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INHIBITION BY WARFARIN OF LIVER MICROSOMAL VITAMIN K-REDUCTASE IN WARFARIN-RESISTANT AND SUSCEPTIBLE RATS

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Absrtact—The NADH-dependent vitamin K-reductase activity of liver microsomes from three closely related rat strains has been studied. One strain (TAS) is susceptible and two strains (HW and HS) resistant to the anticoagulant and lethal effects of warfarin. The effects of cofactors, temperature, detergent and dithiothreitol on vitamin K_1 reduction and solvent extraction of substrate and product have been investigated.

Vitamin K-reductase activity was inhibited by approximately 13 and 8% respectively when microsomal preparations from TAS and HW animals were incubated with 50 μM vitamin K_1 and 10 μM warfarin. In HS rat liver microsomes the enzyme was highly resistant to inhibition by warfarin. Evidence is presented and discussed that suggests that NADH-dependent vitamin K-reductase may be inhibited in the anticoagulant effect of warfarin and may be altered as a result of expression of the warfarin-resistance gene in HS rats. The enzyme activity studied was probably not a DT-diaphorase although both NADH and NADPH acted as cofactors for the reaction.

The anticoagulant warfarin has been extensively used as a rodenticide in Britain for thirty years and warfarin-resistant rats were first observed in Scotland in 1958 [1]. Subsequent pockets of resistance in rats occurred on the Wales–England border [2], in Denmark [3], Holland [4], Germany [5] and the U.S.A. [6]. It was established at an early stage that resistance in rats was due to the inheritance of a single autosomal gene [7]. Warfarin-resistance was tested by feeding wild animals (*Rattus norvegicus*) with normally lethal doses of warfarin. The survivors of this test were interbred with warfarin-susceptible laboratory TAS rats (Tolworth Albino Strain, Wistar derived) to produce strains that are homozygous for either the Scottish (HS) or Welsh (HW) resistance genes but closely related to the laboratory susceptible animals [8–10].

The anticoagulant effect of warfarin is due to an antagonistic effect on the role of vitamin K in post-translational carboxylation of glutamyl residues in blood coagulation factors II, VII, IX and X [reviewed 11]. The γ-glutamyl carboxylase enzyme appears to require the hydroquinone, or reduced form, of vitamin K as a cofactor [12] and vitamin K 2,3-epoxide is a product of the reaction [13]. There is recent evidence [14, 15] to support the hypothesis that an enzyme cycle exists to regenerate vitamin K hydroquinone from the epoxide with the quinone form of vitamin K as an intermediate product. Administration of warfarin to rats resulted in an increase in the ratio of vitamin K 2,3-epoxide to vitamin K [16], an effect that was more pronounced in warfarin-susceptible than in resistant animals and further work suggested that the antagonistic effect of warfarin is to inhibit the enzyme vitamin K 2,3-epoxide-reductase and that this enzyme is altered in resistant animals [17]. It has been confirmed that the level of activity of this enzyme is lower in HS and HW rats than in TAS rats [18] and that warfarin-resistant animals maintain normal blood coagulation activities when warfarin and vitamin K 2,3-epoxide are administered simultaneously [19].

There has been continued discussion on the role of a group of enzymes known as DT-diaphorase (EC 1.6.99.2) in the microsomal reduction of vitamin K to the hydroquinone. The name DT-diaphorase was given to these enzymes [20] because they catalysed the reduction of dichlorophenol–indophenol by NADH (DPNH) and NADPH (TPNH) with equal reaction velocities. Several compounds, including vitamin K_3, were identified as electron acceptors and the activity was totally inhibited by 1μM dicoumarol [20]. A study using purified rat liver cytosolic DT-diaphorase [21] demonstrated that this enzyme can catalyse the NADH-dependent reduction of vitamin K_1 to the hydroquinone. It has already been shown [22], however, that DT-diaphorase activity in liver cytosolic fractions, which contain over 90% of the total liver activity, is essentially the same in three rat strains used in the present study and is not related to warfarin-resistance. Other workers [23] have purified a microsomal DT-diaphorase and shown that this enzyme will catalyse NADH-dependent vitamin K reduction. Studies on dithiothreitol-dependent vitamin K-reductase activity [14, 15] have shown that this enzyme is also inhibited by warfarin. The inhibitory effect on the enzyme was shown to be greater in liver microsomal preparations from susceptible Holtzman rats than in preparations from a warfarin-resistant colony that was originally derived from Welsh resistant animals. A study [10] on the three rat strains in our laboratory showed that

