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VITAMIN K_1 2,3-EPOXIDE AND QUINONE REDUCTION: MECHANISM AND INHIBITION

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The chemical and enzymatic pathways of vitamin K_1 epoxide and quinone reduction have been investigated. The reduction of the epoxide by thiols is known to involve a thiol-adduct and a hydroxy vitamin K enolate intermediate which eliminates water to yield the quinone. Sodium borohydride treatment resulted in carbonyl reduction generating relatively stable compounds that did not proceed to quinone in the presence of base. NAD(P)H:quinone oxidoreductase (DT-diaphorase, E.C. 1.6.99.2) reduction of vitamin K to the hydroquinone was a significant process in intact microsomes, but 1/5th the rate of the dithiothreitol (DTT)-dependent reduction. No evidence was found for DT-diaphorase catalyzed reduction of vitamin K_1 epoxide, nor was it capable of mediating transfer of electrons from NADH to the microsomal epoxide reducing enzyme. Purified diaphorase reduced detergent- solubilized vitamin K_1 10^{-5} as rapidly as it reduced dichlorophenylindophenol (DCPIP). Reduction of 10 μM vitamin K_1 by 200 μM NADH was not inhibited by 10 μM dicoumarol, whereas DCPIP reduction was fully inhibited. In contrast to vitamin K_3 (menadione), vitamin K_1 (phylloquinone) did not stimulate microsomal NADPH consumption in the presence or absence of dicoumarol. DTT-dependent vitamin K epoxide reduction and vitamin K reduction were shown to be mutually inhibitory reactions, suggesting that both occur at the same enzymatic site. On this basis, a mechanism for reduction of the quinone by thiols is proposed. Both the DTT-dependent reduction of vitamin K_1 epoxide and quinone, and the reduction of DCPIP by purified DT-diaphorase were inhibited by dicoumarol, warfarin, lapachol, and sulphaquinoxaline.

KEY WORDS: Vitamin K epoxide, vitamin K reductions, NAD(P)H:quinone oxidoreductase, DT-diaphorase, vitamin K epoxide-diol, vitamin K epoxide-alcohol.

INTRODUCTION

Vitamin K-dependent carboxylase catalyzes the post-translational formation of gamma-carboxyglutamyl residues in the blood coagulation factors II, VII, IX, and X, proteins C, S, and Z, and several other proteins.[1] The reaction occurs in the rough endoplasmic reticulum and requires vitamin K_1 hydroquinone (MH_2), molecular oxygen, carbon dioxide, and a peptide-bound glutamyl substrate. During the reaction, the vitamin is converted to vitamin K 2,3-epoxide (KO). The epoxide can be reduced by a coumarin anticoagulant sensitive microsomal vitamin K epoxide reductase which is assayed using dithiothreitol (DTT) as the reductant *in vitro*.[2] Recent work has shown that thioredoxin, plus thioredoxin reductase, can act as a reductant for the reaction and this system may well represent the physiologically-relevant reductant.[3,4] The quinone can be reduced by either pyridine nucleotide-linked dehydrogenases, including DT-diaphorase, or a disulphydryl-dependent vitamin K reductase[5,6] which appears to be similar to the vitamin K epoxide reductase.

The existence of the microsomal DTT-dependent quinone reduction pathway was

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initially inferred from the observation of anticoagulant-inhibitable (DTT + vitamin-K)-dependent carboxylation.[7] The reaction was subsequently detected by direct HPLC assay for the hydroquinone.[5] Early work on DT-diaphorase (also called vitamin K reductase) while demonstrating NAD(P)H oxidation in the presence of vitamin K_3 showed only a slow rate of reaction with vitamin K_1.[8,9] Indirect evidence for vitamin K_1 reduction was obtained by showing that purified DT-diaphorase could reconstitute (NADH + vitamin K_1)-dependent carboxylation in diaphorase-depleted microsomes.[10] Indirect evidence was also obtained by observation of the coupled reduction of $Fe(CN)_6^{3-}$ entrapped in liposomes containing vitamin K_1 in the membranes and DT-diaphorase, plus NADH on the outside.[11] Direct evidence of DT-diaphorase catalyzed vitamin K_1 hydroquinone formation was eventually obtained by HPLC analysis.[12] The relative importance of the DTT-dependent and NADH-dependent quinone reduction pathways under various therapeutic conditions and in various tissues are continuing subjects of debate.[13-15]

Recently, it has been shown that DT-diaphorase can reduce benzoquinone epoxide to 2-hydroxybenzohydroquinone and suggested that DT-diaphorase also can reduce dimethylnaphthoquinone epoxide.[16]

In this paper, the chemically-feasible pathways of vitamin K epoxide reduction and the potential for DT-diaphorase to catalyze vitamin K epoxide and quinone reduction are explored. The relationship and possible mechanisms of the DTT-dependent vitamin K epoxide and quinone reduction reactions are examined. The mechanism of a number of inhibitors of both DT-diaphorase and vitamin K epoxide reductase is discussed.

