Mechanism of Coumarin Action: Sensitivity of Vitamin K Metabolizing Enzymes of Normal and Warfarin-Resistant Rat Liver†

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ABSTRACT: The in vitro effects of two coumarin anticoagulants, warfarin and difenacoum, on rat liver microsomal vitamin K dependent carboxylase, vitamin K epoxidase, vitamin K epoxide reductase, and cytosolic vitamin K reductase (DT-diaphorase) from the livers of normal and a warfarin-resistant strain of rats have been determined. Millimolar concentrations of both coumarins are required to inhibit the carboxylase and epoxidase activities in both strains of rats. Sensitivity of DT-diaphorase to coumarin inhibition differs when a soluble or liposomal-associated substrate is used, but the diaphorases isolated from both strains of rats have comparable sensitivity. The anticoagulant difenacoum is an effective rodenticide in the warfarin-resistant strain of rats, and the only enzyme studied from warfarin-resistant rat liver that demonstrated a significant differential inhibition by the two coumarins used was the vitamin K epoxide reductase. This enzyme also showed the greatest sensitivity to coumarin inhibition among the enzymes studied. These results support the hypothesis that the physiologically important site of action of coumarin anticoagulants is the vitamin K epoxide reductase.

Vitamin K is required for the postribosomal carboxylation of specific glutamyl residues to γ-carboxyglutamyl residues in microsomal protein precursors to clotting factors II, VII, IX, and X, as well as other vitamin K dependent proteins (Suttie, 1980a). This microsomal carboxylase requires "CO_2", O_2, a glutamyl substrate, and the hydroquinone form of vitamin K (Suttie, 1980b,c). The same crude microsomal preparations that support this carboxylase activity also convert vitamin K to its 2,3-epoxide, and a number of lines of evidence (Suttie et al., 1978; Larson et al., 1981) suggest that the carboxylation and epoxidation reactions are coupled. Liver microsomes also contain a vitamin K epoxide reductase (Matschiner et al., 1974) capable of reducing the 2,3-epoxide to the quinone or possibly the hydroquinone (Fasco & Principe, 1980) and vitamin K reductases that convert the quinone to the hydroquinone. The major vitamin K reductase isolable from solubilized microsomes appears (Wallin et al., 1978; Wallin & Suttie, 1981) to be identical with the cytosolic DT-diaphorase, NAD(P)H dehydrogenase (quinone) (EC 1.6.99.2). It has been demonstrated that DT-diaphorase can use phylloquinone or menaquinones as electron-accepting substrates in vitro when the vitamin is incorporated into liposomes (Martius et al., 1975). The presence of these activities in microsomes suggests that there is cycling of vitamin K among its hydroquinone, epoxide, and quinone forms during the normal course of utilization of the vitamin (Willingham & Matschiner, 1974; Whitlon et al., 1978).

The coumarin anticoagulants (O'Reilly, 1976) are a series of substituted 4-hydroxycoumarins that interfere with the biosynthesis of vitamin K dependent clotting factors by acting as indirect antagonists of the vitamin (Lowenthal & MacFarlane, 1964). Administration of these compounds has been shown to increase the tissue and plasma ratio of vitamin K epoxide/vitamin K (Bell & Matschiner, 1970; Bell et al., 1972) and to inhibit the microsomal epoxide reductase (Willingham & Matschiner, 1974), suggesting that these clinically important drugs act by inhibiting this enzyme and preventing the reutilization of epoxidized vitamin K.

Strains of rats resistant to the rodenticidal action of warfarin have been described (Boyle, 1960; Lund, 1964; Drummond, 1966), and it has been demonstrated (Zimmerman & Matschiner, 1974) that the microsomal epoxide reductase activity from normal rats is much more sensitive to warfarin than the activity from warfarin-resistant rats. Difenacoum, an anticoagulant active in controlling warfarin-resistant rat populations (Hadler & Shadbolt, 1975), is an effective inhibitor of the epoxide reductase obtained from both strains of rats (Whitlon et al., 1978). It was also shown (Whitlon et al., 1978) that the inhibition of vitamin K epoxide dependent carboxylation in intact microsomes from normal or warfarin-resistant rat liver by warfarin or difenacoum was very similar to the pattern observed for the vitamin K epoxide reductase. These data established that a site of coumarin action was in the reconversion of vitamin K epoxide to an enzymatically active form but did not determine if the enzymatic change responsible for warfarin resistance involved only the epoxide reductase. Coumarin anticoagulants also inhibit DT-diaphorase (Ernster et al., 1962), and in this study, the in vitro effects of warfarin and difenacoum on this enzyme and on several other vitamin K related enzymes from the livers of normal and warfarin-resistant rats are compared.

