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METABOLISM *IN VITRO* OF WARFARIN BY ENZYMIC AND NONENZYMIC SYSTEMS

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Abstract—The metabolism of warfarin to 6-, 7- and 8-hydroxywarfarin occurs in rat liver microsomes and requires NADPH and molecular oxygen for activity. The Michaelis constant for the three hydroxylation reactions is $1{\cdot}5 \times 10^{-4}$ M. The enzymic hydroxylation of warfarin by liver differs from the nonenzymic hydroxylation. The enzymic metabolism by rat liver microsomes yields more 7-hydroxywarfarin than 6- or 8-hydroxywarfarin. The nonenzymic hydroxylation of warfarin by Fenton's system results in the formation of 6-hydroxywarfarin as the major hydroxylated product. The formation of an unidentified nonphenolic metabolite is also described.

WARFARIN is a coumarin derivative which is widely used as an anticoagulant in man and also as a raticide. This drug is metabolized *in vivo* in the rat to 6-, 7- and 8-hydroxywarfarin, which are excreted in the urine as pharmacologically inactive metabolites.[1] The present study was initiated to investigate the enzymic hydroxylation of warfarin by liver and also the nonenzymic oxidation of warfarin by certain model systems.

MATERIALS AND METHODS

Materials. A snail digestive juice preparation (commercial name, Glusulase), containing high levels of arylsulphatase (E.C. 3.1.6.1) and β-glucuronidase (E.C. 3.2.1.31), was obtained from Endo Laboratories, Inc. Glucose 6-phosphate dehydrogenase (G-6-P DHase) (E.C. 1.1.1.49) was purchased from Nutritional Biochemicals Corp. The preparation did not metabolize warfarin when assayed as described below. Sodium warfarin was a gift from Endo Laboratories, Inc., U.S.A. and Iptor A.G., Germany. Sodium warfarin-4-^{14}C (4·08 μc/mg) and 6-, 7- and 8-hydroxywarfarin were gifts from Dr. K. P. Link, University of Wisconsin, U.S.A. Silica gel GF 254 according to Stahl was used for TLC and was purchased from Brinkman Instruments, Inc.

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Animals. Weanling male Sprague–Dawley rats from Carworth Farms, weighing about 50 g at the beginning of the experiments, were fed a synthetic diet[2] and water *ad libitum*. The rats were i.p. injected with 37·5 mg/kg of phenobarbital twice daily for 4 days prior to the experiments.

Enzyme assay. Livers of rats were quickly removed, chilled and homogenized with 2 volumes of 0·25 M sucrose in a glass-Teflon homogenizer at 0°. The method used for the subcellular fractionation was essentially the same as that described by Hogeboom.[3] In usual assays, the supernatant fraction obtained after the centrifugation of liver homogenates at 9000 *g* for 15 min was used as the enzyme source. The standard reaction mixture was a modification of that described by Mueller and Miller[4] and contained 1·3 μmole warfarin-4-^{14}C (510000 cpm), 0·54 μmole NADP, 0·62 μmole NAD, 2 μmole ATP, 6·54 μmole glucose 6-phosphate (G-6-P), 120 μmole nicotinamide, 200 μmole KCl, 10 μmole $MgCl_2$, 40 μmole K_2HPO_4–KH_2PO_4 buffer (pH 7·4), 10 μmole Tris-HCl buffer (pH 7·4) and 9000 *g* supernatant fraction equivalent to 166 mg liver in a total volume of 3·5 ml in a 50-ml glass-stoppered tube. The details of the enzyme assays are given elsewhere.[5]

