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CUMENE HYDROPEROXIDE-SUPPORTED MICROSOMAL HYDROXYLATIONS OF WARFARIN—A PROBE OF CYTOCHROME P-450 MULTIPLICITY AND SPECIFICITY*

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Abstract—The hepatic microsomal metabolism of R and S warfarin, supported by NADPH or cumene hydroperoxide, has been investigated to probe the multiplicity and specificity of cytochromes P-450. Microsomes were uninduced, and phenobarbital (PB)-, 3-methylcholanthrene (MC)- or 3β-hydroxy-20-oxo-pregn-5-ene-16-α-carbonitrile (PCN)-induced from rat liver. Cumene hydroperoxide supported the formation of all the NADPH-supported warfarin metabolites (4′-, 6-, 7- and benzylic hydroxywarfarin and dehydrowarfarin), except 8-hydroxywarfarin. Comparisons of the rates of formation of the metabolites supported by NADPH or cumene hydroperoxide (with uninduced and induced microsomes) revealed that cumene hydroperoxide had the following effects: (1) rates of hydroxylation of the phenyl substituent of warfarin (4′-hydroxywarfarin) were increased; (2) rates of metabolism of the aliphatic portion of warfarin (benzylic hydroxywarfarin and dehydrowarfarin) were increased, except with S warfarin and uninduced microsomes; and (3) rates of hydroxylation of the phenyl ring of the coumarin group of warfarin were (a) decreased (7-, 8-hydroxywarfarin) or (b) decreased (6-hydroxywarfarin) with MC-induced microsomes and increased or unchanged with uninduced and PB- or PCN-induced microsomes. We concluded from these studies that multiple cytochromes P-450 are implicated in the metabolism of warfarin; that the cytochromes P-450 catalyzing the formation of 7- and 8-hydroxywarfarin differ from those catalyzing the other metabolites, except for 6-hydroxylation by MC-induced microsomes; that the cytochromes catalyzing 7- and 8- hydroxywarfarin formation differ from one another; that for each metabolite of warfarin, the cytochrome P-450 type predominantly responsible for its formation is the same, irrespective of the mode of induction of the microsomes; and that 6-hydroxylase activity is the exception to the previous point, and is predominantly associated with different cytochromes P-450 in differently induced microsomes. The effects of cumene hydroperoxide have been ascribed to differences in cumene hydroperoxide affinities, differences in cumene hydroperoxide-induced destruction, and differences in cumene hydroperoxide inhibitions of warfarin binding to different cytochromes P-450, together with differences in the situation of cytochromes P-450 in the microsomal membrane.

Elucidation of the mechanism of action and of the number of different cytochromes P-450 in hepatic microsomes, together with their corresponding specificities, is a prerequisite for determination of the role of cytochromes P-450 in chemically induced carcinogenicity, and drug, xenobiotic and endogenous steroid metabolism.

Significant advances toward the resolution of these problems have followed from the numerous reports on the isolation, purification and characterization of a variety of cytochromes P-450 [1–3]. However, investigations into the multiplicity and specificity of cytochromes P-450, which are still retained in the microsomal membrane milieu, are essential to draw correlations with data on purified enzymes. Such comparisons are necessary for evaluation of the role of the microsomal membrane, the situation of the components of the system in the membrane, and the consequences of protein isolation techniques on the properties of cytochromes P-450.

Both enantiomers of the widely utilized oral anticoagulant and rodenticide, warfarin (Scheme I, A), are metabolized by rat hepatic microsomal cytochromes P-450 to yield a variety of products including 6- (scheme I, B), 7- (I, C), 8- (I, D), and 4′-hydroxywarfarin (I, E) and benzylic hydroxywarfarin (I, F)‡ [4, 5], together with the recently identified metabolite, dehydrowarfarin [4-hydroxy-3-(3-oxo-1-phenyl-1-butenyl)-2H-1-benzopyran-2-one] (I, H).§ All of the warfarin metabolites can be analyzed simultaneously by a high-pressure liquid chromatographic

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‡Preliminary mass spectral analysis in our laboratories indicates that the structure of the metabolite identified as benzylic hydroxywarfarin has been incorrectly assigned [5]. The correct structure for this metabolite is probably 10-hydroxywarfarin (IG), and we are presently confirming this structural assignment. The incorrect assignment of the structure does not affect any of the conclusions of the present investigation, and we will continue to refer to this product as benzylic hydroxywarfarin until the alternative structure is confirmed.