Materials and Methods

Materials

Biochemicals and reagents used in these studies were obtained from the indicated sources: vitamin K_1, β-NADH, dichlorophenolindophenol, Tween-20, N^{ϵ}-*t*-Boc-L-lysine, *N*-acetylhomocysteine thiolactone, phosphatidylcholine type VIIE, dicetyl phosphate, and menadione (Sigma, St. Louis, MO); dioxane, 99 mol % acetonitrile, and trifluoroacetic acid (Fisher Scientific, Itasca, IL); dimethyl sulfoxide (Fisher Scientific, Itasca, IL, or Aldrich Chemical Co., Milwaukee, WI); dichloromethane and cyanogen bromide (Aldrich Chemical Co., Milwaukee, WI); dithiothreitol (Calbiochem, Los Angeles, CA); sodium warfarin (Endo, Garden City, NY); $NaH^{14}CO_3$ (Amersham/Searle, Arlington Heights, IL); Boc-Glu-Glu-Leu-OMe (Bachem, Torrance, CA, or gift of S.

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R. Lehrman, University of Wisconsin—Madison); menadione sodium bisulfite (Nutritional Biochemicals, Cleveland, OH); Triton X-100 (Research Products International, Elk Grove Village, IL); Konakion (Hoffmann-La Roche, Nutley, NJ). Technical grade difenacoum [3-(3-*p*-biphenylyl-1,2,3,4-tetrahydronaphth-1-yl)-4-hydroxycoumarin] was a gift of ICI Americas (Goldsboro, NC); ^{3}H-labeled vitamin K_1 was a gift of C. Siegfried, University of Nebraska. Sepharose 4B, DEAE-Sephadex A-50,[1] and Sephadex G-50 were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Silicic acid was Mallinckrodt SilicAR CC4 special; silica gel TLC plates were Machery-Nagel Polygram SIL G/UV (Brinkmann Instruments, Des Plaines, IL).

Methods

Treatment of Animals. Warfarin-susceptible male rats were obtained from the Holtzman Co. (Madison, WI) and fed a commercial rat diet (Purina Lab Chow No. 5012). Warfarin-resistant male rats were obtained from a breeding colony maintained in the laboratory, fed the same diet, and provided with drinking water supplemented with 1 mg/L menadione sodium bisulfite. Vitamin K deficiency was produced by placing rats in coprophagy-preventing cages (Metta et al., 1961) and feeding them a vitamin K deficient diet (Mameesh & Johnson, 1959) and giving them distilled water ad libitum for 10 days (warfarin-susceptible rats) or 3 days (resistant rats). All animals were fasted overnight before decapitation. Livers from a minimum of four animals were combined for each enzyme preparation. The standard error of the mean for triplicate assays of vitamin K dependent carboxylase activity or vitamin K epoxide reductase activity did not exceed 10% of the mean and for vitamin K epoxidase and DT-diaphorase activities did not exceed 5% of the mean. Calculations of the coumarin concentrations required for 50% inhibition of activity did not differ by more than 15% in replicated experiments.

Enzyme Preparations and Assay. Vitamin K epoxide reductase was assayed in freshly prepared liver microsomes. Livers were removed into ice-cold 0.25 M sucrose/0.025 M imidazole hydrochloride, pH 7.2 (SI buffer), blotted and weighed, and then minced and homogenized with a Brinkmann Polytron homogenizer in 4 mL of SI buffer/g of liver. Debris was removed by centrifugation at 10000*g* for 10 min, and microsomes were pelleted from the supernatant by centrifugation at 105000*g* for 60 min. The pellets were drained and resuspended in half the supernatant volume of cold SI buffer with a Dounce glass homogenizer with an A pestle. ^{3}H-Labeled vitamin K_1 2,3-epoxide (10 μg/mL; 4.5 μCi/mg) was added in ethanol to incubations containing 1 mM dithiothreitol and microsomal suspension equivalent to 0.5 g of liver/mL. Sodium warfarin was added in the microsomal buffer. Difenacoum was dissolved in benzene and added to the incubation tube, and the solvent was blown off with nitrogen gas before other additions. Samples (0.55 mL) were incubated at 27 °C for 40 min with rotary mixing before the reaction was stopped and metabolites of the vitamin extracted with 2 volumes of 2-propanol/hexane (3:2). The hexane layer was removed and evaporated, and 40 μL of a carrier solution containing 1–2 mg/mL each of vitamin K_1 and vitamin K_1 epoxide in hexane was added to each tube. A 10-μL aliquot was applied to mineral oil coated, plastic-backed silica gel thin-layer chromatography plates with a fluorescent indicator (Matschiner et al., 1970) and developed in 99 mol % acetonitrile. The vitamin K and vitamin K epoxide spots were cut and placed in scintillation vials with 10 mL of scintillation fluid, and the radioactivity was determined with external standardization on an LKB Ultrobeta 1210 liquid scintillation spectrometer.