Assay of nonenzymic hydroxylation. The systems used for the nonenzymic hydroxylation of warfarin were described by Staudinger and Ullrich.[6] The composition of these systems and the conditions of incubation are described in Table 4. In the case of Fenton's system, the mixture was preincubated with bubbling of a nitrogen stream in the absence of $FeSO_4$ and H_2O_2 for 5 min. A 15-min incubation was then initiated by adding $FeSO_4$ with continuous bubbling of nitrogen. Hydrogen peroxide was added in three equally divided amounts after 0, 5 and 10 min of incubation. Other incubations were for 1 hr with shaking under air. If necessary, the size of the incubation was multiplied in order to detect smaller amounts of metabolites. The reaction was stopped by the addition of 1·0 ml of 2 N HCl, and warfarin and its metabolites were extracted into 60 ml methylene chloride. The methylene chloride layer was transferred into another tube and evaporated; the residue was dissolved in 2 ml methylene chloride and 0·5 ml of this solution was spotted on a silica gel thin-layer plate along a line 12 cm long. Warfarin, 6-hydroxywarfarin, 7-hydroxywarfarin and 8-hydroxywarfarin were analyzed as previously described.[5]

RESULTS

Enzymic metabolism of warfarin

Formation of hydroxywarfarins from warfarin. When warfarin-4-^{14}C was incubated with the 9000 *g* supernatant fraction of liver obtained from phenobarbital-treated rats, the formation of 6-, 7- and 8-hydroxywarfarin and an unidentified metabolite (metabolite X) was observed.[5] These metabolites had R_f values of 0·30, 0·43, 0·57 and 0·12 respectively when the silica gel thin-layer plates were developed as previously described.[5] Since less enzyme activity was found in control livers, experiments were carried out with livers of rats pretreated with 37·5 mg/kg of phenobarbital intraperitoneally twice daily for 4 days. When the 9000 *g* supernatant fraction was incubated under the standard conditions in the presence of various concentrations of warfarin-4-^{14}C, utilization of the double reciprocal plot of Lineweaver–Burk[7] revealed an apparent K_m of $1{\cdot}5 \times 10^{-4}$ M for the formation of 6-, 7- and 8-hydroxywarfarin (Fig. 1). A concentration of warfarin between $3{\cdot}5 \times 10^{-4}$ and 4×10^{-4} M saturated the enzyme with regard to the formation of the hydroxylated metabolites.

Intracellular localization and cofactor requirements for hydroxylation. Table 1 summarizes the localization of enzyme activity in the liver cell. The formation of 6-, 7- and 8-hydroxywarfarin was catalyzed by microsomal fraction, and dropped to about 5 per cent of the original level when the air phase of the incubation was replaced with nitrogen, indicating that the reaction required molecular oxygen as an oxygen

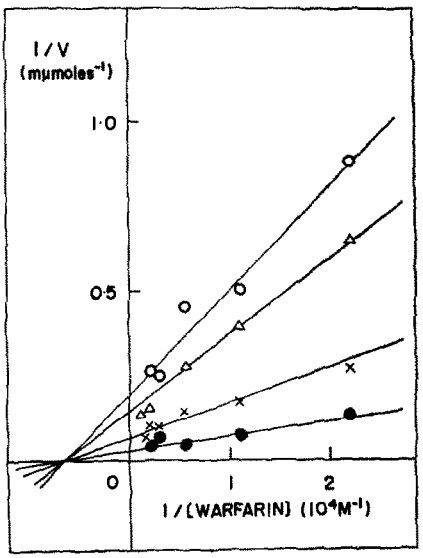

FIG. 1. Determination of the apparent Michaelis constants for the metabolism of warfarin to 6-, 7- and 8-hydroxywarfarin by rat liver. Warfarin-4-^{14}C at the various concentrations was incubated with the 9000 *g* supernatant fraction from 166 mg liver of a phenobarbital-pretreated rat under the standard conditions described in Materials and Methods. Velocity of the reaction is expressed as the amount of metabolite formed during a 30-min incubation. Symbols are: △—△, 6-hydroxywarfarin; ×—×, 7-hydroxywarfarin; ○—○, 8-hydroxywarfarin; ●—●, the sum of 6-, 7- and 8-hydroxywarfarin. The apparent K_m for the formation of 6-, 7- or 8-hydroxywarfarin was calculated as $1{\cdot}5 \times 10^{-4}$ M.