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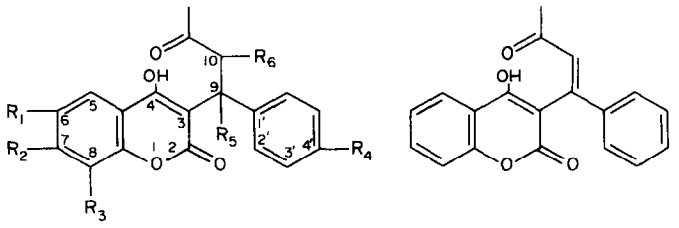

H

A $R_1 = R_2 = R_3 = R_4 = R_5 = R_6 = H$

B $R_1 = OH, R_2 = R_3 = R_4 = R_5 = R_6 = H$

C $R_2 = OH, R_1 = R_3 = R_4 = R_5 = R_6 = H$

D $R_3 = OH, R_1 = R_2 = R_4 = R_5 = R_6 = H$

E $R_4 = OH, R_1 = R_2 = R_3 = R_5 = R_6 = H$

F $R_5 = OH, R_1 = R_2 = R_3 = R_4 = R_6 = H$

G $R_6 = OH, R_1 = R_2 = R_3 = R_4 = R_5 = H$

Scheme 1.

(h.p.l.c.) assay [6]. The relative rates of formation of these metabolites are altered markedly as a consequence of the action of the mixed-function oxidase-inducing agents, such as phenobarbital (PB) and 3-methylcholanthrene (MC) [7]. The metabolism of warfarin thus provides a powerful probe for investigating the function, specificity and multiplicity of cytochromes P-450.

The discovery that cumene hydroperoxide can replace NADPH and O_2 in the cytochrome P-450-catalyzed metabolism of a number of fatty acids, aromatic drugs, and steriods [8–10] provides another approach for the investigation of the mechanism of action of mixed-function oxidases. Hrycay *et al.* [9] have proposed that cumene hydroperoxide inserts the activated oxygen species directly into the substrate–ferricytochrome P-450 complex. This is in contrast to the NADPH-supported mechanism *in vitro*, where the oxygen molecule binds to the substrate–ferrocytochrome P-450 complex, and is activated after incorporation of a further electron from NADPH. Cumene hydroperoxide thus probably substitutes for the early steps in the activation of oxygen in the mechanism of cytochrome P-450 function.

In the present study we have utilized the hepatic microsomal metabolism of R and S warfarin by control, PB-, MC- or 3β-hydroxy-20-oxo-pregn-5-ene-16α-carbonitrile (PCN)-induced microsomes to probe cytochromes P-450. The rates of formation of each metabolite supported by either NADPH or cumene hydroperoxide were compared, and the results used to indicate the multiplicity and specificity of the microsomal cytochromes P-450.

MATERIALS AND METHODS

Materials. PB and MC were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ) and K & K Laboratories, Inc. (Plainview, NY) respectively. PCN was a gift from the Upjohn Co. (Kalamazoo, MI). Racemic warfarin from CalBiochem (La Jolla, CA) was resolved into optically pure R ($[\alpha]_D$ = + 149) and S warfarin ($[\alpha]_D$ = − 149.7) by the method of West *et al.* [11]. Cumene hydroperoxide was obtained from Pfaltz & Bauer (Stamford, CT), and NADP from Sigma Chemical Co. St. Louis, MO. Water was deionized and glass-distilled. All other reagents, including the solvents, were of the highest grades commercially available.