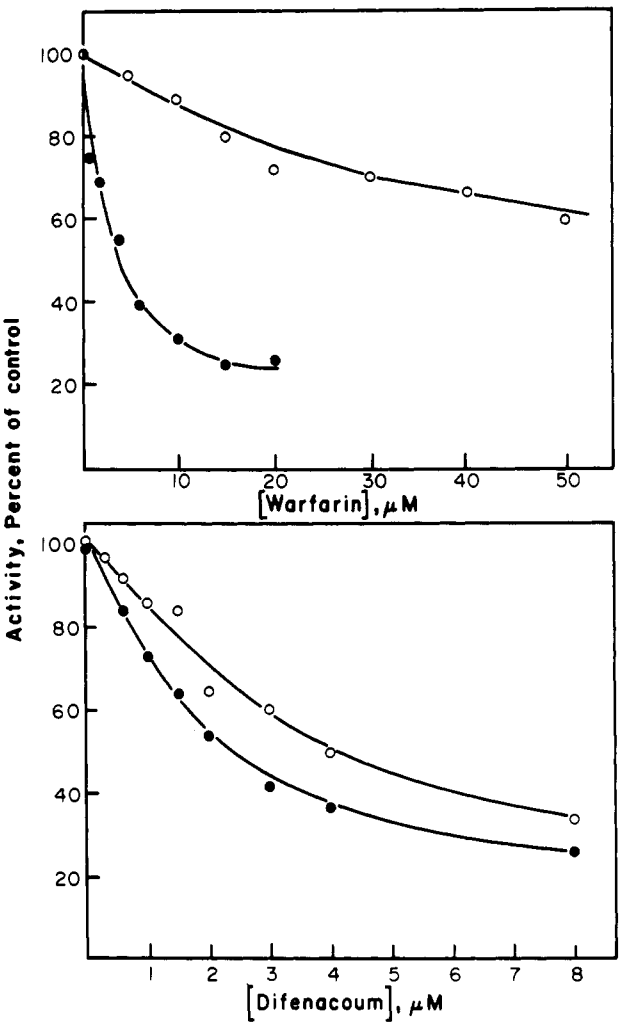

FIGURE 1: Coumarin sensitivity of liver microsomal vitamin K epoxide reductase activity. The conditions of the assay are described in the text, and the values plotted are the means of three separate experiments where each coumarin concentration point was the mean of triplicate assays. Activity was expressed as nanograms of vitamin K formed in 40 min per gram of liver, and the control incubations averaged 1025 for the normal rats and 460 for the warfarin-resistant rats. (●) Normal rats; (○) warfarin-resistant rats.

Detergent-solubilized microsomal preparations were prepared by homogenizing liver of vitamin K deficient rats in 2 mL/g SI buffer with a Potter-Elvehjem homogenizer. Microsomal pellets were obtained as described above, surface-washed with 0.25 M sucrose/0.5 M KCl/0.025 M imidazole hydrochloride, pH 7.2 (SIK buffer), and then quick-frozen and stored in liquid nitrogen. Thawed pellets were solubilized with a Dounce homogenizer in a volume of SIK buffer and 1.5% Triton X-100 equivalent to the supernatant volume and centrifuged at 105000*g* for 60 min. Vitamin K epoxidase activity was measured (Suttie et al., 1980) in incubations containing Triton-solubilized microsomes equivalent to 0.35 g of liver/mL of incubation, 2 mM Boc-Glu-Glu-Leu-OMe, 1 mM dithiothreitol, and 5 μg/mL ^{3}H-labeled vitamin K_1 hydroquinone (2.4 μCi/mg) added in ethanol. Assays were incubated for 15 min at 17 °C, stopped with the addition of 3:2 2-propanol/hexane, and analyzed for metabolites of vitamin K as described above. Sodium warfarin was added to incubations in 0.1 M Tris-HCl, pH 8.0, as 9% of the total

[1] Abbreviations: DEAE, diethylaminoethyl; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane; Me_2SO, dimethyl sulfoxide; NADH, reduced nicotinamide adenine dinucleotide.

