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Warfarin poisoning and vitamin K antagonism in rat and human liver

Design of a system *in vitro* that mimics the situation *in vivo*

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The present paper describes a sytem *in vitro* that has been developed to mimic vitamin K metabolism and vitamin K function in liver. In this system the two pathways that are known to participate in vitamin K reduction are active and the vitamin K-dependent carboxylase accepts a synthetic pentapeptide as substrate. With this system *in vitro* the effect of warfarin on both pathways was examined under conditions which simulated a warfarin-poisoned liver. Identical experiments were completed with rat and human liver. All activities currently associated with vitamin K metabolism and vitamin K function were similar in the rat and human systems. Warfarin neutralized the ability of pathway I (the vitamin K epoxide reductase pathway) to produce reduced and active vitamin K cofactor for the carboxylase. In both the rat and the human system, however, when warfarin was present, reduced vitamin K cofactor was produced by pathway II (the dehydrogenase pathway). The data are consistent with observations *in vivo* on the effect of vitamin K_1 when used as an antidote. This suggests that the system *in vitro* reflects the mechanism *in vivo* by which vitamin K_1 overcomes warfarin poisoning.

INTRODUCTION

Several essential coagulation factors in the haemostatic system are dependent on vitamin K for their biosynthesis (Suttie, 1978; Suttie & Jackson, 1977). Coumarin anticoagulant drugs antagonize the action of vitamin K and are used clinically to control the level of these clotting factors in plasma. The mechanism of action of coumarin drugs has been studied extensively (O'Reilly, 1976; Suttie, 1980; Park & Leck, 1982). Currently, the drugs are believed to exert their anticoagulant effect by interrupting the vitamin K cycle in the liver (Suttie, 1980). This cycle provides reduced vitamin K cofactor for the vitamin K-dependent carboxylase. In the presence of reduced vitamin K_1, the vitamin K-dependent carboxylase converts precursor forms of Factor II, VII, IX, X and protein Z, protein C and protein S to γ-carboxyglutamic acid-containing coagulation factors which can bind Ca^{2+} (Suttie, 1978, 1980).

Coumarin anticoagulant drugs have been shown to irreversibly inhibit the liver enzyme vitamin K epoxide reductase (Fasco & Principe, 1982). This enzyme reduces vitamin K epoxide, and the reductase is essential for maintaining the vitamin K cycle in liver (Suttie, 1980). Vitamin K quinone is also reduced by a microsomal enzyme that has characteristics that are very similar to those of the vitamin K epoxide reductase (Fasco *et al.*, 1982; Preusch & Suttie, 1984). Current available data suggest that reduction of the epoxide and the quinone form of the vitamin is carried out by the same enzyme or enzyme complex (Lee & Fasco, 1984).

Coumarin anticoagulant drugs can neutralize the reductase *in vivo* (Fasco & Principe, 1982), and overdosage with these drugs can result in fatal haemorrhage (O'Reilly & Aggeler, 1976). Suppression of clotting-factor synthesis can, however, be overcome by administration of large doses of vitamin K, and the vitamin is frequently used as an antidote (Van Der Meer *et al.*, 1968; Park *et al.*, 1984).

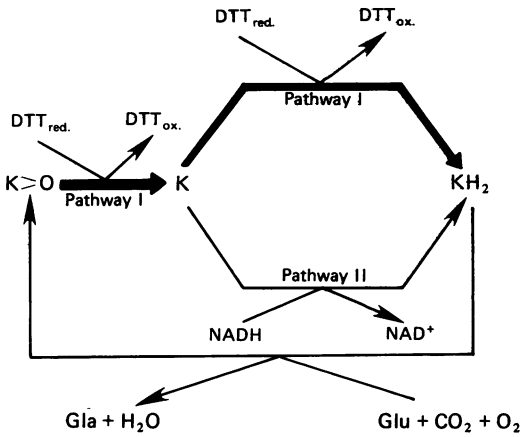
The current model for vitamin K metabolism and vitamin K function in the liver is illustrated in Scheme 1. The depicted model also defines the two pathways in liver (pathways I and II) which can provide the carboxylase with reduced vitamin K cofactor. Since pathway I is irreversibly inhibited by coumarin drugs, it follows that pathway II must be responsible for the antidotic effect of vitamin K. We have recently studied pathway II and have shown that this pathway is little affected by coumarin drugs (Wallin, 1985). The present paper describes a new '*in vitro*' system where we have studied the activity of pathways I and II in concert. Since both pathways for vitamin K reduction are active and the carboxylase accepts a synthetic pentapeptide as substrate, our system represents a distinctive improvement over other carboxylation systems presented in the past (Suttie, 1978, 1980). Using this system we were able to study the action of both pathways in a system resembling a coumarin-poisoned liver. We carried out identical experiments with systems *in vitro* prepared from rat and human livers to see whether or not the current rat model is applicable to the human liver.

EXPERIMENTAL

Chemicals

Vitamin K_1 was purchased from Sigma. The vitamin

Abbreviations used: Vapona, phosphoric acid 2,2-dichloroethenyl dimethyl ester ('2,2-dichlorovinyl dimethyl phosphate'); DTT, dithiothreitol; vitamin K_1H_2, fully reduced vitamin K_1; vitamin K_1 epoxide, vitamin K_1 2,3-epoxide; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]propanesulphonic acid.



