analyses yielded nearly constant background counts (e.g. 8–9 cpm for the known metabolite region of benzylic hydroxywarfarin) which were subtracted from the counting results (108 cpm in this example) to give corrected counts. Autoradiographic studies of duplicate t.l.c. plates disclosed that all of the detectable radio-activity was concentrated in the loci subjected to scintillation counting. Weighed samples of the optically active substrates were counted in the same scintillation mixture to provide calibration standards.

105,000 *g* Supernatant study. The preparation of the ether extract and its application (3-cm streak) to t.l.c. plates were conducted as described above except that the standard solution contained both diastereomeric warfarin alcohols,* 7, each at a concentration of 1 mg/ml. The plates were eluted once with ethylene dichloride–acetone (7:3, 15 cm, R_f, 0 to 0.09), blown

*Although the warfarin alcohols have been established as both plasma and urinary metabolites of warfarin from man (see Ref. 12, 13 and 21), they have not previously been reported as metabolites from the rat either *in vivo* or *in vitro*. However, they may in fact correspond to the unidentified metabolite x, first reported by Ikeda *et al.* [15]. This metabolite, like the warfarin alcohols, was reported to have an absorption curve indistinguishable from that of warfarin, and its formation was catalyzed by the soluble fraction of liver under nitrogen as well as under oxygen.

dry with an air gun (cold), turned 90°, and eluted with MeOH in order to concentrate the fluorescent (u.v. lamp) bands. The plates were eluted once with chloroform–acetic acid (100:1, 5.5 cm), blown dry with an air gun (cold), and eluted once in the same dimension with Et_2O (5.5 cm, R_f, alcohol 1, *R,S*- or *S,R*-configuration, 0.84; alcohol 2, *S,S*- or *R,R*-configuration, 0.56). The loci corresponding to the stereoisomeric alcohols were cut from the plates, placed into scintillation vials, and eluted with MeOH (200 μl, 5 min). This was followed by the addition of Aquasol (10 ml) and scintillation counting. The control analysis yielded nearly background counts, which were substracted from the counting results to give correct counts. Counting efficiency was determined by internal standardization with [^{14}C]toluene.

The rest of the acetone solutions were then combined and applied (11-cm streak) onto activated (15 min at 110°) Eastman 6060 t.l.c. plates. The plates were eluted once with ethylene dichloride–acetone (7:3, 15 cm). The locus corresponding to the warfarin alcohols was cut from the plates, eluted with acetone, filtered, evaporated with N_2, and subjected to electron ionization mass spectrometric analysis (70 eV, 220–230°), *m/e* 310 (M^+), 292 (M^+-H_2O), 263 and 121. These data correspond to warfarin alcohol [21, 26].

10,000 *g* Supernatant study. The preparation of the ether extract and its application to the t.l.c. plates were conducted as described above. The plates were then eluted once with ethylene dichloride–acetone (7:3, 15 cm) and blown dry with an air gun (cold) to yield three distinct broad loci. The slowest moving fraction corresponded to warfarin alcohols (R_f, 0 to 0.09); the middle fraction corresponded to 6-, 7-, 8-, 4′-, and benzylic hydroxywarfarin (R_f, 0.15 to 0.29); and the fastest moving fraction corresponded to warfarin (R_f, 0.37 to 0.52). The plates were cut between the slowest and middle fractions. The warfarin alcohols were analyzed separately by concentrating them in the second dimension (MeOH) and then successively eluting once with chloroform–acetic acid (100:1, 5.5 cm) and once with Et_2O (5.5 cm). The hydroxylated metabolites of *R*- and *S*-warfarin were subsequently separated by concentrating the middle locus (6-, 7-, 8-, 4′-, and benzylic hydroxywarfarin) with MeOH in the first dimension and eluting twice with chloroform–acetic acid (100:1, 15 cm). The plates were then blown dry with an air gun (cold), turned 90°, eluted with MeOH in order to concentrate the fluorescent bands, and finally eluted once with *t*-butanol–benzene–NH_4OH–H_2O (90:40:18:6, 5.5 cm). The locus corresponding to each metabolite was then analyzed by scintillation spectrophotometry as described above.