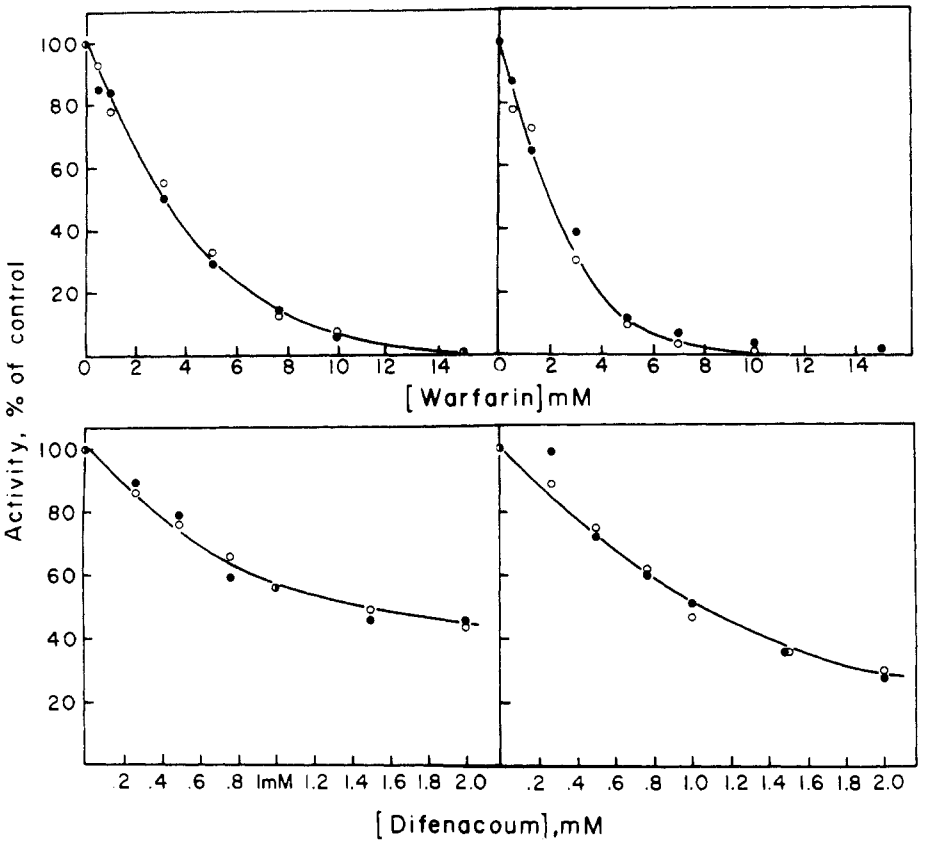

FIGURE 2: Coumarin sensitivity of liver microsomal vitamin K dependent carboxylase activity. The assays for the vitamin KH_2 dependent activity (left two panels) and the (vitamin K + NADH)-dependent activity (right two panels) are described in the text. The values plotted are the means of duplicate experiments where each coumarin concentration point was assayed in triplicate. Activity was expressed as dpm of $^{14}CO_2$ fixed in 15 min per gram of liver, and the control incubations averaged 10 800 for normal rats and 8840 for warfarin-resistant rats in the vitamin KH_2 dependent study and 1190 for normal rats and 3880 for warfarin-resistant rats in the vitamin K + NADH study. (●) Normal rats; (○) warfarin-resistant rats.

volume; difenacoum was added in dimethyl sulfoxide to 1.9% Me_2SO. Vitamin K dependent carboxylase activity was measured (Rich et al., 1981) in stoppered incubation tubes containing Triton-solubilized microsomes equivalent to 0.38 g of liver/mL of incubation, 0.4 mM Boc-Glu-Glu-Leu-OMe, 1 mM dithiothreitol, 25 μCi/mL $NaH^{14}CO_3$ (58 mCi/mmol), and 70 μg/mL vitamin K_1 hydroquinone added in ethanol. In some experiments 70 μg/mL vitamin K_1 quinone (Konakion) and 2 mM NADH were added, and the dithiothreitol was omitted. Samples were incubated for 15 min at 17 °C and then stopped by the addition of 5 volumes of 10% trichloroacetic acid. Precipitated material was pelleted by centrifugation, and fixed radioactivity in the supernatant was determined by bubbling CO_2 through the solution for at least 6 min and then mixing an aliquot with Aquasol and determining radioactivity in a liquid scintillation spectrometer. Additions of sodium warfarin were made in 0.1 M Tris-HCl, pH 8.0, as 9% of the total volume; additions of difenacoum were made in Me_2SO to a final concentration of 1.8% of the solvent.

