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# Vitamin K antagonism of coumarin anticoagulation

## A dehydrogenase pathway in rat liver is responsible for the antagonistic effect

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In the liver, it appears that there are two different pathways for vitamin K reduction. One pathway is irreversibly inhibited by coumarin anticoagulant drugs. The other pathway has been shown in the present study to be composed of enzymes that are not effected by physiological '*in vivo*' concentrations of these drugs. This pathway appears to be responsible for the antidotal effect of vitamin K in overcoming coumarin poisoning. In rat liver the pathway has been shown to be composed of DT-diaphorase (EC.1.6.99.2) and a microsomal dehydrogenase(s). The activity of the microsomal dehydrogenase(s) was 3.6-fold higher with NADH than with NADPH present in the test system. It appears that this enzyme is the physiologically important enzyme in the pathway. In contrast with DT-diaphorase, this enzyme(s) is shown to be tightly associated with the mirosomal membrane. The enzyme(s) is not identical with either of the quinone-reducing enzymes cytochrome *P*-450 reductase or cytochrome-$b_5$ reductase. Our data thus postulate the existence of an as-yet-unidentified microsomal dehydrogenase that appears to have an important function in the pathway.

## INTRODUCTION

In the liver, vitamin K is a necessary cofactor for a microsomal enzyme, the vitamin K-dependent carboxylase, that converts inactive precursor forms of the vitamin K-dependent blood-clotting factors to coagulation zymogens (Suttie, 1978, 1980). This is achieved by carboxylation of specific glutamic acid residues in the precursor proteins, thus converting them to γ-carboxyglutamic acid-containing proteins (Suttie, 1978, 1980). Before it can function as a cofactor, vitamin K must be reduced to its hydroquinone form (Suttie, 1980; Witlon *et al.*, 1978). Concomitant with carboxylation of the protein precursors, the reduced vitamin cofactor is converted into vitamin K 2,3-epoxide (Sadowski *et al.*, 1977; Witlon *et al.*, 1978).

The microsomal enzyme vitamin K epoxide reductase catalyses reduction of vitamin K 2,3-epoxide (Suttie, 1978). It is still a matter of controversy whether the same enzyme or another enzyme with very similar properties to vitamin K epoxide reductase can also reduce vitamin K quinone to the fully reduced hydroquinone form of the vitamin (Fasco *et al.*, 1982; MacNicoll *et al.*, 1984; Lee & Fasco, 1984).

Vitamin K epoxide reductase and the postulated enzyme vitamin K quinone reductase are highly sensitive to inhibition by coumarin drugs, and it is believed that this inhibition is responsible for their anticoagulant effect (Suttie, 1978; Fasco & Principe, 1982*a*). The coumarins are irreversible inhibitors of these 'enzymes' (Fasco & Principe, 1982*a*). It is also known that vitamin K, when given in high doses, can overcome coumarin inhibition of clotting-factor synthesis and thus work as an antidote in cases of coumarin poisoning in animals (Bell *et al.*, 1972; Hart *et al.*, 1984) and humans (Van der Meer *et al.*, 1968).

It appears that the antidotal effect of vitamin K is mediated by a group of nicotinamide-nucleotide-dependent dehydrogenases that are very little effected by coumarin drugs (Suttie, 1980; Wallin & Hutson, 1981). One of these enzymes, DT-diaphorase (EC. 1.6.99.2), has been purified and studied extensively in order to elucidate its role in vitamin K reduction in rat liver (Ernster *et al.*, 1972; Fasco & Principe, 1982*b*; Wallin, 1979; Wallin *et al.*, 1978).

Thus the two pathways for vitamin K reduction in the liver are: (i) reduction pathway I, the physiologically important pathway, consisting of the highly warfarin-sensitive vitamin K-epoxide reductase and possibly vitamin K quinone reductase, and (ii) reduction pathway II, a pathway consisting of several nicotinamide-nucleotide-dependent dehydrogenases. Since coumarin inhibition of pathway I is essentially irreversible (Fasco & Principe, 1982*a*), it follows that pathway II must be responsible for the enhanced clotting-factor synthesis triggered by high concentrations of vitamin K in the presence of these drugs. In order to understand how vitamin K antagonizes these drugs, it is essential to know the properties of pathway II. This was the objective of the present study.

## EXPERIMENTAL

### Materials

Vitamin $K_1$ was purchased from Sigma. The vitamin was reduced to vitamin $K_1$ hydroquinone with dithionite as described by Sadowski *et al.* (1976). AquaMEPHYTON (vitamin $K_1$; 10 mg/ml) was obtained from Merck, Sharp and Dohme. Menadione, type III horse heart cytochrome *c*, NADH, NADPH, Tween 20, Triton X-100, cholic acid, n-octyl β-D-glucopyranoside, warfarin, dicumarol,

Abbreviations used: VAPONA, 2,6-dichlorovinyl dimethyl phosphate; DTT, dithiothreitol; DCPIP, 2,6-dichlorophenol-indophenol; vitamin $K_1H_2$, fully reduced vitamin $K_1$; difenacoum, 3-(3-*p*-biphenylyl-1,2,3,4-tetrahydronaphth-1-yl)-4-hydroxycoumarin; warfarin, 3-(α-acetonylbenzyl)-4-hydroxycoumarin.

salicylic acid and imidazole were from Sigma. The pentapeptide Phe-Leu-Glu-Glu-Leu was purchased from Vega–Fox Biochemicals Division (Tucson, AZ, U.S.A.). $NaH^{14}CO_3$ (60 mCi/mmol) was from Amersham Corp. DCPIP (sodium salt) was from Koch–Light Laboratories (Colnbrook, Bucks., U.K.). Sepharose CL-4B, activated CH-Sepharose 4B, 5′-AMP–Sepharose 4B, 2′,5′-ADP–Sepharose 4B, Blue Sepharose CL-6B and Sephadex G-25 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). The insecticide VAPONA was a gift from Shell Oil Co. (Houston, TX, U.S.A.). Technical-grade difenacoum was kindly provided by ICI American (Goldsboro, NC, U.S.A.). All other chemicals were reagent grade or better.

