Biochemical Pharmacology, Vol. 24, pp. 729–735. Pergamon Press, 1975. Printed in Great Britain.

STUDIES OF THE MICROSOMAL DRUG METABOLISM SYSTEM IN WARFARIN-RESISTANT AND -SUSCEPTIBLE RATS

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(*Received* 17 *June* 1974; *accepted* 25 *September* 1974)

Abstract—A strain of warfarin-resistant rats showed a lower sleeping time response to sodium pentobarbitone than the parent susceptible strain, but this difference did not appear to correlate with resistance in wild rats. Although strain differences in the extent of microsomal drug metabolism were demonstrated they were not sufficient to account for resistance. *In vitro* studies of warfarin metabolism showed that hydroxywarfarins, including 4′-hydroxywarfarin were synthesized. Induction by phenobarbitone led to a greater stimulation of 7-hydroxywarfarin formation compared to that of the 6-, 8-, and 4′-hydroxywarfarins. *In vivo* studies suggested that the rate of metabolism of warfarin by control animals was not the limiting step as far as the lethal effects of warfarin were concerned.

The indirect anticoagulants, such as warfarin, have been in use as rodenticides for some 20 yr. The first report of failure to control a population of rats (*Rattus norvegicus*, Berk.) with either warfarin or diphacinone came from Scotland [1]. Since that time other resistant populations have been found in Wales [2], Denmark [3], The Netherlands [4], Germany [5] and the U.S.A. [6]. Two colonies of warfarin-resistant rats have been developed in this Laboratory using animals originating from either Scotland or Wales [7].

Previous studies [7] suggested that warfarin-resistance in the rat is inherited by a single dominant autosomal gene which also confers resistance to injected warfarin. Thus an absorption defect would appear unlikely. These findings have been confirmed [8] and extended to show no correlation between resistance and the rate of excretion of warfarin or its plasma half-life. It was suggested [8] that resistance occurred at a point between the transfer of warfarin from the binding site on plasma albumin and the active site in the liver cell. These authors did not exclude the possibility that an active metabolite may be produced in smaller quantities by resistant animals. The selection from a randomly-bred Sprague–Dawley strain of animals whose Factor VII depression by warfarin was of short or long duration led to the development of two lines whose plasma warfarin half-life was short or long respectively, and the differences by the fifth generation were highly significant [9]. This suggested that there was a correlation between resistance and the rate of metabolism. Further work showed that the plasma warfarin half-lives of five susceptible strains were different [10] whereas there were no significant differences between those of a Wistar-derived resistant strain and a susceptible Sprague–Dawley strain [11].

Metabolic studies by Link *et al.* [12] showed that after an intraperitoneal injection of [^{14}C]warfarin to Sprague–Dawley rats, 90 per cent of the radioactivity was excreted in 14 days, half appearing in the urine. Analysis of the urine showed that the detoxication products 6-, 7- and 8-hydroxywarfarins accounted for 60 per cent of the total activity and unchanged warfarin was less than 10 per cent. In a later study [13] they reported the presence of 4′-hydroxywarfarin and 2,3-dihydro-2-methyl-4-phenyl-5-oxo-γ-pyrano (3,2-c) (1) benzopyran, which accounted for a further 30 per cent of the urinary activity. The *in vitro* studies of Ikeda *et al.* [14, 15] with rat liver homogenates showed the production of 6-, 7-, and 8-hydroxywarfarins to be associated with the microsomal fraction, and to require molecular O_2 and a source of NADPH. Studies with a Sprague–Dawley derived resistant strain and a susceptible Holtzmann strain showed no differences in the urinary or faecal metabolites following an intraperitoneal injection of warfarin [16]. Work in this laboratory has been directed towards establishing the cause of warfarin resistance at the biochemical level and developing a diagnostic test to identify resistance in wild rat populations. Up to the present, the published data on the biochemistry of warfarin resistance has involved comparisons of strains whose genetic backgrounds have qualitative differences besides that of resistance, and this can lead to false conclusions [17, 18]. In the present investigation we have studied some aspects of the microsomal mixed function oxidase system in relation to resistance in similar strains.