Cytosolic DT-diaphorase was purified by affinity chromatography from the 105000*g* supernatant fraction collected from both vitamin K deficient and normal rats and kept at −20 °C. Thawed supernatant was centrifuged at 135000*g* for 45 min and 0.2% Triton X-100 added to the resulting supernatant before it was dialyzed extensively in 0.05 M Tris-HCl and 0.2% Triton X-100, pH 7.4. After dialysis, the preparation was centrifuged at 135000*g* for 45 min and applied to a menadione–Sepharose 4B affinity column prepared as described by Wallin et al. (1978). The column was washed with 200–300 mL of the dialysis medium and then 200–300 mL of 0.05 M Tris-HCl, pH 7.4, and eluted with 0.05 M Tris-HCl, pH 7.4, containing 50% dioxane. Fractions were assayed spectrophotometrically at 600 nm with 40 μM dichlorophenolindophenol (DCPIP), 100 μM NADH, and 8 mg/mL Tween-20 (Ernster, 1967). Active fractions were pooled and dialyzed into 0.05 M Tris-HCl, pH 8.6, applied to a DEAE-Sephadex A50 column equilibrated in the same buffer, and eluted with a linear gradient from the equilibration buffer to buffer containing 0.5 M NaCl. Active fractions were combined and salt was removed by dialysis. The purified DT-diaphorase was stored at −20 °C, and the thawed activity was measured under steady-state conditions with two different spectrophotometric assays. In the DCPIP assay (Ernster, 1967) an appropriate amount of enzyme was mixed in a cuvette with 14 μM DCPIP, 100 μM NADH, and 8.5 mg/mL Tween 20 in 0.05 M sodium phosphate buffer, pH 7.5. Sodium warfarin was added in 0.1 M Tris-HCl, pH 8.0, to 5% of the total volume; difenacoum was added in dioxane to 5% by volume. Initial rates were determined at 600 nm (ϵ = 21 000 M^{-1} cm^{-1}). For the liposome assay (Martius et al., 1975), membrane vesicles containing 3 mol % vitamin K in a lipid mixture of 95% phosphatidylcholine/5% dicetyl phosphate by weight were prepared by sonication with a Fisher Model 150 sonic dismembrator in 0.2 M $K_3Fe(CN)_6$/0.05 M Tris-HCl, pH 7.7, for 1 h at 20–24 °C under a gentle stream of nitrogen gas. The vesicles were separated from lipid residue by centrifugation at 100000*g* for 45 min and then passed down a Sephadex G-50 desalting column to remove all ferricyanide external to the vesicles. Suspensions of liposomes diluted to A_{436} = 0.50 were stored at 4 °C in the presence of 0.02% NaN_3 and warmed to room temperature for use in the diaphorase assay. Each cuvette contained 70% liposomal suspension, purified cytosolic enzyme,

and 100 μM NADH all in 0.05 M Tris-HCl, pH 7.7. Sodium warfarin was added in the assay buffer. Difenacoum was added in Me_2SO to 1% of the solvent by volume, a concentration shown not to appreciably perturb the liposomal suspension or the enzymatic activity.

Preparations of Vitamin K and Its Derivatives. All manipulations of vitamin K or its derivatives were performed under dim light. Evaporation of solutions was accomplished by warming with steam under a stream of nitrogen gas. Vitamin K concentrations were determined by UV absorbance at 248 nm in hexane solution with an absorptivity of 0.042 mg^{-1} mL^{-1}. Vitamin K 2,3-epoxide was measured at 226 nm with a value for absorptivity of 0.066 mg^{-1} mL^{-1}. Vitamin K_1 for all purposes other than a thin-layer chromatograph carrier was purified by column chromatography on hydrated silicic acid eluted with a stepwise gradient of benzene in hexane from 15 to 45% by volume (Matschiner et al., 1967). Tritiated vitamin K_1 of high specific activity was purified by reverse-phase thin-layer chromatography. Whatman KC-18F plates were prewashed in chloroform, developed with 99 mol % acetonitrile, and eluted with chloroform.

Chemical formation of vitamin K 2,3-epoxide was carried out as described by Fieser et al. (1941). Radioactive vitamin K epoxide was purified by the same thin-layer chromatographic procedure as was used for the tritiated vitamin. Vitamin K was reduced to the hydroquinone with sodium dithionite according to Sadowski et al. (1976). The product was dissolved in 95% ethanol and transferred to a nitrogen-purged serum vial. Low concentration hydroquinone solutions for epoxidation experiments were used immediately; more concentrated solutions for carboxylation experiments could be stored at −20 °C.