Scheme 1. Current model for vitamin K function and vitamin K metabolism in rat liver

Reduction of KO (vitamin K epoxide) and K [vitamin K (quinone)] by pathway I is catalysed by vitamin K epoxide reductase (Suttie, 1980). Reduction of vitamin K by pathway II is catalysed by DT-diaphorase (EC 1.6.99.2) and a microsomal dehydrogenase(s) (Wallin, 1985). Reduced vitamin K cofactor (KH_2) produced by both pathways triggers γ-carboxylation of glutamic acid residues in clotting-factor precursor proteins, converting them to Gla (γ-carboxyglutamic acid)-containing proteins (Suttie, 1980). Further abbreviations: red., reduced; ox., oxidized.

was used to prepare reduced vitamin K_1 hydroquinone and vitamin K_1 2,3-epoxide by the methods of Sadowski *et al.* (1976) and Tishler *et al.* (1940) respectively. Aquamephyton (vitamin K_1; 10 mg/ml) was obtained from Merck, Sharp and Dohme. Warfarin and Chaps were from Sigma. The pentapeptide Phe-Leu-Glu-Glu-Leu was from Vega Fox Biochemicals Division (Tucson, AZ, U.S.A.). $NaH^{14}CO_3$ (60 mCi/mmol) was from Amersham Corp. The insecticide Vapona was a gift from the Shell Oil Company (Houston, TX, U.S.A.). Protein was measured with the Bio-Rad protein assay.

Liver tissues

1. Rat liver. Male Sprague–Dawley rats weighing between 250 and 300 g (Charles River Laboratories) were fed a standard laboratory diet. The rats were fasted for 24 h then killed by decapitation and the livers removed. A second group of rats was given an intraperitoneal injection of warfarin (30 mg/kg) in saline (0.9% NaCl), then fasted for 24 h, killed by decapitation and the livers removed.

2. Human liver. Liver biopsies were obtained from morbidly obese patients undergoing gastric-bypass surgery. The study was approved by the Clinical Investigation Committee of the Milton S. Hershey Medical Center, the Pennsylvania State University. It followed the ethical guidelines of the National Institutes of Health. Subjects participated only after signing an informed-consent form.

Preparation of microsomes (microsomal fractions)

1. Rat liver. Rat livers were rinsed in ice-cold saline and homogenized as described by Suttie *et al.* (1976) in 250 mM-sucrose, 25 mM-imidazole, pH 7.2, containing 1 mM-Vapona (SI-Vapona-buffer). The homogenate was centrifuged at 10000 *g* for 10 min. The pellet was subjected to a second centrifugation at 10000 *g* for 10 min. The postmitochondrial supernatant was then centrifuged at 100000 *g* for 60 min. The supernatant from the 100000 *g* centrifugation was resuspended in the SI-Vapona-buffer and centrifuged a second time at 100000 *g* for 60 min. The supernatant was discarded and the microsomal pellets stored in liquid N_2 with no detectable loss of carboxylase activity or other vitamin K-related activities studied in our system *in vitro*.

2. Human liver. The liver biopsies (8–10 g) were rinsed in ice-cold saline, blotted to remove excess saline, weighed, minced and suspended in 2 vol. (v/w) of the homogenization buffer [50 mM-KCl/200 mM-sucrose/20 mM-Tris/HCl, pH 7.8 (Wallin & Martin, 1985)]. After addition of the insecticide Vapona (final concn. 1 mM), the suspension was dispersed by using a Polytron for 10 s at a low setting. The slurry was then homogenized in a Potter–Elvehjem homogenizer with a tight-fitting pestle. The homogenate was centrifuged twice at 10000 *g* for 10 min as described for rat liver microsomes. The postmitochondrial supernatant was centrifuged for 60 min at 100000 *g*. The microsomal pellets were surface-washed with 250 mM-sucrose/25 mM-imidazole, pH 7.2 (SI-buffer) and stored frozen in liquid N_2 with no detectable loss of the vitamin K-related activities measured in our system *in vitro*. Vitamin K-related activities from specimens obtained from morbidly obese patients did not differ substantially nor did they have DT-diaphorase [NAD(P)H dehydrogenase (quinone)] levels different from those of the occasional specimens obtained from non-morbidly obese patients requiring resection for trauma.

Enzyme activities

1. Vitamin K-dependent carboxylase. Carboxylase activity was measured in rat and human microsomes after resuspending the microsomes in 25 mM-imidazole, pH 7.2, containing 0.5% Chaps (imidazole/Chaps-buffer). The pellets were resuspended in the same volume that was used when the microsomes were suspended in the SI-buffer. Before incubation of the test samples, $MnCl_2$ was added from a concentrated stock solution of the salt to give a final concentration of 1 mM. Carboxylase activity was measured as $^{14}CO_2$ incorporation into the synthetic peptide Phe-Leu-Glu-Glu-Leu as described by Esmon & Suttie (1976). All incubations contained 2 mM of the pentapeptide and 5 mM-DTT. Standard incubations contained 100 μg of vitamin K_1H_2/ml or 100 μg of vitamin K_1 (Aquamephyton)/ml respectively. Standard incubations with NAD(P)H also contained 2 mM-nicotinamide nucleotide. Incubations were carried out for 30 min at 25 °C. The specific activity of the carboxylase varied by $\pm 25\%$ when measured in different microsomal preparations. In order to determine K_m and $V_{max.}$ and compare these kinetic constants from various experiments, data were normalized to the same carboxylase enzyme activity. This activity was the average activity measured in nine individual microsomal preparations when vitamin K_1H_2 was used to support the reaction. Carboxylase activity supported by vitamin K_1+DTT and vitamin K_1+DTT+NADH was then calculated relative to this activity.