The 6- (Scheme I, B), 7- (I, C), 8- (I,D), and 4'-hydroxywarfarin (I, E) metabolites were prepared by the methods of Hermodson *et al.* [12], Pohl *et al.* [13] and Buckle *et al.* [14], modified as described previously [6]. Benzylic hydroxywarfarin (I, F) was isolated as a product of the *in vitro* hepatic microsomal metabolism of R warfarin, as described by Pohl *et al.* [7]. 4-Hydroxy-3-(3-oxo-1-phenyl-1-butenyl)-2H-1-benzopyran-2-one (dehydrowarfarin) (I, H) was synthesized by treatment of warfarin with cuprous chloride and pyridine.*

Methods. Experimental animals were male Wistar rats from the New York State Department of Health Griffin Laboratories, and weighed 250 ± 20 g at the time of sacrifice. The rats were acclimatized at 21 with a 12-hr light cycle before use. Hepatic microsomal cytochromes P-450 were induced with PB (100 mg/kg/day, i.p., in 0.9% saline for 3 days); MC (25 mg/kg/day, i.p., in corn oil for 3 days); or PCN (100 mg/kg/day, i.p., in corn oil suspension for 3 days). Rats were killed by cervical dislocation 24 hr after the final dose of inducing agent. Hepatic microsomes were prepared by the Sepharose 2B chromatography method of Tangen *et al.* [15], which was modified slightly as described previously [6]. The protein concentration of the microsomal suspension was determined by the method of Schacterle and Pollack [16] and the cytochrome P-450 concentration by the method of Omura and Sato [17].

The microsomal incubation system comprised the following (all values are final concentrations): microsomes (2.0 mg protein/ml) in 0.01 M *N*-Tris (hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES) and 0.002 M $MgCl_2$, pH 7.4 (3.0 ml); NADP (0.3 mM) and glucose 6-phosphate (15.5 mM) in the same buffer (1.0 ml); and R or S sodium warfarin (0.75 mM) in

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water (0.1 ml). After a 1-min incubation at 37° in a shaking water bath, glucose 6-phosphate dehydrogenase (yeast, 5 units) was added to initiate the reaction. The reaction mixture was rapidly filtered after a further 10 min, and assayed by a modification* of the h.p.l.c. system described previously [6].

In those experiments where cumene hydroperoxide was added, the reaction was terminated after 5 min. The NADPH-generating system was replaced by buffer, and the reaction was initiated by adding cumene hydroperoxide (1.0 mM) in place of the glucose 6-phosphate dehydrogenase. All other conditions were unaltered. In a control experiment, the microsomes were eliminated from the reaction mixture.

The mean values of the experimental data were compared using the Student's t-test with $P < 0.05$ as a limit of significance.

RESULTS

Cumene hydroperoxide supported the control, PB-, MC- or PCN-induced microsomal metabolism of R and S warfarin to yield all of the previously reported NADPH-supported products (Scheme I, B–H), except for 8-hydroxywarfarin. In the absence of microsomes no products were formed. Cumene hydroperoxide and microsomes do not further metabolize the products of warfarin metabolism. The rates of formation of the various products deviated slightly from linearity with time over 5 min, but relative rates of product formation were unaltered, and reactions were terminated after this period. Sodium periodate also supported the cytochrome P-450-catalyzed formation of these metabolites, but the rates were too low to be quantitated accurately.

Examples of h.p.l.c. patterns of metabolites from R and S warfarin with PB-induced microsomes and NADPH or cumene hydroperoxide are shown in Figs. 1 and 2.

The rates of formation of each of the metabolites of R and S warfarin supported by NADPH or cumene hydroperoxide with control, PB-, MC- or PCN-induced microsomes are presented in Tables 1, 2, 3 and 4 respectively. The ratios of rates with cumene hydroperoxide and NADPH for each metabolite are also presented to facilitate comparisons of the two forms of metabolic reaction. In the case of hydroxylation of the phenyl group of R and S warfarin to yield 4'-hydroxywarfarin, the rate with cumene hydroperoxide was increased or unchanged relative to the rate with NADPH for control, PB-, MC- and PCN-induced microsomes. In contrast, the rates of hydroxylation of the phenyl portion of the coumarin moiety to yield 7- and 8-hydroxywarfarin from both R and S warfarin with induced and uninduced microsomes were decreased with cumene hydroperoxide. In particular, the 8-hydroxylase activity of all the investigated microsomal systems was too low to be quantitated. The third hydroxylation of the coumarin-phenyl ring at the 6-position was, however, more variable, with some results being higher and others lower with cumene hydroperoxide than with NADPH. For the two reactions involving the aliphatic moeity

