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Vitamin K 2,3-epoxide reductase: the basis for stereoselectivity of 4-hydroxycoumarin anticoagulant activity

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1 The administration of S-warfarin (1 mg kg^{-1} i.v.) to rats that were pre-loaded 48 h before with tracer doses (6 μg) of ^{14}C-labelled **R**- or S-warfarin caused the plasma levels of these compounds to increase. This is due to the substitution of the microsomal (vitamin K 2,3-epoxide (K0) reductase) bound **R**- or S-[^{14}C]-warfarin by the unlabelled 4-hydroxycoumarin administered. The rate of reappearance was 3–4 fold higher for **R**- than for S-warfarin; $t_{1/2}$ of release: 1.2 ± 0.04 and 3.7 ± 0.6 h, respectively.

2 Liver microsomes prepared from rats pretreated with **R**- or S-[^{14}C]-warfarin, released these compounds only in the presence of dithiothreitol (DTT; 10 mM). The rate of release was higher for **R**- than for S-warfarin-treated microsomes.

3 Liver microsomes treated *in vitro* with **R**- or S-acenocoumarol could be reactivated by DTT (10 mM). Reactivation was higher for the **R**- than for the S-acenocoumarol-treated microsomes.

4 The microsomal vitamin K0 reductase activity under 'normal' assay conditions ([DTT] = 2 mM) was as sensitive for **R**- as for S-4-hydroxycoumarins. At elevated DTT concentrations (= 42 mM) the rate of vitamin K0 conversion was about 1.5 fold higher in the presence of the **R**-isomers than in the presence of the S-isomers. For instance, at 2 mM DDT the reductase activities in the presence of 2.6 μM **R**- and S-warfarin were about 15% of control. At 42 mM DTT the activities were 90 and 65% of control, respectively.

5 In the *in vitro* experiments acenocoumarol appeared to be more potent than warfarin and phenprocoumon.

6 The following mechanism is proposed: vitamin K0 reductase becomes oxidized during substrate reduction. The oxidized (i.e. inactive) form binds equally to the **R**- and S-enantiomers of 4-hydroxycoumarins. The attached (covalently bound?) coumarin is released by the reactivation (i.e. reduction) of the enzyme. However, the rate of reactivation is strongly attenuated by the attached coumarin. This effect is more pronounced for the S-configuration of the 4-hydroxycoumarin anticoagulants.

Introduction

The oral anticoagulants of the 3 substituted 4-hydroxycoumarins (acenocoumarol, phenrocoumon, warfarin, etc.) are in clinical use as racemic mixtures. For a long time it has been recognized that the stereochemical enantiomers differ in their pharmacological responses. The S-enantiomers of warfarin and phenprocoumon are 2 to 5 times more potent than their **R**-enantiomers in man (O'Reilly, 1974; Jähnchen *et al.*, 1976; Wingard *et al.*, 1978), as well as in experimental animals (Jacobi & Levy, 1974; Schmidt & Jähnchen, 1977). For acenocoumarol, the opposite is true (Meinertz *et al.*, 1978). This difference, however, is a pharmacokinetic related problem. The S-enantiomer of acenocoumarol is cleared 10 times faster than **R**-acenocoumarol (Godbillon *et al.*, 1981; Thijssen *et al.*, 1986a) and close examination showed that for acenocoumarol the S-enantiomer is also intrinsically the most potent enantiomer (Thijssen *et al.*, 1985).

The 4-hydroxycoumarin anticoagulants are thought to act by inhibiting one or two specific dithiol-dependent reductases, i.e. vitamin K 2,3-epoxide (vitamin K0) reductase and vitamin K reductase, which are an integral part of the vitamin

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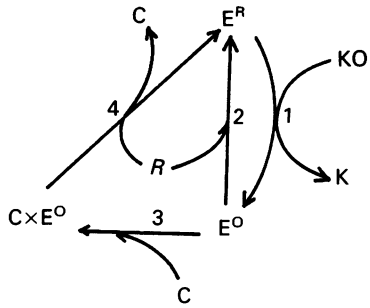

Figure 1 Scheme for the vitamin K 2,3-epoxide (vitamin K0) reductase system and the mechanism of 4-hydroxycoumarin action. E^R and E^O refer to the active (i.e. reduced) and inactive (oxidized) vitamin K0 reductase. K0 and K represent vitamin K0 and vitamin K. C refers to 4-hydroxycoumarin. *R* refers to the endogenous reductor. Reaction steps, 1–4, are the substrate conversion (1), the reactivation of the enzyme (2), the interaction of the coumarin C with the inactive enzyme (3), the reactivation of the coumarin-enzyme complex (4). N.B.: for a susceptible system, the rate of step 4 is smaller than that of step 2.

