**R- and S-Warfarin Inhibition of Vitamin K and Vitamin K 2,3-Epoxide Reductase Activities in the Rat**

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Reduction of vitamin K 2,3-epoxide and vitamin K catalyzed by hepatic microsomal enzymes is required for normal, postribosomal, γ-carboxyglutamate formation in the prothrombin complex Factors II, VII, IX, and X. The R- and S-warfarin enantiomers differentially inhibit (S-warfarin is 2 to 5 times more active) vitamin K function by mechanisms which have not been unambiguously determined. As a step toward determining the physiologically relevant site(s) of warfarin-antivitamin K activity we investigated in Wistar rats the effects of R- and S-warfarin on vitamin K 2,3-epoxide and vitamin K reductase activities and correlated them with effects on plasma concentrations of the Factors II, VII, and X. Based on the results of these studies we conclude that: 1) warfarin inhibition of the vitamin K 2,3-epoxide and vitamin K reductases is essentially irreversible; 2) S-warfarin stereoselectively inhibits both reductases in vivo but not in vitro; 3) the vitamin K reductase which utilizes dithiotohreitol as cofactor in vitro is primarily responsible for vitamin K reduction to vitamin K hydroquinone under physiological conditions; 4) warfarin initially inhibits γ-carboxyglutamate formation by inhibiting simultaneously the vitamin K 2,3-epoxide and vitamin K reductases; and 5) following enantiomer administration there is an apparent lack of correlation between the restoration of the reductase activities and the reintiation of coagulation factor synthesis.

The vitamin K-vitamin K 2,3-epoxide metabolic cycle has been linked to the biosynthesis of calcium-binding γ-carboxyglutamate acid residues in the coagulation Factors II (prothrombin), VII, IX, and X (1–4). Calcium ion binding is required for activation of these coagulation factors to enzymatically active forms (5, 6). Vitamin K also promotes γ-carboxyglutamic acid formation in two other blood proteins of unknown function (7–9) and in other calcium-binding proteins of developing bone (10), kidney (11), lung (12), spleen (13), and placenta (14).

The first step in vitamin K metabolism is its conversion to vitamin K hydroquinone catalyzed by either of two reductases which differ in their cofactor requirement and sensitivity to inhibition by warfarin. One reductase utilizes dithiotohreitol as cofactor in vitro (the physiological cofactor is not known) and is warfarin-sensitive (15, 16); and the other (DT-diaphorase EC 1.6.99.2) requires NADH as cofactor and is relatively warfarin-insensitive (15, 17, 18). The hydroquinone then serves as a cofactor for a γ-carboxylase, which produces γ-carboxyglutamic acid residues in postribosomal precursor protein, and as a substrate for an epoxidase, yielding vitamin K 2,3-epoxide. The requirements of the γ-carboxylation reaction (temperature optimum, oxygen, etc.) are similar to those of the epoxidation reaction and inhibitors such as Chloro-K affect the two reactions similarly (19), thus suggesting an interrelationship. The epoxide is reduced back to vitamin K by vitamin K 2,3-epoxide reductase which also utilizes dithiotreitol as cofactor in vitro and is warfarin-sensitive.

The mechanism(s) whereby warfarin antagonizes vitamin K function has not been unambiguously determined, but considerable evidence indicates vitamin K 2,3-epoxide reductase to be its primary site of action: i) the reductase of warfarin-resistant rats is less sensitive to warfarin than that of normal rats (20), and ii) warfarin increases the normal epoxide/vitamin K ratio in vitro and in vivo which is a consequence of reductase inhibition (4, 21). Presumably epoxide reductase inhibition lowers the hepatic vitamin K concentration below that required to support coagulation factor synthesis (22).

Other evidence, however, suggests that inhibition of the epoxide reductase may not be the sole mode of warfarin-antivitamin K activity. In particular, studies by Bell et al. (23) indicated that warfarin inhibition of coagulation factor synthesis occurs at hepatic vitamin K concentrations which are sufficient to restore coagulant activity to animals made hypoprothrombinemic from vitamin K deficiency (24). Based on these results we considered that the physiological mechanism of warfarin action also includes inhibition of vitamin K hydroquinone formation.