*M. J. Murphy, M. J. Cashin, M. J. Fasco, L. J. Piper, D. N. McMartin and L. S. Kaminsky, manuscript submitted for publication.

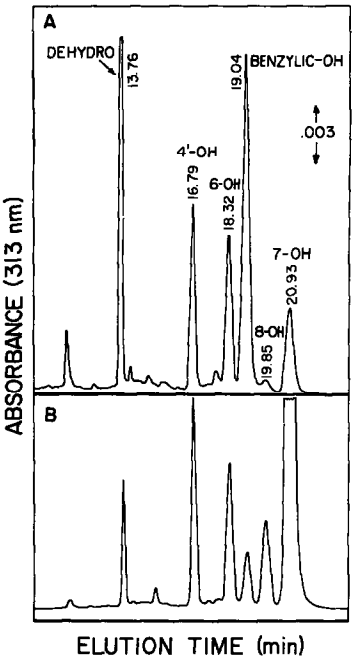

Fig. 1. High pressure liquid chromatographic analysis of the metabolites of R warfarin catalyzed by phenobarbital-induced rat hepatic microsomes (2.0 mg protein/ml; cytochrome P-450, 2.3 nmoles/mg of protein), and either (A) cumene hydroperoxide (1.0 mM) with a column loading of 200 μl of microsomal incubation filtrate or (B) NADPH (0.3 mM) with a column loading of 100 μl of microsomal incubation filtrate. A μBondapak/C_{18} column (4 mm × 30 cm) was used, and products were eluted at a flow rate of 2.0 ml/min.

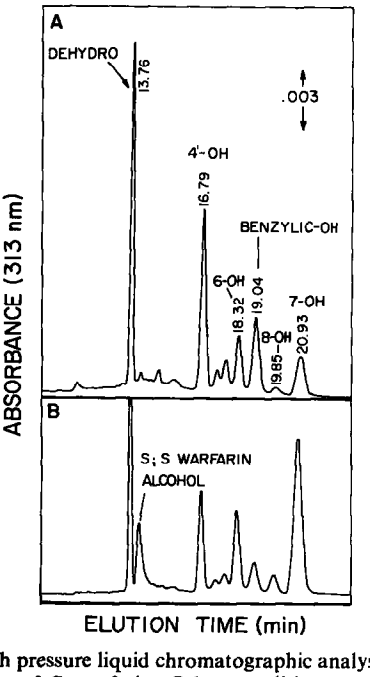

Fig. 2. High pressure liquid chromatographic analysis of the metabolites of S warfarin. Other conditions were as in Fig. 1.

of warfarin, which yield dehydrowarfarin and benzylic hydroxywarfarin, the rates with cumene hydroperoxide were increased, except for S warfarin with uninduced microsomes, which were decreased. For these aliphatic metabolites, the most noticeable increases in cumene hydroperoxide relative to NADPH-supported rates were with PB- or PCN-induced microsomes and R warfarin.

A number of other points are apparent from the metabolite formation rates in Tables 1–4. The most dramatic effect of MC induction was the increase in NADPH-supported 6- and 8-hydroxylase activities with R warfarin relative to control microsomes. With cumene hydroperoxide, however, these activities were differentiated, with the 6-hydroxylase activity being decreased, but remaining high, and the 8-hydroxylase activity at negligible levels. The control, PB, MC- and PCN-induced cytochrome P-450-catalyzed, NADPH-supported rates of metabolism of R warfarin exceeded those of S warfarin. For the corresponding cumene hydroperoxide-supported reactions, this overall stereo-selectivity was retained, notwithstanding changes in the stereoselectivity of some of the individual metabolites. With control and PCN-induced microsomes, R warfarin was favored to a greater extent, and with MC-induced microsomes to a lesser extent when supported by cumene hydroperoxide, than when supported by NADPH. Control and PB-induced microsomes were similar in that both had 7-hydroxywarfarin from R warfarin as the major

