Vitamin K Epoxidase: Properties and Relationship to Prothrombin Synthesis†

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ABSTRACT: Postmitochondrial supernatants from vitamin K deficient rat liver catalyze both the vitamin K dependent conversion of microsomal precursor proteins to prothrombin, and the conversion of vitamin K to its 2,3-epoxide. Requirements for the latter reaction have been studied, and the possible relationship of the two reactions has been investigated. The epoxidase activity is located in the microsomes and, if NAD(P)H is provided, no cytosolic component is required. The reduced pyridine nucleotides are needed to reduce vitamin K to its hydroquinone, and, when the hydroquinone is used as a substrate, no other source of reducing equivalents is required. The reaction requires O_2, and molecular oxygen is incorporated into the epoxide. When the reaction was carried out in ^2H_2O, no deuterium was incorporated into either the vitamin or its epoxide, suggesting that chromanol or methide forms of the vitamin were not intermediates in any of the reactions being studied. The epoxidation of the vitamin was inhibited by direct antagonists of the vitamin, but not by the coumarin anticoagulant Warfarin. In general, the conditions which favor epoxide formation also stimulate the formation of prothrombin. One major exception is the lack of dependence of epoxidation on HCO_3^- concentration, but a requirement of HCO_3^- for prothrombin formation. The data reported here are consistent with, but do not prove, the hypothesis that the epoxidation reaction is coupled in some obligatory manner to the vitamin K dependent carboxylation which is required for prothrombin formation.

Vitamin K functions in the postribosomal modification of liver microsomal protein precursors to form biologically active prothrombin and the other vitamin K dependent plasma clotting proteins, Factors VII, IX, and X (Suttie and Jackson, 1977). This modification involves the carboxylation of specific glutamyl residues in the precursor proteins to form γ-carboxyglutamic acid residues (Stenflo and Suttie, 1977) in these proteins, and we have developed (Esmon et al., 1975) a vitamin K dependent in vitro carboxylase system to study this reaction. Both this microsomal carboxylase (Sadowski et al., 1976; Girardot et al., 1976; Friedman and Shia, 1976), as well as systems (Shah and Suttie, 1974; Jones et al., 1976; Vermeer et al., 1976) described for the in vitro production of prothrombin, appear to require the reduced form of vitamin K and O_2. Studies of the microsomal carboxylase have been facilitated by reports (Esmon and Suttie, 1976; Mack et al., 1976) of the solubilization of the carboxylase activity and the development of a peptide substrate for the enzyme (Suttie et al., 1976).

Other than the recently discovered involvement of the reduced form of vitamin K in the carboxylation reaction, there is no evidence that there is any other "active" metabolite formed from the vitamin. Matschiner et al. (1970) first described the existence of the 2,3-epoxide of vitamin K in liver, and its in vitro formation in microsomal preparations (epoxidase activity) has been described by Willingham and Matschiner (1974). They have observed that there is an increase in hepatic vitamin K epoxidase activity which parallels an increase in the level of microsomal prothrombin precursor(s) (Esmon et al., 1975; Grant and Suttie, 1976) and have suggested that the enzymatic conversion of vitamin K_1 to its 2,3-epoxide is directly coupled to the carboxylation of precursor glutamyl residues and therefore to the synthesis of prothrombin. The initial studies of the epoxidase (Willingham and Matschiner, 1974) indicated that the reaction required microsomes, soluble protein, a heat stable factor, and O_2. Subsequent studies (Willingham et al., 1976) indicated that epoxidase activity was inhibited by tetrachloro-4-pyridinol and 2-chloro-3-phytyl-1,4-naphthoquinone. This investigation was conducted in order to further characterize the vitamin K epoxidation reaction and to determine if there were any other metabolic transformations of vitamin K occurring during the in vitro synthesis of prothrombin. Studies of the epoxidase activity were carried out under conditions that also allowed prothrombin biosynthesis or vitamin K dependent carboxylation to be measured in an attempt to determine the extent to which these activities might be correlated. Preliminary reports of these studies have appeared (Sadowski, 1975; Sadowski and Suttie, 1976).