2. Vitamin K epoxide reductase. Vitamin K epoxide reductase activity was measured in microsomes resuspended in the imidazole/Chaps-buffer. Vitamin K_1 epoxide in 10μl of 95% ethanol was added to 0.5 ml of the microsomal suspension to give a final concentration of 10 μM. After preincubation for 1 min at 25 °C, 20 μl of DTT in the imidazole/Chaps-buffer (final concn. 5 mM) was added, and the suspension incubated in open tubes for 30 min at 25 °C. When human epoxide reductase activity was measured, incubations were carried out at 33 °C (Wallin & Martin, 1985). Reactions were stopped by the addition of 1 ml of propan-2-ol/hexane (3:2, v/w) and vortex-mixed into the test system. The mixture was subjected to a brief centrifugation. A 300 μl portion of the upper hexane phase was removed and evaporated to dryness at room temperature. The residue was dissolved in 100 μl of propan-2-ol and 20 μl was analysed by h.p.l.c. on a Rainin gradient system equipped with a Gilson 704 h.p.l.c. system manager. Separation was achieved on a Rainin Microsorb Short C_{18} reversed-phase column in 100% methanol. Absorbance of eluted materials was measured at 254 nm with an ISCO UM-5 detector equipped with an ISCO HPLC 10 μl flow cell. Retention times for vitamin K_1 epoxide and vitamin K_1 were 3.6 min and 5.8 min respectively, at a flow rate of 2 ml/min. Quantification was based on the integrated absorption peaks when compared with peaks from standards of vitamin K_1 and vitamin K_1 epoxide. The concentration of vitamin K_1 and its epoxide were determined spectrophotometrically in 95% ethanol using molar absorption coefficients of 30800 $M^{-1} \cdot cm^{-1}$ at 266 nm for the epoxide and 18900 $M^{-1} \cdot cm^{-1}$ at 249 nm for vitamin K_1 (Sherman & Sander, 1981).

3. Vitamin K epoxidase. Epoxidase activity was measured at 25 °C as described above for vitamin K epoxide reductase, except that vitamin K_1 (10 μM) replaced vitamin K_1 epoxide and that NADH (2 mM) was present in the test system. Enzymic conversion of vitamin K_1 into vitamin K_1 epoxide was measured by h.p.l.c. as described for the reductase.

RESULTS

Our first objective was to establish a test system where the vitamin K epoxide reductase (pathway I) and pathway II (Scheme 1) could provide the vitamin K-dependent carboxylase with active vitamin K cofactor. For kinetic studies of the system it was also necessary for the carboxylase to accept the exogenously added pentapeptide substrate. Using the zwitterionic detergent Chaps we succeeded in developing a system *in vitro* which fulfilled these requirements set for the system. Most importantly, vitamin K epoxide reductase was active in the presence of Chaps. This membrane enzyme is highly sensitive to inactivation by other detergents at neutral pH (Hildebrandt *et al.*, 1984). The system was found to be most active when microsomes from rat or human liver were suspended in 250 mM-imidazole, pH 7.2, containing 0.5% Chaps (imidazole/Chaps-buffer). Presence of salt (KCl) in the system inhibited the activity.

In order to determine the optimum conditions for vitamin K-dependent carboxylation in the system *in vitro*, we studied the dependence of carboxylase activity on DTT, NADH and vitamin K_1 concentrations. The effect of pH and temperature on carboxylase activity was also determined. We carried out identical studies on the system prepared from rat and human liver microsomes.

The effect of DTT on carboxylase activity was studied over the concentration range 0–20 mM, and it was found that the systems from rat and human liver were both saturated with DTT above 3 mM. Therefore we included 5 mM-DTT in our standard test system. NADH (2 mM), when added to the systems in addition to 5 mM-DTT, increased the activity in the rat and human systems 10 and 33% respectively. At this NADH concentration, both systems were saturated with NADH. NADPH was less efficient than NADH in supporting carboxylase activity.

Both systems exhibited a temperature optimum between 24 and 25 °C and a pH optimum between 8 and 9. Since chemical reduction of vitamin K_1 with DTT occurs at high pH values (Fasco *et al.*, 1983), the systems were studied at pH 7.2, which is the pH value most frequently used to study vitamin K-dependent carboxylation. At pH 7.2 and 25 °C carboxylation of the synthetic pentapeptide substrate was linear during 30 min of incubation.