TABLE 1. WARFARIN METABOLISM BY SUBCELLULAR FRACTIONS OF RAT LIVER*

Fraction	Metabolites formed (mμmoles)			
	Metabolite X	6-Hydroxy-warfarin	7-Hydroxy-warfarin	8-Hydroxy-warfarin
1000 *g* Supernatant	5·9	12·9	24·1	7·3
9000 *g* Supernatant	6·2	10·7	20·8	6·6
Mitochondria	0·1	0·2	0·4	0·3
Microsomes	3·1	11·2	19·9	8·0
Soluble fraction	5·4	1·8	0·3	< 0·1

* Various fractions, equivalent to 166 mg liver from a rat pretreated with phenobarbital, were incubated aerobically at 37° for 30 min with the cofactors as described under Materials and Methods. The nature of metabolite X is discussed in the text. The data are representative of 2 experiments.

source. The hydroxylation reaction was dependent on NADPH (Table 2). In the absence of an NADPH-generating system (NADP, G-6-P and G-6-P DHase), very little hydroxywarfarin was formed.

TABLE 2. NADPH REQUIREMENT FOR WARFARIN HYDROXYLATION*

System	Metabolites formed (mμmoles)		
	6-Hydroxy-warfarin	7-Hydroxy-warfarin	8-Hydroxy-warfarin
Complete	13·1	23·1	4·3
-NAD	9·7	17·6	2·1
-NADP	1·0	1·9	0·4
-NADP, -G-6-P, -G-6-P DHase	0·2	0·2	0·3

* The complete system contained, in addition to 1·3 μmole warfarin-4-^{14}C and cofactors described under Materials and Methods, 10 Kornberg units of G-6-P DHase and liver microsomes. Liver microsomes, equivalent to 500 mg liver, were obtained from a rat pretreated with phenobarbital and were washed twice with 0·25 M sucrose prior to the experiment. Incubation was carried out at 37° for 30 min aerobically. The results shown are typical of 2 experiments.

Characterization of the unknown metabolite. During studies on the metabolism of warfarin by liver, a metabolite (metabolite X) was observed that possessed a different chromatographic mobility from 6-, 7- and 8-hydroxywarfarin (see above). This finding prompted us to investigate the nature of metabolite X. Contrary to the

TABLE 3. COFACTOR REQUIREMENTS FOR METABOLITE X FORMATION*

System	Metabolite X formed (mμmoles)
Complete	10·8
-NAD	9·8
-NADP	4·1
-NADP, -G-6-P	4·8
-NAD, -NADP, -G-6-P	< 0·1

* The composition of the complete system, including 1·3 μmole warfarin-^{14}C, was the same as that described in Materials and Methods, except that liver soluble fraction was used as an enzyme source. The liver soluble fraction was obtained from 166 mg liver from a phenobarbital-pretreated rat and dialyzed extensively against 0·25 M sucrose buffered with 0·01 M KH_2PO_4–K_2HPO_4 buffer (pH 7·4). The results given represent 2 experiments.

formation of hydroxywarfarins, the formation of metabolite X was catalyzed by the soluble fraction of liver (Table 1) and proceeded under nitrogen as well as under oxygen. Cofactor requirement studies (Table 3) revealed that the formation of metabolite X was dependent upon NADP, but not on NADPH. The apparent K_m

for the formation of metabolite X was 4·1 × 10^{-4} M (Fig. 2), which is higher than that for the formation of 6-, 7- and 8-hydroxywarfarin (Fig. 1). A metabolite with the chromatographic properties of metabolite X was also observed in plasma obtained from rats given warfarin.[5] Metabolite X, obtained from plasma, gave a u.v. absorption