Results

Previous studies (Whitlon et al., 1978) demonstrated that the vitamin K epoxide reductase of warfarin-resistant rats was relatively insensitive to warfarin inhibition but was readily inhibited by the anticoagulant difenacoum. The data shown in Figure 1 verify that low micromolar concentrations of either warfarin or difenacoum will inhibit the epoxide reductase of normal rat liver microsomes but that only difenacoum is effective in microsomes of warfarin-resistant rat liver. Increasing the concentration of warfarin 10-fold resulted in only partial inhibition of the resistant rat epoxide reductase. It was repeatedly observed that the enzyme in normal rat liver microsomes gave a 2- to 3-fold greater rate of conversion of vitamin K 2,3-epoxide to its quinone than the comparably prepared enzyme from warfarin-resistant rat liver. The reason for the difference in activity between the two strains is unknown at the present time.

Other vitamin K dependent enzymes did not show this differential response to the two compounds. The liver microsomal vitamin K dependent carboxylase can be inhibited by warfarin only at concentrations unlikely to be present in liver even following large doses. The available data (Esmon & Suttie, 1976; Wallin et al., 1978; Bell & Stark, 1976) were obtained under conditions where (vitamin K + NADH)-supported carboxylation of endogenous protein was measured, and inhibition could represent an effect on either the vitamin K quinone reductase or the vitamin KH_2 dependent carboxylation reaction. Coumarin sensitivity of the carboxylase itself is best measured with vitamin KH_2 as a substrate, and it can be seen (Figure 2, left) that millimolar amounts of either coumarin are needed to produce significant inhibition of vitamin K dependent carboxylation under these conditions. When vitamin K + NADH was used as a substrate (Figure 2, right),

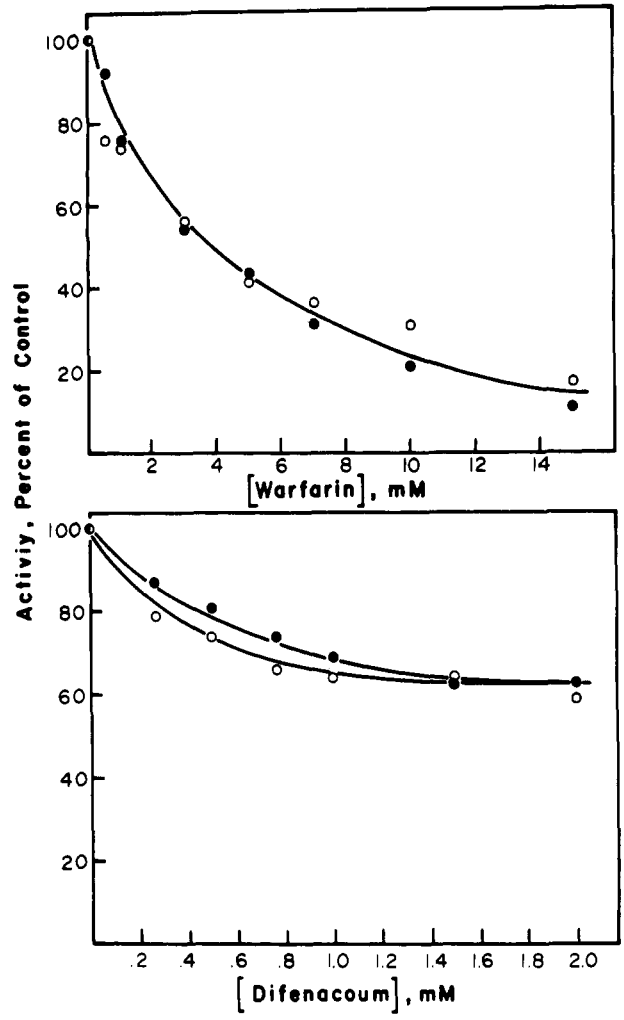

FIGURE 3: Coumarin sensitivity of the liver microsomal vitamin K epoxidase activity. The conditions of the assay are described in the text, and the values are from a single experiment with triplicate assays at each coumarin concentration. Activity was expressed as nanograms of vitamin K epoxide formed in 15 min per gram of liver, and the control incubations averaged 1620 for normal rats and 1350 for warfarin-resistant rats. (●) Normal rats; (○) warfarin-resistant rats.

the sensitivity to the inhibitors was slightly greater, but high concentrations were still necessary. With microsomal preparations obtained from the livers of both normal and warfarin-resistant rats and with either vitamin KH_2 or vitamin K + NADH as a substrate, difenacoum acted as a slightly better inhibitor than did warfarin.