### Preparation of microsomes

Male Wistar rats (250–300 g) (Charles River Laboratories) were given 15 mg/kg of warfarin as intraperitoneal injections. The rats were fasted for 24 h and killed by decapitation. Livers were perfused with ice-cold saline (0.9% NaCl) through the portal vein and and homogenized as described by Suttie *et al.* (1976) in 0.25 M-sucrose/0.025 M-imidazole, pH 7.2 (SI-buffer) containing 1 mM-VAPONA (SI-VAPONA buffer). The homogenate was centrifuged twice at 10000 *g* and then at 100000 *g* for 60 min. The pellets from the 100000 *g* spin were resuspended in the SI-VAPONA buffer and repelleted a second time by centrifugation at 100000 *g* for 60 min. The supernatant was discarded and the pellets stored in liquid $N_2$ with no detectable loss of carboxylase activity.

### Treatment of microsomes

Microsomes were solubilized in 0.25 M-sucrose/0.5 M-KCl/1.5% Triton X-100/0.025 M-imidazole, pH 7.2 (SIK-buffer), containing 1 mM-VAPONA. Undissolved material was removed by centrifugation at 100000 *g* for 60 min. All fractions containing solubilized microsomes were dialysed against a manganese-containing imidazole buffer [10% (v/v) glycerol/0.5 M-KCl/0.5% Triton X-100/10 mM-$MnCl_2$/1 mM-DTT/0.025 M-imidazole, pH 7.2] as recommended by Wallin & Suttie (1980). Before dialysis, 1 mM-VAPONA was added to the buffer.

Washing and sonication of microsomes were performed as follows: microsomes were suspended in a Dounce homogenizer in 0.15 M-KCl/0.025 M-imidazole, pH 7.2, and then centrifuged at 100000 *g* for 45 min. The supernatant was discarded and the pellet subjected to one more wash, followed by centrifugation at 100000 *g*. The pellet was than resuspended in the KCl/imidazole buffer and sonicated on ice for 1.5 min in an Ultrasonic sonciator at maximal setting using a 50% duty cycle.

### Enzyme activities

Vitamin K-dependent carboxylase activity was measured as $^{14}CO_2$ incorporation into the synthetic peptide substrate Phe-Leu-Glu-Glu-Leu as described by Esmon & Suttie (1976). Incubation mixtures contained 2 mM-peptide. Unless stated otherwise, incubations were carried out for 30 min at 25 °C in the presence of either (i) chemically reduced vitamin $K_1$ (100 $\mu$g/ml) or (ii) vitamin $K_1$ (100 $\mu$g/ml) + NAD(P)H (2 mM). The activity of [vitamin $K_1$ + NAD(P)H]-supported carboxylase activity was measured relative to the activity measured with chemically reduced vitamin $K_1$.

DT-diaphorase activity was measured at room temperature as described by Dallner (1963), with DCPIP as electron acceptor. The reaction was monitored at 600 nm. An absorption coefficient ($\epsilon$) of 21000 $M^{-1} \cdot cm^{-1}$ for DCPIP was used to calculate enzyme activity. Cytochrome *c* reductase activity was measured at room temperature in 0.05 M-potassium phosphate, pH 7.5, containing Tween 20 (0.8 mg/ml), 75 mM-cytochrome *c* and 0.1 mM-NADPH. Enzyme activity was measured as the reduction of cytochrome *c* monitored at 550 nm. An $\epsilon$ value of 18500 $M^{-1} \cdot cm^{-1}$ was used (Hollander & Ernster, 1975). Ferricyanide reductase activity was measured at room temperature in 0.1 M-potassium phosphate, pH 7.5, containing Tween 20 (0.8 mg/ml), 0.67 mM-potassium ferricyanide and 0.1 M-NADH. An $\epsilon_{420}$ value of 1,020 $M^{-1} \cdot cm^{-1}$ was used to measure the reduction of ferricyanide (Schellenberg & Hellerman, 1958). The activity of mitochondrial citrate synthase was measured as described by Merrera & Frinkel (1967) after sonication of the samples as described by Oram *et al.* (1975). Lactate dehydrogenase was assayed as described by Kornberg (1955).

### Preparation of affinity resins

DT-diaphorase was purified from rat liver cytosol to electrophoretic homogeneity as described by Wallin & Little (1984). Antibodies against the enzyme were raised in rabbits as previously described (Wallin, 1979). IgG was purified from antisera as described by Fahey (1967) and coupled to Sepharose by using activated CH-Sepharose 4B. The coupling reaction was carried out as outlined in the Pharmacia Instruction Manual. Menadione–Sepharose was prepared as described by Wallin & Little (1984).

### Immunoadsorption

Microsomes solubilized in SIK-buffer were gel-filtrated on Sephadex G-25 in 0.025 M-imidazole/0.5 M-NaCl/0.2% Triton X-100, pH 7.2. DT-diaphorase that was in the void-volume fraction was removed by immunoadsorption on a column of anti-(DT-diaphorase) IgG–Sepharose equilibrated in the same buffer. DT-diaphorase was removed from cytosol using the same procedure, except that Triton X-100 was omitted from the buffer.