We report here our investigations to test this hypothesis by comparing the activities of the warfarin-sensitive vitamin K and vitamin K 2,3-epoxide reductases with the corresponding coagulant activities at various times following warfarin administration to rats. R- and S-Warfarin enantiomers were used to determine whether S-warfarin preferentially inhibits one or both reductases which would be consistent with its greater anticoagulant activity demonstrated in both man (25) and the rat (26).

**EXPERIMENTAL PROCEDURES**

**Materials**—Racemic warfarin was purchased from Calbiochem-Behring, Simplastin from General Diagnostics (Morris Plains, NJ), activated rabbit brain thromboplastin from Dade Diagnostics (Aqua, Puerto Rico), inosithin from Associated Concentrates (Woodside, NY), Russell's viper venom from Miami Serpentarium Labs (Miami, FL), vitamin K1 and dithiotohreitol from Sigma, and Emulgen 911 from Kao Atlas (Tokyo, Japan). The high performance...
liquid chromatography was a Waters Associates (Milford, MA) model 244 equipped with a Spectra Physics (Santa Clara, CA) model 4000 recording integrator and a Waters 710B integrator (Waters Associates). MnBondapak C18 columns and HPLC solvents were obtained from Waters Associates. Water was deionized, glass-distilled, and filtered through a 0.22 μm membrane (Millipore Corp., Bedford, MA) prior to use in the HPLC studies. Coagulation assays were performed with a Bi-O-Mark Coagulation Analyzer (Bayer Co., Westwood, MA). Preparation of Compounds—Optically pure R (α, = 149°) and S (α, = 149°)-warfarin and their sodium salts were prepared by the method of West et al. (27). Vitamin K 2,3-epoxide was prepared by the hydrogen peroxide oxidation of vitamin K by the method of Fieser (28). The vitamin K 2,3-epoxide solutions were prepared to chromatographic homogeneity with detection at 254 nm on a mnBondapak C18 preparative column (7.8 mm inner diameter, 30 cm) using acetoneitrile as the mobile phase at a 3 ml/min flow rate. The solvent was removed in vacuo from collected fractions containing vitamin K and its 2,3-epoxide. Resides of each vitamin were dissolved in aqueous Emulgen 911 (10% v/v) to a final concentration of 20 mg/ml, using the method described by Lowenthal and Jaeger (29). Solutions of vitamin K and epoxide at 2 mg/ml were prepared by dilution of the concentrated solutions with water and were used in metabolic studies.

Animal Experiments—The experimental animals were male Wistar rats (250 ± 10 g) from a colony maintained in this division. An aqueous solution of the sodium salt of R-warfarin was administered intraperitoneally to rats at doses of 20 or 10 mg/kg. S-Warfarin sodium salt was similarly administered at doses of 10 or 1 mg/kg. At various times after administration rats (4/group) were rendered unconscious with N2O and 4% isoflurane, and 100 μl of blood were drawn by cardiac puncture with heparinized 0.3-ml plastic syringe containing 0.5 ml of 3.8% trisodium citrate. Plasma was obtained by centrifugation of the citrated blood at 2000 g for 15 min at 5°C. R- and S-Warfarin concentrations in plasma were determined by HPLC using the rapid chromatographic method, which permits quantitation of warfarin free of its metabolites (30).

The liver of each rat was perfused in situ for 90 s with physiological saline, removed, and stored in cold 20 mM Tris-HCl, 0.15 M KCl buffer, pH 7.4. All further operations were performed at 5°C. Each liver was minced and homogenized in 3 volumes of the Tris-KCl buffer, pH 7.4. The suspensions were centrifuged at 10,000 g for 20 min and microsomes pelleted by centrifugation of the supernatant at 105,000 g for 75 min. The microsomes were resuspended in 3 to 5 ml of the Tris-KCl buffer, pH 7.4, and the protein concentrations were determined by the method of Bradford (31) using commercially available reagents (Bio-Rad). The suspensions were diluted to 10 ml of protein/ml with the same buffer and were stored at −80°C. No change in the activities of the vitamin K or vitamin K 2,3-epoxide reductases occurred for at least 1 month. Control microsomes were prepared identically from a pool of 10 rats.

