# Mechanism of Coumarin Action: Significance of Vitamin K Epoxide Reductase Inhibition†

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ABSTRACT: Vitamin K functions in a microsomal carboxylation reaction that converts glutamyl residues in precursor proteins to γ-carboxyglutamyl residues in the products of this reaction. The same liver microsomal preparations that carry out this carboxylation also convert the vitamin to its 2,3-epoxide (epoxidase activity) and reduce the epoxide to the vitamin (epoxide reductase activity). The effect of the coumarin anticoagulant Warfarin on these reactions has been studied. The vitamin K dependent carboxylase activity in intact microsomes is dependent on either NADH or dithiothreitol as a source of reducing equivalents to form the biologically active reduced form of the vitamin. The dithiothreitol dependent reaction is inhibited by Warfarin, but the NADH dependent reaction is not. When microsomes are solubilized in detergent, dithiothreitol is no longer an effective source of reducing equivalents, and Warfarin inhibition of the carboxylase activity is lost. The vitamin K epoxide reductase will use dithiothreitol, but not NADH as a reductant, and this reaction is strongly inhibited by Warfarin. The vitamin K dependent carboxylase system will utilize vitamin K epoxide as an active form of vitamin K only if dithiothreitol is used as the reducing agent, and, under these conditions, the reaction is inhibited by Warfarin. When microsomes were prepared from a strain of rats resistant to the anticoagulant effect of Warfarin, all three of these dithiothreitol dependent activities—vitamin K dependent carboxylase, vitamin K epoxide dependent carboxylase, and vitamin K epoxide reductase—were relatively insensitive to the inhibitory effect of Warfarin. However, a second coumarin, Difenacoum, which has been shown to be an effective anticoagulant in this strain of rats was an effective inhibitor of these in vitro reactions. These data support the theory that the vitamin K–vitamin K epoxide interconversion is a physiologically important cycle of the vitamin and that the action of Warfarin as an anticoagulant might be to block this cycle. The physiologically important reducing agent which is replaced by dithiothreitol in these in vitro studies has not been identified.

Vitamin K functions in the postribosomal modification of liver microsomal protein precursors to form biologically active prothrombin (factor II) and the other vitamin K dependent plasma clotting proteins, factors VII, IX and X (Suttie & Jackson, 1977). This modification involves the carboxylation of specific glutamyl residues in the precursor proteins to form γ-carboxyglutamyl residues in these proteins (Stenflo & Suttie, 1977), and an in vitro system to study this vitamin K dependent carboxylase has been developed (Esmon et al., 1975). This reaction has now been studied in microsomal suspensions (Sadowski et al., 1976; Girardot et al., 1976; Friedman & Shia, 1976; Shah & Suttie, 1974; Jones et al., 1976; Vermeer et al., 1976) and in detergent solubilized preparations (Esmon & Suttie, 1976; Mack et al., 1976; Friedman & Shia, 1977; Houser et al., 1977). The carboxylase is characterized by a dependence on oxygen and the reduced form of vitamin K, and the lack of dependence on ATP or biotin.

Derivatives of 4-hydroxycoumarin are clinically useful oral anticoagulants (O'Reilly, 1976) which antagonize the action of vitamin K in promoting clotting factor synthesis. Although it appears that coumarins do not directly antagonize the action of vitamin K, the molecular basis for their action has not been established, and a number of hypotheses have been suggested (Suttie, 1977). Some of the vitamin K in liver is present as the 2,3-epoxide of the vitamin (Bell & Matschiner, 1970, 1972; Matchiner et al., 1970) and it has been shown (Willingham and Matschiner, 1974; Willingham et al., 1976; Sadowski et al., 1977a) that microsomal preparations which catalyze the vitamin K dependent carboxylations of glutamyl residues will also metabolize vitamin K to its 2,3-epoxide. It has been postulated (Willingham & Matschiner, 1974) that the "epoxidase" activity is directly coupled to the carboxylase activity. Liver microsomes also contain an enzymatic activity that can reconvert the epoxide back to vitamin K (Matschiner et al., 1974; Zimmerman & Matschiner, 1974). This enzyme is inhibited by Warfarin and it has been postulated (Willingham & Matschiner, 1974) that the cyclic interconversion of the vitamin to its epoxide and back is required for its action, and that Warfarin exerts its effect on prothrombin synthesis through its action on this enzyme. Strains of rats which are resistant to Warfarin were first identified in northern Europe (Boyle, 1960; Lund, 1964; Drummond, 1966) and the epoxide reductase activity in these rats has been shown to be much less sensitive to Warfarin than that obtained from normal rats (Zimmerman & Matschiner, 1974).