Table 1. Rates of formation of the metabolites of R and S warfarin catalyzed by uninduced rat hepatic microsomal cytochromes P-450 supported by cumene hydroperoxide or NADPH

Warfarin metabolite	Rates of formation* (nmoles/mg microsomal protein min) NADPH	CuOOH†	CuOOH/NADPH
R dehydro	0.02 ± <0.005	0.05 ± <0.005	2.27
R benzylic	0.10 ± <0.005	0.20 ± 0.02	2.00
R 4'-OH	0.11 ± 0.01	0.26 ± 0.04	2.36
R 6-OH	0.10 ± 0.02	0.28 ± 0.08	2.80
R 7-OH	0.19 ± 0.06	0.03 ± 0.01	0.16
R 8-OH	0.03 ± 0.01	ND‡	
Total	0.55	0.82	
S dehydro	0.05 ± <0.005	0.04 ± <0.005	0.80
S benzylic	0.04 ± 0.01	0.02 ± 0.01	0.50
S 4'-OH	0.15 ± 0.05	0.28 ± 0.01	1.87
S 6-OH	0.11 ± 0.07	0.09 ± 0.01	0.82
S 7-OH	0.04 ± 0.01	0.02 ± <0.005	0.50
S 8-OH	0.01 ± <0.005	ND‡	
Total	0.40	0.45	

*Sample size, six.
†Cumene hydroperoxide.
‡Not detectable.

Table 2. Rates of formation of the metabolites of R and S warfarin catalyzed by PB-induced rat hepatic microsomal cytochromes P-450 supported by cumene hydroperoxide or NADPH

Warfarin metabolite	Rates of formation* (nmoles/mg microsomal protein/min) NADPH	CuOOH†	CuOOH/NADPH
R dehydro	0.03 ± 0.01	0.15 ± 0.02	5.00
R benzylic	0.17 ± 0.04	1.12 ± 0.24	6.59
R 4'-OH	0.23 ± 0.08	0.18 ± 0.05	0.78
R 6-OH	0.30 ± 0.01	0.23 ± 0.07	0.77
R 7-OH	0.78 ± 0.04	0.09 ± 0.02	0.12
R 8-OH	0.01 ± 0.01	ND‡	
Total	1.52	1.77	
S dehydro	0.08 ± 0.01	0.11 ± <0.005	1.38
S benzylic	0.11 ± 0.03	0.27 ± 0.06	2.45
S 4'-OH	0.12 ± 0.01	0.22 ± 0.02	1.83
S 6-OH	0.15 ± 0.03	0.12 ± 0.02	0.80
S 7-OH	0.19 ± 0.01	0.05 ± 0.01	0.26
S 8-OH	0.03 ± <0.005	ND‡	
Total	0.68	0.77	

*Sample size, nine.
†Cumene hydroperoxide.
‡Not detectable.

Table 3. Rates of formation of the metabolites of R and S warfarin catalyzed by MC- induced rat hepatic microsomal cytochromes P-450 supported by cumene hydroperoxide or NADPH

Warfarin metabolite	Rates of formation* (nmoles/mg microsomal protein/min) NADPH	CuOOH†	CuOOH/NADPH
R dehydro	0.01 ± <0.005	0.05 ± 0.00	5.00
R benzylic	0.03 ± 0.00	0.19 ± 0.01	6.33
R 4'-OH	0.07 ± 0.00	0.21 ± 0.02	3.00
R 6-OH	0.76 ± 0.05	0.22 ± 0.03	0.29
R 7-OH	0.15 ± 0.01	0.02 ± <0.005	0.13
R 8-OH	0.87 ± 0.06	0.02 ± 0.00	0.02
Total	1.89	0.71	
S dehydro	0.02 ± <0.005	0.04 ± 0.00	2.00
S benzylic	0.02 ± 0.00	0.04 ± <0.005	2.00
S 4'-OH	0.09 ± 0.01	0.03 ± 0.01	3.33
S 6-OH	0.30 ± 0.03	0.10 ± 0.01	0.33
S 7-OH	0.06 ± 0.01	0.02 ± <0.005	0.33
S 8-OH	0.06 ± 0.01	0.00 ± 0.00	0.02
Total	0.55	0.50	