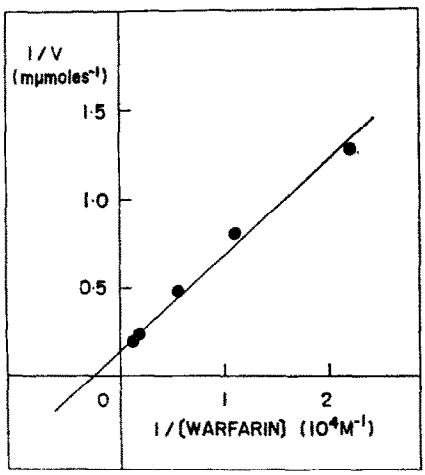

FIG. 2. Determination of the apparent Michaelis constant for the metabolism of warfarin to metabolite X by rat liver. The details of the experiment are given in the legend for Fig. 1. An apparent K_m for the formation of metabolite X was calculated as 4·1 × 10^{-4} M.

spectrum that was identical to that of warfarin, but this metabolite could be distinguished from warfarin by its smaller R_f value (0·12) when developed on a silica gel thin-layer plate as described above. To determine whether metabolite X was a conjugate of warfarin, the metabolite X in plasma was purified on a silica gel thin-layer plate and incubated at pH 5·0 with Glusulase at 37° for 3 hr. The methylene chloride extract from the acidified reaction mixture was spotted on a silica gel thin-layer plate and chromatographed once with chloroform and three times with 8% methanol in benzene as described.[5] Only metabolite X was observed, indicating that it was not a sulfate or glucuronide.

Nonenzymic hydroxylation

Table 4 compares the nonenzymic hydroxylation of warfarin with the enzymic hydroxylation. Fenton's system gave rise predominantly to 6-hydroxywarfarin. Other systems in the table formed large amounts of 6-hydroxywarfarin and 8-hydroxywarfarin, while the amount of 7-hydroxywarfarin formed was relatively small. These findings are in contrast to the results obtained for the enzymic hydroxylation in which the dominant metabolite was 7-hydroxywarfarin. When 6-, 7- or 8-hydroxywarfarin was incubated with Fenton's system or the $SnCl_2$–$Na_4P_2O_7$ system, no measurable decrease in the amount of hydroxywarfarin was observed, indicating that possible secondary reactions, such as the further oxidation of monohydroxywarfarin, were negligible.

TABLE 4. COMPARISON OF THE ENZYMIC AND NONENZYMIC HYDROXYLATION OF WARFARIN*

System	Incubation: Temperature (°C)	Incubation: pH	Incubation: Duration (min)	No. of experiments	Hydroxywarfarin formed (mμmoles): Total†	6-Hydroxywarfarin	7-Hydroxywarfarin	8-Hydroxywarfarin
Rat liver microsomes	37	7·5	30	7	55·0 ± 2·1‡	16·2 ± 0·6 (29)§	29·6 ± 1·2 (54)	9·2 ± 0·3 (17)
Fenton's	37	7·5	15	5	27,920 ± 2260	19,640 ± 1290 (70)	4580 ± 1010 (17)	3700 ± 470 (13)
$SnCl_2$–EDTA	37	7·5	60	5	86·7 ± 23·8	35·3 ± 16·1 (41)	0·3 ± 0·1 (< 1)	51·1 ± 10·4 (59)
$SnCl_2$–EDTA	37	9·5	60	2	730, 790	429, 466 (59)	32, 38 (5)	269, 286 (36)
$SnCl_2$–$Na_4P_2O_7$	37	9·5	60	6	1367 ± 188	841 ± 110 (61)	53 ± 12 (4)	473 ± 76 (35)
$FeSO_4$–EDTA	0	7·5	60	2	532, 697	295, 421 (58)	50, 32 (7)	187, 244 (35)
Cu_2Cl_2	37	7·5	60	2	941, 968	472, 500 (51)	50, 71 (6)	419, 397 (43)