K cycle in the endoplasmic reticulum of the hepatocyte (Suttie, 1980). The reductase(s) is (are) believed to contain two sulphhydryl groups in its active centre which become oxidized to form a disulphide bond during substrate reduction (Silverman, 1980; Fasco *et al.*, 1983; Lee & Fasco, 1984).

The reductase(s) can be assayed in *in vitro* systems, for instance in liver microsomes, using dithiothreitol (DTT) as the donor for reducing equivalents. When tested in such systems, no differences in sensitivity for **R**- and S-warfarin were observed by Fasco & coworkers (Fasco & Principe, 1982; Wilson & Fasco, 1986). We also found no differences in the ID_{50}s of the **R**- and S-enantiomers of acenocoumarol and of phenprocoumon in *in vitro* rat liver microsomal vitamin K0 reductase systems (unpublished results).

The inhibition of the reductase(s) has (have) been described as being essentially irreversible (Fasco & Principe, 1982). This conclusion was based on the observations that the reductase activity of warfarin-treated (*in vitro* or *in vivo*) microsomes cannot be reactivated by either wash procedures or dialysis. However, we recently suggested that the interaction between the coumarin drugs and vitamin K0 reductase is not irreversible in the strict sense, but *in vivo* a dynamic equilibrium exists between release of the coumarin due to reactivation of the enzyme, and recomplexation after reoxidation of the enzyme due to substrate reduction (Figure 1) (Thijssen & Baars, 1987). The model, depicted in Figure 1, incorporates the presence of two forms of vitamin K0 reductase; an active (i.e. reduced, containing two sulphhydryl groups) and an inactive (i.e. oxidized, containing a disulphide bound) form. The 4-hydroxycoumarins are assumed to interact only with the oxidized form (Fasco *et al.*, 1983; Thijssen, 1987). The model, among others, emanated from *in vivo* experiments in rats showing that [^{14}C]-warfarin is displaced from its microsomal binding site (i.e. vitamin K0 reductase) by 4-hydroxycoumarin administration. *In vitro* the microsomal bound [^{14}C]-warfarin was released only in the presence of high amounts of DTT (Thijssen & Baars, 1987).

We investigated whether this unique system is also the basis for the stereoselectivity of the 4-hydroxycoumarin activity. The results show that the reactivation of the vitamin K0 reductase-4-hydroxycoumarin complex proceeds faster for the **R**- than for the S-configuration.

Methods

In vivo *displacement experiments*

Rats were provided with PE-10 catheters in the left femoral artery and the right femoral vein. The catheters were exteriorized at the neck. The rats were allowed to recover for at least 2 days. This technique is routinely used in our laboratory and as judged from physical examination (i.e. weight gain and motoric activity) the rats were in good condition. **R**-/S-[^{14}C]-warfarin (2×10^6 d.p.m., ca. 6 μg) in saline was administered as a bolus injection via the femoral vein catheter. Blood (100 μl) was sampled at regular time intervals via the arterial catheter. At 48 h, the rats received 1 mg kg^{-1} S-warfarin i.v. and the reappearance of **R**-/S-[^{14}C]-warfarin in blood was followed. The rate of reappearance was calculated as described previously for racemic [^{14}C]-warfarin (Thijssen & Baars, 1987). Briefly, assuming the reappearance to be exponential (i.e. first order process) the plasma **R**-/S-[^{14}C]-warfarin curve following upon the administration of S-warfarin is the result of two log-linear curves; the reappearance or release curve and the elimination curve. By curve stripping, i.e. method of residuals (Rowland & Tozer, 1980), the individual rate constants can be analysed.