*Sample size, six.
†Cumene hydroperoxide.

Table 4. Rates of formation of the metabolites of R and S warfarin catalyzed by PCN-induced rat hepatic microsomal cytochromes P-450 supported by cumene hydroperoxide or NADPH

Warfarin metabolite	Rates of formation* (nmoles/mg microsomal protein/min) NADPH	CuOOH†	CuOOH/NADPH
R dehydro	0.09 ± <0.005	0.42 ± 0.02	4.67
R benzylic	0.30 ± 0.02	1.57 ± 0.06	5.23
R 4'-OH	0.07 ± <0.005	0.19 ± 0.01	2.71
R 6-OH	0.08 ± 0.01	0.25 ± 0.05	3.13
R 7-OH	0.17 ± 0.02	0.02 ± <0.005	0.12
R 8-OH	0.04 ± 0.01	ND‡	
Total	0.75	2.45	
S dehydro	0.19 ± <0.005	0.20 ± 0.01	1.05
S benzylic	0.14 ± 0.01	0.25 ± 0.02	1.79
S 4'-OH	0.10 ± 0.01	0.21 ± <0.005	2.10
S 6-OH	0.11 ± <0.005	0.09 ± 0.02	0.82
S 7-OH	0.05 ± <0.005	0.01 ± <0.005	0.20
S 8-OH	0.02 ± 0.01	ND‡	
Total	0.61	0.76	

*Sample size, six.
†Cumene hydroperoxide.
‡Not detectable.

NADPH-supported metabolite. This was not duplicated in the cumene hydroperoxide-supported reaction, where with control microsomes 6-hydroxywarfarin was the major R warfarin metabolite, while PB-induced microsomes yielded benzylic hydroxywarfarin as the major product.

DISCUSSION

The pH optimum for cumene hydroperoxide-supported microsomal hydroxylations has been reported to be at approximately pH 8 [18, 19]. In order to facilitate comparisons between NADPH- and cumene hydroperoxide-supported reactions, however, we performed the peroxide-supported reactions at the same pH (pH 7.4) used for the NADPH-supported reactions.

The differences in mechanism between NADPH- and cumene hydroperoxide-supported cytochrome P-450-catalyzed reactions have not been fully elucidated. A current proposal [9, 20], based on comparisons of observed spectral intermediates after addition of cumene hydroperoxide to rabbit liver microsomes with spectra obtained by addition of H_2O_2 to ferrimyoglobin, catalase, horseradish peroxidase or chloroperoxidase, is that cumene hydroperoxide reacts directly with the substrate–ferricytochrome P-450 complex to form a product which undergoes heterolytic oxygen–oxygen bond scission to yield a ferryl ion complex (or a resonance form thereof).

In contrast, in the NADPH-supported reaction mechanism, the oxygen molecule is incorporated into the substrate–ferrocytochrome P-450 complex. The resultant ternary complex accepts an electron and, after internal electron displacements, splits off an atom of oxygen to form water with conversion of the complex to a ferryl ion of the form $S\text{–}Fe^{4+}\text{-}O^{-}$, where S is the substrate and Fe^{4+} is the iron component of the heme of cytochrome P-450. Thus, cumene hydroperoxide reacts directly with the cytochrome P-450 molecule, while NADPH requires the intercession of at least NADPH cytochrome P-450 reductase and phospholipid, and possibly other protein molecules. These differences in mechanism have been supported by studies using purified and reconstituted cytochrome P-450 preparations [21]. The results of these studies also indicate the existence of a common ternary activated intermediate for the NADPH and cumene hydroperoxide-supported reactions, although not necessarily the ferryl ion intermediate.