The imidazole/Chaps-buffer only partially solubilized the microsomal vesicles. We therefore conducted experiments in order to determine the solubilizing effect of 0.5% Chaps on the various microsomal enzymes that were involved in our system *in vitro*. Rat and human microsomes were suspended in the imidazole/Chaps-buffer and subjected to centrifugation at 100000 ***g*** in order to separate soluble and insoluble materials. Carboxylase and reductase activities were measured in pellets and supernatants as percentages of the activities measured in microsomes suspended in the imidazole/Chaps-buffer (Fig. 1). When chemically reduced vitamin K_1 was used as cofactor for the carboxylase, 380 and 470% of carboxylase activity was recovered in the pellets from rat and human microsomes respectively. All carboxylase activity was retained in the pellets. When carboxylase activity was supported by pathway I or pathways I + II, less than 100% of the initial activity was recovered (Fig. 1). This loss of activity coincided with loss of reductase activity in the pellet (Fig. 1). Reductase activity, however, was present in the supernatant (Fig. 1). Thus, in contrast with the carboxylase, a significant portion of the reductase was extracted from the microsomes and brought into solution in an active form with Chaps. When the microsomes were treated with 0.5% Chaps, 56% of the reductase activity in rat liver microsomes and 33% of the reductase activity in human microsomes was measured in the fraction of solubilized protein. Recovery of all reductase activity from rat and human microsomes after centrifugation was 136 and 87% respectively. About 80% of the activity of the rat enzyme was recovered in the pellet and 56% in the supernatant. Thus extraction of microsomes with 0.5% Chaps appeared to enhance the activity of the membrane-bound enzyme.

We could not measure DT-diaphorase activity (Wallin, 1985) in the pellets. This enzyme is easily removed from the membrane by detergent extraction (Wallin, 1986) and was found in this study to be present in the Chaps supernatant. Thus, in order to preserve an intact carboxylation system for studies on pathways I and II, we could not prepare our system *in vitro* from the pellets, which have higher carboxylase activity.

Table 1 shows various enzyme activities related to vitamin K function and vitamin K metabolism measured

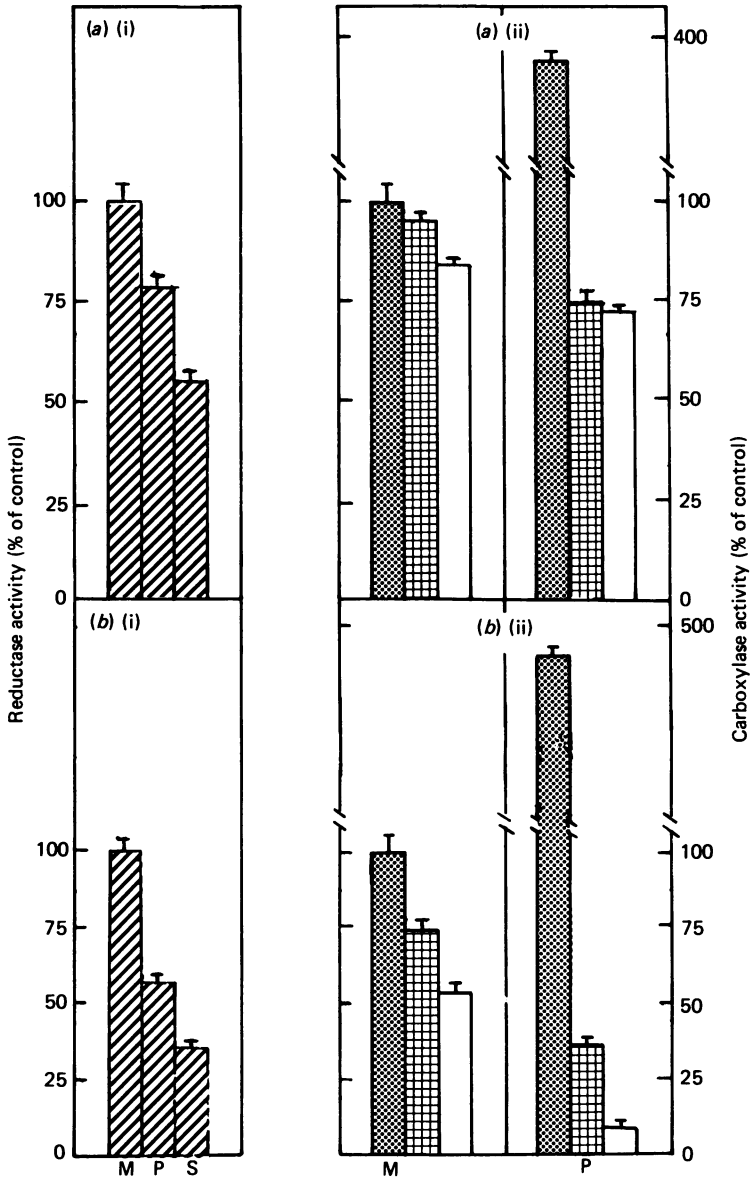


Fig. 1. Effect of Chaps on the vitamin K-dependent carboxylase and the vitamin K epoxide reductase in rat and human liver microsomes

Microsomes from rat (*a*) and human (*b*) liver were suspended in imidazole/Chaps-buffer and pellets and supernatants were separated after centrifugation at 100000 *g* for 60 min. Vitamin K epoxide reductase (i) and carboxylase activities (ii) were measured as described in the Experimental section in the various fractions. Before measurements were made, the pellets were suspended in the imidazole/Chaps-buffer in a volume that was equal to the starting volume. Activities are presented as percentages of the activity measured in microsomes suspended in the imidazole/Chaps-buffer. The experiment was carried out with microsomes from a pool of eight rat livers and a pool of eight human liver biopsies with approximately the same weight (5 g). Results are the means ± S.D. for three parallel incubations. Carboxylase activity was supported by chemically reduced vitamin K_1 (▨), pathway (I+II) (▦) and pathway I (□). Abbreviations used: M, microsomes; P, pellet; S, supernatant.