During our studies on the mechanism of action of Warfarin, Friedman & Shia (1976) and Mack et al. (1976) reported that the addition of dithiothreitol to incubations of rat liver microsomes stimulated the vitamin K dependent incorporation of $H^{14}CO_3^-$ into protein and replaced the requirement for NADH. Initially, we were not able to reproduce this reported effect of dithiothreitol on the vitamin K dependent carboxylase. The apparent discrepancy between our findings and those previously published prompted us to reexamine the effect of dithiothreitol and Warfarin on hepatic microsomal protein carboxylation, and to further investigate the hypothesis that the vitamin K epoxide reductase is the physiologically im-

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TABLE I: Effect of Warfarin on Microsomal Vitamin K Dependent Carboxylase.[a]

| Reducing agent added | Protein carboxylation (dpm/g of liver × $10^{-3}$) | | | |
|---|---|---|---|---|
| | Vitamin $K_1$ | | Vitamin $K_1H_2$ | |
| | Control | Warfarin | Control | Warfarin |
| A. Intact microsomes | | | | |
| None | 7.2 | 1.2 | 24.2 | 25.0 |
| NADH | 42.3 | 39.5 | 28.9 | 27.3 |
| Dithiothreitol | 82.0 | 2.8 | 38.9 | 22.7 |
| Dithiothreitol + NADH | 80.5 | 43.1 | 41.8 | 25.6 |
| B. Solubilized microsomal extract | | | | |
| None | 0 | 0 | 15.3 | 14.6 |
| NADH | 20.3 | 20.2 | 14.8 | 15.2 |
| Dithiothreitol | 0.5 | 0.5 | 14.0 | 16.3 |
| Dithiothreitol + NADH | 20.3 | 20.3 | 12.9 | 14.0 |

[a] Microsomes were prepared and vitamin K dependent carboxylation was measured as described in Materials and Methods. Concentrations of additions were: dithiothreitol, 1 mM; Warfarin, 50 μg/mL; and NADH, 1 mg/mL. The four experiments were performed with different microsomal preparations, and control values cannot be compared. The data in the intact microsomal experiments are from single incubations with duplicate assays of the extent of $H^{14}CO_3^-$ incorporation. The data in the solubilized microsomal experiments are the means of duplicate incubations and duplicate assays.

portant site of coumarin action. A preliminary report of this study has appeared (Sadowski et al., 1977b).

## Materials and Methods

*Treatment of Animals.* Normal, male Holtzman rats and rats homozygous for the Warfarin resistant trait (Hermodson et al., 1969) were used. Vitamin K deficiency was produced in normal rats by feeding a vitamin K deficient diet (Mameesh & Johnson, 1959) for 7–10 days to rats housed in cages that prevented coprophagy (Metta et al., 1961). The Warfarin resistant rats were vitamin K deficient in 3 days. The animals were fasted for 18 h prior to killing.

*Incubation Conditions.* Microsomes were prepared as previously described (Sadowski et al., 1977a). The surface washed microsomes were resuspended with a large clearance Dounce homogenizer in 0.25 M sucrose–0.025 M imidazole–0.08 M KCl, pH 7.2, to give a final concentration of microsomes equivalent to 0.5 g of liver per mL. Triton X-100 solubilized microsomes were prepared by resuspending surface washed microsomes in 0.25 M sucrose–0.025 M imidazole–0.5 M KCl–1.5% Triton X-100 with a large clearance Dounce homogenizer to give a final concentration of microsomes equivalent to 0.5 g of liver/mL. The solubilized microsomes were centrifuged at 105 000*g* for 60 min and the resulting supernatant was used in the incubations. The standard incubation mixture consisted of sufficient microsomes or Triton X-100 solubilized microsomes to give a final concentration equivalent to 0.33–0.4 g of liver/mL incubation after additions were made. An ATP generating system (Sadowski et al., 1976) and cycloheximide (66 μg/mL) were included in each microsomal incubation but not in the solubilized microsomal incubations (Esmon & Suttie, 1976). When vitamin K-dependent protein carboxylation was measured, $H^{14}CO_3^-$ (59.5 mCi/mmol of $NaH^{14}CO_3^-$, Amersham/Searle) was added to the incubations to give final concentrations of 5–20 μCi/mL. The reactions were started by the addition of either vitamin K or vitamin K epoxide in ethanol (0.05 mL) to a final concentration of 4 μg of vitamin/mL of incubation (8.89 μM) unless otherwise stated. Incubations were routinely carried out at 27 °C for 30 min with rotary mixing in 13 × 100 mm disposable borosilicate glass culture tubes open to the atmosphere (epoxidase activity measured), sealed with parafilm (carboxylase activity measured), or under a nitrogen atmosphere (epoxide reductase activity measured). The carboxylation was essentially finished in 30 min under these conditions (Sadowski et al., 1976). When reductants other than lipoic acid were added, they were dissolved in buffer immediately prior to addition to the incubation mixtures. Reduced lipoic acid was dissolved in ethanol.