MATERIALS AND METHODS

Chemicals. Phenobarbitone sodium (BP) was obtained from B.D.H. Ltd., Poole and their Folin–Ciocalteu reagent was titrated with standard NaOH (phenolphthalein as indicator) and diluted to 2 N with respect to H^+. Aniline hydrochloride was recrystallized from ethanol, dried over P_2O_5 and 0·1-g portions (m.p. 200–201°) stored under nitrogen in sealed vials. Ethylmorphine was obtained from May & Baker Ltd., Dagenham. Warfarin, 3-(α-acetonylbenzyl)-4-hydroxycoumarin, and warfarin sodium were gifts from Ward Blenkinsop Ltd., Widnes and 3-(α-acetonylbenzyl [α-^{14}C])-4-hydroxycoumarin (sp. act. 7·1 mCi/m-mole)

was purchased from the Radiochemical Centre, Amersham. 2,5-Diphenyloxazole (PPO) and 1,4-di-[2-(5-phenyloxazolyl)]-benzene (POPOP) were obtained from Koch Light Ltd., Colnbrook. Crystalline bovine serum albumin was obtained from Armour Laboratories, Eastbourne. D-Glucose-6-phosphate dehydrogenase (Torula yeast, type XII) and all other biochemicals were from Sigma (London) Ltd. and general laboratory reagents were the highest grade commercially available. Pentobarbitone sodium solution (Nembutal) was obtained from Abbot Laboratories Ltd., Queensborough, Kent.

Syntheses. 1,1-Di(*p*-chlorophenyl)-2-dichloroethylene (*p,p'*-DDE) was prepared as described previously [19]. The syntheses of 6-, 7-, and 8-hydroxywarfarin were based on published methods [20, 21] and yielded products whose uncorrected melting points were 204–208°, 197–199° and 185–187° respectively. The intramolecular dehydration product of warfarin, 2-methyl-4-phenyl-5-oxo-γ-pyrano [3,2-c] [1] benzopyran (I) was prepared by the method of Seidman *et al.* [22] and yielded a product with m.p. 147–148°. The analogous derivative from warfarin alcohol, 2,3-dihydro-2-methyl-4-phenyl-5-oxo-γ-pyrano [3,2-c] [1] benzopyran (II) was prepared by low pressure hydrogenation of (I) and had a m.p. 188–190°.

Animals. The Wistar-derived warfarin-susceptible strain (TAS) has been maintained in this laboratory for some 20 yr and the development of the homozygous warfarin resistant colonies, HS and HW, from matings between TAS animals and wild, resistant rats from either Scotland or Wales respectively, has been described [7]. Only male animals were used in this study and they were caged in groups of six at about 100 g body wt. They were maintained on diet 41B (Oxoid) and water *ad libitum* until they were sacrificed at 200–230 g body wt, corresponding to 10–11 weeks of age and attainment of sexual maturity. Control animals were fed normal food and water and the experimental animals received either diet containing 250 ppm DDE for 3 weeks [23] or drinking water containing 0·1% sodium phenobarbitone for 1 week [24]. Wild resistant rats trapped in a rural area were individually caged and acclimatized in the laboratory for 1 month before use.

Enzyme assays. All animals were killed between 9.00 and 10.00 hr by cervical dislocation and bled from the carotid arteries. The livers were rapidly excised and post-mitochondrial supernatants (PMS) and microsomal suspensions prepared as described previously [23]. The hearts were removed from some groups of animals, rinsed with saline, blotted dry and weighed prior to estimation of the DDE residues.

Cytochrome P-450 levels were estimated in the freshly prepared microsomal suspension by the method of Omura and Sato [25] using their extinction value of 91 $mM^{-1}\,cm^{-1}$, with a Unicam SP800 spectrophotometer coupled to an external recorder to allow scale expansion. All the assays were linear with respect to both time and protein concentration over the range studied. The PMS was stored when necessary at −10° for up to 2 days with no significant loss of enzyme activity. Duplicate incubations were carried out in 25-ml conical flasks containing a marble to facilitate aeration [26] in a shaking water bath at 37°.