### Affinity chromatography

Microsomes were solubilized in SIK-buffer and DT-diaphorase removed by immunoadsorption as described above. The immunoadsorbed material was gel-filtrated on Sephadex G-25 in 0.025 M-imidazole/0.2% Triton X-100, pH 7.2, before being loaded on to a column of 2′,5′-ADP–Sepharose, 5′-AMP–Sepharose or Blue Sepharose equilibrated in the same buffer. Cytochrome *c* reductase and ferricyanide reductase activities were measured in fractions eluted from the columns. These activities were used to evaluate the specificity and efficiency of the various affinity resins to retain NADH- and NADPH-dependent enzymes.

### Other methods

Rat liver mitochondria were isolated by standard techniques of differential centrifugation (Schneider & Hogeboom, 1950) in a buffered (pH 7.0) medium containing 0.225 M-mannitol/0.075 M-sucrose/5 mM-Mops and 50 $\mu$M-EDTA. Protein was measured by using the Bio-Rad protein assay.

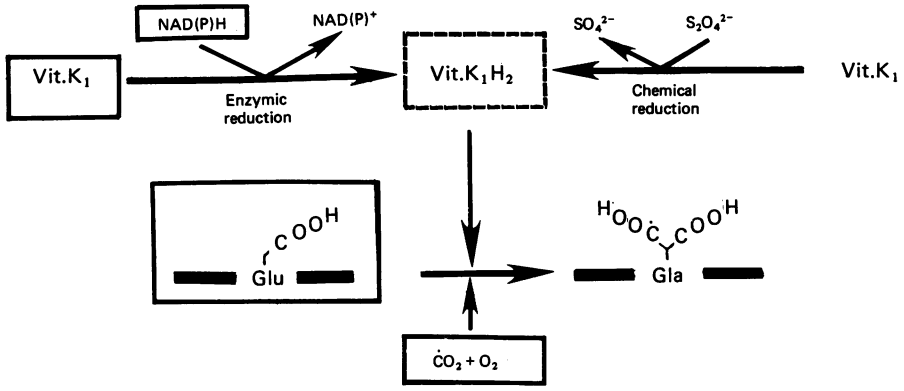


**Fig. 1. Carboxylation test system**

Vitamin $K_1H_2$ cofactor can either be produced by pathway II (see the text) or the chemically reduced cofactor can be added to the test system. The compounds that are enclosed by rectangles are necessary for a functional test system. Abbreviations used: $\dot{C}O_2$, $^{14}C$ radioactively labelled $CO_2$; Gla, $\gamma$-carboxyglutamic acid; Vit., vitamin.

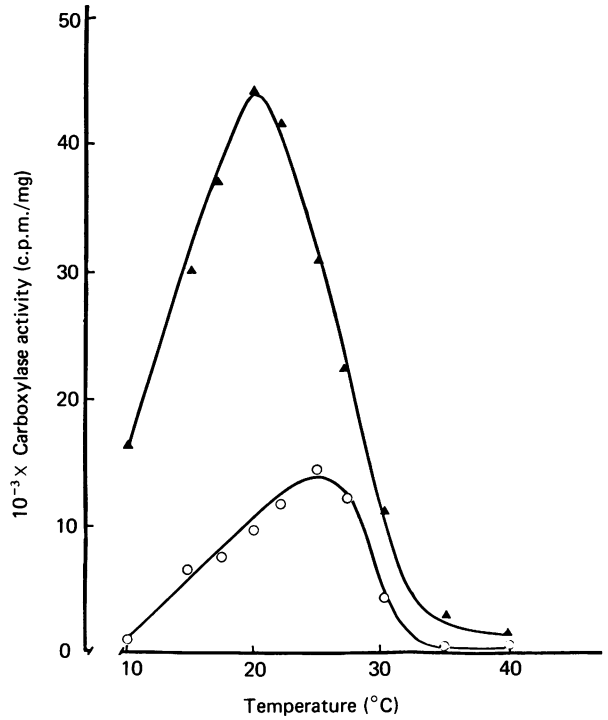


**Fig. 2. Dependence of carboxylation on temperature**

Carboxylase activity supported by vitamin $K_1H_2$ (▲) and vitamin $K_1$+NADH (○) respectively was measured at the different temperatures shown. Each value is the average for duplicate incubations, the duplicates differing by less than 5%.

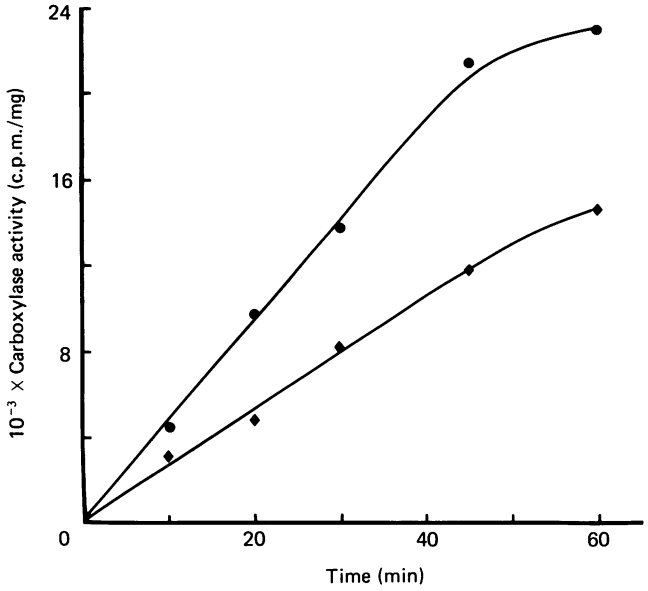


**Fig. 3. Effect of VAPONA on carboxylase activity**

Carboxylase activity supported by vitamin $K_1$+NADH was measured at 25 °C at the various time points shown. Activity was measured in the absence (◆) or presence (●) of 1 mM-VAPONA. Each value is the average for duplicate incubations, the two values differing by less than 5%.