* The results with rat liver microsomes were obtained with warfarin-4-^{14}C and were described elsewhere.[5] The composition of the nonenzymic systems are as follows (all are dissolved or suspended in a final volume of 6·0 ml): Fenton's: ascorbic acid, 187·5 μmole; $FeSO_4$, 31 μmole; H_2O_2, 150 μmole; EDTA, 47 μmole; phosphate buffer, 250 μmole; warfarin, 175 μmole. $SnCl_2$–EDTA: $SnCl_2$, 500 μmole; EDTA, 750 μmole; phosphate buffer, 250 μmole; warfarin 175 μmole. $SnCl_2$–$Na_4P_2O_7$: $SnCl_2$, 250 μmole; $Na_4P_2O_7$, 500 μmole; warfarin, 175 μmole. $FeSO_4$–EDTA: $FeSO_4$, 250 μmole; EDTA, 375 μmole; phosphate buffer, 300 μmole; warfarin, 175 μmole. Cu_2Cl_2: Cu_2Cl_2, 250 μmole; acetate buffer, 600 μmole; warfarin, 175 μmole.

† The sum of 6-, 7- and 8-hydroxywarfarin.

‡ Mean ± S.D.

§ The figure in parenthesis indicates the percentage of each hydroxywarfarin to total hydroxywarfarin.

DISCUSSION

The studies presented here demonstrate the enzymic and nonenzymic conversion of warfarin to 6-, 7- and 8-hydroxywarfarin. No evidence for 5-hydroxywarfarin was obtained, although this compound would probably have a mobility between that of 8-hydroxywarfarin and warfarin on the TLC plate.[8] The conversion of warfarin to 6-, 7- and 8-hydroxywarfarin *in vitro* provides the enzymic mechanism for the metabolism *in vivo* of warfarin to these hydroxylated metabolites.[1, 5] No evidence was obtained for the participation of separate enzyme systems for the three hydroxylation reactions to form 6-, 7- and 8-hydroxywarfarin. The apparent Michaelis constants for the hydroxylation of warfarin in the 6-, 7- and 8-positions are identical. Furthermore, pretreatment of rats with phenobarbital increased the rate of the three hydroxylation reactions to the same extent.[5]

Although several organic compounds of smaller molecular size are hydroxylated *in vitro* by Fenton's system[6, 9] as well as by Udenfriend's system,[10] with a pattern similar to that found *in vivo*, the present results show that the isomer ratios of hydroxylated products of warfarin obtained from Fenton's system are different from those obtained from the rat liver microsomal system. The liver microsomal system yielded more 7-hydroxywarfarin than 6- or 8-hydroxywarfarin. This is in agreement with the studies *in vivo* by Link *et al.* on warfarin,[1] and by Kaighen and Williams[11]

and Fink and Kerékjártó[12] on coumarin. In addition, no 5-hydroxycoumarin formation was observed in rats.[12] The differences in results between Fenton's system and other nonenzymic systems (Table 4) support the postulation by Staudinger and Ullrich[6, 13] and Ullrich *et al.*[14] that two different mechanisms participate in the nonenzymic hydroxylation. Judging from the calculation by Samuel,[15] it is reasonable that Fenton's system yield predominantly 6-hydroxywarfarin. In other systems, nearly equal amounts of 6-hydroxywarfarin, 7-hydroxywarfarin and 8-hydroxywarfarin were expected to be formed[13, 14] when warfarin was incubated. The low yield of 7-hydroxywarfarin cannot be attributed to further metabolism, since the experimental results proved that further metabolism of 7-hydroxywarfarin under the condition employed was negligible. Recently, Dearden *et al.* reported that the pattern of phenolic products from metal ion–oxygen systems was dependent on the concentration of the metal ion.[16]

The nature of metabolite X still remains to be investigated. Since metabolite X has the same ultraviolet absorption spectrum as warfarin, it is unlikely that the warfarin skeleton is modified. The requirements for the formation of this metabolite *in vitro* are similar to those for the conversion from *p*-nitrobenzyl alcohol to *p*-nitrobenzoic acid[17] and from 3-hydroxymethylhexabital to 3-ketomethylhexabital,[18] although it is apparent that hydroxywarfarin is not an intermediate for metabolite X formation since it can be formed even in the absence of microsomes.

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