Data analysis. K_m and V_{max} studies. Data from the ^{14}C above ^{3}H channel were used in all calculations. The expected recovered activity in cpm was computed for each incubation mixture. The actual recovered activities from all the loci, corrected for background, were summed, and the ratio of the experimentally determined total activity to the expected activity determined the portion recovered. Approximately 70 per cent of the expected radioactivity was recovered. The data were corrected for recovery losses and the rates of production of each metabolite in nmoles/mg of microsomal protein/min were calculated. Kinetic parameters were determined by a weighted least squares fit [27, 28] of the experimental rate (V) and substrate concentration (S) data to an S/V vs S plot. Replicate experiments established that errors in V were approximately normally distributed. Errors in S were about an order of magnitude less than errors in V; therefore S was assumed to be error-free in all calculations. The *t*-distribution with *n*-degrees of freedom was used to compute 95 per cent confidence limits.

Two separate experiments, each consisting of two or three replicate incubations for each of five substrate concentrations, were analyzed and values for K_m and V_{max} were computed. The paired experiments were found to give estimates of K_m and V_{max} which did not differ significantly at the 95 per cent confidence level (Student's *t*-test) [29]. The data from both experiments were therefore pooled, and combined estimates of K_m and V_{max} were obtained*.

RESULTS

Experiments performed in Albany

The recoveries of individual hydroxylated metabolites of the warfarin enantiomers by rat liver microsomes are summarized in Table 1. The *R*-isomer of warfarin was hydroxylated to a much greater extent than the *S*-isomer, a finding in agreement with the shorter half-life found [10, 11, 17] for the *R*-isomer after administration *in vivo*. The production of 7-hydroxywarfarin, the major metabolic reaction, was

Table 1. Comparative biotransformation of *R* and *S* warfarin by normal microsomes from rat liver (Wistar)*

Warfarin metabolite	*R* warfarin	*S* warfarin
6-Hydroxywarfarin	23 ± 2	22 ± 6.3
7-Hydroxywarfarin	56 ± 3	17 ± 2
8-Hydroxywarfarin	10 ± 2	†
4′-Hydroxywarfarin + benzylic hydroxywarfarin‡	26 ± 0	34 ± 4.2

* Metabolite concentrations are expressed as nmoles produced after 20 min incubation at a cytochrome P-450 concentration of 4.1×10^{-6} M. Each value is an average of three separate determinations (±) S. D.

† The amount of metabolite (< 5 nmoles) produced was too small for reliable analysis.

‡ Benzylic hydroxywarfarin was not quantitated in this study, as the compound was not separable from 4′-hydroxywarfarin on the t.l.c. systems used. In an experiment run in Seattle (in duplicate), benzylic hydroxywarfarin was formed by the lyophilized microsomes from male Wistar rats with the same stereochemical preference, $R/S = 3.0$, as that displayed by the Sprague–Dawley. Moreover, the amount of benzylic hydroxywarfarin produced from the *R* isomer is approximately 30 per cent of the amount of 4′-hydroxywarfarin formed from this isomer. If Table 1 is corrected in light of these results, the same stereoselectivity is seen to prevail, regardless of the source of the microsomes.

*Paired comparisons of single elements of highly correlated data (K_m, V_{max}) are not as rigorous as comparisons of joint probability regions. Therefore, there may be significant differences in addition to those reported in Results.

Table 2. Comparative kinetics of the oxidation of *R* and *S* warfarin by normal microsomes from rat liver (Sprague–Dawley)*

Warfarin metabolite	Apparent K_m (mM) *R*-warfarin	d.f.	*S*-warfarin	d.f.	Apparent V_{max} (nmoles/mg protein after 10-min incubations) *R*-warfarin	d.f.	*S*-warfarin	d.f.
6-Hydroxywarfarin	0.096 ± 0.028	22	0.032 ± 0.011	23	0.754 ± 0.049	22	0.644 ± 0.023	23
7-Hydroxywarfarin	0.046 ± 0.024	22	0.050 ± 0.010	23	1.653 ± 0.113	22	0.455 ± 0.013	23
8-Hydroxywarfarin	0.093 ± 0.051	22	0.198 ± 0.048	19	0.448 ± 0.052	22	0.200 ± 0.015	19
4′-Hydroxywarfarin	0.109 ± 0.038	19	0.067 ± 0.021	23	0.500 ± 0.042	19	0.652 ± 0.036	23
Benzylic hydroxywarfarin	0.803 ± 0.400	19	0.221 ± 0.157	19	1.693 ± 0.527	19	0.236 ± 0.060	19