## RESULTS

Vitamin K epoxide reductase and the postulated vitamin K quinone reductase are inactive in Triton X-100. Thus solubilization of the microsomes in Triton X-100 allowed us to study vitamin $K_1$ cofactor production by pathway II independent of pathway I. Our test system is illustrated in Fig. 1. Vitamin $K_1$+NAD(P)H, or vitamin $K_1H_2$, were added to the system in concentrations that have been shown to be saturating for the carboxylation reaction (Suttie *et al.*, 1979). Optimum carboxylase activity measured with vitamin $K_1H_2$ and (vitamin $K_1$+NADH) was at 20 and 25 °C respectively (see Fig. 2). A temperature optimum of 20 °C for vitamin $K_1H_2$-supported carboxylation has also been reported by others (Suttie *et al.*, 1979). A higher temperature optimum (25 °C) was found for (vitamin $K_1$+NADH)-supported carboxylation, which involves the pathway-II enzymes. However, significant activities were measured with both forms of the vitamin at 25 °C and this allowed us to carry out our studies at the temperature optimum for pathway-II-supported carboxylation.

A plot of pathway-II-supported carboxylation versus time with and without the addition of the proteinase inhibitor VAPONA is shown in Fig. 3. VAPONA (1 mM) was added to the reconstitution buffer before dialysis of

**Table 1. Effect of immunoadsorption on carboxylase activity**

Carboxylase activity supported by vitamin $K_1$+NAD(P)H was measured in solubilized microsomes before and after immunoadsorption on anti-(DT-diaphorase) IgG–Sepharose (see the Experimental section). All activities were measured relative to the activity measured in solubilized microsomes with vitamin $K_1$+NADH. The results obtained after addition of purified DT-diaphorase (2.4 $\mu$g) to the test system are also shown. The results are averages for triplicate incubations and did not differ more than 5%. The control activity (100%) was 72000 c.p.m. of product/min per mg of protein.

| | Carboxylase activity (% of control) | | | |
|---|---|---|---|---|
| | Before immunoadsorption | | After immunoadsorption | |
| Additions | NADH | NADPH | NADH | NADPH |
| None | 100* | 28 | 50 | 16 |
| DT-diaphorase | 178 | 130 | 173 | 155 |

* All activities were measured relative to this control activity.

the test samples. The presence of VAPONA resulted in a significant increase in carboxylase activity, which suggested that proteolytic degradation was occurring in our test system with the Triton X-100-solubilized microsomes. Fig. 3 also illustrates that the reaction was linear for 30 min. Linear rates were also observed when DT-diaphorase was removed from the test system (results not shown). Thus a 30 min assay could be used to measure initial velocities.

Antibodies against DT-diaphorase were immobilized on Sepharose and used to remove the enzyme from the carboxylation test system. Immunoadsorption of Triton X-100-solubilized microsomes was carried out in the presence of 0.5 M-KCl. At this salt concentration, cytochrome *c* reductase and ferricyanide reductase were eluted unretarded through the immunoaffinity column, which indicated that non-specific adsorption of microsomal protein to the column resin was insignificant.

The immunoaffinity column removed all DT-diaphorase activity. Removal of this enzyme was verified by showing that dicumarol-sensitive diaphorase activity was absent in the unretarded fraction (Ernster, 1967).

We then studied how removal of DT-diaphorase from pathway II affected the carboxylation test system. As shown in Table 1, immunoadsorption resulted in a 50% loss of pathway-II-supported activity. Consistent with previous observations (Wallin & Hutson, 1981), immunoadsorption had no inhibitory effect on carboxylase activity supported by vitamin $K_1H_2$ (results not shown). When an identical sample was eluted through a non-immune-IgG–Sepharose matrix, a 100% recovery of pathway-II-supported activity was measured. Also the ratio between carboxylase activities triggered with vitamin $K_1H_2$ and (vitamin $K_1$+NADH) in soluble microsomes before and after chromatography was unchanged. This control showed that the IgG ligand was responsible for loss of pathway-II activity. This experiment provided evidence that the carboxylation system *in vitro* contains a vitamin $K_1$-reducing dehydrogenase(s) that is immunologically different from DT-diaphorase. This enzyme(s) provided 50% of the reduced vitamin $K_1$ cofactor in our carboxylation system *in vitro*. The enzyme(s) apparently has a preference for NADH over NADPH, since carboxylase activity was 3.6-fold higher with NADH present in the test system (Table 1).

The participation of DT-diaphorase in pathway II is demonstrated clearly in Table 1. The addition of 2.4 $\mu$g of purified enzyme to solubilized microsomes and immunoadsorbed microsomes enhanced (vitamin $K_1$+NADH)-supported activity 78% and 123% respectively. DT-diaphorase is a flavoprotein that uses NADH and NADPH equally well as reductants (Ernster, 1967). This is reflected in the experiments with added DT-diaphorase, where NADPH becomes a significantly better reductant for vitamin $K_1$ reduction (Table 1).