The sensitivity of vitamin K epoxidase activity in Triton-solubilized microsomes from normal and warfarin-resistant rat liver to the two coumarins was also similar (Figure 3). The vitamin K epoxidase activity was somewhat less sensitive than the vitamin K dependent carboxylase to the two inhibitors. Bell & Stark (1976) found similar results for warfarin inhibition of epoxidation in a postmitochondrial supernatant. Difenacoum is a rather hydrophobic compound, and its limit of solubility in the aqueous-detergent solutions used in these experiments is approximately 2 mM. Higher concentrations could therefore not be tested.

The coumarin sensitivity of purified cytosolic DT-diaphorase is very much dependent on the assay used. The conventional assay employs a soluble dye, DCPIP, as a cosubstrate for NADH in the presence of the activator Tween 20. Under these conditions (Figure 4, left) there were no apparent differences between the normal rat enzyme and the enzyme from warfarin-resistant rats with respect to warfarin inhibition and only a minor difference in their sensitivities to difenacoum.

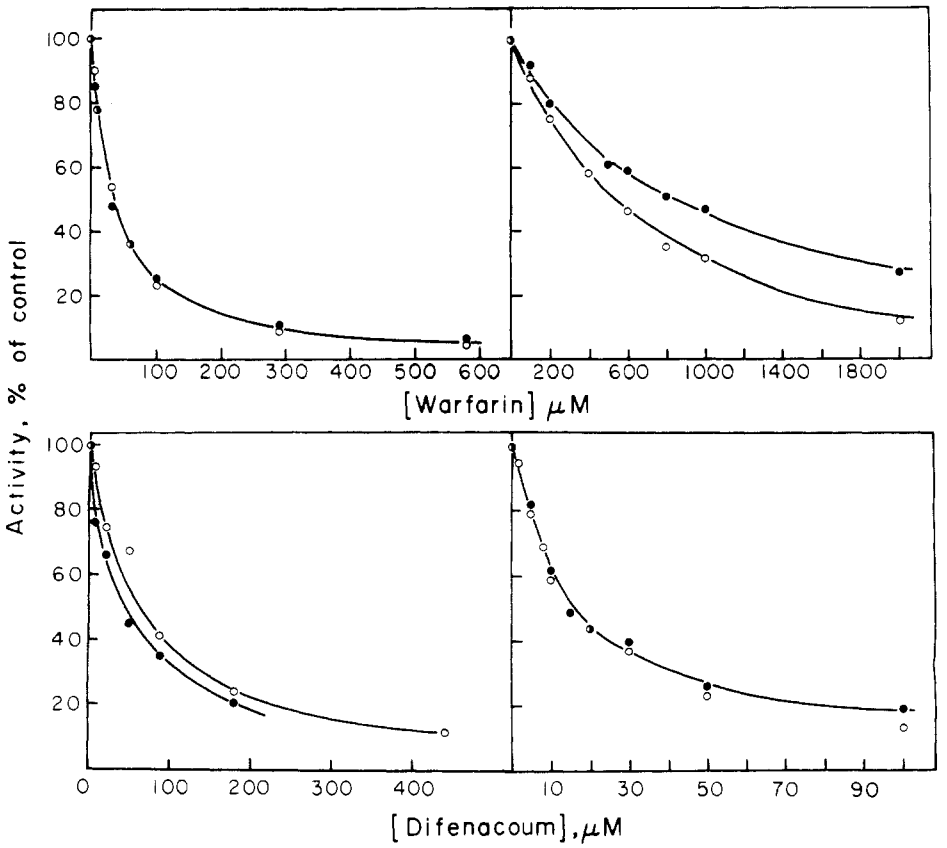

FIGURE 4: Coumarin sensitivity of liver DT-diaphorase activity. The assays utilizing DCPIP as a substrate (left two panels) and liposomal vitamin K_1 as a substrate (right two panels) are described in the text. The values plotted are from a single experiment with triplicate assays at each coumarin concentration. Sufficient amounts of the enzyme purified from either normal or warfarin-resistant rat liver to give convenient and accurate initial rates were used. (●) Normal rats; (○) warfarin-resistant rats.