To examine the tissue distribution of **R**-/S-[^{14}C]-warfarin and the effect of displacement by S-warfarin thereupon, rats were provided with a femoral vein catheter. The [^{14}C]-warfarin enantiomers (1.5×10^6 d.p.m.) were administered as a bolus injection. Twenty-four hours later, control rats received saline (i.v.), the experimental rats received S-warfarin (1 mg kg^{-1} in saline). The **R**-[^{14}C]-warfarin rats were killed 3 h later, the S-[^{14}C]-warfarin ones 12 h later. The rats were killed under light ether anaesthesia by bleeding via the abdominal aorta. Blood was col-

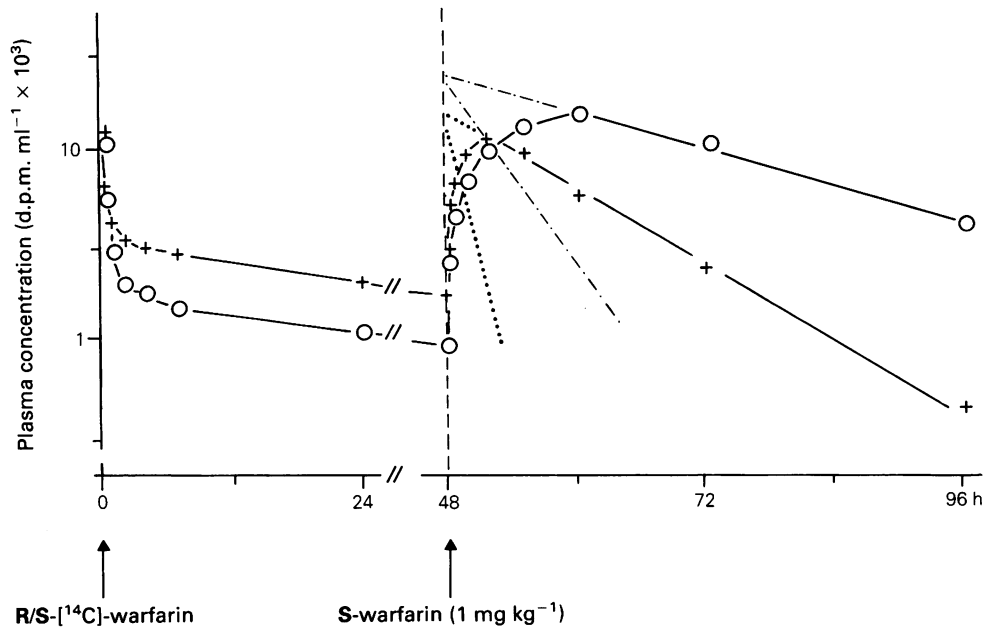

Figure 2 The effect of the administration of unlabelled S-warfarin (1 mg kg^{-1}, i.v.) at 48 h on the time course of plasma R-[^{14}C]-warfarin (+) and S-[^{14}C]-warfarin (○) concentrations. R-/S-[^{14}C]-warfarin (2 × 10^6 d.p.m. = 6 μg) were administered i.v. at time 0. Broken lines are construction lines obtained via curve stripping to estimate the rate of reappearance of the compounds in the circulation (see Methods).

lected in a heparinized syringe. Liver was rinsed *in situ* with ice-cold saline via the portal vein. The kidneys were rinsed via the abdominal aorta. The tissues were excised, rinsed and homogenized (Potter-Elvehjem) in 3 vol (w/v) of ice-cold buffer (0.02 M Tris-HCl, 0.25 M sucrose, 0.15 M KCl, pH 7.4).