Ethylmorphine demethylase was assayed with PMS that had been stored overnight. Each flask contained 400 μmoles Tris–HCl buffer pH 7·4, 20 μmoles $MgSO_4$, 40 μmoles semicarbazide, 4 μmoles ethylmorphine, 20 μmoles glucose-6-phosphate and 1·8 μmoles NADP in a total volume of 3 ml. After preincubating for 5 min the reaction was initiated by adding 1 ml PMS. The incubations were terminated and the formaldehyde formed was determined using a modification of the method of Nash [27] as described previously [28].

Aniline hydroxylase was measured with PMS that had been stored for 2 days. The incubation mixture was a modification of that used by Kato and Gillette [29] and was similar to that for ethylmorphine *N*-demethylase except that 32 μmoles aniline hydrochloride replaced ethylmorphine and semicarbazide was omitted. The *p*-hydroxyaniline produced during the 30-min incubation was determined by a modification [28] of the method of Imai *et al.* [30].

Warfarin metabolism was measured with PMS that had been stored overnight using an incubation mixture similar to that described above, except that 0·7 μmoles warfarin replaced the aniline hydrochloride. The [^{14}C]warfarin (20–30 nCi) was added in 100 μl ethyl acetate to empty flasks and the solvent allowed to evaporate prior to the addition of the aqueous solutions. The incubation was terminated after 30 min by quantitatively transferring the contents of the flasks into test tubes of boiling water. The resulting suspensions were adjusted to below pH 2 with 5 N HCl and extracted for 24 hr with ether using the test tube as the centre piece of an upward displacement extractor. Each ether extract was reduced to low volume at room temperature, dried by passage through an ether washed column of anhydrous sodium sulphate and made to 5·0 ml. Duplicate aliquots were used to assess the total recovery of ^{14}C-compounds and the remainder was transferred as a band to one half of a 34 × 20-cm glass t.l.c. plate which had been coated with a 0·25-mm layer of silica gel GF_{254} (Merck) and activated at 120° for 1 hr. The previously described hydroxywarfarin metabolites [12] and the two intramolecular dehydration products (I) and (II) were each added in 20-μg quantities to each band and also to a separate centre strip as markers. The plate was supported at an angle of 15° in a loose-layer tank and developed with chloroform–methanol–glacial acetic acid::300–10–8. The plates were viewed under a u.v.-lamp at 254 nm and 350 nm and the six marker compounds located. The areas corresponding to the markers and those in between were quantitatively removed and eluted with 10 ml diethyl ether directly into a glass scintillation vial, as we showed by a spectrophotometric assay that warfarin and its metabolites were quantitatively recovered. The ether was evaporated at room temperature and each vial received 10 ml scintillant mixture (4 g PPO and 0·1 g POPOP per litre toluene) and the ^{14}C-content assayed by a Nuclear Enterprise NE 8310 liquid scintillation spectrometer. The observed count rate was corrected for quenching effects by the channels ratio method. The activity of each of the ten elutions from one chromatogram was expressed as a percentage of the total recovered and converted to nmoles of compound. In order to determine 4'-hydroxywarfarin the areas of silica gel corresponding to the 6- and 7-hydroxywarfarin chromatographic markers were eluted with ether and each extract was applied to a 50 × 4-cm Whatman No 1 paper strip. These descending chroma-

tograms were developed overnight using a solvent system composed of *t*-butanol–benzene–concentrated ammonia–water::45–20–9–3 [13]. The metabolites were located using a Tracerlab SC-525B strip scanner, the appropriate portion of the paper shredded into a stoppered centrifuge tube containing 10 ml 1% sodium pyrophosphate and allowed to soak for 3 hr. The suspension was acidified with 5 N HCl and extracted with 2 × 10-ml volumes of diethyl ether with a vortex mixer and the phases separated by centrifugation. The ^{14}C-content of the ether extracts were assayed as described above. For confirmation of the identity of the warfarin metabolites, the products formed during an *in vitro* study of six phenobarbitone-treated TAS rats were extracted, pooled and subjected to both thin-layer and paper chromatography. The resulting ether solutions were reduced to low volume under N_2 at room temperature and each transferred to the solid probe inlet of an AEI MS30 mass spectrometer, and the spectra compared with those from the synthetic standards.