*Assay for Protein Carboxylation.* Incorporation of $H^{14}CO_3^-$ into protein was determined as previously described (Sadowski et al., 1976; Esmon & Suttie, 1976).

*Analysis for Metabolites of Vitamin $K_1$.* Vitamin K epoxide reductase assays were performed under a nitrogen atmosphere created by blowing nitrogen over the incubation mixture for 15 min. The reaction was started by the addition of $^3$H-labeled vitamin $K_1$ epoxide and incubated under a stream of nitrogen at 27 °C for 30 min. Epoxidase assays were started by the addition of $^3$H-labeled vitamin $K_1$ and incubated in air. The reaction was stopped by the addition of two volumes isopropyl alcohol:hexane (3:2) and the distribution of radioactivity in vitamin $K_1$ and vitamin $K_1$ epoxide determined as previously described (Sadowski et al., 1977a). Blank values (less than 10% of control incubations) were determined by extracting the microsomal preparation immediately after addition of the radioactive vitamin.

*Vitamin $K_1$, Vitamin $K_1$ 2,3-Epoxide, and Inhibitors.* Vitamin $K_1$ (6,7,8,9-$^3$H-label) was synthesized as described by Matschiner (1970). Vitamin K epoxide (labeled and unlabeled) was prepared by oxidation of the vitamin with hydrogen peroxide in alkaline aqueous ethanol as described by Fieser et al. (1941). Vitamin K hydroquinone (vitamin $KH_2$) was prepared as previously described (Sadowski et al., 1976). Unlabeled vitamin K was obtained from Sigma (St. Louis, Mo.) and purified by silicic acid chromatography before use (Matschiner et al., 1967). Sodium Warfarin (Endo) was dissolved in buffer immediately before use. Difenacoum [3-(3-*p*-diphenyl-1,2,3,4-tetrahydronaphth-1-yl)-4-hydroxycoumarin] was obtained from Sorex (London) Limited and dissolved in benzene. The approporate amount of Difenacoum in benzene was added first to the incubation tube and the benzene solvent then evaporated off.

*Chemicals.* Creatine phosphokinase, ATP, NADH, NADPH, cysteine, glutathione, ascorbate, and reduced lipoic acid were purchased from Sigma (St. Louis, Mo.). Creatine phosphate was obtained from Pierce (Rockford, Ill.). Dithiothreitol was purchased from Calbiochem. Unless otherwise stated, all chemicals used in these studies were of reagent grade or the highest purity available from local supplies.

TABLE II: The Effect of Warfarin on Microsomal Vitamin K Epoxidase.[a]

| Reducing agent added | Vitamin $K_1$ epoxide formed (nmol/g of liver) | | | |
|---|---|---|---|---|
| | Vitamin $K_1$ | | Vitamin $K_1H_2$ | |
| | Control | Warfarin | Control | Warfarin |
| A. Intact microsomes | | | | |
| None | 0.71 ± 0.06 | 0.60 ± 0.05 | 6.40 ± 0.18 | 7.27 ± 0.36 |
| NADH | 9.54 ± 0.21 | 9.88 ± 0.11 | 10.90 ± 0.4 | 11.70 ± 0.10 |
| Dithiothreitol | 0.89 ± 0.04 | 0.57 ± 0.20 | 2.40 ± 0.11 | 7.65 ± 0.23 |
| Dithiothreitol + NADH | 0.66 ± 0.06 | 9.96 ± 0.04 | 2.05 ± 0.09 | 11.30 ± 0.10 |
| B. Solubilized microsomal extract | | | | |
| None | 0.69 ± 0.01 | 0.45 ± 0.07 | 6.29 ± 0.12 | 6.27 ± 0.22 |
| NADH | 5.87 ± 0.19 | 6.09 ± 0.09 | 7.25 ± 0.17 | 6.97 ± 0.18 |
| Dithiothreitol | 0.77 ± 0.02 | 0.74 ± 0.07 | 7.27 ± 0.23 | 6.48 ± 0.32 |
| Dithiothreitol + NADH | 6.48 ± 0.07 | 6.71 ± 0.04 | 7.42 ± 0.18 | 7.36 ± 0.08 |

[a] Microsomes were prepared and epoxide formation was measured as described in Materials and Methods. Concentrations of additions as in Table I. The results are the average of triplicate incubations ± standard error of the mean.