* Data were derived from weighted least-squares linear regression of $[S]/V$ vs $[S]$. The data are expressed as the means ± standard errors with degrees of freedom (d.f.) as shown.

highly stereoselective for the *R*-isomer while in contrast *S*-warfarin was stereoselectively and stereospecifically reduced to the *S,S*-warfarin alcohol by the microsomal fraction. Although the reduction was not quantitated, the experiments were repeated in Seattle and the same results were obtained, that is, only the *S,S*-alcohol could be detected. This is in marked contrast to the results (see below) obtained from fresh microsomes from Sprague–Dawley rats where no reduction could be detected after incubation.

Experiments performed in Seattle

K_m and V_{max} studies. When five different concentrations of *R*- and *S*-[^{14}C]warfarin were incubated with liver microsomes from normal rats, all reactions were linear with respect to both the 10-min incubation period and the microsomal protein concentration. The results of the Michaelis–Menten analysis of the initial velocity data are given in Table 2. A high degree of stereoselectivity is evident. For each metabolite formed except 6-hydroxywarfarin one of the optical isomers of warfarin is transformed at a significantly faster rate. The production of the 7-, 8- and benzylic hydroxy metabolites from *R*-warfarin occurred with significantly greater maximum velocities ($P < 0.05$) (Fig. 1). Conversely the maximum velocity for the production of the 4′-hydroxy metabolite was significantly greater from *S*-warfarin ($P < 0.05$).

On the basis of statistical analysis, the apparent K_m values for the production of the five metabolites

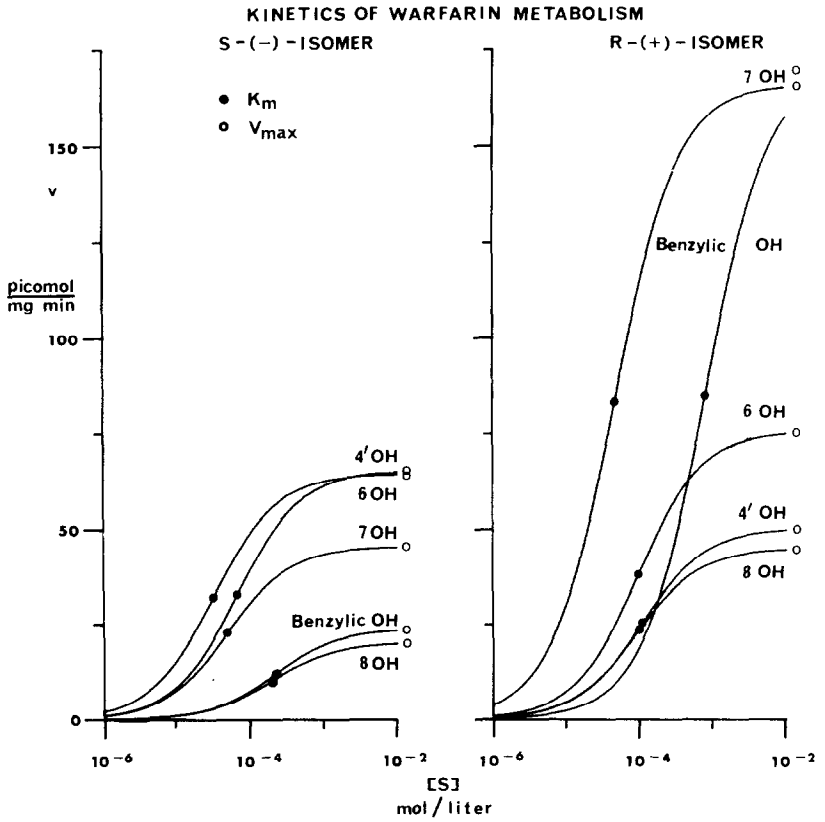

Fig. 1. The theoretical velocity for the formation of each of the indicated products was calculated from the experimentally determined K_m and V_{max} values and plotted as a function of log $[S]$. Such plots approach V_{max} asymptotically and have inflection point $[S] = K_m$.