The second enzyme in pathway II appears to be relatively unaffected by coumarin drugs and the platelet inhibitor salicylate (see Fig. 4). When these drugs were added to solubilized microsomes in which DT-diaphorase had been removed by immunoaffinity chromatography, neither of the drugs inhibited pathway-II-supported carboxylation significantly (Fig. 4). At high concentrations all drugs affected the vitamin K-dependent carboxylase enzyme itself, consistent with what has been reported by Hildebrandt & Suttie (1982). Thus the inhibition of carboxylation seen at high concentrations of the drugs does not appear to result from inhibition of the second enzyme, but rather from inhibition of the carboxylase enzyme. Difenacoum was the most potent inhibitor of the carboxylase enzyme and caused a 50% inhibition at a concentration of 0.5 mM (Fig. 4*c*).

We also studied the kinetics of the effect of the drugs on purified DT-diaphorase. All drugs exhibited competitive inhibition with NADH. From Lineweaver–Burk plots we calculated the inhibition constants ($K_i$) shown in Table 2.

The data from these experiments suggest that these drugs should have no significant effect on pathway-II-supported carboxylation *in vivo*. Liver concentrations of coumarin drugs have been measured in the lower micromolar range (Bachmann & Sullivan, 1983; Zimmerman & Matschiner, 1974). These concentrations are significantly lower than the drug concentrations we have shown to effect pathway II.

The next objective in characterizing pathway II was to investigate the mode of attachment of the pathway-II enzymes to the microsomal membrane. Subjecting the microsomal pellet to three washes in ice-cold saline and sonication caused a complete loss of dicumarol-sensitive diaphorase activity. Therefore this treatment resulted in removal of DT-diaphorase from the membrane vesicles. When pathway-II-supported carboxylation activity was

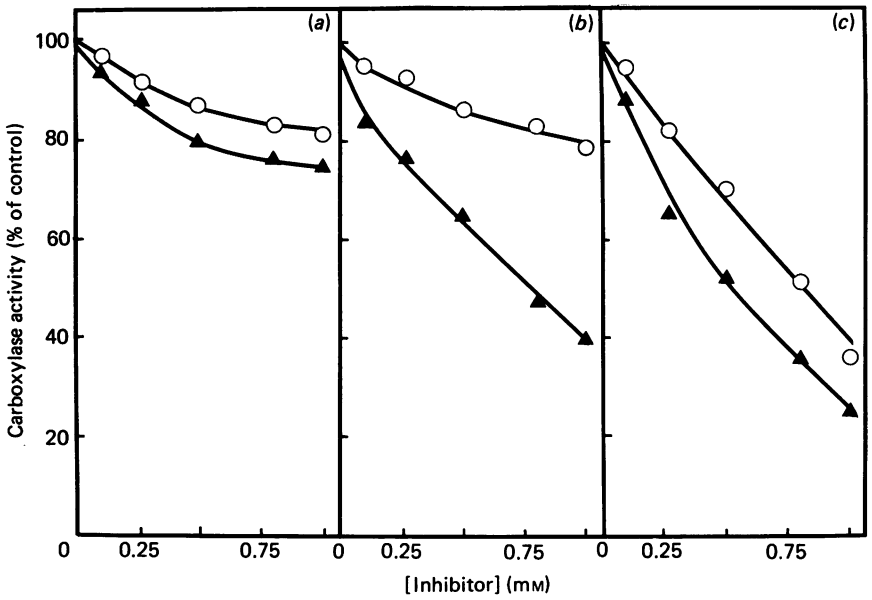


**Fig. 4. Effect of salicylate, warfarin and difenacoum on carboxylation in immunoadsorbed microsomes**

The effect of the anticoagulants on vitamin $K_1H_2$ (○)- and vitamin $K_1$+NADH (▲)-supported carboxylation was measured at 27 °C. Difenacoum was added to the test system in dimethyl sulphoxide (final concn. 2%) which by itself resulted in 8 and 12% inhibition of vitamin $K_1H_2$- and (vitamin $K_1$+NADH)-supported activity respectively. Inhibition by difenacoum was measured relative to a control containing 2% dimethyl sulphoxide. Each value is the average for triplicate incubations, the three results differing by less than < 5%. (*a*) Salicylate inhibition; (*b*) warfarin inhibition; (*c*) difenacoum inhibition.

**Table 2. Inhibition of DT-diaphorase by salicylate and coumarin drugs**

The effect of salicylate, warfarin and difenacoum on purified DT-diaphorase was measured in the diaphorase test system described in the Experimental section. Inhibition constants ($K_i$) were obtained from Lineweaver–Burk plots. For these plots the concentration of NADH was varied while the concentration of DCPIP was kept at 40 μM.

| Inhibitor | Inhibition constant, $K_i$ (μM) |
|---|---|
| Salycilate | 24 |
| Warfarin | 8 |
| Difenacoum* | 12 |

*Difenacoum was delivered to the test system in dioxan (final concn. 0.55%), which by itself resulted in 3% inhibition of activity.

measured in the washed and sonicated microsomes, 50% of the carboxylation activity was lost. A 50% loss of activity was also seen after immunoadsorption of microsomes (Table 1). Thus it appears that the activity of the second enzyme(s) was unaffected by the wash-and-sonication procedure, which suggests a tight association of the enzyme(s) with the membrane. On the other hand, the results also suggest that DT-diaphorase is only loosely associated with the membrane.

Contamination of microsomes by cytosol and mitochondria was measured by using marker enzymes for these subcellular fractions. Lactate dehydrogenase and citrate synthase activities were used as markers for cytosol and mitochondria respectively. On the basis of the specific activities of the enzymes in cytosol and purified mitochondria, contamination of microsomes by the respective subcellular fractions were calculated to be 1.4% and 2.5% respectively (Table 3). When purified mitochondria and cytosol were added to washed and sonicated microsomes, the effect on carboxylase activity was as shown in Table 4. Mitochondria had a small activating effect on the carboxylase, but inhibited (vitamin $K_1$+NADH)-supported carboxylation by 50%. Cytosol from which DT-diaphorase had been removed (see the Experimental section) had no effect on (vitamin $K_1$+NADH)-supported carboxylase activity. The results from these experiments indicate that the second enzyme(s) is located in the endoplasmatic reticulum, which is also the site of location for the vitamin K-dependent carboxylase (Wallin & Prydz, 1979).