TABLE III: Effect of Warfarin on Vitamin K Epoxide Reductase.[a]

| Reducing agent added | Vitamin K formed (nmol/g of liver) | | | | Solubilized microsomes |
|---|---|---|---|---|---|
| | Intact microsomes | | | | |
| | $N_2$ atmosphere | | Air atmosphere | | $N_2$ atmosphere |
| | Control | Warfarin | Control | Warfarin | Control |
| None | 0.75 ± 0.05 | 0.03 ± 0.02 | 0.80 ± 0.03 | 0.08 ± 0.03 | 0.11 ± 0.06 |
| NADH | 0.34 ± 0.03 | 0.04 ± 0.03 | 0.20 ± 0.08 | <0.01 | 0.16 ± 0.03 |
| Dithiothreitol | 12.6 ± 1.0 | 0.31 ± 0.06 | 7.90 ± 0.12 | 0.30 ± 0.01 | 0.30 ± 0.08 |
| Dithiothreitol + NADH | 11.5 ± 0.2 | 0.10 ± 0.02 | 5.77 ± 0.08 | <0.01 | 0.34 ± 0.09 |

[a] Microsomes were prepared and epoxide reductase was assayed as described in Materials and Methods. Concentration of additions as in Table I. The results are an average of triplicate incubations ± standard error of the mean. In the absence of any enzyme preparations, there was no reduction of vitamin K epoxide by dithiothreitol.

## Results

The incubation conditions initially used in our laboratory, which failed to demonstrate that dithiothreitol could replace NADH and stimulate vitamin K dependent carboxylation, were also used to study vitamin K epoxidation (Sadowski et al., 1977a) and, because of this, they contained a low concentration of Warfarin to block epoxide reduction. It was therefore decided to test the hypothesis that Warfarin specifically inhibited the dithiothreitol supported vitamin K dependent carboxylation. The results of a typical experiment presented in Table I demonstrate that Warfarin did not significantly influence microsomal protein carboxylation when either NADH + vitamin K or vitamin $KH_2$ were used as a source of vitamin. The carboxylase is dependent on reduced vitamin K, and, in the absence of Warfarin, dithiothreitol could replace NADH as a source of reducing equivalents for hydroquinone formation and also elicit a marked stimulation of the carboxylation seen in the presence of either vitamin $KH_2$ or vitamin K + NADH. The observation that dithiothreitol + vitamin K could substitute for NADH + vitamin K only in the absence of Warfarin suggested that dithiothreitol, in contrast to NADH, was supplying reducing equivalents to the vitamin in a Warfarin-sensitive reaction. The effect of Warfarin on the dithiothreitol dependent carboxylation was found (data not shown) to be independent of the vitamin K concentration used. That dithiothreitol may have an additional function other than the reduction of the quinone was suggested by the observation that dithiothreitol was more effective than NADH for vitamin K dependent carboxylation and, in contrast to NADH, stimulated vitamin $KH_2$ dependent carboxylation. It has previously been demonstrated (Esmon & Suttie, 1976) that vitamin K dependent carboxylation in a Triton X-100 solubilized microsomal system is supported equally by NADH + vitamin K or by vitamin $KH_2$ and that Warfarin has no inhibitory effect on this system. However, the data in Table I indicate that dithiothreitol was ineffective as a source of reducing equivalents for carboxylation in detergent solubilized microsomes.

The epoxidation of vitamin K to its 2,3-epoxide is a microsomal reaction which also requires NADH (Sadowski et al., 1977a), and it has been suggested that the epoxidation of the vitamin is coupled to the vitamin K dependent carboxylation of protein. As dithiothreitol could substitute for NADH in the microsomal vitamin K dependent carboxylation reaction, the ability of dithiothreitol to replace NADH for the microsomal epoxidation of vitamin $K_1$ was examined. The data in Table II suggested that dithiothreitol could not replace the NADH requirement for epoxidation, and, unlike the carboxylation reaction which was stimulated by dithiothreitol, the epoxidation of vitamin K + NADH or vitamin $KH_2$ appeared to be inhibited by dithiothreitol. It was also found that dithiothreitol did not stimulate or support epoxidation of vitamin $K_1$ in solubilized microsomes. However, unlike the situation in the intact, resuspended microsomes, the epoxidation reaction in solubilized microsomes did not appear to be inhibited by the presence of dithiothreitol. When vitamin $KH_2$ was used in the solubilized system, the epoxidation was independent of the source of reducing equivalents. The effect of Warfarin on the epoxidase reaction was also dependent on the source of reducing equivalents. When vitamin K + NADH was used in the presence of dithiothreitol, there was an apparent stimulation of intact microsomal vitamin K epoxidation by Warfarin, and, when vitamin $KH_2$ was used, dithiothreitol and Warfarin resulted in an apparent stimulation of epoxidation over that observed with dithiothreitol alone.