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HW rats maintain normal blood coagulation activity when warfarin and vitamin K_1 are administered in drinking water whereas both HS and TAS rats have decreased coagulation factors. The present study was undertaken using the same three closely related rat strains to investigate the possibility that the anticoagulant activity of warfarin, and the expression of the warfarin-resistance gene, effect the activity of vitamin K-reductase in liver microsomes

MATERIALS AND METHODS

Animals

Animals of the TAS, HW and HS strains bred in our laboratory were fed a standard diet of pelleted 41B (Oxoid Ltd., Basingstoke, Hampshire, U.K.) and tap water *ad libitum*. Water for HW strain animals was supplemented with 0.1% menadione sodium bisulphite (Sigma London Chemical Co. Ltd., Poole, Dorset, U.K.). All animals were fasted for 16 hr before sacrifice and were 50–70 days old.

Detection and determination of reduced vitamin K_1

Reduced vitamin K_1 was separated from vitamin K_1 by high performance liquid chromatography (HPLC). The apparatus used consisted of two M6000A pumps, a 710B autoinjector, a 740 data module, a 720 system controller (Waters Associates, Hartford, Cheshire, U.K.), a Pye-Unicam LC3 ultraviolet absorbance detector (Pye-Unicam Ltd, Cambridge, U.K.), set at 254 nm and 0.04 absorbance units full scale deflection, and a fraction collector (2111 Multirac; L.K.B. Instruments Ltd. South Croydon, Surrey, U.K.). Samples (10–200 μl) were chromatographed at 30° on columns of Zorbax ODS (4.8 × 250 mm, Du Pont (U.K.) Ltd, Hitchin, Herts, U.K.) packed at a pressure of 6000 psi in dichloromethane (HPLC grade; Rathburn Chemical Co., Walkerburn, Peeblesshire, U.K.) using a CP III slurry packer (Jones Chromatography Ltd., Llanbradach, Glamorgan, U.K.). Elution of samples at 2 ml/min was carried out using a convex gradient (Waters #3, 0–6 min) from 100% acetonitrile (HPLC grade; Rathburn Chemical Co.) to acetonitrile:isopropanol (50:50; v/v: HPLC grade; Rathburn Chemical Co.). The retention times of vitamin K_1 hydroquinone and vitamin K_1 quinone were 5.4 and 7.4 min, respectively. When appropriate, eluant fractions were collected between 5.1–6.1 and 7.1–8.1 min and radioactivity in these fractions determined by liquid scintillation counting in Unisolve 100 (5 ml; Koch Light Laboratories Ltd., Haverhill, Suffolk, U.K.) using a model 4530 scintillation counter (Packard Instrument Co., Caversham, Berks. U.K.) with absolute activity calculation facilities.

Ice-cold isopropanol (1.25 ml) was routinely added to microsomal incubation mixtures (750 μl) to terminate the reaction. Experiments were also carried out using HPLC grade (Rathburn Chemical Co.) acetonitrile, methanol, tetrahydrofuran, ethyl acetate and hexane/isopropanol (1:1; v/v). Samples were vortexed for 20 sec after the addition of organic solvent, centrifuged at 5000 *g* for 5 min and the supernatant examined by HPLC. Standard reduced vitamin K_1 was synthesised by chemical reduction with sodium dithionite under nitrogen as previously described [12].

Preparation of liver microsomes

The livers of five male rats of the same strain were excised after perfusion via the portal vein with ice-cold 0.1 M phosphate buffer (pH 7.4), minced and homogenised with a Potter-Elvehjem homogeniser in four volumes (by weight) of the same buffer. Cellular debris, nuclei and mitochondria were removed by centrifugation at 10,000 *g* for 20 min (HS 18; MSE Instruments Ltd., Crawley, Sussex, U.K.) and the microsomes sedimented from the supernatant by centrifugation at 100,000 *g* for 60 min (Prepspin 50; MSE Instruments Ltd.). The microsomes were washed by resuspending in two volumes (by liver weight) of phosphate buffer. The microsomes were finally resuspended in two volumes (by weight) of buffer and aliquots stored at −70° until use.