in rat and human microsomes suspended in the imidazole/Chaps-buffer. The greatest difference was found between the specific activities of rat (325 pmol/mg) and human (125 pmol/mg) vitamin K epoxide reductase. The other specific activities measured were similar in the two systems. When measured in intact microsomes, the specific activity of vitamin K epoxide reductase was found to be higher in human microsomes than in rat microsomes (Wallin & Martin, 1985). Thus Chaps appears to inactivate the human enzyme more than the rat enzyme.

As shown in Fig. 2, warfarin (0.01–1.0 mM) inactivated pathway-I-supported carboxylase activity in the rat and the human systems *in vitro*. Thus, in the presence of warfarin, the carboxylase activity remaining in the test system when supported by vitamin K_1+NADH+DTT resulted from pathway-II activity (Fig. 2). High concentrations of warfarin resulted in some inhibition of the vitamin K-dependent carboxylase from rat liver, but had insignificant effect on the carboxylase from human liver (Fig. 2). Since warfarin neutralized pathway I in the rat and human systems *in vitro*, this provided us with test systems where the ability of pathway II to overcome warfarin inhibition of clotting-factor synthesis could be studied.

We studied recovery of carboxylase activity in the rat

Table 1. Vitamin K-related enzyme activities in rat and human liver

The various enzyme activities listed in the Table are the means from nine individual experiments ± S.E.M. Activities were measured as described in the Experimental section and are calculated as product formed/30 min of incubation per mg of microsomal protein. Pathway-I + II- and pathway-I-supported activity refer to the presence in the test system of vitamin K_1 + NADH + DTT and vitamin K_1 + DTT respectively.

Liver	10^{-2} × Carboxylase activity (c.p.m./mg) supported by:			Vitamin K epoxidase activity (pmol/mg)	Vitamin K epoxide reductase activity (pmol/mg)
	Vitamin K_1H_2	Pathway I + II	Pathway I		
Rat	60 ± 2.7	58 ± 2.6	55 ± 3.2	60 ± 4	325 ± 20
Human	56 ± 2.8	44 ± 1.9	30 ± 1.3	85 ± 6	125 ± 9

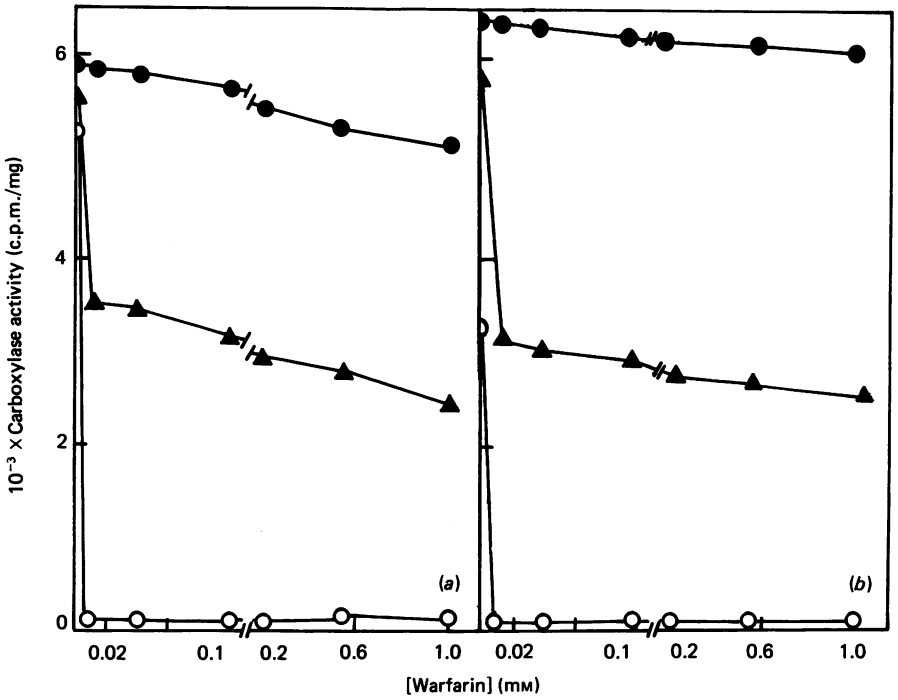


Fig. 2. Warfarin inhibition of carboxylase activity in the systems *in vitro* from rat and human livers

Carboxylase activity was measured in rat (*a*) and human (*b*) microsomes suspended in the imidazole/Chaps-buffer as described in the Experimental section. Activity was measured in the presence of the various warfarin concentrations shown in the Figure. The experiment was carried out with microsomes from the same pools of rat and human liver tissue as described in the legend to Fig. 1. Results are the average of triplicate incubations differing by less than 5%. Carboxylase activity is presented as quantity of product formed/30 min per mg of microsomal protein. The activity was supported by chemically reduced vitamin K_1 (●), pathway I + II (▲) and pathway I (○).

and the human system when the systems were loaded with 1 mM-warfarin. Figs. 3(*a*) and 3(*b*) show the results from these studies in the rat and the human systems *in vitro*. Recovery of carboxylase activity was measured as a function of vitamin K_1 concentration. Data from five individual experiments were normalized as described in the Experimental section to the same carboxylase enzyme activity to create each point. We found this normalization procedure to be acceptable, because the ratios between the carboxylase enzyme activity (vitamin K_1H_2-supported) and the activity when supported by either of the pathways were constant in the various microsomal preparations. The data fitted simple Michaelis–Menten kinetics. From the double-reciprocal plots, we calculated the apparent-K_m and $V_{max.}$ values for the various vitamin K-dependent reactions. The kinetic constants are listed in Table 2.