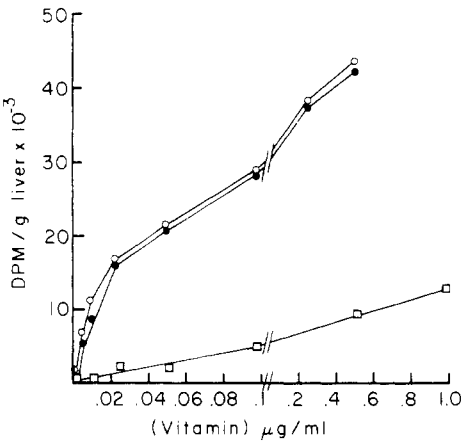


FIGURE 1: Effect of vitamin concentrations on vitamin K dependent carboxylation. Microsomes were prepared and vitamin K dependent carboxylation was determined as described in Materials and Methods in the presence or absence of 1 mM dithiothreitol or 1 mg/mL NADH. (O- - - -O) vitamin K epoxide + dithiothreitol; (●- - - -●) vitamin K + dithiothreitol; (□- - - -□) vitamin K + NADH.

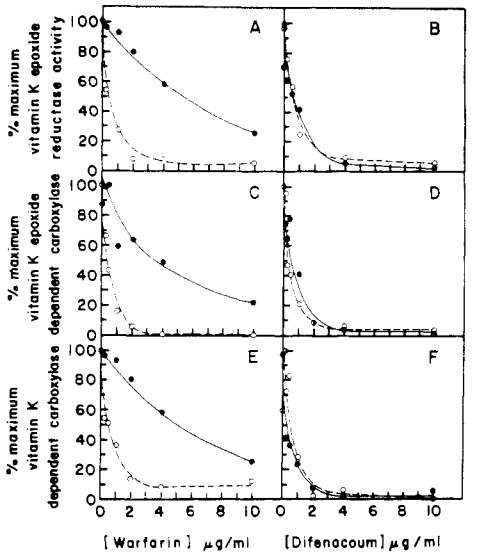


FIGURE 2: Inhibition of vitamin K epoxide reductase, vitamin K epoxide dependent carboxylation, and vitamin K dependent carboxylation, by Warfarin and Difenacoum in normal or Warfarin resistant rat liver. Microsomes were prepared from normal or Warfarin resistant rat liver and the various activities determined as indicated in Materials and Methods. The source of reducing equivalents in all reactions was 1 mM dithiothreitol. (O- - - -O) microsomes from normal rats; (●- - - -●) microsomes from Warfarin resistant rats.

TABLE IV: Effect of Warfarin on Vitamin K Epoxide Dependent Carboxylation of Microsomal Protein.[a]

| Reducing agent added | Protein carboxylation (dpm/g of liver × $10^{-3}$) Control | Warfarin |
|---|---|---|
| None | 0.8 | <0.1 |
| NADH | 5.2 | 0.2 |
| Dithiothreitol | 73.0 | <0.1 |
| Dithiothreitol + NADH | 69.2 | 2.0 |

[a] Intact microsomes were prepared and vitamin K epoxide dependent carboxylation measured as described in Materials and Methods. Concentration of additions as in Table I. The results are means of duplicate incubations.

The apparent ability of dithiothreitol to replace the NADH requirement for carboxylation but not for epoxidation would seem to contradict the hypothesis that the epoxidation of vitamin K is an obligatory molecular event during carboxylation. However, a Warfarin-sensitive conversion of the epoxide back to vitamin K has been demonstrated in rat liver (Matschiner et al., 1974). The epoxidase assay used could only measure the final steady state concentration of epoxide, and, if dithiothreitol were stimulating the conversion of epoxide to vitamin K, then its presence during the incubation could have prevented the accumulation of the epoxide. The effects of dithiothreitol on the epoxide reductase activity were therefore examined. The data in Table III indicate that NADH was inefficient as a source of reducing equivalents for the epoxide reductase in rat liver microsomes. However, the microsomal epoxide reductase showed a marked stimulation in the presence of dithiothreitol that was almost completely blocked by Warfarin. As can be seen, incubations carried out under nitrogen are a better reflection of the epoxide reductase activity due to the inability of the vitamin to reform the epoxide in the absence of oxygen (Sadowski & Suttie, 1976).