In vitro *experiments*

Liver microsomes were prepared as described previously (Thijssen, 1987). Microsomal vitamin K0 reductase activity was assayed as described previously (Thijssen, 1987), except that vitamin K0 (5 mM) in 2% Triton-X 100 in Tris-HCl buffer was used as a substrate solution. Thus, 20 μl of microsomes (about 0.3 mg of protein) and 75 μl of buffer (0.02 M Tris-HCl, 0.15 M KCl, pH 7.4) were mixed in a reaction vessel. Following a 3 min (30°C) preincubation period, DTT, 2 μl of a 0.1 M solution in Tris buffer, was added and the mixture incubated for another 3 min. The reaction was started by the addition of 2 μl of the vitamin K0 stock solution and was stopped by the addition of 0.9 ml of ice-cold isopropanol. Extraction and analysis by h.p.l.c. were as described previously (Thijssen, 1987). When following the effects of 4-hydroxycoumarins and of DTT on the reductase with time, the assay conditions (i.e. reaction volume) were adjusted accordingly. At selected time points aliquots (0.1 ml) of the reaction mixture were assayed for the amounts of vitamin K formed. Further details are given in the text.

Materials

Racemic [^{14}C]-warfarin (46 mCi mmol^{-1}) was obtained from Amersham (The Netherlands). The isolation of its isomers was performed according to Banfield & Rowland (1983) with some modifications; N-t-BOC-L-proline (Sigma Chemicals) was used instead of N-CBZ-L-proline because the diastereoisomers of the former showed a more favourable chromatographic behaviour on thin layer chromatography (silica-gel t.l.c. plates with concentration zone; Merck, Darmstadt, F.R.G.; eluting phase: petroleum ether, ethylacetate, methanol; 75:25:1; v:v:v). The enantiomers of warfarin were prepared according to the procedure of West *et al.* (1961). The enantiomers of acenocoumarol and of phenprocoumon were a gift from Ciba-Geigy Ltd. (Basel, Switzerland) and Hoffmann-La Roche (Basel, Switzerland), respectively. Dithiothreitol (DTT) was purchased from Boehringer (F.R.G.), vitamin K0 was prepared from vitamin K1 (phylloquinone, Merck) according to the procedure of Tishler *et al.* (1940).

Table 1 The effect of *in vivo* administration of unlabelled S-warfarin on the tissue distribution of R- and S-[^{14}C]-warfarin

	Concentration (d.p.m. g^{-1} of tissue)					
	R-warfarin[a]			*S-warfarin*[b]		
	Control ($n = 2$)	*Exp* ($n = 3$)	r[c]	*Control* ($n = 3$)	*Exp* ($n = 3$)	r[c]
Plasma	1470	8290 ± 1400	5.64	750 ± 130	10,050 ± 400	13.40
Liver homogenate	35,800	21,850 ± 4300	0.60	39,525 ± 4570	17,700 ± 270	0.45
Cytosol	5270	17,900 ± 4600	3.40	1500 ± 570	6090 ± 570	4.07
Microsomes	15,800	3900 ± 680	0.25	21,525 ± 3130	6300 ± 240	0.29
Kidney homogenate	12,700	5560 ± 800	0.44	11,290 ± 725	5090 ± 200	0.45

[a] Twenty-four hours after the i.v. administration of 1.5×10^6 d.p.m. R-[^{14}C]-warfarin, saline (Control) or S-warfarin (1 mg kg^{-1}, i.v.) (Exp) was administered. Rats were killed 3 h later, i.e. at maximal effect (see Figure 2).
[b] Twenty-four hours after the i.v. administration of 1.5×10^6 d.p.m. S-[^{14}C]-warfarin, saline (Control) or S-warfarin (1 mg kg^{-1}, i.v.) (Exp) was administered. Rats were killed 12 h later (see Figure 2).
[c] Ratio between experimental and control data.

All other chemicals were of anlytical grade and were obtained from regular suppliers.

Male Wistar rats (supplied by Winkelman, Borchen F.R.G.) were used. Their weights were 270–300 g. The animals had free access to water and food.

Tissue radioactivity was assayed by liquid scintillation counting. The fraction of radioactivity associated with unchanged warfarin was estimated by the h.p.l.c. method used to assay 4-hydroxycoumarins (Thijssen *et al.*, 1985). Where appropriate, differences in results were analysed for significance by Student's unpaired t test; $P < 0.05$ was taken as being significant.