Plasma Coagulation Factor Assays—All clot formation times are the average of at least two determinations. Factors II, VII, and X plasma concentrations in rats administered the warfarin enantiomers are expressed as per cent normal concentration. Pooled control plasma was obtained from 10 to 15 untreated rats and per cent normal curves were produced by dilution of the control plasma. Factor-deficient plasmas were prepared from species described in the assay references (32–34).

One-stage prothrombin time tests were performed with Simplastin according to manufacturer's direction.

Factor II concentrations in plasma were determined by the method of Magnin and Lawson (32).

Factor VII assays were performed by a modification of the method of Nemerson and Clyne (33). The imidazole-saline buffer used was 0.01 M imidazole-HCl, 0.15 M NaCl, 0.88% (w/v) PEG 6000 buffer, pH 7.4, and the thromboplastin used was Dade rabbit brain diluted with 2 parts of saline and 2 parts PEG buffer. All reagents were stored at 0°C prior to use. Factor VII-deficient plasma (0.1 ml) and test plasma (0.1 ml) (diluted with the imidazole-PEG buffer) were warmed in a water bath at 37°C for 5 min. After this 5 min, 0.5 ml of 25 mM CaCl2 in the imidazole-PEG buffer was added at 37°C. Clot times were recorded from the time of CaCl2 addition.

Factor X-deficient plasma was prepared by the method of Hougé (34). The imidazole-PEG and imidazole-PEG-CaCl2 buffers were the same as those used in the Factor VII assay. Thromboplastin was prepared by mixing 0.5 ml of Russell’s viper venom (0.1 mg/ml of water), 1 ml of inosinat (1 mg/ml of NaCl), and 8.5 ml of 200 mM Tris-HCl (pH 7.4) to 20 ml with 0.5 ml of PEG buffer. Factor X-deficient plasma, diluted test plasma, thromboplastin, and CaCl2 buffer were added as described for the assay of Factor VII. Clot times were recorded from the time of CaCl2 addition.

Warfarin Binding to Microsomal Reductases—All operations were performed at 1 to 5°C. Two ultracentrifuge polycarbonate tubes (2.5 × 8.9 cm) each contained 9.8 ml of 200 mM Tris-HCl, 0.15 M KCl buffer, pH 7.4, and 4.2 ml of microsomes (10 mg of protein/ml). Warfarin sodium salt (28 nmol) was included in the Tris-KCl buffer added to one of the tubes. After 1 h, two 3-ml aliquots were removed from each tube and were assayed for vitamin K and vitamin K 2,3-epoxide reductase activities as described below. The remaining 10-ml portions were diluted with sufficient 20 mm Tris-HCl, 0.15 M KCl buffer, pH 7.4, to fill the tubes (approximately 25 ml) and the microsomes were pelleted by centrifugation at 105,000 g for 75 min. The supernatants were decanted, and the pellets carefully washed 3 times with 30 to 35 ml of the 20 mm Tris-KCl buffer, pH 7.4. Each pellet was resuspended by homogenization with a Teflon pestle in sufficient 200 mm Tris-HCl, 0.15 M KCl buffer, pH 7.4, to restore the volume to 10 ml. Aliquots (2 ml) were assayed for vitamin K and vitamin K 2,3-epoxide reductase activities in the same way as the ultracentrifuged pellets.

Vitamin K and Vitamin K 2,3-Epoxide Reductase Assays—Microsomal vitamin K and vitamin K 2,3-epoxide reductase activities in normal rats and rats administered the warfarin enantiomers were determined as previously described (16) with slight modification. Reaction mixtures were 2 ml and contained in order of addition: 1.35 ml of 200 mM Tris-HCl, 0.15 M KCl buffer, pH 7.4, with or without R- and S-warfarin sodium salt added; 40 nmol of vitamin K or vitamin K 2,3-epoxide substrate in 0.01 ml of 2% (v/v) Emulgen 911; and 0.6 ml of microsomes (10 mg of protein/ml). Mixtures were incubated at 25°C for 1 min and 0.04 ml of dithiothreitol (100 mm) was added with mixing. After 5 additional min at 25°C, reaction was terminated with isopropanol/hexane and concentrations of vitamin metabolites were determined by HPLC as described (16). Variations in this procedure are cited in the text.