MATERIALS AND METHODS

Microsomes and cytosol in 0.25 M sucrose, 0.01 M *Tris*-HCl, 1 mM EDTA buffer, pH 7.6, and solubilized microsomes in 0.25 M sucrose, 0.01 M sodium AMPSO, 0.8% sodium cholate buffer, pH 8.8, were prepared as previously described.[17] DT-diaphorase was purified from the cytosol by chromatography on azodicoumarol-Sepharose[18] and Sephadex G-100. Activity was assayed spectrophotometrically[8] at 23°C in 1 ml of 0.05 M *Tris*-HCl buffer, pH 7.4, containing 8.5 mg/ml Tween-20 15 μM DCPIP, and 200 μM NADH. The purified enzyme had a specific activity of 0.66 μmol/min/μg-protein, and a flavin content of 25 pmol/μg-protein assuming an extinction coefficient of 11.3 mM^{-1}.

Vitamin K_1 epoxide and quinone reduction were assayed by HPLC analysis on an Alltech Econosil 10 μ C-18 column (4.2 × 250 mm) using either 2 ml/min of 100% methanol (retention times (min): KH_2 = 3.00, KO = 7.05, and K = 11.2) or a gradient method. Initial conditions (2 mL/min of 9% H_2O/25% isopropanol/66% acetonitrile) were held for one minute, then the solvent was linearly changed to 25% isopropanol/75% acetonitrile over 4 minutes and the final condition was held for 10 minutes (retention times (min): KH_2 = 8.0, KO = 9.8, K = 12.6). Peaks were detected by absorbance at 254 nm. Incubations typically contained 1.0 ml of microsomes, vitamin K epoxide or quinone added in 1/100th vol of 1% Emulgen 911, and DTT added in 1/40th vol of 2.5 M KCl. Reactions were stopped by extraction with 2 vol of isopropanol/hexane (1:1). The hexane phase was evaporated, redissolved in 100 μL of mechanol, and 50 μL was injected for HPLC analysis.

Vitamin K_1 and menaquinone-4 epoxide, quinone, and hydroquinone substrates

and standards were prepared as previously described.[17] All other materials were obtained from Fisher, Sigma, or Aldrich Chemical Companies.

Reductions with Sodium Borohydride

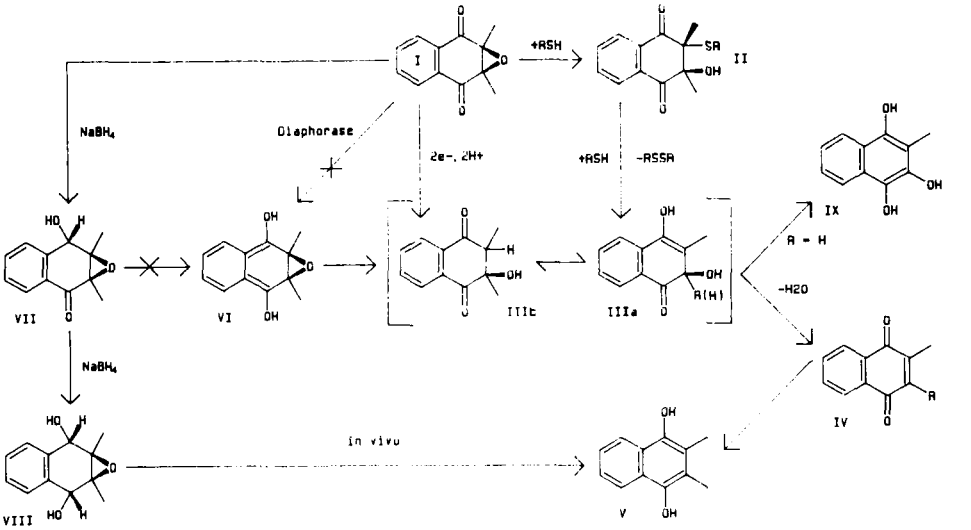
Synthesis of vitamin K epoxide-diol[19] Vitamin K_1 epoxide (25 μmol) was treated with 2 mmol of $NaBH_4$ in 1 ml of ethanol resulting in the immediate disappearance of the epoxide and appearance of several new early eluting low intensity peaks on analytical HPLC in 100% methanol. All were clearly distinct from the hydroxyvitamin K intermediate in thiol reduction of the epoxide (rt = 4.2 min). Minimal further changes were observed with up to 4 hrs reaction. The principal material eluting at 3.4 min was isolated by hexane extraction of the acidified reaction mixture and semi-preparative chromatography on an Alltech Econosil 10 μ C-18 column (10 × 250 mm) using 4 mL/min of 97.5% methanol/2.5% water. The product had a UV spectrum similar to the literature spectrum[19] and clearly distinguished from that of any other vitamin K derivatives. UV (hexane) λ nm (log ε): 238 min (1.81); 254 sh (2.27); 260 max (2.36); 264 min (2.21); 266 max (2.24). The ^{1}H-NMR spectrum (300 MHz), $CDCl_3$): δppm 7.6 (*m* 2H); 7.3 (*m*, 2H); 5.1 (*m*, 1H); 4.76 (*d*, J = 10.3 Hz, 1H); 4.65 (*d*, J = 10.6 Hz, 1H); 3.31 (*dd*, J_1 = 14.5, J_2 = 9.3 Hz, 1H); 2.18 (*s*, 2H); 2.1 (*dd*, obscured); 1.97 (*t*, J = 7.8 Hz, 2H); 1.73 (*s*, 3H); 1.68 (*s*, 3H); 0.9–1.6 (19H); 0.85, 0.83, 0.81, 0.79 (*s*, 3H); is consistent with the structure of 1,2,3,4-tetrahydro-vitamin K_1 hydroquinone 2,3-epoxide (vitamin K epoxide-diol). A ^{13}C-NMR attached proton test showed singlet peaks at 70.269 and 67.047 ppm corresponding to the 1- and 4-benzylic alcohol carbons.