In the absence of warfarin, only small differences were found between the K_m and the $V_{max.}$ (per mg of microsomal protein) for the carboxylation reaction in the rat and the human system when supported by either pathway I or the combination of pathways I and II. However, in the presence of 1 mM-warfarin, the K_m values for the combined pathway-I-and-II-supported activity increased 6-fold and 2-fold in the rat and the human system respectively. Also, as shown in Fig. 3, vitamin K_1 was unable to overcome warfarin inhibition of pathway I. Thus pathway II was solely responsible for

Table 2. Apparent kinetic constants for pathway-I-and-II-supported carboxylation in rat and human liver

The apparent kinetic constants for carboxylation as a function of vitamin K_1 concentration listed in the Table are the means calculated from five individual experiments performed with five different microsomal preparations. The data were calculated from Lineweaver–Burk plots after normalization of the data to the same carboxylase enzyme activity as described in the Experimental section. The various microsomal preparations used from rat and human livers are described in the legend to Fig. 3. Experiments were carried out in the absence and presence of 1 mM-warfarin. Variability is shown as ±S.E.M. Pathway I and II and pathway I are explained in the legend to Table 1.

		Apparent kinetic constants			
		Control		Warfarin-treated	
Liver	Carboxylase activity supported by:	K_m (μM)	$10^{-2} \times V_{max.}$ (c.p.m./mg)	K_m (μM)	$10^{-2} \times V_{max.}$ (c.p.m./mg)
Rat	Pathway I+II	10±1.1	67±6.4	63±6.8	28±3.0
	Pathway I	8.5±0.92	52±6.0	0±0	0±0
Human	Pathway I+II	11±1.2	51±4.4	20±2.1	21±2.3
	Pathway I	7.4±0.85	32±4.0	0±0	0±0

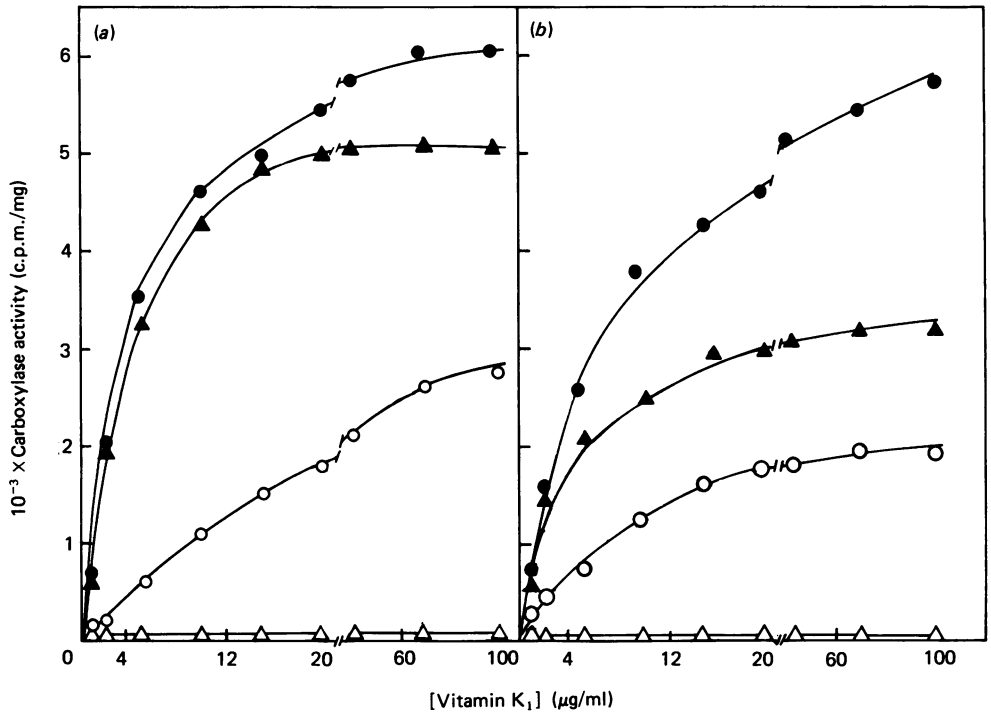


Fig. 3. Dependence of rat and human carboxylase activities on vitamin K_1 concentration

Carboxylase activity was measured as described in the Experimental section in rat (*a*) and human (*b*) microsomes suspended in the imidazole/Chaps-buffer. The dependence of activity on vitamin K_1 concentrations was measured in the presence and absence of 1 mM-warfarin. Each point represents the mean from five individual experiments after normalization of the data as described in the Experimental section. The experiments with rat microsomes were carried out with five different microsomal preparations each obtained from pools of four rat livers. The experiments with human microsomes were carried out with individual preparations obtained from five different liver specimens. Carboxylation activity is presented as quantity of product formed/30 min per mg of microsomal protein. Each measurement was the average of three parallel samples differing by less than 5%. Pathway-I+II-supported activity in the absence (●) or presence (○) of 1 mM-warfarin and pathway-I-supported activity in the absence (▲) or presence (△) of 1 mM-warfarin are shown.

providing reduced vitamin K_1 cofactor for the carboxylase in the warfarin-poisoned system *in vitro*.