Thus, dithiothreitol can function as a source of reducing equivalents both for the epoxidation of the vitamin and for reduction of the epoxide. Epoxide formed from NADH + vitamin K cannot be converted back to vitamin K due to the relative inactivity of the epoxide reductase. In the presence of dithiothreitol, the epoxide formed can be reconverted to the vitamin resulting in a much lower accumulation of epoxide. When the vitamin $KH_2$ is used, and the Warfarin sensitive dithiothreitol reduction of vitamin K is bypassed, the addition of Warfarin causes an apparent stimulation (Table II) of epoxidase activity because of inhibition of the epoxide reductase.

The observation (Table III) that NADH was ineffective as a source of reducing equivalents for the epoxide reductase, a Warfarin inhibitable activity, suggests why NADH dependent vitamin K carboxylation is relatively insensitive (Sadowski et al., 1976) to Warfarin. The data in Table III also explain the apparent lack of effect of dithiothreitol in the solubilized microsomes in Table II. It can be seen that the solubilized microsomes exhibited no measurable epoxide reductase activity either in the presence or absence of dithiothreitol. This lack of reductase activity could cause the accumulation of epoxide in the solubilized microsomes shown in Table II.

Under conditions where the epoxide reductase is active, vitamin K epoxide should be an active form of the vitamin. Table IV shows that vitamin K epoxide was relatively ineffective in supporting carboxylation when no reductant was added and was only a poor form of the vitamin when NADH was used as a reductant. However, when dithiothreitol was used as a reductant, vitamin K epoxide was effective in supporting carboxylation. Warfarin completely inhibited the carboxylation due to vitamin K epoxide and dithiothreitol. As expected (data not shown), vitamin K epoxide did not support carboxylation in solubilized microsomes regardless of the reductant.

The nature of the vitamin K–vitamin K epoxide interconversions predicts that an increased concentration of the vitamin might be required to drive carboxylation under conditions where the vitamin K epoxide could not be reconverted to vitamin K. Figure 1 shows that, in the presence of dithiothreitol, and thus an active vitamin K epoxide reductase, vitamin K and vitamin K epoxide are both effective in supporting carboxylation. However, in the presence of NADH, and thus a relatively inactive vitamin K epoxide reductase, more vitamin is needed to drive the carboxylation.

The observation that normal vitamin K-deficient rat liver microsomes possess Warfarin-sensitive dithiothreitol depen-

TABLE V: Effect of Various Reducing Agents on Vitamin K Dependent Carboxylation, Vitamin K Epoxide Dependent Carboxylation, and Epoxide Reductase Activity.[a]

| Reducing agent | Carboxylase activity | | | | Epoxide reductase | |
|---|---|---|---|---|---|---|
| | Vitamin K | | Vitamin K epoxide | | | |
| | Control | Warfarin | Control | Warfarin | Control | Warfarin |
| None | 11 | <1 | 10 | <1 | 8 | <1 |
| Dithiothreitol | 100 | <1 | 100 | <1 | 100 | 3 |
| NADH | 49 | 45 | 16 | 3 | 12 | 1 |
| NADPH | 49 | 37 | 10 | <1 | 9 | <1 |
| Cysteine | 16 | 4 | 13 | <1 | 12 | 4 |
| Ascorbate | 12 | 3 | 11 | <1 | 8 | <1 |
| Glutathione | 18 | 2 | 8 | 2 | 9 | <1 |
| Lipoic acid | 55 | <1 | 42 | <1 | 53 | 3 |

[a] Microsomes were prepared and assays performed as indicated in Materials and Methods. All reducing agents other than NAD(P)H were added at a concentration of 1 mM. NAD(P)H was present at 1 mg/mL. Values are means of from two to five experiments utilizing each reducing agent and are expressed as a percentage of the activity obtained with dithiothreitol.