In investigations of the microsomal metabolism of a substrate to multiple products [e.g. Refs. 9, 19, 20], any differential in the effects of the replacement of NADPH with cumene hydroperoxide on the product formation rates has been interpreted to indicate involvement of more than one form of cytochrome P-450. The differences in the effects of cumene hydroperoxide on the various warfarin metabolite formation rates thus confirm the previous suggestions of the participation of a number of cytochromes P-450 in warfarin metabolism. The present results permit an analysis of the multiplicity and the related specificity of these cytochromes P-450. Thus, the cytochromes P-450 predominantly involved in the formation of 7- and 8-hydroxywarfarin from R and S warfarin must differ from those catalyzing the other warfarin hydroxylations, with the possible exception of the 6-hydroxylase activity of MC-induced microsomes, because of their depressed formation rates with cumene hydroperoxide. Furthermore, these cytochromes P-450 associated with the warfarin 7- and 8-hydroxylase activities must also differ from one another. This follows from the fact that the rate of R 8-hydroxywarfarin formation is markedly enhanced by MC-induction of the microsomes (Table 3), which indicates the involvement of a cytochrome P-448 enzyme, while the formation rate of R 7-hydroxywarfarin is markedly increased by PB induction of the microsomes, implying the involvement of a cytochrome P-450. The fact that R 6-hydroxywarfarin rates are also markedly enhanced by MC induction indicates that its formation is also mediated by a cytochrome P-448. Comparisons of cumene hydroperoxide-supported with NADPH-supported rates of formation indicate that, with MC-induced microsomes, R 6-hydroxywarfarin and R 8-hydroxywarfarin formation may be catalyzed by the same form of cytochrome P-448, based on depressed rates for both metabolites. However, the fact that the extents of depression of the rates with cumene hydroperoxide differ, with 8-hydroxywarfarin rates being depressed to a much greater extent than 6-hydroxywarfarin rates, suggests that 6-hydroxywarfarin formation may be catalyzed by two enzymes, one of which is the same as that catalyzing 8-hydroxywarfarin formation. In contrast, however, by utilizing similar comparisons of cumene hydroperoxide- and NADPH-supported rates, it is clear that, with control, PB- and PCN-induced microsomes, 6- and 8-hydroxywarfarin formation must be catalyzed by different cytochromes P-450. The results with 6-hydroxywarfarin thus indicate that the formation of this metabolite is apparently catalyzed by different cytochromes P-450 which predominate to different extents depending on the state of induction of the microsomes. However, by utilizing similar comparisons, 8-hydroxywarfarin is apparently synthesized by only one form of cytochrome P-450 in the induced and uninduced microsomes. This is supported by our unpublished results on the metabolism of warfarin by reconstituted purified rabbit liver cytochromes P-450.* The formation of some of the hydroxylated metabolites was shown to be catalyzed by more than one enzyme, while other hydroxylated products arose from only one enzyme.

In the case of both the NADPH- and cumene hydroperoxide-supported microsomal reactions, the rate-controlling step for the metabolism of warfarin must precede the formation of the common ferryl ion intermediate. The postulation of an earlier rate-controlling step is necessary to explain the increase in some metabolite formation rates, and the decrease in others, when supported by cumene hydroperoxide (Tables 1–4). There are three possible explanations for these decreases in the formation of rates of some of the warfarin metabolites. Purified and microsomal cytochromes P-450 have been demonstrated to be susceptible to destruction by cumene hydroperoxide [10, 21], probably through degradation of the heme group. The extent of the destruction is apparently diminished by the presence of substrates, and varies with the structure of the substrates [21]. Thus, *N*-methylaniline diminishes the extent of cumene hydroperoxide-induced destruction of a purified cytochrome P-450 to a much greater extent than does benzphetamine. The diminished rates for the cumene hydroperoxide-supported formation of 7- and 8-hydroxywarfarin may thus be a consequence of the diminished protection of the cytochromes P-450 against cumene hydroperoxide-induced destruction afforded by warfarin, when bound in the orientation required to produce 7- and 8-hydroxylation. An alternative possibility is that cumene hydroperoxide or its degradation product 2-phenyl-2-propanol (cumenol) [22] competes with warfarin, in that orientation, for the catalytic sites on the cytochromes P-450. A third possible explanation for the diminished rates of formation of 7- and 8-hydroxywarfarin with cumene hydroperoxide is that the cytochromes P-450 responsible for the formation of these metabolites exhibit a relatively low affinity for cumene hydroperoxide. The last possibility is unlikely, however, in view of the known support of cumene hydroperoxide for the microsomal and purified cytochrome P-448-catalyzed metabolism of benzpyrene [23] and biphenyl [24].