The concentrations of either warfarin or difenacoum required to inhibit diaphorase activity were both in the micromolar range. The concentrations of warfarin or difenacoum needed to inhibit the diaphorase were quite different when vitamin K that has been incorporated into liposomes is used as the electron-accepting substrate (Figure 4, right). Some 20- to 30-fold greater concentrations of warfarin were required to effectively inhibit the diaphorase when the liposome assay was used than when the DCPIP assay was used. Conversely, the amount of difenacoum needed to cause inhibition was decreased by a factor of 3–4. Under the conditions of the liposome assay, a small difference in the warfarin sensitivity of the normal and warfarin-resistant rat enzymes became apparent.

Table I: Inhibitor Concentrations Giving 50% Inhibition

	warfarin		difenacoum	
	normal rat liver	resistant rat liver	normal rat liver	resistant rat liver
vitamin K dependent carboxylase				
vitamin KH_2 dependent	3 mM	3 mM	1.4 mM	1.4 mM
(vitamin K + NADPH)-dependent	2 mM	2 mM	1.0 mM	1.0 mM
epoxidase	4 mM	4 mM	>2.0 mM	>2.0 mM
DT diaphorase				
soluble assay	33 μM	30 μM	49.0 μM	67.0 μM
liposomal assay	840 μM	540 μM	17.0 μM	17.0 μM
epoxide reductase	4 μM	>50 μM	2.5 μM	4.0 μM

Discussion

A summary of the concentrations of warfarin and difenacoum required to produce 50% inhibition of each of the enzymatic activities measured is presented in Table I. The data presented in Table I and Figures 1–4 support the hypothesis that the inhibitory effect of the coumarin anticoagulants on vitamin K dependent clotting factor synthesis is due to their inhibition of the vitamin K epoxide reductase. This enzyme was the most sensitive of the various vitamin K dependent activities studied and was the only enzyme whose coumarin sensitivity was clearly altered when the enzyme was obtained from the livers of a warfarin-resistant strain of rats. These data are consistent with the previous indications of Whitlon et al. (1978) that an alteration in this enzyme is the major genetic alteration in the warfarin-resistant rat strain. It has been suggested (Fasco & Kaminsky, 1980; Fasco & Principe, 1980) that warfarin has an effect on reactions other than the epoxide reductase, but the data presented here are more consistent with a single site of action. There may, however, be undiscovered vitamin K reductase activities in microsomes that were not measured in this study. Their relative sensitivity to the two coumarins is not known. How the levels of coumarin needed to inhibit the vitamin K epoxide reductase in vitro compare to those found in anticoagulant-treated animals is difficult to assess, owing to complications resulting from intracellular distribution, partitioning between membrane and soluble phases, and binding to proteins. Although some differences in the coumarin sensitivities of the normal and warfarin-resistant DT-diaphorases were evident, these differences were of insufficient magnitude to be useful in explaining warfarin resistance. Similar results have been obtained by Friedman & Griep (1980). Strain differences in liver DT-diaphorase activity in warfarin-resistant rats have been reported by Ernster et al. (1972), but the lower activity in the resistant strain was subsequently found (Lind et al., 1973) to be due to their Wistar heritage. Whether this dif-

ference represents an altered enzyme form or merely a change in the steady-state amount of enzyme present in the liver has not been determined. The results obtained in this study suggest that the enzyme itself is somewhat altered in the strain of rats from which the resistant animals were derived.

The microsomal vitamin K reductase activity that participates in (Vitamin K + NADH)-dependent carboxylation has been shown to be comparable to the cytosolic DT-diaphorase in its warfarin sensitivity (Wallin et al., 1978). In this study the concentrations of warfarin or difenacoum that inhibit (vitamin K + NADH]-dependent carboxylation are appreciably higher than the amounts needed to inhibit the purified cytosolic DT-diaphorase, implying that the vitamin K reductase is not rate limiting with respect to carboxylation under the conditions at which these assays were carried out.

The ability of many detergent and lipid "activators" to modulate the kinetic properties of DT-diaphorase is well-known (Ernster et al., 1960; Hollander & Ernster, 1975). It is interesting that the properties of the enzyme with respect to inhibition by warfarin and difenacoum are so different when the liposome assay is used instead of the conventional DCPIP assay. With Tween 20 present as activator, warfarin and difenacoum were about equally effective as inhibitors, but when phospholipid vesicles replaced the detergent, the more lipophilic inhibitor difenacoum became a much better inhibitor than warfarin. These observations seem to be in keeping with the important but unexplained role of detergents and lipids in the kinetics of DT-diaphorase.

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