Results

As has been demonstrated previously for racemic [^{14}C]-warfarin, the administration of a high (macro) dose of a 4-hydroxycoumarin (here S-warfarin) to rats which had received tracer doses of R- or S-[^{14}C]-warfarin 48 h before caused the plasma levels of radioactivity to increase immediately. Figure 2 shows a typical example of such an experiment. The radioactivity reappearing in the circulation was more than 90% intact warfarin. The reappearance of R-/S-[^{14}C]-warfarin following the administration of unlabelled S-warfarin has to be ascribed to the replacement of the [^{14}C]-warfarin compounds that were bound to vitamin K0 reductase (Thijssen & Baars, 1987; see also Figure 1). The experiments showed R-[^{14}C]-warfarin to be released 3–4 times faster than the S-enantiomer (Figure 2). The half-lives of release were 1.2 ± 0.04 and 3.7 ± 0.6 h (mean ± s.d., $n = 4$) for R- and S-[^{14}C]-warfarin, respectively. The differences were significant, $P < 0.001$.

The distribution of tracer doses of R- and S-[^{14}C]-warfarin in the liver and kidney of controls and S-warfarin-treated rats are presented in Table 1. The times taken for examination were when maximal displacement effects had occurred, i.e. at 3 h for R-[^{14}C]-warfarin and at 12 h for S-[^{14}C]-warfarin (see Figure 2). In controls differences in the steady-state plasma concentrations between the isomers were obvious; they were about 2 fold higher for R-[^{14}C]-warfarin (see also Figure 2), and the levels in liver cytosol were about 3 fold higher for the R-isomer. Microsomes of the R-[^{14}C]-warfarin-treated rats contained about 45% of the total amount in the liver, for S-[^{14}C]-warfarin this was about 55%. The *in vivo* administration of S-warfarin (1 mg kg^{-1}) displaced about 70–75% of the microsomal bound R- and S-[^{14}C]-warfarin. This resulted in increased cytosol (3 to 4 fold) and plasma levels. The latter was more pronounced for S-[^{14}C] warfarin (13 fold) than for R-[^{14}C]-warfarin (5 to 6 fold). On the whole, however, no gross differences between the distributions of R- and S-warfarin in the liver were observed, either in the control or in the experimental group. For non-hepatic tissue, like kidney, a displacment of the R-/S-[^{14}C]-warfarin content was also observed (Table 1).

The control microsomes of the foregoing experiment were used to investigate the *in vitro* release of microsomal bound R-/S-[^{14}C]-warfarin. The results (Table 2) show hardly any release of radioactivity by mere dilution of the microsomes. In the presence of DTT (10 mM), however, microsomal bound [^{14}C]-warfarin was released in a time-dependent manner and this was more pronounced for the R- than for the S-enantiomer of warfarin (Table 2).

Table 3 shows the results of experiments performed to see if the DTT-dependent release of micro-

Table 2 The effect of dithiothreitol (DTT) on the release of [^{14}C]-warfarin from rat liver microsomes[a]

Time (h)	Wash conditions	[^{14}C]-warfarin d.p.m. in microsomes (% of starting material)[b] R	(release)[c]	S	(release)[c]
1	Buffer	86.5		92	
	Buffer, 10 mM DTT	77.5	(9)	86	(6)
4	Buffer	87		92	
	Buffer, 10 mM DTT	58	(29)	75	(17)

[a] One ml microsomes were diluted with 10 ml buffer (0.02 M Tris-HCl, 0.15 M KCl, pH 7.4) with and without 10 mM DTT. The mixtures were incubated at room temperature with gentle shaking on a rotary shaker (20 r.p.m.). After the incubation time microsomes were repelleted (60 min, 100,000 *g*) and resuspended up to the original volume.
[b] **R**, microsomes of **R**-[^{14}C]-warfarin-treated animals; **S**, microsomes of **S**-[^{14}C]-warfarin-treated animals (see Table 1).
[c] The amount released due to DTT treatment was obtained by subtracting blank (buffer) values.

somal bound 4-hydroxycoumarin was associated with reactivation of the vitamin K0 reductase activity. **R**- and **S**-acenocoumarol were used as the reductase inactivators. As can be seen, DTT (10 mM) treatment for 1 h resulted in some reactivation of the reductase activity and this was more pronounced for the **R**-acenocoumarol-treated microsomes (about 2 fold more).