RESULTS

Addition of 2 μM warfarin to microsomes inhibited vitamin K reductase activity to 5% of normal and vitamin K 2,3-epoxide reductase activity to 25% of normal (Fig. 1). Dilution of the microsomes with buffer, followed by ultracentrifugation and repeated washing of the resultant pellet did not even partially restore the activity of either enzyme. Control microsomes containing no warfarin but otherwise identically treated lost only 28 and 4%, respectively, of their vitamin K and vitamin K 2,3-epoxide reductase activities after 5 h of the washing treatment. Warfarin inhibition of both reductases is thus irreversible by these criteria. These results can be compared with those of Lorusso and Suttie (35) which demonstrated that rat hepatic microsomes bind warfarin in an essentially irreversible manner. Since the warfarin concentrations required to saturate the microsomal binding site(s) (0.1 to 5 μM) (35) were similar to the concentrations which we previously demonstrated inhibited the vitamin K and epoxide reductases (16), it is probable that the irreversible binding occurred at the enzymes involved in vitamin K and vitamin K 2,3-epoxide reduction.

The effects of R- or S-warfarin (0.75 μM) on quantities of vitamin K hydroquinone formed from vitamin K by microsomes with dithiothreitol as electron donor as a function of time are illustrated in Fig. 2. The rate of hydroquinone formation, in the absence of warfarin enantiomers, was linear for at least 20 min, then decreased to values of 0.22 nmol/mg of protein/min. R- and S-Warfarin were equally effective as inhibitors of hydroquinone formation, diminishing the linear rate to 0.11 nmol/mg of protein/min. Initial hydroquinone formation rates were linear for at least 20 min under conditions of warfarin inhibition by 75% or greater.

R- and S-Warfarin were also equivalently effective as inhibitors of the reduction of vitamin K 2,3-epoxide to vitamin K.
Antivitamin K Activity of R- and S-Warfarin

Fig. 1. Effects of warfarin addition to microsomes on percent normal vitamin K and vitamin K 2,3-epoxide reductase activities before and after centrifugation and washing of microsomes. Control microsomes were treated identically except that warfarin was omitted. Details were as described under "Experimental Procedures." Total vitamin K 2,3-epoxide metabolism was calculated from the sum of the concentrations of vitamin K and vitamin K hydroquinone formed during 5 min of incubation at 25 °C.

Fig. 2. Effects of R(+) or S(-)-warfarin (sodium salts, 0.75 μM) on normal (---) quantities of vitamin K hydroquinone formed during microsomal metabolism of vitamin K as a function of time. Reaction mixtures were prepared and the product was assayed as described under "Experimental Procedures" except the time of incubation at 25 °C was varied.

Fig. 3. Microsomal metabolism of vitamin K 2,3-epoxide to vitamin K and vitamin K hydroquinone in the presence and absence of warfarin (0.75 μM) as a function of time. Vitamin K (----) and vitamin K hydroquinone (---) concentrations formed during incubation at 25 °C in the absence of the warfarin enantiomers. Concentrations of vitamin K formed in the presence of R(+) or S(-)-warfarin; and of vitamin K hydroquinone formed in the presence of R(+) or S(-)-warfarin during incubation at 25 °C. Reaction mixtures were prepared and the products were assayed as described under "Experimental Procedures."