Studies of kinetic constants. For the determination of the kinetic constants, the preincubation mixtures described above were supplemented with 4 i.u. glucose-6-phosphate dehydrogenase and the PMS was replaced by a microsomal suspension. The enzyme activities were determined over the range 0·1–1·0 mM ethylmorphine and 0·06–0·6 mM aniline and Lineweaver–Burk plots were obtained from the results by subjecting them to a linear regression analysis.

Chemical analysis. Residue levels of *p,p'*-DDE were measured in the freshly excised hearts of certain groups of animals using a g.l.c. assay previously described [23]. Protein levels in the microsomal suspensions were measured by the method of Lowry *et al.* [31] using bovine serum albumin as a standard.

Sleeping times. The effect of sodium pentobarbitone on the righting reflex was determined by the method of Wenzel and Lal [32], with each animal receiving an intraperitoneal injection of 3 mg sodium pentobarbitone in 0·2 ml per 100 g body wt. The phenotyping of wild rats for resistance was performed 7 days later by determining the lethal response to a s.c. dose of 200 mg warfarin/kg body wt.

Table 1. The responses of rat strains to an intraperitoneal injection of 3 mg sodium pentobarbitone/100 g body wt

Strain	Number of animals	Body wt (g)	Sleeping time (min)
TAS	12	219 ± 6	54·3 ± 5·4
HS	17	218 ± 4	42·9 ± 1·5*
Wild	20	303 ± 23	77·4 ± 20·9

Values are given as mean ± S.E.M.
* $P < 0.05$ compared to TAS.

Warfarin toxicity. This was assessed by feeding diet 41B containing 50 ppm or 250 ppm warfarin to control and DDE or phenobarbital-induced animals.

Statistical analysis. All the comparisons were made using Student's *t*-test.

RESULTS

A comparison of the in vivo *effects of sodium pentobarbitone.* The time for the loss of the righting reflex following the treatment of male rats with a barbiturate is shown in Table 1. As previously reported [33] a difference ($P < 0.05$) exists between the laboratory strains suggesting that they may also show differences in their drug metabolic capacity. The varied response of the wild rat is reflected by the large standard error of the mean.

Comparison of the control levels for some parameters associated with liver drug metabolism. The results for some parameters of hepatic drug metabolism given in Table 2 show a comparison between a warfarin-susceptible (TAS) and two warfarin-resistant (HS or HW) strains of rat. When compared with the TAS strain, the relative liver weight (RLW) for the HS strain is significantly higher and the specific value for the microsomal protein is reduced more than can be wholly attributed to the increased liver weight. Although the HW strain shows no difference in the RLW, there is a significant reduction in the protein level. The HW strain shows the lowest level of cytochrome P-450 and the liver totals for the TAS strain were higher than those for the

Table 2. Comparison of parameters associated with liver drug metabolism in warfarin-resistant and -susceptible strains of rats

Strain	TAS	HS	TAS	HW
Body wt (g)	223 ± 4	224 ± 5	224 ± 7	220 ± 6
Relative liver wt (g liver/100 g body wt)	4·28 ± 0·07	4·51 ± 0·09*	4·04 ± 0·06	4·10 ± 0·07
Microsomal protein (mg protein/g liver)	36·8 ± 1·5	30·5 ± 0·6†	36·9 ± 0·9	35·1 ± 0·4*
Cytochrome P-450 (nmoles/g liver)	38·0 ± 2·7	33·7 ± 1·3	32·2 ± 0·8	31·2 ± 0·7
Ethyl morphine§	4335 ± 412	4353 ± 504	4310 ± 129	4526 ± 322
Aniline‖	688 ± 30	345 ± 15‡	671 ± 75	468 ± 19‡
Warfarin¶	533 ± 48	460 ± 25	460 ± 17	424 ± 17
Warfarin**	277 ± 28	285 ± 18	248 ± 16	243 ± 11

* $P < 0.05$.
† $P < 0.02$.
‡ $P < 0.01$.
§ nmoles HCHO formed per g liver per 15 min.
‖ nmoles *p*-hydroxyaniline formed per g liver per 30 min.
¶ nmoles warfarin metabolized per g liver per 30 min.
** nmoles hydroxywarfarins formed per g liver per 30 min.
Values are given as mean ± S.E.M. obtained from groups of six animals.