Assay of vitamin K-reductase

The assay method was adapted from that described by Fasco and Principe [14]. Reaction mixtures were 0.75 ml of Tris–HCl buffer (0.2 M, pH 7.4) with 0.15 M KCl, 0.2% (w/v) Emulgen 913 (Kao Atlas, Tokyo, Japan) and contained microsomes equivalent to 0.08 g wet wt liver. NADH (Sigma London Chemical Co.) or NADPH (Sigma London Chemical Co.) were dissolved in 0.2 M Tris–HCl buffer pH 7.4 at a concentration of 10 mM. Dithiothreitol (DTT; Sigma London Chemical Co.) was dissolved in the same buffer at a concentrations of 100 mM and solutions of Emulgen 913 were 1.0% (w/v). [1′,2′^{3}H] Phylloquinone (vitamin K_1) was a gift from Hoffman La Roche Ltd. (Basle, Switzerland).

Radiolabelled vitamin K_1 was purified by HPLC as described above and diluted to a specific activity of 18.9 μCi/μmol with unlabelled vitamin K_1 (Sigma London Chemical Co.). Emulgen 913 (200 μl; 1% w/v in diethyl ether, glass distilled grade, Rathburn Chemical Co.) was added to radiolabelled vitamin K_1 (451 μg) dissolved in a small volume of diethyl ether. The solution was evaporated to dryness under oxygen-free nitrogen. Tris–HCl buffer (2 ml; 0.2 M, pH 7.4, 0.15 M KCl) was added dropwise with continuous vigorous stirring. The final concentration of the solution was 500 μM vitamin K_1 and 0.1% (w/v) Emulgen 913 in Tris–HCl buffer (pH 7.4).

Optimisation of assay conditions

All buffers and solutions were deoxygenatd before use by degassing under vacuum and replacement of air with oxygen-free nitrogen. Enzyme assays and preparation of samples for HPLC were carried out under oxygen-free nitrogen. All experiments were carried out under reduced lighting.

The effect of temperature on vitamin K-reductase activity was investigated in the range 25–45°. Reaction mixtures contained 1 mM NADH, 5 mM DTT and 33 μM radiolabelled vitamin K_1.

The effect of DTT concentration (0–20 mM) on vitamin K-reductase activity was investigated. Reaction mixtures contained 1 mM NADH and 33.3 μM radiolabelled vitamin K in Tris–HCl buffer (0.2 M, pH 7.4, 0.15 M KCl) containing 0.2% (w/v) Emulgen

913. Incubations were carried out at 37° for 5 min and the amounts of reduced vitamin K_1 formed were determined by HPLC and liquid scintillation counting.

The role of reduced dinucleotides as cofactors for vitamin K-reductase activity was investigated using NADH and NADPH at concentrations of 0–1.7 mM. Reaction mixtures contained 5 mM DTT and 33.3 μM radiolabelled vitamin K_1 and incubations were carried out at 37° for 5 min.

Kinetic studies on vitamin K-reductase

All studies were carried out for 5 min at 37° under nitrogen and reduced lighting in deoxygenated Tris–HCl buffer (0.2 M, pH 7.4) containing 0.15 M KCl, 0.2% (w/v) Emulgen 913, 5 mM DTT and 1 mM NADH. Sodium warfarin (Ward Blenkinsop and Co. Ltd., London, U.K.) was added in Tris–HCl buffer at final concentrations of up to 2 mM when appropriate. Radiolabelled vitamin K_1 was added at concentrations of 20–100 μM to initiate the reaction.

Results of analyses of vitamin K_1-reductase activity were plotted as the inverse of enzyme velocity ($1/v$) against the inverse of vitamin K_1 concentration ($1/[S]$: Lineweaver–Burk plots). Best fit curves were calculated using a desk-top calculator and maximal velocities (V_{max}) for each microsomal preparation were calculated from intercepts on the $1/v$ axis. Standard errors (± S.E.) of intercept and slope were calculated by least squares regression analysis. The value for intercept and slope were used for calculation of the Michaelis constant (K_m) where the slope of the uninhibited curve is K_m/V_{max}. Values for the inhibitor-enzyme dissociation constant (K_i) were calculated as for a competitive inhibitor in experiments with TAS and HS liver microsomes where the slope of inhibited curves are equal to $\{1 + ([I]/K_i)\}(K_m/V_{max})$.

In experiments with HW liver microsomes warfarin inhibition was considered to be non-competitive and the intercepts $1/v$ are equal to $\{1 + ([I]/K_i)\}(1/V_{max})$.