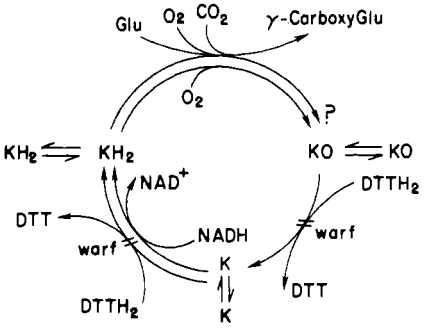


FIGURE 3: Vitamin K related metabolic activities in rat liver microsomes (DTT, dithiothreitol).

dent pathways for vitamin K dependent carboxylation, vitamin K reduction, and vitamin K epoxide reduction prompted us to compare these reactions in microsomes prepared from vitamin K deficient Warfarin resistant rat liver. The results of these experiments are presented in Figure 2. As little as 0.5 $\mu$g/mL Warfarin sodium (1.5 $\mu$M) significantly inhibited vitamin K dependent carboxylation in microsomes from normal rat liver when either vitamin K or vitamin K epoxide was used as a source of vitamin. However, microsomes prepared from Warfarin resistant rat liver required 10–20 times more Warfarin in the incubation to achieve the same degree of inhibition. Likewise, the epoxide reductase was 10–20 times less sensitive to the effects of Warfarin in resistant rats compared with normal rats. If the primary physiological function of Warfarin is to inhibit the vitamin K epoxide reductase, then coumarin anticoagulants that are effective against Warfarin resistant rats should be effective against the epoxide reductase in resistant rats. Difenacoum, a newly synthesized coumarin, has been reported (Hadler & Shadbolt, 1975) to be effective in this Warfarin resistant strain of rats. Although Difenacoum had no effect on NADH dependent carboxylation (data not shown), it completely inhibited the dithiothreitol-dependent carboxylation. A comparison between Figures 2A and 2B shows that Difenacoum is more effective than Warfarin against the resistant rat epoxide reductase and that with Difenacoum the inhibition is similar in normal and Warfarin-resistant rats. Figures 3C–3F show that the resistance of dithiothreitol dependent vitamin K and vitamin K epoxide carboxylation to coumarin anticoagulants is lost in Warfarin resistant rats when Difenacoum is used.

In an attempt to determine what the physiologically important Warfarin sensitive reductant for the epoxide reductase might be, a number of different biological reducing agents were tested in the microsomal system to determine their ability to substitute for dithiothreitol. The effects of these reducing agents on vitamin K and vitamin K epoxide dependent carboxylation, and on the vitamin K epoxide reductase, are shown in Table V. Of the reducing agents tested, the most effective in supporting carboxylation (at 1 mM) was reduced lipoic acid which functioned as well as NADH and supported about 50% of the extent of carboxylation as did dithiothreitol. All the reducing agents, expect NAD(P)H, that effectively supported carboxylation were Warfarin inhibitable. Only lipoic acid showed any appreciable ability (about 50% of the dithiothreitol activity) to serve as a reductant in the conversion of the epoxide to the vitamin.

## Discussion

A model for vitamin K metabolism consistent with the data reported here is presented in Figure 3. The model proposes that there are two vitamin K quinone reductase activities in the microsomes: a NADH supported, Warfarin insensitive activity, and a dithiothreitol supported, Warfarin sensitive reductase. What relationship these have to previously studied microsomal reductases is not clear. A microsomal TPNH-cytochrome *c* reductase will reduce menadione (Masters et al., 1965) as will DT-diaphorase (Hosoda et al., 1974). It has been suggested by Wallin (1978) that the microsomal bound portion of the latter enzyme is the activity responsible for driving the vitamin K dependent carboxylation reaction by NADH. The coumarin sensitivity of these flavoprotein quinone reductases varies with incubation conditions and relative purity of the preparation used.

The model in Figure 3 also indicates that vitamin K can be metabolized to vitamin K epoxide but leaves open the question (Sadowski et al., 1977a) of whether or not the epoxidation reaction is coupled to the carboxylation reaction. As indicated (?) the form of the vitamin which is the product of the $KH_2$ dependent carboxylation is not known, nor is the rate of conversion of vitamin $KH_2$ to vitamin K relative to the rate of carboxylation known. The model postulates that the epoxide can be recycled to vitamin K for possible reuse by a Warfarin sensitive enzyme which requires a reductant replaceable by dithiothreitol. Recycling of the epoxide and vitamin K is apparently a very effective way to form the reduced vitamin K required for carboxylation. When dithiothreitol is not supplied, this cycle is not active, and 100 times more vitamin was required to achieve the same amount of carboxylation (Figure 1).