Table 3. Comparison of the effects of DDE or phenobarbitone on liver drug metabolism in warfarin-resistant and -susceptible rat strains

Strain	TAS			HS		
Treatment	None	DDE	Phenobarbitone	None	DDE	Phenobarbitone
Body wt (g)	220 ± 3 (27)	216 ± 4 (6)	226 ± 6 (24)	218 ± 8 (24)	195 ± 10 (6)	213 ± 6 (24)
Relative liver wt (g liver/100 g body wt)	4·12 ± 0·05 (27)	4·79 ± 0·05 (6)	5·33 ± 0·08 (24)	4·35 ± 0·04 (24)	5·34 ± 0·04 (6)	5·72 ± 0·01 (24)
Microsomal protein (mg/g liver)	36·3 ± 1·1 (17)	46·6 ± 0·6 (6)	48·4 ± 1·1 (8)	30·5 ± 0·6 (16)	44·6 ± 1·0 (6)	40·6 ± 2·5 (6)
Cytochrome P-450 (nmoles/g liver)	35·7 ± 1·5 (19)	61·5 ± 1·7 (6)	79·8 ± 2·3 (14)	33·6 ± 0·9 (21)	78·6 ± 2·4 (6)	74·1 ± 3·4 (21)
Heart residue level (ppm DDE)	—	3·45 ± 0·1 (6)	—	—	3·57 ± 0·3 (6)	
Ethylmorphine*	4323 ± 193 (12)	6510 ± 246 (6)	11,254 ± 317 (6)	4353 ± 504 (6)	6720 ± 271 (6)	8310 ± 474 (6)
Aniline†	680 ± 36 (12)	1485 ± 44 (6)	1111 ± 29 (6)	345 ± 15 (6)	1007 ± 11 (6)	905 ± 46 (6)
Warfarin†	477 ± 26 (14)	512 ± 24 (6)	1208 ± 74 (6)	445 ± 39 (14)	959 ± 70 (6)	1128 ± 49 (6)
Warfarin§	260 ± 15 (14)	314 ± 11 (6)	877 ± 70 (12)	220 ± 19 (14)	689 ± 18 (6)	840 ± 46 (11)

* nmoles HCHO formed per g liver per 15 min.
† nmoles *p*-hydroxyaniline formed per g liver per 30 min.
‡ nmoles warfarin metabolized per g liver per 30 min.
§ nmoles hydroxywarfarins formed per g liver per 30 min.
Values are given as mean ± S.E.M. with the number of animals in parenthesis.

resistant strains, that for the HW being the lowest. The control levels for the *N*-demethylation of ethylmorphine, a type I substrate [34], were very similar for all strains. The study of the metabolism of substrates showing a type II binding spectrum was performed using aniline [35] and warfarin [36], although warfarin is unusual in that the spectrum is not abolished by treatment with dithionite. Vainio and Hanninen [37] found that 0·8 M acetone produced a 3·5-fold increase in the hydroxylation of aniline but a slight inhibition of the hydroxylation of 3,4-benzyprene or the *N*-demethylation of ethylmorphine. We confirmed this finding for aniline but found that warfarin hydroxylation by control rats was completely inhibited. The values for *p*-hydroxyaniline production showed a strain variation in that those for the two resistant strains were lower than that of the TAS strain.