Synthesis of vitamin K epoxide-alcohol Vitamin K_1 epoxide (20 μmol) was reacted with 5 μmol of $NaBH_4$ in 1 ml of ethanol resulting in the immediate disappearance of the epoxide and the appearance of single peak at 4.02 min on analytical HPLC in 100% methanol. The product obtained after semi-preparative HPLC had a UV spectrum completely different from either the epoxide, hydroxy vitamin K, or the epoxide-diol. UV (hexane) λ nm (log ε): 225 min (3.53); 244 max (3.98); 274 min (2.90); 287 max, sh298 (2.98); 315 min (1.60); 333 max (1.78). The ^{1}H-NMR spectrum (300 MHz, $CDCl_3$) was consistent with a 4:1 mixture of carbonyl reduction products: δppm (major isomer) 7.33–7.87 (*m*, 4H); 5.04 (*t*, J = 6.3 Hz, 1H); 4.78 (*d*, J = 11.5 Hz, 1H); 3.09 (*dd*, J_1 = 6.7 Hz, J_2 = 10.9 Hz, 1H); 2.32 (*dd*, J_1 = 7.0 Hz, J_2 = 14.9 Hz, 1H); 2.1 (*s*, 1H); 1.89 (*t*, J = 7.7 Hz, 2H); 1.70 (*s*, 3H); 0.86–1.51 (19H); 0.74–0.8 (12H); (minor isomer) 4.85 (*d*, J = 11.54, 1H).

RESULTS AND DISCUSSION

Chemical Pathways of Vitamin K Epoxide Reduction

Scheme 1 illustrates several potential pathways of vitamin K epoxide reduction. The reaction of the epoxide (I) with thiols has previously been shown to involve a thiol adduct intermediate (II) and a hydroxyvitamin K enol intermediate (IIIa) which can eliminate water upon treatment with base to yield the quinone (IV).[20,21,22] The keto tautomer of this intermediate (IIIb) can be isolated as a by-product of the DTT-dependent reduction of the epoxide by warfarin-resistant rat liver microsomes, suggesting this is also an intermediate in the enzymatic reaction mechanism.[23] This same



SCHEME 1 Potential pathways of vitamin K epoxide reduction. The pathway shown for diaphorase reduction occurs for benzoquinone epoxides, but is shown here to be negligible for vitamin K epoxide.

2,3-dihydro-3-hydroxy-vitamin K derivative could also be obtained by direct reduction of the oxirane ring, however, a reaction following this pathway has not been demonstrated. The quinone undergoes further reaction with thiols to generate the hydroquinone (V) under conditions similar to those for epoxide reduction.[5,22,23]

In an attempt to synthesize standards for the expected initial products of DT-diaphorase catalyzed reduction of the epoxide, the reaction of the epoxide with sodium borohydride was investigated. The reaction of 20 mM vitamin K_1 epoxide with 5 mM $NaBH_4$ in ethanol is virtually instantaneous and complete. The product of the reaction was stable to air reoxidation and treatment with the base triethylamine for over 24 hours. The NMR spectrum of the product was clearly inconsistent with the structure of the proposed 1,6-reduction product (VI), but would be consistent with a mixture of the products of 1,2-carbonyl reduction (VII). The base stability of this product suggests that it could not be an intermediate in the reported DT-diaphorase catalyzed reduction of epoxides. In the presence of excess $NaBH_4$ rapid reduction of the second carbonyl group occurs to yield vitamin K epoxide diol (VIII). The suggested, but only partially supported, structure of this previously reported material[19] was confirmed by ^{1}H-NMR spectroscopy and a ^{13}C-NMR attached proton test. This material was also found to be base stable, suggesting it cannot undergo isomerization to open the oxirane ring and eliminate water to yield the hydroquinone (V) directly. It was, however, previously shown to be a weakly active form of the vitamin *in vivo*,[19] suggesting that some pathway for its conversion to the hydroquinone must occur, perhaps involving its reoxidation to the epoxide.

The reported reduction of benzoquinone epoxide by DT-diaphorase presumably must involve an initial 1,6-reduction reaction to generate an intermediate similar to VI, but lacking the fused benzenoid ring. This reaction may be more facile for benzoquinone than for naphthoquinone epoxides since reduction of the former would not result in loss of aromaticity. Isomerization of the structure would open the

oxirane ring to form an intermediate similar to III. Formation of the reported product, a hydroxy-substituted hydroquinone (IX), would result from enolization driven by the aromaticity gained in the hydroquinone. For dialkyl-substituted quinone epoxides, such as vitamin K_1 epoxide, no such enolization is possible, and only quinone product should be observed.

DT-Diaphorase Reduction of Vitamin K_1 but not Vitamin K_1 Epoxide

Table 1 shows the results obtained by HPLC analysis for the direct assay of DT-diaphorase catalyzed vitamin K_1 reduction in comparison with the rate for spectrophotometric assay of DCPIP reduction. Conditions known to stimulate the DT-diaphorase reduction of other quinones were tested in an attempt to optimize the rate of reaction. Whereas BSA and TWEEN-20 are well known stimulators of DCPIP and vitamin K_3 (menadione) reduction,[8] they had a minimal effect or actually inhibited vitamin K_1 (phylloquinone) reduction. Vitamin K_1 reduction was five orders of magnitude slower than DCPIP reduction under the conditions tested. No vitamin K_1 epoxide reduction was detected under any of the conditions tested and using two different HPLC analysis methods.