Table 3. Comparative reduction of *R* and *S* warfarin by the 105,000 *g* supernatant fraction from rat liver

Warfarin alcohol metabolite	Product formed from rat 105,000 *g* supernatant* (nmoles/mg protein after 10-min incubation)					
	No cofactor added		NADH added (1.3 mM)		NADPH added† (1.0 mM)	
	R-warfarin	*S*-warfarin	*R*-warfarin	*S*-warfarin	*R*-warfarin	*S*-warfarin
R,S alcohol	0.18		0.28		0.71	
R,R alcohol	0.04		0.03		0.08	
S,S alcohol		0.01		0.01		0.07
S,R alcohol		0.03		0.02		0.14
Total	0.22	0.04	0.31	0.03	0.79	0.21

* Each value is the average of two analyses. A warfarin concentration of 0.65 mM was employed in these studies.
† NADPH-generating system containing NADP.

for each isomer of warfarin are indistinguishable except in two cases: it was found that the K_m for 8-hydroxylation of *S*-warfarin was greater than the K_m for both 6- and 7-hydroxylation of *S*-warfarin ($P < 0.010$) and the K_m for 6-hydroxylation of *R*-warfarin was greater than the K_m for 6-hydroxylation of *S*-warfarin ($P < 0.05$). In all other cases, if the K_m values for a given hydroxylation reaction are compared for each enantiomer of if the K_m values for all hydroxylation reactions are compared for a given enantiomer, the values found experimentally do not differ significantly ($P < 0.05$).

105,000 *g* Supernatant study. Reduction of warfarin to its alcohols could not be detected using the microsomal fraction of liver but was observed when the 105,000 *g* supernatant was employed as the source of the enzyme. The cofactor requirement and stereochemical course of this reaction are presented in Table 3. Significant reduction occurs even in the absence of the added cofactor. NADPH appears to be a more effective cofactor for the reduction than NADH. This finding agrees with previous investigations of the reduction of ketones by the cytosol fraction [30–34]. The reduction of warfarin is clearly a stereoselective process: *R*-warfarin is reduced to the *R,S*-alcohol with a high degree of stereospecificity. The *S*-isomer of warfarin is reduced with a lower degree of stereospecificity and predominantly to the *R*-configuration.

10,000 *g* Supernatant study. The same basic stereoselectivity that was observed in the microsomal study and in the 105,000 *g* supernatant study was also obtained employing the 10,000 *g* supernatant fraction (Table 4). *R*-warfarin is still stereoselectively hydroxylated to 7-, 8-, and benzylic hydroxywarfarin and is stereoselectively and stereospecifically reduced to *R,S*-warfarin alcohol. The relative quantities of the 6-, 7-, and 8-hydroxy metabolites of both *R*- and *S*-warfarin also are in close agreement with the results of the microsomal study. The smaller amount of the benzylic hydroxy metabolites obtained in this single concentration study relative to the quantities of the other metabolites is suggestive of a larger K_m for benzylic hydroxylation.

Table 4. Comparative biotransformation of *R* and *S* warfarin by the 10,000 *g* supernatant fraction from rat liver

Warfarin metabolite	Product formed from rat 10,000 *g* supernatant* (nmoles/mg protein/10 min)			
	R-warfarin		*S*-warfarin	
	Concn	Per cent	Concn	Per cent
6-Hydroxy-warfarin	0.21	14.1	0.16	23.5
7-Hydroxy-warfarin	0.53	35.6	0.13	19.1
8-Hydroxy-warfarin	0.11	7.4	0.04	5.9
4′-Hydroxy-warfarin	0.17	11.4	0.20	29.4
Benzylic hydroxywarfarin	0.08	5.4	0.02	2.9
R,S alcohol	0.33	22.1		
R,R alcohol	0.06	4.0		
S,S alcohol			0.08	11.8
S,R alcohol			0.05	7.4
Total	1.49	100.0	0.68	100.0

* Each value is the average of two analyses. A warfarin concentration of 0.65 mM was employed. The incubation reaction mixtures were fortified with an NADPH-generating system containing NADP (1.0 mM) and NADH (1.3 mM).