The effect of pretreatments on microsomal drug metabolism. Both DDE and phenobarbitone are inducers of hepatic drug metabolism and the effects of *in vivo* pretreatment were studied with the warfarin-susceptible (TAS) and resistant (HS) strains (Table 3). The percentage increase in RLW, microsomal protein and cytochrome P-450 following phenobarbitone treatment were similar in the two strains and although their heart residue levels of DDE were comparable, the liver responses differed in that the microsomal protein and cytochrome P-450 increases were higher for the HS animals. With the three substrates studied, phenobarbitone produced a 2- to 3-fold increase in metabolism in both strains, but the response to DDE was more variable. Both strains showed an increase in the metabolism of ethylmorphine and aniline but only the HS strain showed an increase in the production of hydroxywarfarins.

Qualitative and quantitative analysis of warfarin metabolites. The thin layer chromatography solvent system adopted separated warfarin from the hydroxywarfarin metabolites described by Link *et al.* [12]. With the conditions employed it appeared that there were several 'solvent' fronts as had been reported previously for a similar system [38]. As the R_f values for the metabolites varied with temperature and the length of time that the activated plate was exposed to the air during sample application, unlabelled markers were always used to locate the metabolic products. The positive identification of the metabolites was obtained by mass spectrometry and the mass ratio of the main

Table 4. The effect of phenobarbitone treatment on the production of hydroxywarfarins by rat liver preparations

Position of the hydroxyl group	TAS		HS	
	Control	Phenobarbitone	Control	Phenobarbitone
6	2·76 ± 0·22	4·73 ± 0·62	2·43 ± 0·10	4·86 ± 0·35
4′	2·10 ± 0·19	4·55 ± 0·50	2·49 ± 0·24	4·22 ± 0·42
7	2·65 ± 0·28	11·90 ± 2·10	2·98 ± 0·50	10·95 ± 0·64
8	1·95 ± 0·26	5·65 ± 0·79	2·67 ± 0·51	4·80 ± 0·57

The values are the mean ± S.E.M. obtained with six animals and expressed as nmoles product formed per mg microsomal protein per 30 min at 37°.

Table 5. The effect of phenobarbitone treatment on the kinetic constants of microsomal oxidases in various strains of rat

Strain	Treatment	0·1–1·0 mM Ethylmorphine K_m (mM)	0·1–1·0 mM Ethylmorphine V_{max}*	0·06–0·6 mM Aniline K_m (mM)	0·06–0·6 mM Aniline V_{max}†
TAS	None	0·278	70	0·124	18·7
	Phenobarbitone	0·272	213	0·191	67·6
HS	None	0·258	196	0·070	16·6
	Phenobarbitone	0·821	466	0·141	32·3

* nmoles HCHO formed per mg microsomal protein per 15 min.
† nmoles *p*-hydroxyaniline formed per mg microsomal protein per 30 min.

fragments were identical with those previously reported [39] although small differences were found in their relative intensities. Thin layer chromatography followed by paper chromatography indicated that the 6- and 8-hydroxywarfarins separated as pure compounds during t.l.c. and their identities were confirmed by mass spectrometry and u.v. spectra. The material eluted from the thin layer chromatograms, which corresponded to the 7-hydroxywarfarin marker, showed the presence of two radioactive compounds when subjected to paper chromatography. The major component was identified as 7-hydroxywarfarin and the other as 4'-hydroxywarfarin. The total production of hydroxylated metabolites is shown in Table 3, and the results reflected those based on the loss of warfarin from the incubation mixtures. The values for the individual hydroxylated warfarins are shown in Table 4. In the control animals of both strains the ratio of the quantities of the four products approaches unity, but following phenobarbital treatment although there is an increased production of all these products the 7-hydroxywarfarin now accounts for nearly half the total.

Effect of phenobarbitone treatment on the in vitro *kinetic constants.* The Lineweaver–Burk plots were constructed by subjecting the results to a regression analysis and as the regression constants all exceeded 0·9550 the constants were calculated on the assumption that the graphs were linear; the results are shown in Table 5. With ethylmorphine as the substrate, phenobarbitone treatment of TAS animals did not change the K_m but increased the V_{max}, whereas for the HS strain induction increased both the K_m and the V_{max}. The results with aniline as the substrate appear more complex as the K_m values differ for each strain and treatment. For both strains, phenobarbitone treatment increased the K_m and V_{max} values when compared with their respective controls.