We have previously observed (Sadowski et al., 1976) that the NADH dependent carboxylase is resistant to Warfarin at

high concentrations of vitamin K and showed only limited inhibition at lower concentrations. However, the present study had demonstrated that the dithiothreitol driven carboxylase can be inhibited by the presence of Warfarin at any concentration of vitamin K. In addition to lowering the apparent vitamin K requirement, the addition of dithiothreitol consistently stimulated the carboxylation observed at higher concentrations of vitamin K where recycling would not be expected to significantly contribute to the total pool of vitamin K available to the carboxylase. Data (Ren et al., 1977) correlating the relative effectiveness of various coumarins as in vivo anticoagulants and as in vitro inhibitors of the epoxide reductase would also support the importance of this enzyme in the action of the coumarin anticoagulants.

From the data presented, the Warfarin effect and the dithiothreitol effect appear to be interrelated. A loss of Warfarin sensitivity of the vitamin K dependent carboxylase in solubilized microsomes has been previously reported (Esmon & Suttie, 1976) and this loss of Warfarin effect might well reflect the loss of the ability to utilize dithiothreitol in the solubilized system. The general hypothesis that Warfarin exerts its effect by the inhibition of the epoxide reductase (Willingham & Matschiner, 1974) has been supported by the data presented here and the reported dithiothreitol requirement for epoxide reductase activity in microsomes (Zimmerman & Matschiner, 1974) has been confirmed. In addition, these data have suggested that there is also a Warfarin sensitive dithiothreitol dependent reduction of vitamin K occurring in these preparations.

The physiological importance of the dithiothreitol dependent reduction of vitamin K and of vitamin K epoxide to the action of Warfarin seems to be supported by the observed activity (see Figure 2) of the coumarin anticoagulant, Difenacoum. Although Difenacoum had little effect in vitro against NADH supported reactions, it was as effective against the dithiothreitol supported epoxide reductase, vitamin K and vitamin K epoxide dependent protein carboxylation in the Warfarin resistant rats as in normal rats. It is possible that the dithiothreitol dependent reductions of both vitamin K and vitamin K epoxide are a property of the same enzyme. Both reactions are Warfarin resistant in the mutant rats and the probability of two simultaneous mutations occurring in proteins having to do with Warfarin binding seems low. The higher in vivo requirement for vitamin K in the resistant rats has been postulated (Hermodson et al., 1969) to be due to the synthesis of a protein with a lower binding affinity for both vitamin K and Warfarin. This protein was located in the microsomes by Lorusso & Suttie (1972) and has now been purified by Searcey et al. (1977). The model in Figure 3 would, however, suggest that the increased vitamin K requirement might result because more of the vitamin used would have to be furnished from a vitamin K pool rather than being partially furnished by the epoxide reductase which is less active (Matschiner et al., 1974) in the resistant rat.

The model in Figure 3 is also compatible with the observations that caused Lowenthal (1970) to suggest the existence of two vitamin K transport routes, one a diffusion pathway and the other a Warfarin sensitive active transport process. Instead of alternate transport routes, the data could be explained by vitamin K reductases with differing sensitivities to Warfarin. Increasing the dose of a constant ratio of vitamin to Warfarin would result in inhibition of carboxylation at low doses by blocking the dithiothreitol dependent pathway. Further increases in dosage would not block the NADH pathway but would have the effect of increasing the vitamin K concentration to the point where carboxylation could be driven through the NADH route.

These data suggest that some physiological reducing agent, other than NAD(P)H, is able to support the microsomal reduction of vitamin K epoxide and vitamin K. These data do not distinguish between direct action of dithiothreitol as a primary reductant or its ability to reduce some other compound which is then acting as the in vitro reducing agent. Of the biological reducing agents tested, only reduced lipoic acid had significant activity in the reduction of vitamin K epoxide. Its ability to serve as a reductant in this cycle was also inhibited by Warfarin. Lipoate normally functions as an oxidizing and acyl group transferring coenzyme and it may be that in this reaction it is merely acting in the same manner as dithiothreitol.

Whether or not the dithiothreitol dependent vitamin K epoxide reductase studied here is the important physiological site of Warfarin action cannot be determined with certainty from this data. The data, however, strongly support the view that this is how Warfarin acts in the in vitro carboxylase system. Although animals treated with Warfarin have a high liver ratio of epoxide to vitamin, there is still a large amount of free vitamin present in the liver and this should be sufficient to drive prothrombin synthesis without the need to recycle the epoxide. An explanation compatible with the data presented here would be that all of the vitamin is not in the same pool and that diffusion of the vitamin within the microsomal membrane might be a relatively slow process. If this is the case, the large amount of vitamin present might not be available for utilization by the carboxylase. A final determination of the physiological significance of this Warfarin sensitive reaction will therefore depend on a closer understanding of the pools of vitamins that might exist under both in vitro and in vivo conditions.

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