DISCUSSION

Microsomal studies

Considering the different methodologies and species utilized and the different microsomal preparations (fresh vs lyophilized), the similarity of the results in terms of stereochemical preference from the two series of independent studies lends credence to the validity of these results.

Michaelis–Menten analysis of microsomal enzyme kinetics has become an accepted method in describing mixed-function oxidase activity. However, since the Michaelis–Menten method was derived for isolated, purified enzymes, restrictions and limitations are encountered in its application to the more complex microsomal system [35–37]. For example, the kinetic parameters, K_m and V_{max}, are not true kinetic constants of a discrete enzymatic step but, more accurately, are a reflection of some rate-limiting step [37]. This limitation, nevertheless, does not prevent meaningful interpretation of many experiments, particularly in comparative studies such as the comparative biotransformation of enantiomers or the biotransformation of a substrate into more than one analyzed product.

The results in Table 2 are in reasonably good agreement with those obtained by Ikeda *et al.* [15]

for racemic warfarin incubated with phenobarbital-induced 9000 *g* supernatant (K_m 1.5×10^{-4} M). However, the use of optically active substrates not only reveals the complexity of the system but allows its more meaningful analysis.

Hydroxylation of R-warfarin

The apparent K_m values for the 6-, 7- and 8-hydroxylation of *R*-warfarin are statistically indistinguishable at the 95 per cent confidence level. Since a single substrate is being transformed in a chemically and spatially discrete part of the molecule (coumarin ring) into three structurally distinct products, it is reasonable to assume that the products are formed at a single enzymatic site and that product formation is rate limiting. If this assumption is valid, then the observed differences in V_{max} only reflect differences in the various activation energies for product formation irrespective of mechanism.

However, these data do not exclude the possibility that similar, but independent, enzymes are involved in the production of the coumarin-hydroxylated products that are observed. Indeed, recent work from our own laboratories in which we find that microsomes from phenobarbital- and 3-methylcholanthrene-pretreated animals have differentially induced coumarin hydroxylation processes argues in favor of such an interpretation, since the same enzymes might also be present in untreated animals but at relative activities such that they function as an apparent singular entity.

Since the K_m values for both 4′-and benzylic hydroxylation are indistinguishable from the K_m value for coumarin ring hydroxylation of *R*-warfarin, no additional insight into the multiplicity of the microsomal enzymes can be gained. If a single hemoprotein is responsible for all of the aromatic hydroxylations of *R*-warfarin, the indistinguishability of K_m values implies that product formation is rate limiting.

The lack of availability of a standard to facilitate the t.l.c. assay of benzylic hydroxywarfarin is responsible for the imprecision of the reported K_m values (Table 2). However, other evidence suggests that the K_m for benzylic hydroxylation is larger than the K_m for any of the aromatic hydroxylations. For example, a smaller quantity of the benzylic hydroxy metabolite is obtained in the single concentration study relative to the quantities of aromatic hydroxylation products (Table 4). Moreover, it can be shown statistically that the V_{max} for benzylic hydroxylation is greater than the V_{max} for either 4′- or 8-hydroxylation (Table 2). This can only be true if the K_m for benzylic hydroxylation is indeed larger than the K_m for either 4′- or 8-hydroxylation. The fact that the K_m values for 6- or 7-hydroxylations are no larger than the K_m values for 4′- or 8′-hydroxylation implies that the K_m for benzylic hydroxylation must be larger than the K_m for any of the aromatic hydroxylation processes. In addition, benzylic hydroxylation results from a fundamentally different chemical reaction and involves oxidation at a sterically hindered aliphatic site. These data can be most readily accommodated by a model which postulates more than one enzymatic process. Such a model is in accord with recent evidence for the existence of multiple forms of microsomal monooxygenases in normal animals [38–44].