Further characterization of pathway II and its enzymes depended on a successful separation of these enzymes from the vitamin K-dependent carboxylase. As shown in the present study, separation of DT-diaphorase from the carboxylase is easily achieved (Table 1). To separate the second enzyme in the pathway from the carboxylase we tried various affinity supports with specificity for NAD(P)H-dependent enzymes. Chromatography on 5′-AMP–Sepharose, 2′,5′-ADP–Sepharose and Blue Sepharose is shown in Fig. 5. Chromatography was carried out on solubilized microsomes where DT-diaphorase had been removed by immunoaffinity chromatography. Recoveries of enzyme activities in the pooled fractions from the column eluates are shown in Table 5. 2′,5′-ADP–Sepharose (Fig. 5*b*) removed all cytochrome *c* reductase activity from the carboxylation test system. However, 32% of the ferricyanide reductase was

**Table 3. Contamination of microsomes by cytosol and mitochondria**

Microsomes were washed and sonicated as described in the Experimental section. The activities of the marker enzymes lactate dehydrogenase (LDH) and citrate synthetase (CS) were measured in cytosol, in purified mitochondria and in washed and sonicated microsomes (see the Experimental section).

| Fraction | Marker enzyme | Specific activity of marker enzyme (μmol/min per mg) | Contamination of microsomes* (%) |
|---|---|---|---|
| Cytosol | LDH | 3.89 | 11.2† |
| | | | 3.6‡ |
| | | | 1.4§ |
| Mitochondria | CS | 0.20 | 1.4† |
| | | | 1.9‡ |
| | | | 2.5§ |

* Contamination of microsomes by cytosol and mitochondria was calculated on the basis of the measured specific activities of the marker enzymes in cytosol, purified mitochondria and the various microsomal preparations.
† Routine microsomal preparation.
‡ Microsomes subjected to one wash in 0.15 M-KCl.
§ Microsomes subjected to three washes in 0.15 M-KCl and sonication.

**Table 4. Effect of mitochondria and cytosol on carboxylation**

Carboxylase activity supported by vitamin $K_1H_2$ and vitamin $K_1$ + NAD(P)H was measured at 25 °C in washed and sonicated microsomes before and after addition of purified mitochondria (1.4 mg/ml) and cytosol (0.6 mg of cytosolic protein/ml) respectively. Washed and sonicated microsomes represent microsomes subjected to the wash and sonication procedure described in the Experimental section. Results are averages for triplicate incubations and differed by less than 5%. Carboxylase activity is presented as c.p.m./mg of microsomal protein.

| Additions | $10^{-3}$ × Carboxylase activity (c.p.m./mg) | | |
|---|---|---|---|
| | Vitamin $K_1H_2$ | Vitamin $K_1$ + NADH | Vitamin $K_1$ + NADPH |
| None | 52.1 | 12.5 | 3.1 |
| Mitochondria | 55.2 | 7.3 | 2.0 |
| Cytosol* | 49.3 | 11.2 | 3.0 |

* Cytosol was made deficient in DT-diaphorase as described in the Experimental section.

recovered in the pooled unretarded fraction (Fig. 5*b*, fraction B). When chromatographed on 5′-AMP–Sepharose (Fig. 5*a*) cytochrome *c* reductase and ferricyanide reductase activities were recovered in 70 and 15% yield in the unretarded pooled fraction (Fig. 5*a*, fraction A). For both columns a fraction of retained protein with high ferricyanide reductase activity could be eluted with 1.5 M-NaCl in the equilibration buffer. The different abilities of these affinity supports to bind cytochrome *c* reductase and ferricyanide reductase agree well with their specificities towards NADPH- and NADH-specific enzymes. Blue Sepharose (Fig. 5*c*) retained less of the two activities than expected from its known binding capacity for nucleotide-requiring enzymes (Subramanin, 1984). The unretarded pooled fraction from this column (Fig. 5*c*, fraction C) contained 20 and 60% of the ferricyanide reductase and cytochrome *c* reductase activities that were applied to the column.

The effect of chromatography on carboxylase activity in the unretarded fractions from the affinity columns is shown in Table 6. Carboxylase activity was also measured in fraction A (Fig. 5*a*) after addition of 1000 units of ferricyanide reductase from reaction D (Fig. 5*a*). The ratio between the activities measured with vitamin $K_1H_2$ and pathway II (vitamin $K_1$ + NADH) was constant in all fractions studied, and was the same as the ratio measured with solubilized microsomes (Table 6). We found that this ratio was unchanged in dilutions of solubilized microsomes (results not shown). These dilutions were adjusted to give the same protein concentrations as those measured in the unretarded fractions (range 4–5 mg/ml). Since the ratio was the same in the unretarded fractions, this suggests that none of the affinity resins bound the second enzyme(s) in pathway II. Also, the data strongly suggest that cytochrome *c* reductase and ferricyanide reductase are not involved in vitamin $K_1$ cofactor production by pathway II.

We also tried chromatography on menadione–Sepharose. Although this affinity resin efficiently removed DT-diaphorase from solubilized microsomes (Wallin *et al.*, 1978), removal of the second vitamin $K_1$-reducing enzyme(s) in pathway II was not successful. A variety of different chromatographic conditions were tried, but the enzyme would not bind to the menadione affinity ligand.