Dicoumarol is the classic inhibitor of DT-diaphorase[8] and is significantly more potent than the related 4-hydroxycoumarin, warfarin. Surprisingly, although these compounds inhibited DT-diaphorase reduction of DCPIP, they had no effect on reduction of vitamin K_1. This may be reasonable in view of the action of these compounds as competitive inhibitors versus NADH binding to the oxidized form of the enzyme.[24] The low activity of vitamin K_1 may result in very low levels of the oxidized enzyme being present in the steady state.

Table 2 presents the results for vitamin K_1 epoxide and quinone reduction after incorporation into microsomal membranes and in the presence of DTT, NADH, or NADH + DT-diaphorase as reductants. The bulk of the NADH-dependent quinone reduction can be inhibited by high (100 μM) dicoumarol. It is clear that DT-diaphorase can catalyze quinone reduction under these conditions. However, it is not

TABLE 1
Activity of DT-diaphorase with various substrates, inhibitors, and stimulators (μmol/min/mg)[a]

Assay conditions	Substrate		
	40 μM DCPIP	10 μM K_1	10 μM K_1O
Buffer	198	0.0030	ND[b]
+ 1 mg/mL BSA	286	0.0021	ND
+ 8.5 mg/mL Tween-20	327	0.0010	ND
+ 10 μM Warfarin	106	0.0030	-
+ 10 μM Dicoumarol	1	0.0030	-

[a] Assays were at 23°C and contained buffer (0.2 M Tris-HCl, 0.15 M KCl, pH 7.4), 200 μM NADH, and DCPIP added in buffer, or vitamin K_1 epoxide or quinone added in 1/100th vol of 1% Emulgen 911. Incubations with DCPIP contained 17 ng of purified diaphorase in a 1 m total volume and were continuously monitored by spectrophotometry. Incubations with the vitamins contained 8.65 μg of purified diaphorase in 0.5 ml and were for 10 minutes.

[b] ND — Not detectable. No new peaks observed (< 0.0005 AU peak height). For comparison, the incubations with buffer product KH_2 peaks of 0.004 AU peak height. In another test, vitamin K epoxide was incubated with 0.173 mg/ml purified DT-diaphorase for 7 hrs, sufficient to reduce 0.024 mol of DCPIP or 109 nmol of vitamin. No new peaks were detected even when the reaction mixture was directly injected for HPLC analysis without extraction.

TABLE 2
Effect of DT-diaphorase on microsomal vitamin K epoxide and quinone reduction (nmol/min/g-liver).[a]

Incubation	Vitamin K_1	Vitamin K_1O
Microsomes + DTT	0.50	1.0
Microsomes + NADH	0.09	ND[b]
+ Diaphorase	0.11	ND

[a] Assays contained microsomes equivalent to 0.25 g-liver/ml, 2 mM DTT or 200 μM NADH, and vitamin added in 1/100th vol of 1% Emulgen 911 and were incubated at 23°C for 10 min. Diaphorase when added was purified enzyme to yield a final concentration equivalent to 10 g-liver/ml.
[b] ND – Not detected.

capable of reducing the epoxide nor of providing reducing equivalents to the membrane bound DTT-dependent epoxide reductase. Cytosolic DT-diaphorase, as opposed to membrane associated enzyme, may not normally contribute significantly to vitamin K_1 reduction *in vivo* since addition of a very large excess of purified enzyme stimulated reduction only minimally. The DTT-dependent reduction of vitamin K_1 is significantly greater than the NADH-dependent reaction and the thiol-dependent reaction probably represents the major reduction pathway *in vivo* under normal conditions. The NADH-dependent reduction may be important during treatment of anticoagulant overdose with high levels of vitamin K as has been discussed by other workers.[13,14]

Vitamin K_3 (menadione) is known to stimulate microsomal oxidation of NADPH via both 2-electron reduction and 1-electron reduction/semiquinone reoxidation pathways. The latter pathway is stimulated upon dicoumarol inhibition of the DT-diaphorase catalyzed 2-electron reduction.[25] Addition of 10 μM vitamin K_3 to microsomes equivalent to 5 mg-liver/mL and 200 μM NADPH in 0.25 M sucrose, 0.025 M Tris-HCl, 0.1% Triton X-100 buffer, pH 7.4, containing 10 μM dicoumarol, resulted in a greater than stoichiometric decrease in NADPH absorbance at 340 nm with an initial rate of 0.025 AU/min. No detectable decrease in absorbance (< 0.002 AU/min) occurred for addition of 10 μM vitamin K_1 in the presence or absence of dicoumarol. These results suggest that vitamin K_1 is a poor substrate for redox cycling via NADPH:cytochrome P450 reductase, as well as a poor substrate for microsomal DT-diaphorase, in comparison with vitamin K_3.