The enhanced rates of formation of some of the warfarin metabolites supported by cumene hydro-

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peroxide are possibly a consequence of the relatively high affinity of cumene hydroperoxide for the corresponding cytochromes P-450. The most marked effects of cumene hydroperoxide in increasing the rates of formation of warfarin metabolites are with dehydrowarfarin* and benzylic hydroxywarfarin, which involve metabolism of the aliphatic moeity of the warfarin molecule. With PB- and PCN-induced microsomes the rates of cumene hydroperoxide-supported formation of dehydrowarfarin and benzylic hydroxywarfarin are much greater than the corresponding rates with control and MC-induced microsomes, which implies that the pertinent cytochrome(s) P-450 are induced by PB and PCN. However, the fact that the corresponding NADPH-supported rates do not exhibit comparable effects of this induction by PB and PCN suggests that a high affinity of cumene hydroperoxide for these cytochrome(s) P-450 cannot be the only explanation for the elevation of rates of cumene hydroperoxide-supported over NADPH-supported reactions. An alternative explanation is based on a model for the microsomal mixed-function oxidase proposed by Peterson *et al.* [25]. In this model the catalytic portion of the NADPH cytochrome P-450 reductase protrudes above the surface of the membrane, and the reductase is surrounded by a cluster of cytochromes P-450. The NADPH-dependent reduction of these cytochromes is rapid because no translational motion of the cytochromes P-450 in the membrane is required. A second group of cytochromes P-450 is situated at a distance from any reductase molecule, and the NADPH-supported reduction of these cytochromes P-450 is slow because of the requirement for translational motion to the reductase. Since cumene hydroperoxide interacts directly with the cytochromes P-450, it does not differentiate between the cytochromes P-450 clustered around the reductase and those at a distance from the reductase. The cytochromes(s) P-450 responsible for formation of dehydrowarfarin and benzylic hydroxywarfarin could thus possibly fall into the group which is situated at a distance from the reductase. PB and PCN induction increases the levels of the enzyme(s), but their catalytic rates supported by NADPH were still low because of the requirement for translational motion. With cumene hydroperoxide, however, without any requirement for translational motion the rates of product formation were markedly increased, and more closely reflected the increased enzyme levels.

The fact that warfarin 4′-hydroxylase activity is generally increased by cumene hydroperoxide suggests that this activity could be related to those associated with hydroxylations at the aliphatic carbons of warfarin.

In summary, the use of cumene hydroperoxide to support the metabolism of warfarin has clearly indicated the role of a number of cytochromes P-450 in the microsomal hydroxylation of the drug. The fairly uniform results with control, and PB- MC- or PCN-induced microsomes, with respect to the relative rates with NADPH or cumene hydroperoxide, indicate that for each metabolite of warfarin, the cytochrome P-450 type predominantly responsible for its formation is the same, irrespective of the mode of induction of the microsomes. The exception to this is the 6-hydroxylase activity, which is apparently predominantly associated with different cytochromes P-450 in differently induced microsomes.

*The dehydrowarfarin metabolite probably arises by spontaneous dehydration of an intermediate which is hydroxylated on one of aliphatic carbons of the warfarin molecule. Studies to confirm this mechanism of formation are presently under way.

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