Hydroxylation of S-warfarin

Unlike the results obtained for *R*-warfarin, the K_m value for 8-hydroxylation from *S* warfarin is significantly larger than the K_m values for either 6- or 7-hydroxylation. Thus, it is clear that two kinetically distinct enzymatic processes must be involved despite the proximity of the sites of metabolic attack. If product formation is rate limiting, the contribution of the rate constant for product formation to K_m should be negligible; hence, all the K_m values should be the same while the V_{max} values may be different. If product formation is not rate limiting but a common intermediate is involved, two cases are possible: the rate of product formation is comparable to the rate of dissociation of the common intermediate back to substrate or the rate of formation of the common intermediate itself may be rate limiting. In the first case, the product which is formed with the greatest V_{max} must also have the greatest K_m, while in the second case the K_m for all products must be the same.

Since 8-hydroxylation occurs with the largest K_m coupled to the smallest V_{max}, none of the above conditions are fulfilled. Hence, there can be no common intermediate in the formation of 8-hydroxywarfarin and either 6- or 7-hydroxy metabolites. Since the K_m values are different, if a single enzymatic site is involved, the observed products must arise from at least two chemically distinct and irreversible pathways each of which may involve one or more different intermediate steps.

As was the case for *R*-warfarin, the K_m values for 4′- and benzylic hydroxylation yield little new information by themselves. Although the evidence is less convincing in this case the data suggest that K_m for benzylic hydroxylation is larger than the rest.

Comparative hydroxylation of the enantiomers

A high degree of stereoselectivity as evidenced by the differing V_{max} values for each enantiomeric pair of products is displayed by the microsomal system. The indistinguishability of the K_m values for 6- and 7-hydroxylation of a given enantiomer suggests on first analysis that a common enzymatic site is responsible for the generation of the 6- and 7-hydroxylated products with the differences in V_{max} attributable to stereochemical effects on the transition state. When the K_m values for the two 6-hydroxylation products are compared, they are found to just differ at the 95 per cent confidence level. This is not true for 7-hydroxylation. If 6- and 7-hydroxylation occur via a common intermediate, a stereoselective difference in K_m values for the 6-hydroxy metabolites should be reflected in a corresponding identical difference in the K_m values for the 7-hydroxy metabolites. This does not appear to be the case if the K_m differences are real. Therefore, for at least one of the enantiomers, 6- and 7-hydroxylation cannot occur via a common intermediate.

Arene oxides are well-established intermediates in aromatic hydroxylation processes mediated by microsomal mixed-function oxidases [45]. Hence, an attractive common intermediate for the formation of the 6- and 7-hydroxylated products of warfarin would be the 6–7 epoxide. Subsequent ring opening and aromatization would give the hydroxylated products.

Studies of the aromatizations of arene oxides have shown that the rate-limiting step of this process involves the spontaneous opening of the arene oxide. The direction in which the opening occurs depends on the stability of the carbocationoid transition state [46–53]. In the case of the postulated arene oxide, this would imply that 6-hydroxylation would occur preferentially to 7-hydroxylation, due to the ortho- and para-directing effect of the lactone oxygen. However, since 7-hydroxylation yields the major product for the *R*-enantiomer, it is most reasonable to conclude that a common intermediate is not involved in the formation of the 6- and 7-hydroxy metabolites of this isomer.

Similar electronic arguments permit one to conclude that a 7–8 epoxide cannot be an intermediate in the 7-hydroxylation of *R*-warfarin. As discussed above, no common intermediate for the formation of the 7- and 8-hydroxy metabolites of *S*-warfarin is possible based on kinetic evidence. Therefore, it is safe to conclude that if 7–8 epoxidation occurs in the metabolism of either isomer it must yield 8-hydroxywarfarin exclusively.

Unlike coumarin hydroxylation, 4'-hydroxylation is stereospecific for the *S*-enantiomer but these results are consistent with either the single site or multisite model. The apparently larger K_m values for benzylic hydroxylation of both isomers suggest that, if product formation is rate limiting, aliphatic hydroxylation probably occurs at a different site from aromatic hydroxylation.