Table I

<table>
<thead>
<tr>
<th>Time after administration</th>
<th>Concentration in citrated plasma</th>
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<tr>
<td></td>
<td>R-Warfarin (μg/ml)</td>
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<tr>
<td>1</td>
<td>16.2 ± 6.1</td>
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<tr>
<td>6</td>
<td>6.4 ± 1.1</td>
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<td>16</td>
<td>2.9 ± 1.3</td>
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<tr>
<td>24</td>
<td>0.3 ± 0.2</td>
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<td>32</td>
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Antivitamin K Activity of R- and S-Warfarin

At a R- or S-warfarin concentration of 0.25 μM (data not shown), the rate of epoxide to vitamin K conversion was not detectably inhibited while the rate of hydroquinone formation was inhibited by 33%. Warfarin concentrations greater than 1.0 μM produced complete inhibition of hydroquinone formation and with increasing warfarin concentrations a progressive decrease in the rate of vitamin K formation.

The plasma concentration of R- and S-warfarin administered separately at doses of 20 and 10 mg/kg, respectively, and determined at various times thereafter are presented in Table I. These doses produced essentially equivalent plasma concentrations of the enantiomers at most of the times investigated. The effects of these doses on the per cent normal plasma concentrations of the enantiomers at most of the times investigated. The effects of these doses on the per cent normal levels of Factors II, VII, and X in plasma as a function of time are illustrated in Fig. 4. Both R- and S-warfarin diminished Factor VII below detectable levels at 16 h after administration; but at 24 h Factor VII levels were partially restored only in rats treated with R-warfarin. In contrast to Factor VII, Factors II and X reached minimum levels at 24 h after administration of either enantiomer. At this time Factor II levels were equivalent in rats administered either enantiomer, but Factor X levels were slightly more depressed in S- than in R-warfarin-treated rats. Factors II, VII, and X each initially decreased from plasma at a rate which was independent of treatment with either R- or S-warfarin. At 48 h after dosing with the warfarin enantiomers the average plasma concentrations of Factors II, VII, and X had extensively recovered and were higher in R- than in S-warfarin-treated rats.

A single oral dose of R-warfarin (20 mg/kg) or S-warfarin (10 mg/kg) inhibited the hepatic microsome catalyzed reduction of vitamin K to hydroquinone in vitro to undetectable levels (less than 2% of normal concentrations) over a period of 1 to 24 h after administration (Fig. 5). No difference in the extent of R- and S-warfarin inhibition of the reductase was observed during this period. At 48 h after administration, however, microsomes from S-warfarin-treated rats exhibited substantially less reductase activity than did microsomes from R-warfarin-treated rats, thus demonstrating a stereoselective inhibition at equivalent plasma concentrations of the enantiomers. The rates of recovery of reductase activity were essentially parallel in R- and S-warfarin-treated rats and normal values were not attained even at times of 96 and 144 h after administration, respectively. The reductase thus recovers from the warfarin inhibition very slowly even during a period when plasma levels of warfarin are no longer detectable. A comparison of the time courses of reductase inhibition and anticoagulation measured by one-stage prothrombin as-

![Figure 4](http://www.jbc.org/)

**FIG. 4.** Effects of the warfarin enantiomers on per cent normal Factor VII (○—○), Factor X (●—●), and Factor II (□—□) concentrations in rat plasma at various times after administration of a single oral dose. A, R-warfarin at 20 mg/kg; B, S-warfarin at 10 mg/kg. Factor concentrations were determined as described under "Experimental Procedures" and are the average ± S.D. value obtained from four rats.

![Figure 5](http://www.jbc.org/)

**FIG. 5.** Effects of a single oral dose of R-warfarin (sodium salt, 20 mg/kg, ○—○) or S-warfarin (sodium salt, 10 mg/kg, □—□) on vitamin K reduction catalyzed by hepatic microsomes and on one-stage prothrombin times (R-warfarin, ●—●; S-warfarin, □—□) at various times after administration. Coagulation and reductase assays were performed as described under "Experimental Procedures." Normal values were established from plasma and hepatic microsomes obtained from 10 rats.
Antivitamin K Activity of R- and S-Warfarin

say following warfarin administration (Fig. 5) demonstrates that maximal inhibition persists beyond times when anticoagulation levels have reached their maximum and have begun to return to normal values.