DTT-Dependent Microsomal Vitamin K Epoxide and Quinone Reduction

Figure 1 shows the progress curve for the reduction of vitamin K_1 epoxide at a high concentration of microsomes in the presence of DTT. Vitamin K formation reaches a steady state after several minutes whereas vitamin K hydroquinone formation only becomes significant after accumulation of the quinone. In contrast, the formation of vitamin K hydroquinone from exogenously added quinone is linear over the same time interval (data not shown). These results are consistent with a sequential two step reduction pathway in which the quinone is an intermediate in hydroquinone formation from the epoxide. The total extent of epoxide reduction is given by the sum of the quinone plus hydroquinone concentrations and the total amount of reductive activity by the quinone plus twice the hydroquinone concentration.

Figure 2 shows the extent of quinone and hydroquinone formation at long incubation times as a function of vitamin K epoxide concentration whereas Figure 3 shows the extent of hydroquinone formation from vitamin K. The inset in Figure 3 compares

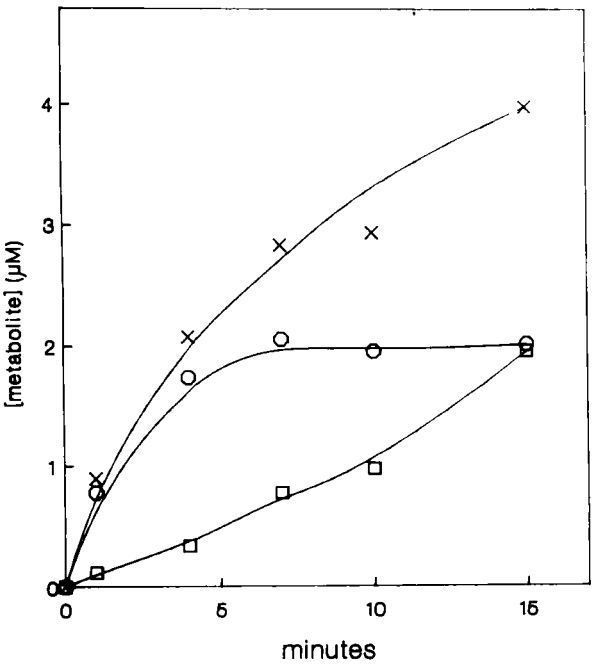


FIGURE 1 Time course for reduction of 10 μM vitamin K_1 epoxide by whole rat liver microsomes equivalent to 0.5 g-liver/ml at 23°C in the presence of 2 mM DTT. Concentrations (μM) of vitamin K formed (circles), vitamin K hydroquinone formed (squares), and vitamin K quinone + hydroquinone = epoxide reduced (crosses).

the extent of hydroquinone formation from exogenously added quinone with that for quinone generated *in situ* from the epoxide. For this plot, the quinone concentration present at the end of the incubation was used. At low epoxide concentrations, the quinone generated *in situ* is a more effective substrate than exogenously added quinone. This phenomenon most likely reflects diffusion limited channeling of the intermediate. At high epoxide concentration, a decrease in the extent of hydroquinone formation is observed, despite the continuing increase in the steady state concentration of quinone substrate. This result suggests possible inhibition of quinone reduction by the epoxide.

At shorter incubation times, secondary reduction of the quinone formed from the epoxide may be neglected and valid initial velocity data for epoxide reduction can be obtained in the absence of the competing reaction. Under these conditions, the epoxide and the quinone are fairly comparable substrates for microsomal reduction (K_M = 14 μM K_1O, V_{MAX} = 1.2 nmol/min/g-liver; K_M = 8 μM K_1, V_{MAX} = 0.5 nmol/min/g-liver).

Several lines of evidence suggest that both the epoxide reduction and the quinone reduction occur at the same enzymatic site.[26] We have previously reported the use of menaquinone-4 epoxide and quinone (geranylgeranyl-side chains) as competing substrates versus vitamin K_1 epoxide (phytyl-side chain) to demonstrate quinone inhibition of epoxide reduction.[27] In the following experiments, both the quinone and

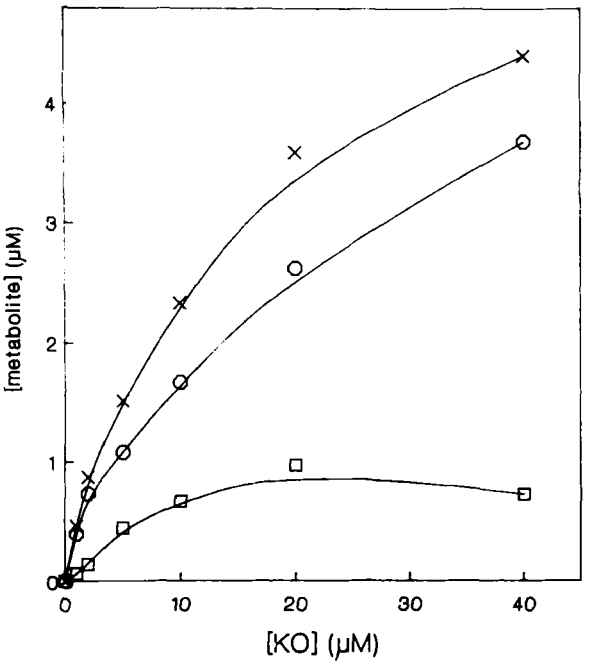


FIGURE 2 Metabolite profile after 10 minute incubations as a function of initial vitamin K epoxide concentration (μM). Conditions and symbols are as for Figure 1.

hydroquinone products derived from each substrate have been quantitated by reverse phase HPLC. Due to potential nonenzymatic interconversion of the quinones and hydroquinones ($KH_2 + MK \leftrightarrow K + MKH_2$), it is necessary to assume that total hydroquinone ($KH_2 + MKH_2$) formation may represent the true extent of exogenous quinone reduction. The data in Table 3 show that both quinone inhibition of epoxide reduction and epoxide inhibition of quinone reduction can be observed. Figure 4 shows the effect of increasing concentrations of menaquinone-4 on the extent of vitamin K_1 epoxide reduction. Figure 5 shows the effect of increasing concentrations of menaquinone-4 epoxide on vitamin K_1 reduction.