105,000 g Supernatant study

The enzymatic reduction of ketones by mammalian biological systems is a well-established biotransformation route. The primary site for these reductions appears to be in the cytosol fraction of the liver cell [30–34], although aromatic ketones and aldehydes are also reduced by the soluble fraction of rabbit kidney [34]. Unlike alcohol dehydrogenase, which requires NADH as cofactor, most of the reported ketone reductions appear to utilize NADPH more effectively [30–34].

When the reduction *in vitro* or *in vivo* of nonsymmetrically substituted ketones occurs, optically active metabolites are commonly produced [30, 32, 54, 55], thus establishing the stereospecific nature of this reaction. Moreover, such reductions appear to occur in the same stereochemical sense. For example, the reduced metabolites of acetophenone [30] and prostaglandins [32] have been shown to possess the *S*-configuration.

The biological reduction *in vivo* of the ketone group of warfarin in man also follows this same stereochemical sense: *R*-warfarin is preferentially reduced to the *R,S*-alcohol, while *S*-warfarin is preferentially reduced to the *S,S*-alcohol [12, 13, 23, 47]. This reduction appears to be stereoselective for *R*-warfarin. To further characterize the stereochemistry of this enzymatic reaction in mammalian systems, investigations *in vitro* in rat were conducted. These studies were also performed in order to determine if warfarin alcohol is, in fact, a metabolite of warfarin in the rat. Previous studies in the rat [14] and in the guinea pig [56] resulted in the isolation and characterization of cyclic dehydrated warfarin alcohol, *8*. However, these workers did not observe the presence of the warfarin alcohols themselves. Since it seemed likely that cyclic dehydrated warfarin alcohol is derived from warfarin alcohol, either during isolation or *in vivo* via an activation process, identification of its formation *in vitro* would support this contention. The results in this study (Table 3) show that significant reduction occurs even in the absence of added cofactors; however, added NADPH appeared to be more effective than added NADH. The reduction is a stereoselective process: *R*-warfarin is reduced to the *R,S*-alcohol with high stereospecificity. This finding conforms closely to the results obtained in man [12, 13, 21, 23]. Unlike the results obtained from man, *S*-warfarin is reduced with less stereospecificity and predominantly to the *R*-configuration. This reversal in expected configurations seems to imply that the configuration of warfarin must have a significant effect on the stereochemical course of reduction, as well as on the degree of reduction. If this were not the case, one would expect *S*-warfarin to be stereospecifically reduced to the *S,S*-alcohol.

10,000 g Supernatant study

In order to study the stereochemistry of the biotransformation of warfarin in a system more closely representing the intact animal, the 10,000 *g* supernatant of rat liver was studied. The similarity between the results using the separated microsome and cytosol fractions and the 10,000 *g* combined fraction indicates that no significant secondary stereoselective biotransformation of the oxidized metabolites of warfarin has occurred in the cytosol component. Similarly, no apparent secondary stereoselective biotransformation of *R,S*- and *R,R*-warfarin alcohols has taken place in the microsomal component. However, an apparent reversal in the stereospecific reduction of *S*-warfarin occurred in the 10,000 *g* supernatant (Table 4). In this system, *S*-warfarin was reduced to the *S,S*-warfarin alcohol while in the 105,000 *g* supernatant, *S*-warfarin was stereoselectively reduced to the *S,R*-warfarin alcohol (Table 3). The basis for these differences is not known.

The results of the 10,000 *g* supernatant study are helpful in interpreting the pharmacokinetic properties *in vivo* of warfarin. For example, a number of workers have independently reported [9, 10, 11, 17] that *R*-warfarin is cleared from the plasma of rats at a rate nearly twice that of the *S*-isomer. This difference appeared to reflect a difference in the rate of metabolism of these two isomers. The results in Table 4 seem to establish that this supposition is probably true in that *R*-warfarin is biotransformed approximately twice as rapidly as *S*-warfarin. Moreover, the reversed stereoselectivity displayed by man (*S*-enantiomer) and rat (*R*-enantiomer) for 7-hydroxylation, the major metabolic route in both species, would account for the species difference between man and rat in the relative kinetics of elimination of the warfarin enantiomers.

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