At these doses R- and S-warfarin were also very potent inhibitors of vitamin K 2,3-epoxide reduction and the profile of the inhibition at various times after enantiomer administration is illustrated in Fig. 6. The corresponding one-stage prothrombin coagulation times are again included for the purpose of comparison. In general epoxide reduction was not as extensively inhibited as was vitamin K reduction (cf. Fig. 5). Further, S-warfarin was significantly more effective an inhibitor than was R-warfarin at all the time points investigated. Maximum inhibition of epoxide reduction occurred at 6 h after administration of either enantiomer with average values of 7.5 and 2.5% normal, respectively, for R- and S-warfarin-treated rats. During the ensuing period to 24 h there was a slow recovery phase followed by a more rapid phase with essentially normal values being attained at 96 h in R-warfarin-treated rats and 144 h in S-warfarin-treated rats. Reinitiation of coagulation factor synthesis occurred at 16 h in the former group with approximately 9 to 11% normal epoxide reductase levels, and at 24 h in the latter group with approximately 3 to 8% normal epoxide reductase levels.

A dose of 1 mg/kg of S-warfarin or 10 mg/kg of R-warfarin produced essentially equal changes in the percent normal plasma concentrations of Factors II, VII, and X (Fig. 7) thus producing an equivalent anticoagulant response. Factor VII was again most affected followed by Factors X and II. Synthesis of Factor VII was initiated sometime before 16 h after enantiomer administration since at this time its percent normal plasma concentration was greater than at the higher doses (Fig. 5).

The effects of these doses on sensitive vitamin K reductase activity are illustrated in Fig. 8. Included in the figure are the profiles of the one-stage prothrombin times. S-Warfarin was a better inhibitor of reductase activity than was R-warfarin and levels of activity during the first 6 h after administration were approximately 1 and 3.5% of normal, respectively. Inhibition produced by R-warfarin persisted for a much shorter time than that by S-warfarin resulting in more rapid recoveries to normal activity. At 16 h after administration, when coagulation factor synthesis was occurring, reductase activities were approximately 10 and 1% normal in R- and S-warfarin-treated rats, respectively.
Antivitamin K Activity of R- and S-Warfarin

The effects of R- and S-warfarin administration on hepatic microsomal vitamin K and vitamin K 2,3-epoxide reductase activities were compared with effects on coagulant activity to probe the physiologically relevant sites of warfarin-antivitamin K function. Since the extent of R- and S-warfarin inhibition of reductase activities in vivo could only be determined by an in vitro assay in hepatic microsomes, it was necessary to assess the effects of microsome isolation on the extent of inhibition. Experiments designed to simulate these effects demonstrated that warfarin inhibition of vitamin K and epoxide reductase activities is essentially irreversible and is probably not altered during microsome isolation. Results obtained from the in vitro assay therefore reflect the situation in the intact animal.

DISCUSSION

The effects of R- and S-warfarin administration on hepatic microsomal vitamin K and vitamin K 2,3-epoxide reductase activities were compared with effects on coagulant activity to probe the physiologically relevant sites of warfarin-antivitamin K function. Since the extent of R- and S-warfarin inhibition of reductase activities in vivo could only be determined by an in vitro assay in hepatic microsomes, it was necessary to assess the effects of microsome isolation on the extent of inhibition. Experiments designed to simulate these effects demonstrated that warfarin inhibition of vitamin K and epoxide reductase activities is essentially irreversible and is probably not altered during microsome isolation. Results obtained from the in vitro assay therefore reflect the situation in the intact animal.

The coagulation data presented here agree well with results reported by Vainieri and Wingard (36) for Factors II, VII, and X following acute dosage with warfarin. Factor VII has the shortest plasma half-life and therefore its loss is the most pronounced. Factor VII is also the most sensitive to variations in plasma warfarin levels (36), presumably because of its relatively greater depression and only small changes in concentration are required to overcome its decline. The profile of the one-stage prothrombin assay per cent normal activity versus clot time curve is hyperbolic and large changes in clot formation times accompany relatively small changes in low per cent normal coagulation factor concentrations. Based on coagulation results described here, the assay is primarily reflecting changes in Factor VII concentrations. Indeed, the earliest times detected for restoration of Factor VII activity in R- and S-warfarin-treated rats all agree with those detected by one-stage assay.