Initial velocity patterns for vitamin K_1 epoxide reduction by DTT are parallel, consistent with a Ping-Pong kinetic mechanism.[17,27] Product inhibition patterns for dihydroxydithiane (oxidized DTT) are competitive versus the epoxide, and noncompetitive versus DTT under appropriate conditions, consistent with a single site, rather than multisite Ping- Pong mechanism.[27] Sulphhydryl modification with NEM indicates the presence of a reducible active site disulphide which may be protected from modification by either the epoxide or quinone substrate.[28] Both the epoxide reduction and the quinone reduction are inhibited by coumarin anticoagulants, and several other classes of inhibitor, and both reactions are less sensitive to these inhibitors when assayed in microsomes derived from warfarin-resistant strain rats.[29-31] The quinone inhibition of epoxide reduction was previously shown to be competitive versus the epoxide and linear non-competitive versus DTT, consistent with alternate substrate inhibition and indicating the lack of product inhibition by the quinone.[27]

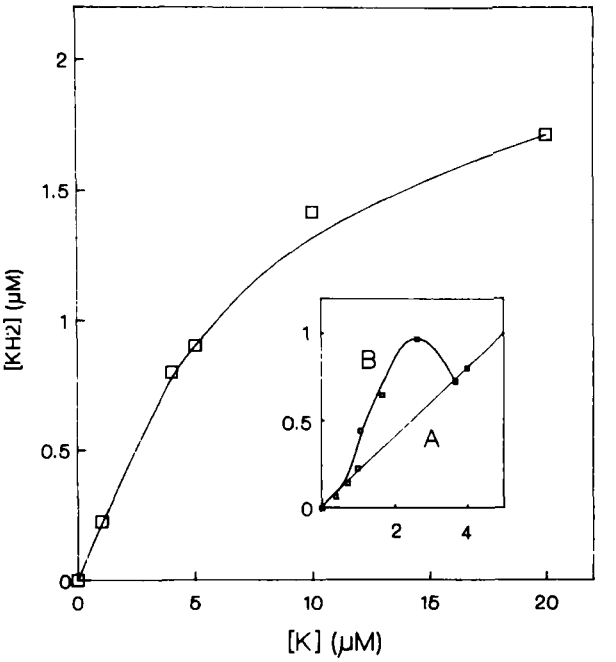


FIGURE 3 Vitamin K hydroquinone concentration (μM) after 10 minute incubations as a function of initial exogenously added vitamin K concentration (μM). Inset: A) Expansion of low range data. B) Hydroquinone formed from the epoxide plotted versus the concentration of vitamin K present at the end of the incubation, based on data from Figure 2.

TABLE 3
Microsomal reduction of epoxide and quinone substrates: competition[a]

	nmol product formed				nmol (% control)	
Substrates	K	KH_2	MK	MKH_2	Epoxide reduction[b]	Quinone reduction[c]
KO	1.49 ± 0.06	0.61 ± 0.11	–	–	2.11 (100) ± 0.16	–
K	–	1.02 ± 0.10	–	–	–	1.02 (100) ± 0.10
MKO	–	–	3.71 ± 0.06	0.35 ± 0.08	4.05 (100)′ ± 0.13	–
MK	–	–	–	1.35 ± 0.21	–	1.35 (100)′ ± 0.21
KO + MK	0.43 ± 0.07	ND	–	1.01 ± 0.12	0.43 (19)	1.01 (75)′ ± 0.12
MKO + K	–	0.30 ± 0.07	1.41 ± 0.07	0.25 ± 0.06	1.67 (41)′ ± 0.12	0.55 (54) ± 0.03

[a] Reaction mixtures containing 1 ml whole microsomes equivalent to 0.13 g-liver/ml and 2 mM DTT were incubated for 5 min at 23°C. Vitamins (10 μM each) were added as premixed micelles in 1/100th vol of 1% Emulgen 911. Data are the average for duplicate incubations.
[b] ($K + KH_2$) or ($MK + MKH_2$).
[c] ($KH_2 + MKH_2$).

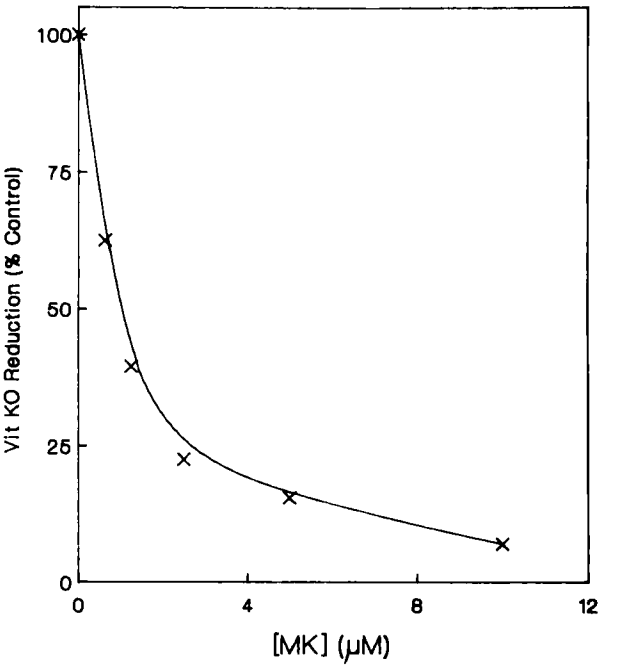


FIGURE 4 Inhibition of 10 μM vitamin K_1 epoxide reduction by menaquinone-4.