The results of *in vivo* experiments (Figure 2) suggested that the mechanism of stereoselectivity of the 4-hydroxycoumarins is linked to the reactivation of the oxidized enzyme-4-hydroxycoumarin complex (step 4, Figure 1). The data from *in vitro* experiments (Tables 2 and 3) are in agreement with this. However, the *in vitro* results were not as dramatic as expected. We, therefore, studied the effect of time on microsomal vitamin K0 reductase activity and investigated the effect of 4-hydroxycoumarin enantiomers and the effect of DTT thereupon. Figure 3 shows the results from a typical experiment in which the effects of **R**-/**S**-acenocoumarol (2.3 μM) and **R**-/**S**-warfarin (2.6 μM) on microsomal vitamin K0 reductase activity were investigated. At a DTT concentration (2 mM) optimal for the control reaction (K_M of DTT at the vitamin K0 concentration used is about 0.2 mM; own observation and that of others), no differences in reductase activity were observed for the **R**- and **S**-4-hydroxycoumarin-treated microsomes (incubation time 0–20 min). Following the addition of extra DTT (final concentration 42 mM) the rate of the vitamin K0 conversion in the 4-hydroxycoumarin-treated microsomes increased drastically. The final reductase rates obtained were higher for the microsomes treated with the **R**-coumarins. The reductase activity of the control microsomes was not affected by the addition of DTT (Figure 3). The observed ratios between the **R**- and **S**-isomers for the vitamin K0 reductase activity in the presence of 42 mM DTT were (mean $\pm$ s.d.): 1.53 $\pm$ 0.06 ($n = 3$, significantly different from 1, $P < 0.01$), 1.40 $\pm$ 0.1 ($n = 3$, significantly different from 1, $P < 0.05$), and 1.3 ($n = 1$) for acenocoumarol, warfarin and phenprocoumon, respectively.

Two other facts became apparent from these experiments: (1) the inhibition can be reversed completely by increasing the amount of DTT in the reaction mixture (Figure 3). N.B.: No vitamin K0 conversion was ever observed in the absence of microsomes or in the presence of denaturated (10 min, 100°C) microsomes; (2) the 4-hydroxycoumarin congeners are not equipotent, i.e. acenocoumarol was more potent than warfarin (see Figure 3). Phenprocoumon and warfarin were equipotent.

Table 3 The effect of dithiothreitol (DTT) on the reactivation of **R**-/**S**-acenocoumarol-treated rat liver microsomes[a]

Addition	Vitamin K0 reductase activity (% of control)[b] Start	Wash	Wash + DTT (10 mM)
0	100	100	92
R-acenocoumarol	<2	4	15.5
S-acenocoumarol	<2	3	9

[a] One ml of microsomal suspension (15 mg protein ml^{-1}) was incubated in the presence of **R**- or **S**-acenocoumarol (5 μg ml^{-1}) at room temperature for 30 min. Aliquots (2 × 20 μl) were removed for reductase assay. The remainder was diluted with 10 ml of buffer (0.02 M Tris-HCL, 0.15 M KCl, pH 7.4) with and without 10 mM DTT and incubated for 1 h on a rotary shaker (20 r.p.m.) at room temperature. The microsomes were repelleted (60 mm, 100,000 *g*) and resuspended up to the original volume.
[b] The results are the mean of two separate experiments. All reductase assays were performed in duplicate.