The toxicity of warfarin and warfarin-inducer mixtures. It is not very meaningful to express the chronic toxicity data of warfarin in the form of a LD_{50} therefore the comparison shown in Table 6 is based on the time required to produce a 50 per cent kill. With TAS animals fed 0·005% warfarin this time was 5 days, and was not prolonged by prior induction with DDE or phenobarbitone. The toxicity of 0·025% warfarin to TAS rats was decreased by prior treatment with phenobarbitone as the time for a 50 per cent kill was increased from 4 days to 10 days. No deaths occurred in the HS strain with feeding 0·005% warfarin for 32 days, although 50 per cent kill occurred at 10–11 days with feeding 0·025% warfarin. Phenobarbitone induction of the HS strain did not decrease the toxic effect of 0·025% warfarin but following DDE induction, a 50 per cent kill was obtained after 6 days.

DISCUSSION

The selection of the laboratory strains of warfarin resistant rats involved a procedure in which the frequency of incrosses was greater than 95 per cent [7], nevertheless the results obtained in this comparative study suggest that the resistant HS strain differs from the susceptible TAS in several respects.

The difference in the sleeping time response of the TAS and HS strains following sodium pentobarbitone treatment suggested that this might form the basis of a diagnostic test for warfarin-resistance in wild animals. However, this is precluded because the response of wild resistant and susceptible rats to this treatment (Table 1) varied between 13 and 342 min with those for three animals subsequently identified as resistant, being 31, 44 and 143 min.

The *in vitro* studies of the microsomal mixed function oxidase system of these strains showed some significant differences that are not immediately explicable. The HS strain has a higher RLW and a lower total microsomal protein content than the other two strains and the cause of this increase has not been identified. The control levels of ethylmorphine *N*-demethylase and warfarin metabolism were similar in all strains, but those for aniline hydroxylation were not.

Table 6. The effect of DDE or phenobarbitone pretreatment on the chronic toxicity of warfarin to strains of rats

Pretreatment	Treatment	Time to reach 50% kill (days) TAS	Time to reach 50% kill (days) HS
None[a]	0·005% warfarin	5	>30
250 ppm DDE[b]	0·005% warfarin + 250 ppm DDE	5	—
0·1% phenobarbitone[c]	0·005% warfarin + 0·1% phenobarbitone	5	—
None[a]	0·025% warfarin	4	10
250 ppm DDE[b]	0·025% warfarin + 250 ppm DDE	5	6
0·1% phenobarbitone[c]	0·025% warfarin + 0·1% phenobarbitone	10	10

Before treatment animals received (a) control diet, (b) 250 ppm DDE in the food for 3 weeks, (c) 0·1% sodium phenobarbitone in the drinking water for 1 week. Warfarin was added to the food at the levels indicated.

Phenobarbitone treatment produced similar percentage increases in RLW, microsomal protein, cytochrome P-450 and warfarin hydroxylation in the TAS and HS strain, but the increase in the *N*-demethylase was higher and that for aniline hydroxylase lower in the TAS than in HS animals. The induction effects of 250 ppm DDE were greater with the HS strain, although the heart residue levels were similar. In a previous study of some of these parameters [33] 50 ppm DDE produced higher relative levels in the TAS animals, but then they were associated with higher residue levels. The lack of effect of DDE on warfarin metabolism in the TAS strain was surprising in view of the fact that extracts of the same animals showed an increase in the RLW, microsomal protein, cytochrome P-450 and the metabolism of ethylmorphine and aniline.

Our preliminary studies of warfarin hydroxylation confirmed the conclusion of Ikeda *et al.* [14] that the properties of the enzyme system fulfil the criteria for a mixed function oxidase in that it was associated with the microsomal fraction, required a source of NADPH and oxygen and was inhibited by carbon monoxide. During subcellular fractionation although we obtained increases in the enzyme activity per mg protein, in contrast to the findings of Ikeda *et al.* [14] the activity per gram of liver decreased, similar to that reported for glucose-6-phosphatase [40]. Reconstitution of the PMS from microsomal pellet and soluble supernatant of control or phenobarbitone-induced animals of either strain resulted in a loss of up to 50 per cent of the warfarin hydroxylation activity although an almost quantitative recovery of ethylmorphine *N*-demethylase and aniline hydroxylation were obtained.