These results, together with the demonstration of epoxide inhibition of quinone reduction, are consistent with reduction of both the epoxide and the quinone by a single enzyme via a Ping-Pong mechanism involving oxidized and reduced forms of an active site disulphide. Release of the quinone in this model is irreversible, assuring that the overall two step reduction sequence can occur. Scheme 2 shows a proposed model for vitamin K epoxide reduction by vitamin K epoxide reductase based on the chemical model studies and observation of hydroxy vitamin K formation by the warfarin-resistant rat strain enzyme. Scheme 3 shows a potential model for vitamin K reduction at the same active site utilizing the same catalytic groups.

Inhibitors of Vitamin K Epoxide Reductase and DT-Diaphorase

Table 4 compares the sensitivity of vitamin K epoxide reductase and DT-diaphorase to a number of inhibitors. In warfarin-resistant-strain rats, the epoxide reductase is decreased, but that of the diaphorase is not, distinguishing the epoxide reductase as the pharmacologically important site of coumarin anticoagulant action.[32] The diaphorase is uniquely sensitive to dicoumarol, whereas the sensitivity of the epoxide reductase is similar for various derivatives having large hydrophobic side chains (data not shown). Inhibition of DT-diaphorase by dicoumarol and warfarin is known to be competitive versus NADH, K_i = 10 nM dicoumarol, K_i = 23 μM warfarin in the presence of TWEEN-20.[24]

It has been difficult to demonstrate reversibility of coumarin anticoagulant inhibition of the epoxide reductase, and until recently kinetic studies of the inhibition did

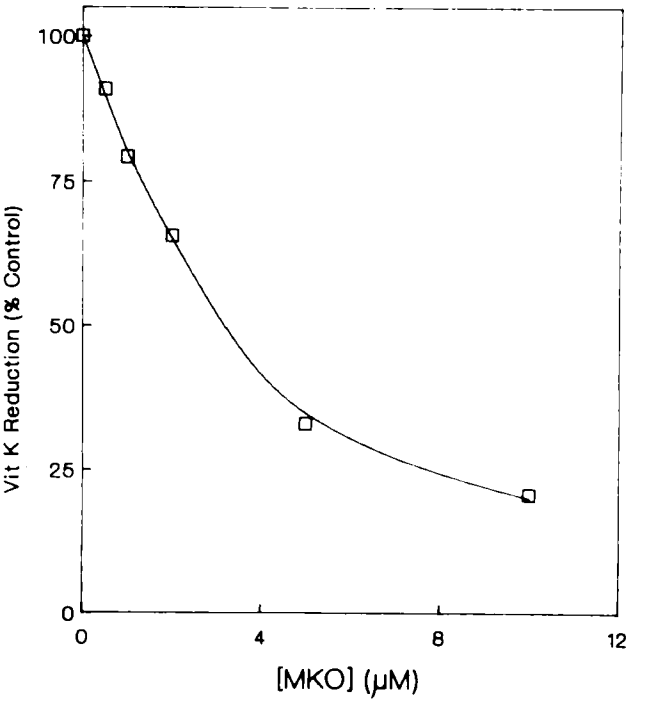
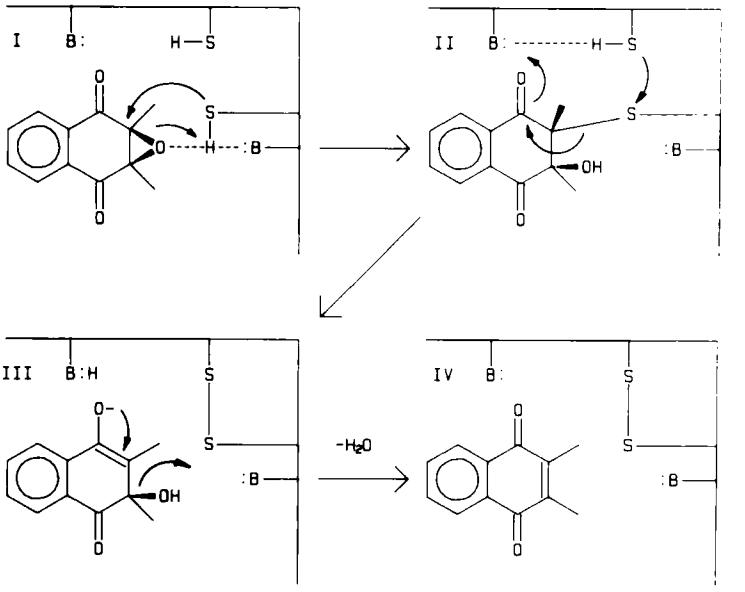
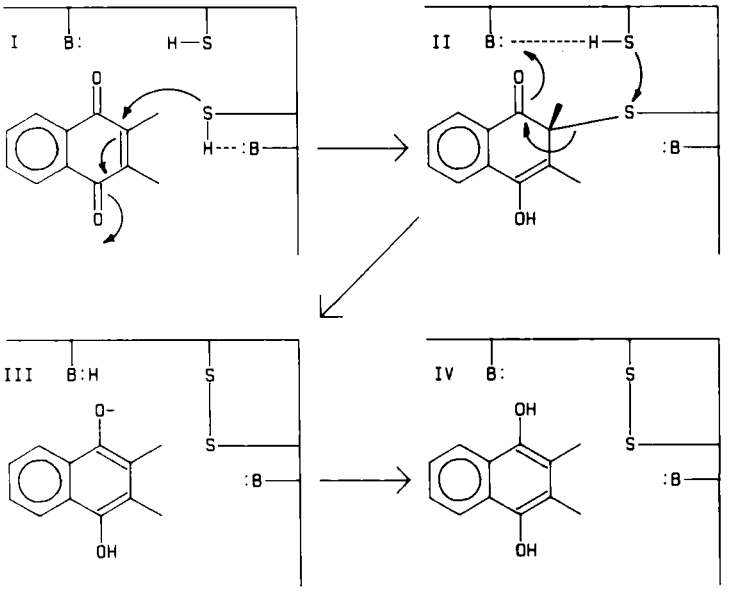