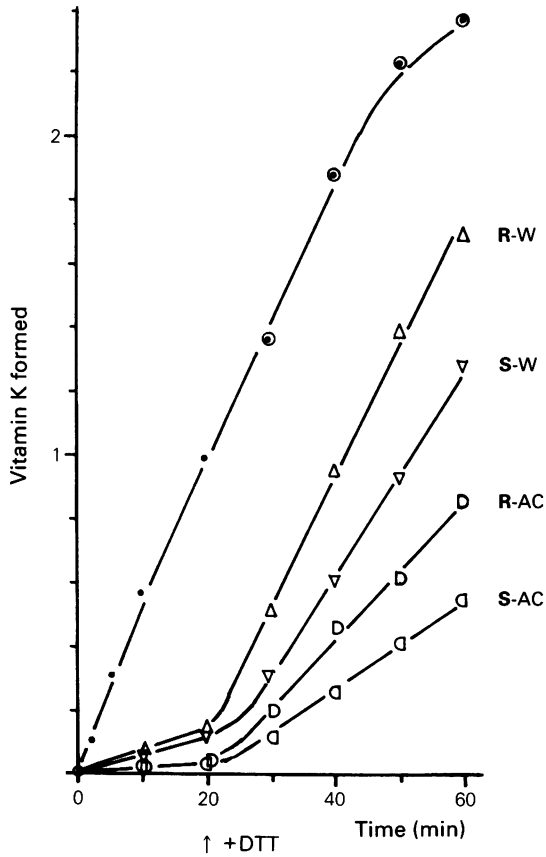

Figure 3 The effect of dithiothreitol (DTT) on the microsomal vitamin K 2,3-epoxide (vitamin K0) to vitamin K conversion in the presence of **R**-/**S**-warfarin (**R**-W, S-W) and in the presence of **R**-/**S**-acenocoumarol (**R**-AC, S-AC). Microsomes were preincubated with the 4-hydroxycoumarins (4 $\mu g\,ml^{-1}$ microsomes) for 20 min. Then 0.2 ml microsomes were mixed with 0.8 ml Tris-HCl buffer and preincubated with DTT (2 mM) at 30°C. The reaction was started by the addition of vitamin K0 (see Methods section). At 20 min DTT was enhanced to 42 mM. Control reaction at 2 mM DTT (●); at 42 mM DTT (⊙). Ordinate scale: amount of vitamin K formed in arbitrary (vitamin K peak/internal standard peak) units.

Discussion

In a recent paper (Thijssen & Baars, 1987) we proposed that the tight complex between 4-hydroxycoumarins and the oxidized (i.e. inactive) form of the enzyme vitamin K0 reductase dissociates following reduction (i.e. reactivation) of the disulphide bond in the active centre of the enzyme. The rate of reduction of the disulphide bond of the 'susceptible' enzyme is low when complexed with 4-hydroxycoumarin, thus explaining the mechanism of action of the oral anticoagulants (Figure 1). Support for this model is given by the mechanism on which the 4-hydroxycoumarin resistance of the Scottish warfarin resistant genotype is based. The oxidized vitamin K0 reductase of this resistant strain, notwithstanding that a 4-hydroxycoumarin is tightly (irreversibly) complexed with it, has been shown to undergo reactivation and thereby to release the coumarin under relatively mild reducing (i.e. 2 mM DTT) conditions (Thijssen, 1987). Thus, the reactivation of an enzyme of this genotype is hardly retarded in the presence of the classical 4-hydroxycoumarins, which explains the resistance to these drugs.

With this model in mind, it seemed obvious to investigate the reactivation pathway (step 4, Figure 1) as the possible basis for the stereoselectivity of the 4-hydroxycoumarins. The displacing experiments showed that the *in vivo* rate of reactivation of the oxidized vitamin K0 reductase-warfarin complex was 3 to 4 times faster for the **R**-warfarin-enzyme complex. The results from *in vitro* experiments demonstrated basically the same characteristics, a higher rate of release (Table 2) and a higher rate of reactivation (Table 3, Figure 3) for the **R**-4-hydroxycoumarin-enzyme complex. The differences between the **R**- and S-enantiomers in the *in vitro* experiments, however, were not as high as was observed for the *in vivo* displacement experiments. One reason could be that the endogenous reductor is more selective for stereochemical configurations. Another reason could be that the association rate between the 4-hydroxycoumarin and the oxidized enzyme (step 3, Figure 1) exhibits stereoselectivity. However, computer simulations of the model (Figure 1) showed that step 3 is of little influence on the steady-state level of active enzyme as long as its rate is not low in comparison to step 2 (unpublished results).

Besides the stereoselectivity, the model also explains the higher **R**-warfarin liver cytosol and plasma levels (Table 1); i.e. the higher rate of reactivation of the enzyme-R-warfarin complex (step 4, Figure 1) assures a higher 'free' **R**-warfarin steady-state level. Also the observation that rat liver microsomal vitamin K and vitamin K0 reductase activity recovered more quickly from the **R**-warfarin administration than from the S-warfarin administration, *in vivo*, (Fasco & Principe, 1982) can be explained by this model.