RESULTS

Optimisation of recovery of vitamin K_1 hydroquinone and its determination by HPLC

A number of problems were encountered in this study due to the labile nature of the reaction product. Reduced vitamin K_1 could not be detected by HPLC following addition of chemically reduced vitamin K_1 hydroquinone to aqueous samples or aqueous–organic mixtures in the absence of DTT. When DTT (1–5 mM) was included in these mixtures recovery of reduced vitamin K_1 was 80–90% and was independent of the concentration of DTT. Repeated HPLC analysis was carried out on a sample that originally contained 25 μM reduced vitamin K and 5 mM DTT in isopropanol:water (5:3; v/v). This indicated that approximately half of the reduced vitamin K_1 initially present was oxidised to vitamin K_1 in 3.5 hr at room temperature under nitrogen and in subdued lighting. All samples subsequently contained 5 mM DTT and were analysed by HPLC within 10 min of the end of the incubation.

The extraction from microsomal preparations of mixtures of vitamin K_1 and chemically reduced vitamin K_1 hydroquinone was investigated using several organic solvents (organic:aqueous; 5:3, v/v). Relatively polar solvents such as acetonitrile and acetone were not suitable since only 40–60% of the vitamin and its reduced form were extracted from the precipitated protein. Less polar solvents such as tetrahydrofuran and isopropanol gave good recoveries (95–100%) of vitamin K_1 but with tetrahydrofuran 50–60% of the reduced vitamin K_1 present was oxidised relative to the results obtained with isopropanol. When ethyl acetate was used the chromatograms obtained after injection of 100–200 μl of the organic phase showed extremely mis-shapen elution profiles and it was impossible to estimate the recoveries of vitamin K_1. Extraction with 2 vol. of isopropanol:hexane (1:1; v/v), as described by Fasco and Principe [14], gave good recovery of the vitamin but an apparent oxidation of 25–30% of reduced vitamin K_1 present relative to the results obtained using isopropanol alone. Brief investigations indicated that the step involving evaporation of the organic phase under a stream of nitrogen at 35° caused a decrease in the amount of reduced vitamin K_1 detected. Extraction of vitamin K_1 quinone and metabolically formed hydroquinone from microsomal incubations (750 μl) was subsequently carried out using isopropanol (1.25 ml).

Optimisation of microsomal incubation conditions

Previous studies on vitamin K_1-reductase [14, 15] involved incubation of vitamin K_1 with rat liver microsomes at 25°. We have investigated the effect of temperature on the rate of reduction of radiolabelled vitamin K_1 and the results are shown in Fig. 1. The rate of vitamin K_1 reduction at temperatures between 30° and 37.5° in the presence of microsomes from TAS rat livers (●—●) was more than twice the rate at 25°. The rate of the reaction was lower when incubations were carried out above 40°. It was also observed that vitamin K_1 reduction occurred in the absence of enzyme and that the apparent rate of

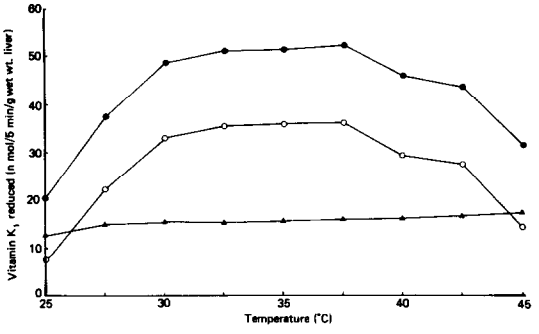

Fig. 1. Effect of temperature on vitamin K_1-reductase in rat liver microsomal incubations. The figure shows the apparent K_1-reductase activity observed in the absence (▲—▲) and presence (●—●) of microsomes from 0.08 g (wet wt) TAS rat livers in incubations with radiolabelled vitamin K_1 carried out at different temperatures (25–45°) as described in the text. The results obtained in the absence of microsomes were subtracted from those obtained with microsomes at the same temperature and plotted as vitamin K-reductase activity (○—○).