We also studied carboxylase activity supported by pathway I and II in a system *in vitro* prepared from livers of warfarin-injected rats. In this system *in vitro* we were unable to measure pathway-I-supported carboxylase activity (Fig. 4). We calculated a K_m value of 55.4 μM and a $V_{max.}$ value of 4300 c.p.m./mg for carboxylation supported by vitamin K_1+NADH+DTT. In warfarin-injected rats the pool of precursors for the vitamin K-dependent clotting factors increases, which results in enhanced carboxylase activity (Suttie, 1980). The K_m and

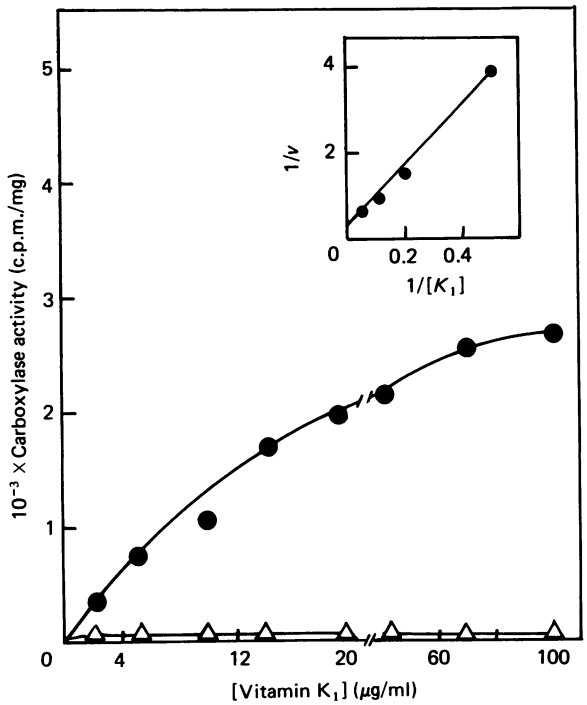


Fig. 4. Dependence of carboxylase activity on vitamin K_1 concentration in microsomes prepared from livers of warfarin-injected rats

Carboxylase activity was measured as described in the Experimental section in microsomes prepared from livers of rats that had been injected with warfarin (30 mg/kg) 24 h before they were killed. Microsomes were suspended in the imidazole/Chaps-buffer as described in the text. The dependence of activity on vitamin K_1 concentration was measured. Carboxylase activity is presented as quantity of product formed/30 min per mg of microsomal protein. The experiment was carried out with microsomes prepared from a pool of four rat livers obtained from warfarin-injected rats. Each measurement was the average of three parallel samples differing by less than 5%. A (double-reciprocal) plot of 1/velocity {[10^{-2} × carboxylase activity (c.p.m./30 min per mg)]$^{-1}$} versus 1/vitamin K_1 concentration ($1/[K_1]$; μg/ml^{-1}) is shown in the inset. Symbols are: ●, pathway I+II; △, pathway I.

$V_{max.}$ values determined in the systems *in vitro* prepared from livers of normal and warfarin-injected rats are therefore not comparable.

DISCUSSION

In order to study the effect of warfarin on the pathways that provide the carboxylase with reduced vitamin K cofactor *in vitro*, it was necessary to develop a system where both pathways for vitamin K reduction were active and the pentapeptide substrate could be used as substrate for the carboxylase. In a previous paper (Wallin & Martin, 1985) we have shown that such a system is feasible. The present paper presents a detailed study on this system prepared from both rat and human liver. Previously, pathway-I-supported carboxylation has been studied in systems where microsomal precursor proteins for the vitamin K-dependent clotting factors were used as substrates for the carboxylase (Suttie, 1980; Whitlon *et al.*, 1978). This was necessary because only the intact microsomes provided systems *in vitro* with vitamin K epoxide reductase activity. Use of the endogenous protein precursors as substrates, however, makes it difficult to carry out any reliable kinetic analysis of the carboxylation reaction because: (1) carboxylation of the protein precursors is an extremely fast reaction (Kappel & Olson, 1984); (2) it is difficult to control the substrate concentration for the enzymic reaction under study.

Our systems *in vitro* required the presence of DTT to express maximal carboxylase activity. The vitamin K-dependent carboxylase is known to be highly stimulated by DTT (Suttie, 1980), which was clearly the reason for this requirement. Because DTT was used in our system to provide the vitamin K epoxide reductase (pathway I) with reduction equivalents, the activity of pathway II could not be measured separately from pathway I in the absence of warfarin. DTT is an artificial substrate for the vitamin K epoxide reductase, but is commonly used as substrate for this enzyme, since the naturally occurring substrate is not known (Suttie, 1985).