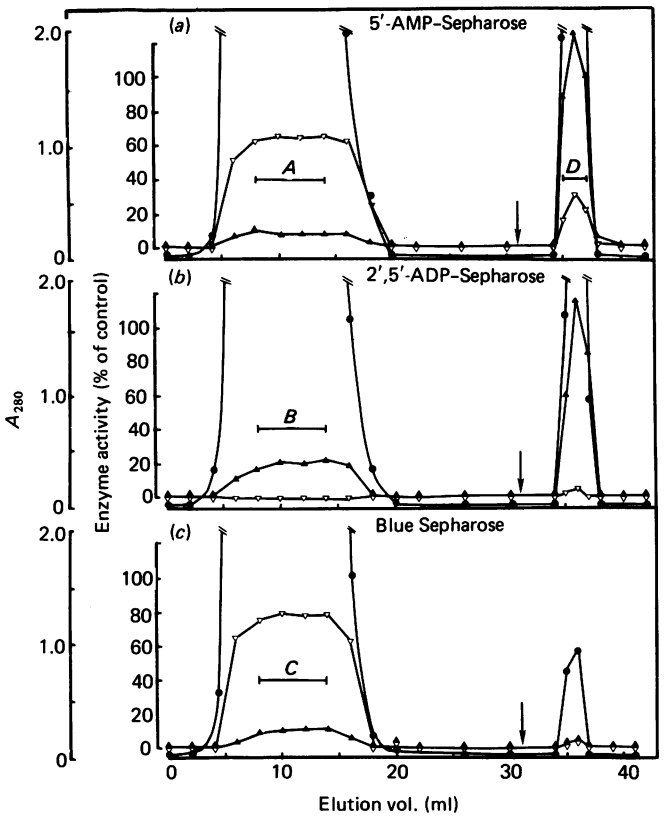


**Fig. 5. Affinity chromatography of immunoadsorbed microsomes**

Solubilized microsomes were immunoadsorbed on anti-(DT-diaphorase) IgG–Sepharose and subsequently subjected to chromatography on 5'-AMP–Sepharose (*a*), 2',5'-ADP–Sepharose (*b*) and Blue Sepharose (*c*) respectively as described in the text. Samples (10 ml) were applied to each of the affinity columns, all of which had a bed volume of 8.8 $cm^3$. A, B, C and D indicate pooled fractions from the various peaks of protein eluted from the columns. ▽, Cytochrome *c* reductase activity; ▲, ferricyanide reductase activity; ●, $A_{280}$. The arrow indicates start of elution with 1.5 M-NaCl in the column equilibration buffer. Enzyme activity is presented as a percentage of that measured in the sample applied to the column.

## DISCUSSION

The ability of vitamin $K_1$ to function as an antidote in cases of poisoning with coumarin drugs is clinically important. In order to understand the mechanism underlying this effect we used the solubilized carboxylation system *in vitro* described by Esmon & Suttie (1976). In this system, Triton X-100 is the solubilizing agent. The detergent inactivates the pathway-I enzymes and thus restricts reduction of vitamin $K_1$ to other enzymes in the liver. In addition, use of this system allowed us to use chromatography to study the enzymes involved in vitamin $K_1$ metabolism and provided an excellent medium for antigen–antibody reactions. Our data suggest that vitamin $K_1$ cofactor production by pathway II is not affected by difenacoum and warfarin at physiological concentrations of the drugs. Also salicylate had no effect on pathway-II-supported carboxylation. This platelet inhibitor has been reported to cause a coumarin-like effect by producing hypoprothrombinaemia in experimental animals (Park & Leck, 1981). On the basis of our data, it is unlikely that this effect is mediated via an effect of salicylate on the pathway-II enzymes. Park & Leck (1982) have estimated that difenacoum is 100-fold more potent than warfarin as an anticoagulant. The greater potency of difenacoum *in vivo* cannot be explained by an effect of the drug on the pathway-II enzymes.

Our data indicate that vitamin $K_1$ should work as an antidote to warfarin and difenacoum poisoning. This has been shown to be true in animals (Park & Leck, 1982) as well as in humans (Van Der Meer *et al.*, 1968; Barlow *et al.*, 1982).

In rat liver, pathway II consists of at least two different enzymes. Of these enzymes, DT-diaphorase is inhibited by coumarin drugs and might, therefore, be a potential site for the action of diaphorase inhibitors stronger than warfarin and difenacoum. Dicumarol has been shown to inhibit strongly DT-diaphorase *in vivo* (Schor *et al.*, 1983). However, it is known that dicumarol does not block the activity of pathway II *in vitro* (MacNicoll *et al.*, 1984). Furthermore, it is also known that vitamin $K_1$ will work as an antidote to overcome dicumarol poisoning (Dreisbach, 1980). This supports our hypothesis that the

**Table 5. Recovery of enzyme activities after affinity chromatography**

Cytochrome *c* reductase, ferricyanide reductase and carboxylase activities were measured in the pooled unretarded fractions eluted from the three affinity columns described in Fig. 5. Fractions A, B, C and D refer to the pooled fractions shown in Fig. 5. Values are averages for duplicate determinations. Carboxylase activity was measured with chemically reduced vitamin $K_1$. One enzyme unit equals one nmol of substrate transformed/min by 1 ml of enzyme solution.

| Fraction | Volume (ml) | [Protein] (mg/ml) | Enzyme activity [units/ml (% recovery)] Cytochrome *c* reductase | Ferricyanide reductase | Carboxylase recovery (%) |
|---|---|---|---|---|---|
| Microsomes | 10 | 6.5 | 22.5 (100%) | 931 (100%) | 100 |
| A (5'-AMP–Sepharose) | 8 | 4.5 | 19.6 (70%) | 175 (15%) | 69 |
| B (2'5'-ADP–Sepharose) | 8 | 3.8 | ~0 (0%) | 298 (32%) | 68 |
| C (Blue Sepharose) | 7 | 5.1 | 19.0 (59%) | 269 (20%) | 63 |
| D (5'-AMP–Sepharose) | 2 | 3.0 | 3.3 (3%) | 968 (21%) | 0 |

* Carboxylase activities in the various fractions are shown in Table 6.