However, the kind of the interaction between the 4-hydroxycoumarins and the oxidized vitamin K0 reductase is still speculative. The irreversible character of the complex suggests a covalent bond. Apparently this bond is reversed by the reductor (DTT) without destruction of the coumarin. This and the fact that the 4-hydroxycoumarins interact only with

the oxidized enzyme might point to a chemical interaction, possibly with the disulphide bond of the enzyme. In the literature many authors still share the view that vitamin K (K0) and the 4-hydroxycoumarins are structurally related and the absence of any competition between the substances is therefore surprising. However, we recently made the observation that the 4-hydroxycoumarins, unlike vitamin K and vitamin K0, did not give a signal at reductive conditions (up to -1.6 V) in an electrochemical h.p.l.c. cell, but strongly responded at oxidative conditions ($1/2\,E_{max} = 0.5$ V) (unpublished results). Principally these results show the absence of any relationship between vitamin K and the 4-hydroxycoumarins. Although not conclusive, we also feel that these data support our hypothesis with regard to the interaction mechanism.

The fact that R-/S-[^{14}C]-warfarin in kidneys responded similarly, to the administration of a 'macro' dose of S-warfarin (displacer), to liver tissue is in agreement with our earlier findings showing that extrahepatic tissues, like the kidney, possess vitamin K0 reductase activity (Thijssen *et al.*, 1986b).

The discussion so far has been conducted on the following assumption: (1) vitamin K0 reductase switches from a reduced (active) form to an oxidized (inactive) form during substrate reduction. Reactivation needs a reductor and in *in vitro* systems DTT serves that purpose. The presence of two active site sulphhydryl groups which are converted to a disulphide fit into this mechanistic model. (2) The 4-hydroxycoumarins interact with the oxidized enzyme. This hypothesis, first formulated by Fasco *et al.* (1983) is based on the observation that following DTT pretreatment the initial velocity of microsomal vitamin K0 reduction is insensitive to 4-hydroxycoumarin inhibition. In accordance with this finding are recent observations in our laboratory which show that the saturable microsomal [^{14}C]-warfarin binding is abolished by pretreatment with DTT (results to be published elsewhere).

However, Silverman & coworkers (1988) have recently questioned the presence of active site sulphhydryl groups. Experiments with solubilized bovine microsomes showed that the inhibitory effect of thiol blocking agents on vitamin K0 reductase activity is independent of DTT pretreatment. These and other results led Silverman *et al.* (1988) to postulate an alternative mechanism for vitamin K0 reduction, instead of acting as a reducing agent DTT is a substrate for the reductase. DTT binding induces a conformational change which activates the enzyme and so assumes the proper alignment of vitamin K0, the dithiol and a functional group on the enzyme. The dithiol is the electron donor for the reduction. This model will explain the present results as well as the assumption that 4-hydroxycoumarins interact only with the inactive enzyme, i.e. in the absence of the dithiol substrate, and that this interaction is somehow irreversible. The 4-hydroxycoumarin binding stereoselectively reduces the interaction i.e. affinity, of the dithiol with its binding site. However, once DTT binding is accomplished, the resulting conformational change will cause the release of the 4-hydroxycoumarin.

The competition described between 4-hydroxycoumarins and DTT (Silverman *et al.*, 1988) is not compatible with this extension of the model. Competition in the strict sense presumes an interaction equilibrium for the 4-hydroxycoumarins. However, all data up to now refute such an equilibrium (Fasco & Principe, 1982; Thijssen, 1987; Thijssen & Baars, 1987). Hence, it remains to be determined whether the observed apparent competition is inherent in the model presented in Figure 1; i.e. whether a competitive interaction occurs between the DTT or 4-hydroxycoumarin concentration-dependent rates of steps 2 and 3, respectively.

As well as the purification of vitamin K0 reductase, knowledge of the endogenous reductor will enlarge our understanding of the system and the mechanism of 4-hydroxycoumarin inhibition.

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