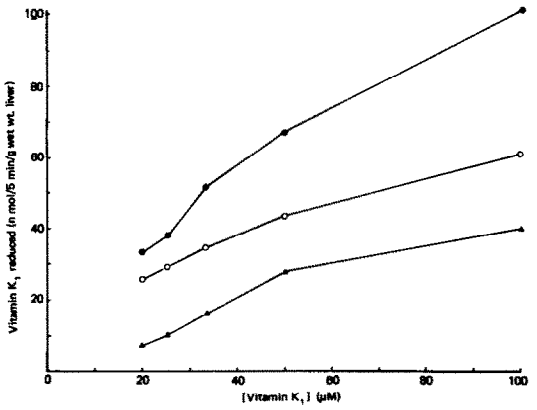

Fig. 2. Effect of vitamin K_1 concentrations on its reduction by rat liver microsomal incubations. The figure shows the apparent vitamin K_1-reductase activity observed in the absence (▲—▲) and presence (●—●) of microsomes from 0.08 g (wet wt) TAS rat livers at several vitamin K_1 concentrations in incubations carried out as described in the text. The results obtained in the absence of microsomes were subtracted from those obtained with microsomes at the same concentration of radiolabelled vitamin K_1 and plotted as vitamin K-reductase activity (○—○).

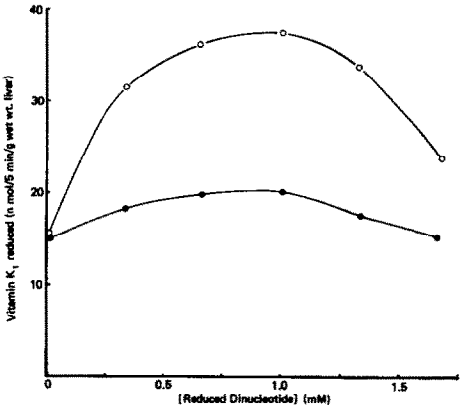

Fig. 3. Effect of NADH and NADPH concentration on vitamin K_1-reductase activity of rat liver microsomes. The figure shows vitamin K-reductase activity when NADH (○—○) or NADPH (●—●) was present at concentrations of 0–1.7 mM in incubations of TAS rat liver microsomes with radiolabelled vitamin K_1 carried out as described in the text.

reaction due to non-enzymic reduction (Fig. 1, ▲—▲) increased from a rate that was equivalent to 12.5 nmol/5 min/g wet wt liver to 17.3 nmol/5 min/g wet wt liver between 25 and 45°. The original radiolabelled substrate solution contained 0.4% reduced vitamin K_1 and would only account for 1.25 nmol/5 min/g wet wt liver of this apparent non-enzymic reduction. The rate of non-enzymic reduction was subtracted from the total at each temperature and the difference plotted as microsomal vitamin K_1-reductase activity (○—○). This indicated an optimal incubation temperature of 32.5–37.5° and all subsequent incubations were carried out at 37°

Studies on the rate of radiolabelled vitamin K_1 reduction at substrate concentrations between 20 and 100 μM (Fig. 2) indicated that the total (●—●) and non-enzymic (▲—▲) reaction rates increased at higher substrate concentrations. Vitamin K_1-reductase activity of TAS rat liver microsomes was plotted (Fig. 2, ○—○) after subtraction of the non-enzymic rate from the total rate at each substrate concentration. For the results shown in Fig. 3 and Table 1, the non-enzymic reduction appropriate to each substrate concentration was subtracted from the total observed reduction to give a value of enzyme catalysed vitamin K_1 reduction.

In the presence of 1 mM NADH, both the enzymic and non-enzymic rates of vitamin K_1 reduction, were found to be independent of the concentration of DTT in the range 1–20 mM. The non-enzymic reduction rate measured in the absence of microsomes was not altered by the addition of warfarin at concentrations of up to 100 μM.

Table 1. Kinetic parameters of vitamin K-reductase from liver microsomes of three rat strains

Rat strain	Warfarin concentration (μM)	$1/V_{max}$(±S.E.) (μmol^{-1} vitamin K_1 reduced/5 min/g wet wt liver)	Slope(±S.E.) $[(1/v)/(1/[S])]$	V_{max} (nmol vitamin K_1 reduced/5 min/g wet wt liver)	K_m (μM vitamin K_1)	K_i (μM warfarin)
TAS	0	11.17 (0.50)	0.58 (0.16)	89.5	51.1	–
	10	9.71 (0.43)	0.82 (0.015)	103	84.2	23.2
	50	10.50 (0.55)	1.03 (0.030)	95.2	93.1	62.2
HW	0	8.26 (0.67)	0.80 (0.018)	121	96.3	
	10	9.01 (0.59)	0.86 (0.017)	111	92.9	113
	50	10.81 (0.55)	1.07 (0.015)	92.5	99.3	163
HS	0	2.77 (0.42)	0.46 (0.014)	361	167	
	50	2.82 (0.43)	0.46 (0.014)	355	175	
	2000	2.25 (0.81)	0.81 (0.020)	444	334	3130