Several investigators have not obtained linear Lineweaver–Burk plots when determining the kinetic constants of microsomal oxidation [41, 42] and in one instance these values for ethylmorphine *N*-demethylase have been shown to be dependent upon substrate concentration [42]. Our results gave linear plots and strain differences such as the change in K_m for ethylmorphine in phenobarbitone induced HS rats became apparent. The results for aniline hydroxylation confirmed the more complex strain differences of this system.

The *in vivo* metabolism of warfarin by the rat results in the formation of six metabolites [13], three of which have been chromatographically identified during *in vitro* studies [15]. Barker *et al.* [13] were the first to describe the formation of 4′-hydroxywarfarin in the rat and it would appear that this product does not separate from one of the metabolites in the chromatographic solvent system used by Ikeda *et al.* [15]. Warfarin alcohol, a reduction product of warfarin in man [39] has some anticoagulant activity although it is concluded that at the level found in plasma during warfarin therapy its presence is of little consequence in the pharmacological action of warfarin [43]. Ikeda *et al.* [15] reported the presence of an unknown metabolite produced by an enzyme system whose properties in the rat resemble those of a soluble aromatic reductase [44]. In the present study of the microsomal oxidation of warfarin, the warfarin alcohols were not found, although we found that warfarin alcohol had similar chromatographic properties to the unknown described by Ikeda *et al.* [15]. The ratio of the quantities of the 4′-, 6-, 7-, and 8-hydroxywarfarins was found to be unity for control animals of the strains used in this study whereas with the studies of Ikeda *et al.* [15] with Sprague–Dawley derived rats and Barker *et al.* [13] with Holtzman-derived rats, the 7-hydroxywarfarin predominated. Following phenobarbitone induction of TAS or HS rats this ratio did not remain constant and there was a specific stimulation of 7-hydroxywarfarin production. It appears that there may be two enzyme systems producing hydroxylated derivatives as a similar situation has recently been reported for biphenyl hydroxylation in hamster and rats [45].

In humans, phenobarbital pretreatment decreased the plasma warfarin half-life following a single oral dose of the anticoagulant [46]. It has been shown with rats that prior induction with phenobarbitone produced a 10-fold increase in the single dose LD_{50} for warfarin and an increased *in vitro* metabolism of warfarin [15]. Although we found phenobarbitone to be an inducer of warfarin metabolism *in vitro* (Table 3) it did not decrease the chronic toxicity of 0·005% warfarin (Table 6). Phenobarbitone treatment did protect the TAS strain against the toxic effect of 0·025% warfarin but neither phenobarbitone nor DDE was an antagonist with the HS strain.

It would appear from the results for normal susceptible animals that warfarin metabolism is not the rate-limiting step as far as its lethal effects are concerned. Recent work has shown that when a Sprague–Dawley derived resistant strain was interbred with a Holtzman susceptible strain, selection of the resistant line produced animals with a higher vitamin K requirement than the parent Holtzman strain [16] and less warfarin was associated with their liver microsomes following *in vivo* or *in vitro* treatment [47, 48]. Vitamin K is metabolized to an epoxide, vitamin K oxide, which is recycled by reduction to vitamin K in normal animals. In the presence of warfarin, the epoxide accumulates [49] and appears to be a competitive inhibitor of the role of vitamin K in the synthesis of certain blood clotting factors. Preliminary studies indicate that less vitamin K oxide accumulates in the liver of warfarin-resistant rats as the reductase is less sensitive to inhibition [50]. Work in this laboratory [51] with our HW and HS strains has confirmed the greater requirement of resistant animals for vitamin K [16] and further investigation of the strain differences in the site of action and metabolism of vitamin K will be likely to lead to a biochemical explanation for anticoagulant resistance in rats.

Acknowledgements—We are grateful to Mr K. A. Tarrant for his skilled technical assistance, Mr. S. Bailey for performing the mass spectral analyses and Mr. B. Rennison for use of computing facilities.

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