When warfarin was used to neutralize pathway I, it was possible to assess the relative contributions of pathway I and II to the total vitamin K_1 cofactor production in our systems *in vitro*. Pathway I was clearly the most active vitamin-K-cofactor-producing pathway, providing greater than 90% of the activity for the rat and 65% of the activity for the human system.

The addition of 0.5% Chaps to microsomes in 250 mM-imidazole, pH 7.2, did not release the vitamin K-dependent carboxylase from the microsomal membrane. However, the vitamin K epoxide reductase was partially solubilized by this treatment. Thus it appears that the carboxylase has a tighter association with the microsomal membrane than has the reductase. Since 53% of the reductase activity from rat liver was measured in solution at pH 7.2, our system allows the reductase to be solubilized at neutral pH in quantities that might be sufficient for further purification and characterization of the enzyme. In the past, this has been a problem for researchers (Hildebrandt *et al.*, 1984).

The vitamin K cycle (Whitlon *et al.*, 1978) was active in our system *in vitro* prepared from rat and human liver. We could demonstrate (results not shown) that vitamin K_1 epoxide as well as vitamin K_1 could trigger carboxylation in the presence of DTT. Therefore our system also reflects the system previously studied in intact microsomes (Whitlon *et al.*, 1978).

In systems prepared from rat and human liver, warfarin (1 mM) neutralized pathway-I-supported carboxylation. We have shown previously that this concentration of warfarin neutralizes vitamin K epoxide reductase in human microsomes (Wallin & Martin, 1985). A warfarin concentration of 1 mM is also known to inactivate the rat enzyme (Fasco & Principe, 1982). Thus inactivation of pathway-I-supported carboxylation in the rat and human systems paralleled inactivation of reductase activity. This is consistent with the current concept of the biological function of this enzyme (Suttie, 1980). Given that coumarin anticoagulant drugs and vitamin K_1 compete for essential thiol groups on the reductase (Fasco *et al.*, 1983), our data also support the hypothesis that coumarins irreversibly inhibit the reductase. Clearly, increasing the vitamin K_1 concentration in our system *in vitro* did not reverse the inhibition of pathway-I-supported carboxylation. On the other hand, high concentrations of vitamin K_1 triggered

pathway-II-supported carboxylase activity when warfarin was present. This shows that clotting-factor synthesis can be triggered by reduced vitamin K_1 cofactor produced by this pathway when the reductase is neutralized with coumarin anticoagulant drugs. This suggests that pathway II is important as a salvage pathway for the antidotic effect of vitamin K_1 in cases of poisoning with these drugs.

We studied pathway-I- and pathway-II-supported carboxylation in the absence and presence of warfarin and found that carboxylation of the pentapeptide following Michaelis–Menten kinetics when measured as a function of vitamin K_1 concentration. This simple kinetic pattern was also observed by Larson & Suttie (1978) when chemically reduced vitamin K_1 was used as cofactor for the carboxylase. It is difficult to envisage a model for this complex enzyme system which can explain the simple enzyme kinetics observed. On the other hand, the kinetic constants calculated from our data provided information which appears to be consistent with results obtained from studies *in vivo* on coumarin anticoagulation and vitamin K antagonism (Van Der Meer *et al.*, 1968; Park *et al.*, 1984). The K_m values for carboxylation supported by pathway I and II increased 6-fold and 2-fold in the rat and human systems respectively, after addition of 1 mM-warfarin. Thus the higher vitamin K_1 levels required to maintain clotting-factor synthesis in coumarin-poisoned rats (Bell *et al.*, 1972), rabbits (Park *et al.*, 1984), and humans (Van Der Meer *et al.*, 1968) are consistent with our observation that higher vitamin K_1 concentrations will trigger carboxylation by pathway II. The significantly lower K_m value calculated for the reaction in the presence of warfarin in the human system *in vitro* also suggests that the vitamin is a better antidote in the human liver than in rat liver.

In order to show that our system *in vitro* mimics the situation *in vivo* in a coumarin-poisoned liver, we prepared our rat system from animals that had been injected with high concentrations of warfarin. In this system, when no warfarin was added to the test system, we were unable to measure pathway-I-supported carboxylation. This suggests that our system *in vitro* reflects vitamin K metabolism and vitamin K function in the intact liver.

Since the content of vitamin K_1 in liver is low (8 ng/g of rat liver; Haroon & Hauschka, 1983), it can be questioned whether or not our system responds to the concentrations of vitamin K_1 *in vivo*. Our system requires vitamin K_1 at a concentration in excess of 5 μM to express significant carboxylase activity. Vitamin K, however, is a fat-soluble vitamin that will concentrate in the membranes of the cell. Thus it is not unlikely that the liver enzymes involved in vitamin K function and vitamin K metabolism are surrounded by a local vitamin K concentration that is effective in our system *in vitro*.

In conclusion, these studies show that human liver as well as rat liver contain two independent pathways for vitamin K reduction and that these pathways behave very similarly *in vitro*. This suggests that the mechanisms of action for coumarin anticoagulant drugs and the antidotic effect of vitamin K are similar in the rat and the human liver. Thus it appears that the current model (Suttie, 1985) for vitamin K metabolism and vitamin K function in rat liver is applicable to the human liver.

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