The kinetic parameters presented for vitamin K-reductase of three rat strains were calculated from best-fit curves of Lineweaver–Burk plots of results obtained from incubations of radiolabelled vitamin K_1 with liver microsomal preparations, carried out as described in the text. Standard errors (S.E.) of the intercept ($1/V_{max}$) and slope were calculated by the least-squares method. V_{max} represents the maximum activity of the enzyme, K_m is the Michaelis constant and is inversely proportional to the affinity of the enzyme for vitamin K_1. The inhibitor–enzyme dissociation constant K_i is inversely proportional to the affinity of the enzyme for warfarin. in calculating K_i warfarin was considered to be a competitive inhibitor in preparations from TAS and HS rats, and as a non-competitive inhibitor with HW rat liver preparations.

The effect of reduced dinucleotide on enzymic vitamin K_1 reduction was investigated using NADH (Fig. 3, ○—○) and NADPH (●—●) at concentrations of 0–1.7 mM. The optimum enzymic reduction rate was obtained using a 1 mM concentration of either cofactor with 33.3 μM radiolabelled vitamin K_1, but NADH was more effective as a cofactor than NADPH. The reaction rate with 1 mM NADH was 37.2 nmol/5 min/g wet wt liver compared with 15.1 nmol/5 min/g wet wt liver in the absence of added reduced dinucleotide.

The results of enzymic vitamin K_1 reduction in incubations at 37° with NADH (1 mM), DTT (5 mM) and radiolabelled vitamin K_1 concentrations of 20–100 μM with liver microsomes from TAS and HW rats are summarised in Table 1. These experiments were carried out at concentrations of 0, 10 and 50 μM warfarin. The best fit curves were calculated from all 5 points at 0 and 10 μM warfarin but the two values of $1/[S]$ equals 40 and 50 mM^{-1} were omitted for calculation of the curve at 50 μM warfarin for TAS rats and $1/[S]$ equals 50 mM^{-1} at 50 μM warfarin for HW rats. The regression coefficients r were greater than 0.95 for all curves.

Results (Table 1) were obtained from similar experiments using liver microsomes from HS rats at 0, 50 μM and 2 mM warfarin. The best fit curves were calculated from all five points both in the presence and absence of warfarin and $r \geq 0.95$. Experiments were also carried out at 50, 100, 200 and 400 μM warfarin and the results obtained were identical to those obtained in the absence of warfarin.

Three kinetic parameters were calculated from the intercepts and slope of each curve and are presented in Table 1. The maximal velocity (V_{max}) for vitamin K_1-reductase activity of TAS rat liver microsomes was similar both in the presence and absence of warfarin. The Michaelis constant (K_l), however, increased from 51.1 μM vitamin K_1 without warfarin to 84.2 and 98.1 μM at 10 and 50 μM warfarin, respectively. The results calculated for the warfarin-enzyme dissociation constant (K_i) for a competitive inhibitor indicate a value of less than 100 μM warfarin.

With liver microsomes from HW rats, V_{max} decreased from 121 nmol/5 min/g wet wt liver without warfarin to 92.5 nmol/5 min/g wet wt liver with 50 μM warfarin and the Michaelis constant remained at approximately the same value (96.3–99.3 μM vitamin K_1). The inhibitor–enzyme dissociation constants were therefore calculated for a non-competitive inhibitor and suggest a value of between 100 and 200 μm warfarin.

The results calculated for vitamin K-reductase of HS rat liver microsomes in the absence of warfarin indicate a relatively high V_{max} of 361 nmol/5 min/g wet wt liver and a Michaelis constant of 167 μM vitamin K_1. The warfarin concentration used for the inhibited curve was 2 mM and resulted in apparent increases in both K_m and V_{max}. The figure for K_i was calculated for a competitive inhibitor and indicated a value of over 3 mM warfarin.

The results shown in Table 2 are from experiments investigating the effect of 1 μM dicoumarol on vitamin K_1-reductase activity in liver microsomes from TAS and HS rats. These results indicate that at 33.3 μM vitamin K_1 and 1 mM NADH the inhibitory effect of 1 μM dicoumarol on the enzyme from TAS rats is approximately 10% whereas the enzyme from HS rat liver microsomes is uninhibited.