**Table 6. Effect of affinity chromatography on carboxylase activity**

Carboxylase activity was measured in the pooled unretarded fractions eluted from the three affinity columns described in Fig. 5. Fractions A, B, C and D refer to the pooled fractions shown in Fig. 5. Results are averages for triplicate samples and differed by less than 5%. A background activity of 4500 c.p.m./ml was measured in the absence of vitamin $K_1$ and was subtracted.

| Fraction | $10^{-3}$ × Carboxylase activity (c.p.m./ml) | | |
|---|---|---|---|
| | (*a*) Vitamin $K_1H_2$ | (*b*) Vitamin $K_1$+NADH | (*a*)/(*b*) |
| Microsomes | 309 | 83.8 | 3.7 |
| A (5′-AMP–Sepharose) | 266 | 73.2 | 3.6 |
| B (2′,5′-ADP–Sepharose) | 261 | 72.6 | 3.6 |
| C (Blue Sepharose) | 276 | 75.1 | 3.7 |
| A+D from 5′-AMP–Sepharose* | 221 | 60.5 | 3.7 |

* 1000 units (see Table 5) of ferricyanide reductase activity from fraction D (Fig. 5*a*) was added to 3 ml of fraction A from the same column.

second enzyme(s) in pathway II is not affected by coumarin drugs.

The enzymes that constitute pathway II in rat liver are bound differently to the microsomal membrane. The wash and sonication treatment removed DT-diaphorase from the vesicles, but apparently left the second enzyme(s) intact in the membrane. These data question the current notion of a specific microsomal DT-diaphorase (Hildebrandt & Suttie, 1982; Lind & Höjeberg, 1981). The enzyme activity that has been reported to be present in microsomes might be the result of contamination by cytosol. By using the marker enzyme lactate dehydrogenase, cytosolic contamination was shown to be 11% in our routine preparations. However, our routine microsomal preparations contained < 10% of the DT-diaphorase activity that should have been present according to the calculated cytosolic contamination. One possible explanation for this discrepancy is inhibition of DT-diaphorase when it is present in a microsomal environment. Another possibility is that DT-diaphorase and other cytosolic proteins could be removed more easily from the microsomal preparations than lactate dehydrogenase. The latter possibility would imply that our calculated cytosolic contamination is overestimated. On the other hand, the data strongly suggest that the second enzyme(s) is of microsomal origin and not due to mitochondrial contamination. The significant inhibition of (vitamin $K_1$+NADH)-supported carboxylation caused by purified mitochondria was apparently due to competition between pathway II and mitochondrial NADH dehydrogenase for NADH.

Our data strongly suggest that cytochrome *c* reductase and ferricyanide reductase do not participate in vitamin $K_1$ cofactor production in the liver. These enzymes, which are synonymous with cytochrome *P*-450 reductase and cytochrome $b_5$ reductase, can reduce vitamin $K_3$ and are believed to play a significant role in quinone reduction in the liver (Thor *et al.*, 1982). Chromatography on 2′,5′-ADP–Sepharose and 5′-AMP–Sepharose removed 100 and 80% of cytochrome *c* reductase and ferricyanide reductase respectively from the carboxylation system without affecting the activity of pathway II. If one of these enzymes had been identical with the second enzyme(s) in pathway II, an effect on the pathway-II activity should have been observed. In contrast, removal or addition of DT-diaphorase had significant effects on the test systems.

Our data demonstrate that rat liver DT-diaphorase can provide the vitamin K-dependent carboxylase with reduced vitamin K cofactor. In support of this observation is the work of Fasco & Principe (1982*b*), which shows that purified DT-diaphorase can reduce the quinone form of the vitamin to vitamin K hydroquinone. Thus, rat liver DT-diaphorase appears to contribute significantly to the activity of pathway II. Recently, we (Wallin & Martin, 1985) have shown in human liver that the coumarin-sensitive DT-diaphorase activity is only 0.8% of the activity measured in rat liver. It appears that pathway II in human liver is mainly catalysed by the other NADH-specific enzyme(s) in the pathway. The reason for the lack of DT-diaphorase activity in human liver could be that the human enzyme, in contrast with the rat enzyme, is dependent upon enzyme induction to obtain a reasonable basal activity. It has been shown that rat liver DT-diaphorase is easily induced by a variety of chemical agents (Talalay & Benson, 1981).

DT-diaphorase is an ubiquitous enzyme (Benson *et al.*, 1980) with a broad substrate specificity (Ernster, 1967). This suggests that the enzyme is involved in cellular functions other than the reduction of vitamin K. It is possible that the biological function of the second enzyme in pathway II is vitamin K reduction and that the involvement of DT-diaphorase in this reaction results from its broad substrate specificity. Vitamin K epoxide reductase has been shown to exhibit a high degree of substrate specificity towards vitamin K epoxide reduction (Liptay-Reuter *et al.*, 1985). The existence of a dehydrogenase with similar specificity for vitamin K quinone reduction seems reasonable.

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