Of the doses employed in these investigations, S-warfarin at 10 mg/kg produced the greatest anticoagulant response (determined by one-stage prothrombin assay) attaining the peak of maximum effect at 24 h after administration. This dose is in large excess of that required to completely block vitamin K dependent-coagulation factor synthesis in rats (36) and thus rates of change of one-stage prothrombin times prior to the peak reflect only coagulation factor loss from plasma due to degradation. Since the same rates of change were initially exhibited following administration of the other R- or S-warfarin doses it can be concluded that they also completely blocked coagulation factor synthesis. For R-warfarin at 20 mg/kg complete blockage persisted for 16 h after administration and for R- and S-warfarin at 10 and 1 mg/kg, respectively, for 6 h after administration.

We have previously demonstrated (16) that large quantities of vitamin K hydroquinone are formed by Wistar rat hepatic microsomes in the presence of dithiothreitol with either vitamin K or the epoxide as substrate, and that only relatively insignificant quantities of hydroquinone are formed when vitamin K and NADH (the cofactor for DT-diaphorase) are added. The time course of dithiothreitol-supported epoxide metabolism to vitamin K and hydroquinone (Fig. 3) is typical of a coupled enzyme system indicating that the two warfarin sensitive-reductases are closely associated in the microsomal membrane. Since the vitamin K concentrations normally present in hepatic tissue are very low, highly active coupled enzyme systems would best be able to perform the multistep conversions of the vitamin K-vitamin K 2,3-epoxide metabolic cycle. It thus follows that hydroquinone formation in vivo arises preferentially via the sensitive reductase pathway and not via DT-diaphorase as has been suggested (18, 37). Hydro-
quinone formed by cytosolic DT-diaphorase would reach the carboxylase/epoxidase enzymes only by diffusion and would be expected to play only a minor role in coagulation factor synthesis. Support for these conclusions is derived from the experiments of Lowenthal and Birnbaum (38) who studied warfarin and vitamin K effects on Factor VII synthesis in liver slices isolated from vitamin K-deficient rats. They concluded that coumarin anticoagulants irreversibly block vitamin K transport to its site of action and that at high vitamin K concentrations the inhibition can be overcome because vitamin K can reach its site of action by an alternate route. We have demonstrated here that warfarin inhibition of the sensitive vitamin K reductase is essentially complete and irreversible at the warfarin doses used. Further, no detectable increase in sensitive vitamin K reductase activity occurs in warfarinized rats during the period when coagulation factor synthesis is proceeding at a rapid rate (Fig. 5). In view of these data and recent findings demonstrating that hydroquinone formation is necessary for γ-carboxyglutamic acid synthesis in hepatic precursor protein (17, 39), it is highly probable that the irreversible warfarin block observed by Lowenthal and Birnbaum was actually at the site of sensitive hydroquinone formation. At higher vitamin concentrations sufficient hydroquinone was supplied via the alternate pathway catalyzed by DT-diaphorase to overcome the warfarin inhibition. Whitlon et al. (15) also reached similar conclusions based on in vitro studies of γ-carboxyglutamic acid formation in hepatic microsomes driven by vitamin K in the presence of dithiothreitol or NADH.

The data from numerous investigations have demonstrated that after warfarin administration there is an increase in the normal vitamin K 2,3-epoxide/vitamin K ratio. This increase has only been observed after administration of vitamin K, however, and the extent of change under physiological conditions is not known. Vitamin K administration also restores coagulant activity to warfarin-treated rats in a dose-dependent manner (23). Since the sensitive vitamin K reductase is blocked by warfarin, the exogenous vitamin K is probably metabolized to hydroquinone (and subsequently epoxide) by the normally alternate route catalyzed by DT-diaphorase. Warfarin inhibition of the epoxide reductase causes an accumulation of epoxide with a resultant lowered vitamin K concentration. At low concentrations of the vitamin, it cannot sustain DT-diaphorase-catalyzed hydroquinone formation sufficiently to overcome the coagulation factor degradation rate and no synthesis is apparent as was the case in the studies of Bell et al. (23). At higher concentrations of vitamin K, DT-diaphorase-catalyzed hydroquinone formation should be more effective thus producing a measurable net increase in coagulation factor synthesis.