Table 2. Inhibition of vitamin K_1-reductase activity of rat liver microsomes by dicoumarol

Rat strain	Vitamin K_1-reductase activity (nmol vitamin K_1 reduced/5 min/g wet wt liver) No dicoumarol	1 μM dicoumarol
TAS	34.8	31.7
HS	60.5	60.5

The results presented are the average of duplicate analyses of vitamin K_1-reductase activity in rat liver microsomes in the presence and absence of 1.0 μM dicoumarol. The radiolabelled vitamin K_1 concentration was 33.3 μM and the incubations were carried out as described in the text.

DISCUSSION

This study was undertaken to investigate the possible connection between liver microsomal vitamin K-reductase and warfarin-resistance in rats, and the effects of inhibition of this enzyme in the anticoagulant activity of warfarin. However, it was necessary to optimise the assay conditions before any kinetic studies could be carried out. Preliminary studies on the effect of substrate concentration on the rate of vitamin K_1 reduction indicated that increased concentrations of Emulgen 913 increased the rate of reaction. This suggests a partial solubilisation of the membrane–protein complex of the microsomes and may be expected to cause an enhanced rate of reaction. All subsequent incubation mixtures contained 0.2% (w/v) Emulgen 913.

The non-enzymic reduction of vitamin K_1 observed in the absence of microsomal preparations was probably due to the reducing effects of 5 mM DTT. Indeed Fasco and Principe [14] reported a method for chemical synthesis of vitamin K_1 hydroquinone using 100 mM DTT that gave a yield of 95% reduced form. Results of experiments on the extraction of vitamin K_1 with organic solvents in the present study indicated that DTT was essential to ensure that vitamin K_1 hydroquinone was not oxidised in aqueous solutions. HPLC grade tetrahydrofuran does not contain stabilisers to prevent peroxide formation and it may be that these peroxides caused oxidation of reduced vitamin K_1, even in the presence of 5 mM DTT, when this solvent was used for extraction.

If microsomal DT-diaphorase is involved in vitamin K reduction as part of the vitamin K cycle then the vitamin K-reductase activity should, by definition [20], be stimulated equally by NADH and NADPH. We have observed (Fig. 3), however, that although there is vitamin K_1 reduction occurring in the absence of added reduced nucleotide, when NADH is present at a concentration of 1 mM the reaction rate is considerably enhanced and that NADPH is less effective than NADH. Furthermore, the enzyme activity we have investigated is not inhibited by 1 μM dicoumarol

(Table 2) and should not be classified as a DT-diaphorase [20]. The relatively large NADH concentration probably does not reflect the requirement of the vitamin K-reductase studied but rather the overall requirement of other NADH-dependent microsomal systems.

The results of kinetic studies (Table 1) were obtained using crude microsomal preparations, which were used because we are studying a membrane-bound system, and it would be unwise to attach any significance to the precise values obtained for the kinetic parameters of vitamin K-reductase. General trends can be observed, however, and there are apparent differences in the enzyme of liver microsomes from the three strains studied. The NADH-dependent enzyme present in the TAS liver microsomes has a relatively high affinity for vitamin K_1 and warfarin and inhibition by warfarin is competitive with respect to vitamin K_1. The vitamin K-reductase of HW rat liver microsomes has a lower affinity for both vitamin K_1 and warfarin and the inhibition is apparently non-competitive with respect to vitamin K_1. Studies on DTT-dependent liver-microsomal vitamin K-reductase [15] showed that the enzyme was inhibited by warfarin and that the enzyme of warfarin-susceptible Holtzman rats was inhibited to a greater extent than the enzyme of a resistant strain that was originally bred from wild Welsh rats (J. H. Greaves, personal communication). We have used a direct assay of vitamin K hydroquinone formation similar to that previously described [15] but have observed that the optimum incubation temperature is 32.5–37.5° and that 5 mM DTT causes non-enzymic vitamin K reduction (Figs. 1 and 2). Although there is vitamin K-reductase activity in the absence of added reduced dinucleotide, the enzyme activity we have investigated is clearly NADH-dependent (Fig. 3). It is possible that the warfarin-sensitive vitamin K reduction observed previously [15] and in the present study is catalysed by the same enzyme but using a different electron donor. We have also investigated NADH-dependent enzyme activity in a second warfarin-resistant strain (HS) that has a low affinity for vitamin K_1 and is uninhibited at concentrations of up to 400 μM warfarin (Table 1). The HS strain is less resistant to warfarin than the HW strain [8, 9] and is susceptible to the anticoagulant effect of warfarin when also receiving a supplementary dose of vitamin K_1, whereas the HW animals are not [10]. We cannot explain why the highly warfarin-resistant vitamin K-reductase activity in HS rat liver microsomes cannot utilise the administered vitamin K_1 to produce the hydroquinone form required as a cofactor for the carboxylation of blood coagulation factors. This suggests that although vitamin K-reductase is inhibited by warfarin, and warfarin-resistant animals have a less susceptible enzyme, the major anticoagulant effect of warfarin is not that observed on vitamin K-reductase and the effect of expression of the warfarin-resistance gene is on another enzyme or binding protein. This hypothesis may be correct for animals of the HW strain but the vitamin K-epoxide reductase of HS rats has not been investigated and may not be inhibited by warfarin. Thus the expression of the warfarin resistance gene in HS rats may result only in alterations in vitamin K-reductase activity. We are conducting similar studies to investigate the inhibitory effects of warfarin on vitamin K dependent γ-glutamyl carboxylase and vitamin K-epoxide reductase in TAS, HS and HW animals to determine the biochemical expression of warfarin resistance.