FIGURE 5 Inhibition of reduction of 10 μM vitamin K_1 by menaquinone-4-epoxide.



SCHEME 2 Proposed mechanism of enzymatic vitamin K epoxide reduction.



SCHEME 3 Postulated mechanism for reduction of vitamin K utilizing the same active site residues as proposed for vitamin K epoxide reduction.

not seem appropriate. Using a solubilized enzyme preparation,[17] it has been possible to show that inhibition by 3-ethyl-4-hydroxycoumarin can be reversed by Sephadex G-25 chromatography. Warfarin inhibition was only partially reversible, in the absence of DTT, but almost fully reversible by addition of DTT prior to chromatography (data not shown). The kinetics of 3-ethyl-4-hydroxycoumarin inhibition were found to be approximately competitive versus DTT (K_i = 5.7 μM) and uncompetitive versus the epoxide (data not shown). These results are consistent with binding of 4-hydroxycoumarins to the oxidized form of the enzyme as has previously been suggested.[33] Similar trends were found for warfarin inhibition of the epoxide reductase, but simple linear inhibitions were not observed. Inhibition of the warfarin-resistant enzyme by high concentrations of warfarin was freely reversible and remained approximately competitive versus DTT, but with a much higher K_i. Lapachol (K_i = 0.18 μM)[30] and sulphaquinoxaline 9K_i = 1 μM)[31] inhibitions of the epoxide

TABLE 4
Inhibition of rat liver microsomal vitamin K epoxide reductase and cytosolic DT-diaphorase. I_{50} (μM).[a]

	Epoxide reductase	Diaphorase
Dicoumarol	2	0.05
Warfarin	2	20
Lapachol	2	0.3
Sulfaquinoxaline	8	10
Ticrynafen	5000	3

[a]Vitamin K epoxide reductase activity was assayed using microsomes equivalent to 0.1 g-tissue/ml, 10 μM KO, and 1 mM DTT incubated for 10 min at 23°C. DT-diaphorase was assayed at 23°C using 15 μM DCPIP and 50 μM NADH in the presence of 8.5 mg/mL tween-20.

reductase are similar in their behavior to 4-hydroxycoumarin anticoagulant inhibition.

We have previously suggested that these compounds act as analogues of the proposed hydroxy vitamin K enolate intermediate (III) bound to the oxidized form of the enzyme shown in Scheme 2.[23,34] A lower affinity for the enolate, as well as for these inhibitors, would account for the formation of hydroxy vitamin K (Scheme 1, IIIb) by the warfarin-resistant rat enzyme.

That these compounds can assume similar electronic and steric structures is emphasized by the observation that they are all also inhibitors of DT-diaphorase. Lapachol was previously shown to be competitive versus NADH, $K_i = 0.15\,\mu M$.[35] This observation is of particular note given the recent report that DT-diaphorase can reduce quinone epoxides to hydroxy-substituted hydroquinones.[16] We have subsequently shown that sulphaquinoxaline is also an inhibitor of DT-diaphorase, competitive versus NADH, $K_i = 6\,\mu M$ (submitted). Whether all three classes of compound bind to the same site on the oxidized form of DT-diaphorase in the same relative orientations as suggested for inhibition of the epoxide reductase remains to be determined.

Ticrynafen is unique in that it inhibits DT-diaphorase, but not vitamin K epoxide reductase. Inhibition of the diaphorase is competitive versus NADH, $K_i = 1.3\,\mu M$, and mutually exclusive with warfarin.[36] The orientation and structural similarity of this compound to the other inhibitors is not clear, and its local interaction with the enzyme may be different. This inhibitor has been used to demonstrate that DT-diaphorase has no role in microsomal vitamin K epoxide reduction using cytosol as the reductant.[37] Thus far no specific inhibitors are known which inhibit vitamin K epoxide reductase, but not DT-diaphorase.

Clearly the inhibitor binding sites of vitamin K epoxide reductase and DT-diaphorase share some common features and it is likely that additional mutual inhibitors will be identified.

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