Since R- and S-warfarin differentially inhibit vitamin K function, it follows that if the sensitive vitamin K and/or vitamin K 2,3-epoxide reductases are the primary site(s) of anticoagulant action, enantiomer inhibition of them should be correspondingly differentiated. While R- and S-warfarin were equivalently potent inhibitors of the sensitive vitamin K and epoxide reductases in vitro, S-warfarin was a much better inhibitor of them when administered in vivo both at equivalent plasma concentrations and at equivalent anticoagulant effect. The in vivo stereoselectivity of the inhibition coupled with its magnitude strongly suggests that warfarin antagonism of these reductases is an essential part of its antivitamin K activity. Assuming that the DT-diaphorase has a minor role in the physiological metabolism of vitamin K for the reasons discussed above, blockade of vitamin K and epoxide reduction by warfarin essentially prevents vitamin K metabolism in the liver. This situation thus offers an explanation for Bell's observation (22) that hepatic vitamin K concentrations in rats treated with anticoagulants are sufficient to restore coagulant activity to rats made hypoprothrombinemic from vitamin K deficiency.

Following each dose of R- or S-warfarin, levels of vitamin K 2,3-epoxide reductase activity were depressed maximally during the period from 1 to 6 h and recovered slightly before restoration of coagulation factor synthesis was detected. Assuming that hepatic reductase concentrations are in excess of those needed to maintain normal coagulant function, only partial recovery of these activities would permit coagulation factor synthesis and thus recovery of coagulation. There is, however, no apparent correlation between extents of reductase inhibition in R- and S-warfarin-treated rats and coagulation factor synthesis activity. From the data of Fig. 6, coagulation factor synthesis was detected in S-warfarin-treated rats at 24 h and at epoxide reductase levels which were equal to or less than the 1 to 6 h levels in R-warfarin-treated rats. Since at 1 to 6 h no coagulation factor synthesis can be detected, these reductase levels at 24 h should also produce complete inhibition. From Fig. 9, reductase activity levels in R-warfarin-treated rats were substantially higher than in S-warfarin-treated rats, yet the anticoagulant responses were indistinguishable. Since the anticoagulant response at 16 h was less than that produced by the higher R- and S-warfarin doses (cf. Fig. 6), then coagulation factor synthesis must be occurring at this time. Assuming that the epoxide reductase is normally an essential part of this process, then in R-warfarin-treated rats (Fig. 9), the levels of epoxide reductase activity at 16 h must be at least partially functional. If this is in fact the case, then the lower levels of epoxide reductase activity in S-warfarin-treated rats (Fig. 9) must be less functional and should therefore produce a greater anticoagulant response. Finally, even if vitamin K formation via the epoxide reductase is sufficient, how does hydroquinone formation occur rapidly enough when the sensitive-vitamin K reductase is also inhibited to levels which are apparently below those required to sustain coagulation factor synthesis? The results of these investigations do not provide a satisfactory explanation, but do suggest that currently proposed relationships between the vitamin K-vitamin K 2, 3-epoxide metabolic cycle, γ-carboxyglutamic acid formation in precursor protein and coagulant activity may be oversimplified with respect to in vivo events.

In summary we have provided evidence that warfarin initially inhibits vitamin K-dependent coagulation factor synthesis by blocking simultaneously vitamin K and vitamin K 2,3-epoxide metabolism. We have proposed that DT-diaphorase has a minor role in the reduction of vitamin K under physiological conditions and have shown that the results of a number of other investigations are consistent with this conclusion. Lastly, we have demonstrated that following warfarin administration there is a lack of correlation between the restoration of vitamin K-dependent coagulation factor synthesis and the recovery of the sensitive reductases. Coagulant activity recovers as plasma warfarin levels diminish, but reductase activities only fully recover very much later. Experiments are currently in progress to elucidate more completely the roles of the enzymes involved in the vitamin K-vitamin K 2,3-epoxide metabolic cycle and the mechanisms of warfarin antivitamin K activity.

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