REFERENCES

1. C. M. Boyle, *Nature, Lond.* **188**, 517 (1960).
2. D. C. Drummond and E. W. Bentley, Report on International Conference on Rodents and Rodenticides, E.P.P.O., Paris, 1965, p. 57 (1967).
3. M. Lund, Report on the International Conference on Rodents and Rodenticides, E.P.P.O., Paris, 1965, p. 49 (1967).
4. A. J. Ophof and D. W. Langveld, *Schr. Reihe. Ver. Wass.-Boden-u. Lufthyg.* **32**, 39 (1969).
5. H. J. Telle, *Auz. Schaedlingsk. Pflanzenschutz.* **45**, 17 (1972).
6. W. B. Jackson and D. Kaukeinen, *Science (N.Y.)* **176**, 1343 (1972).
7. J. H. Greaves and P. B. Ayres, *Nature, Lond.* **215**, 877 (1967).
8. J. H. Greaves and P. B. Ayres, *Lab. Anim.* **7**, 141 (1973).
9. J. H. Greaves and P. B. Ayres, *Genet. Res. (Cambridge)* **28**, 231 (1976).
10. J. H. Greaves and P. B. Ayres, *Genet. Res. (Cambridge)* **40**, 59 (1982).
11. J. W. Suttie, *C.R.C. Crit. Rev. Biochem.* **8**, 191 (1980).
12. J. A. Sadowski, C. T. Esmon and J. W. Suttie, *J. biol. Chem.* **251**, 2770 (1976).
13. A. E. Larson, P. A. Friedman and J. W. Suttie, *J. biol. Chem.* **256**, 11032 (1981).
14. M. J. Fasco and L. M. Principe, *Biochem. biophys. Res. Commun.* **97**, 1487 (1980).
15. M. J. Fasco, E. F. Hildebrandt and J. W. Suttie, *J. biol. Chem.* **257**, 11210 (1980).
16. R. G. Bell and P. T. Caldwell, *Biochemistry* **12**, 1759 (1973).
17. J. T. Maschiner, A. Zimmerman and R. G. Bell, *Thromb. Diath. Haem. Suppl.* **57**, 45 (1974).
18. M. G. Townsend, E. M. Odam and A. K. Nadian, in *Vitamin K metabolism and Vitamin K-dependent proteins* (Ed. J. W. Suttie) p. 361. University Park Press, Baltimore, MD (1979).
19. A. D. Martin, L. C. Steed, R. Redfern, J. E. Gill and L. W. Huson, *Lab. Anim.* **13**, 209 (1979).
20. L. Ernster, M. Ljunggren and L. Danielson, *Biochem. biophys. Res. Commun.* **2**, 88 (1960).
21. M. J. Fasco and L. M. Principe, *Biochem. biophys. Res. Commun.* **104**, 187 (1982).
22. C. Lind, B. Rase, L. Ernster, M. G. Townsend and A. D. Martin, *FEBS Lett.* **37**, 147 (1973).
23. R. Wallin, O. Gebhardt and H. Prydz, *Biochem. J.* **169**, 95 (1978).