Materials and Methods

Treatment of Animals. Male 250–300-g Holtzman strain rats were used throughout the study. Vitamin K deficiency was produced by feeding a vitamin K deficient diet (Mameesh and Johnson, 1959) for 7–10 days to rats housed in cages that prevented coprophagy (Metta et al., 1961). The animals were fasted for 18 h before they were killed.

Incubation Conditions. The deficient animals were killed by decapitation and their livers quickly removed, minced, and homogenized in two parts (w/v) ice-cold 0.25 M sucrose–0.025 M imidazole (pH 7.2) buffer (buffer A). A postmitochondrial supernatant was obtained by centrifugation of the homogenate at 10 000*g* for 10 min in a Beckman Model J21B refrigerated centrifuge equipped with a JA-20 rotor. Microsomes were prepared from the postmitochondrial supernatant by centrifugation at 105 000*g* for 60 min in a Beckman Model L-2 ultracentrifuge. The microsomal pellet was surface-washed once with a supernatant equivalent volume of 0.25 M sucrose–0.025

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M imidazole–0.08 M KCl (pH 7.2) buffer (buffer B) and gently resuspended in buffer B with a large clearance Dounce homogenizer to give a final concentration of microsomes equivalent to 0.5 g of liver per mL.

The standard incubation mixture consisted of sufficient postmitochondrial supernatant or microsomes resuspended in buffer B to give a final concentration of 0.4 g of liver per mL of incubation after additions were made. An ATP-generating system (Sadowski et al., 1976) and cycloheximide (100 μg/mL) were included in each incubation unless stated otherwise. When vitamin K dependent protein carboxylation was measured, $H^{14}CO_3^-$ (59.5 mCi/mmol of $NaH^{14}CO_3^-$, Amersham/Searle) was added to the incubation to give a final concentration of 5 μCi/mL. The total HCO_3^- concentration in the incubation following the addition of radioactive HCO_3^- was found to be approximately 1 mM. The reactions were started by the addition of tritium-labeled vitamin K_1 (2.0×10^4 dpm/μg) in ethanol (0.05–0.10 mL) to give a final concentration of vitamin K equal to 4.0 μg/mL (8.89 μM). Final incubation volumes ranged from 1.0 to 5.0 mL. When pyridine nucleotides were added to the incubations, they were dissolved (1 mg/mL) in the ATP-generating system immediately prior to addition to the incubation mixtures. Incubations were routinely carried out at 27 °C for 30 min with rotary mixing in 18 × 150 mm or 13 × 100 mm disposable borosilicate glass culture tubes open to the atmosphere or sealed with parafilm if carboxylation was also measured. The same postmitochondrial preparations that epoxidize the vitamin also contain an enzymatic activity that reduces the epoxide to the vitamin. This activity is extremely sensitive to Warfarin (Matschiner et al., 1974), and, unless otherwise indicated, 20 μg/mL (0.066 mM) Warfarin was added to the incubations to block the reconversion of epoxide to vitamin. At the level of vitamin used in these experiments, this concentration of Warfarin has no effect on in vitro prothrombin synthesis (Shah and Suttie, 1974), the vitamin K dependent microsomal carboxylase (Sadowski et al., 1976), or the epoxidation reaction (Bell and Stark, 1976). The activity of this reductase in surface-washed resuspended microsomes was found to be sufficiently low and this addition was not necessary.

Assay for Prothrombin, Protein Carboxylation, and Cytochrome P450. Following incubation, an aliquot of the incubation mixture was centrifuged at 105 000*g* for 60 min at 4 °C. The microsomal pellet obtained was surface-washed, resuspended in buffer B, and then made 0.25% in Triton X-100 to give a final concentration of 0.6 g of liver per mL. This suspension was centrifuged at 105 000*g* for 1 h, and the soluble extract was assayed for prothrombin as previously described (Shah and Suttie, 1974). A minus vitamin K control value, which ranged from 2.0 to 3.0 units per g of liver, has been subtracted from all values.

Incorporation of $H^{14}CO_3^-$ into protein was determined by repetitive trichloroacetic acid precipitation of aliquots of the remaining microsomal pellet (resuspended) and of the microsomal Triton X-100 extract as detailed earlier (Sadowski et al., 1976). In some experiments, the total protein of the incubation was precipitated with trichloroacetic acid without prior separation of the microsomes. Whenever ^3H-labeled vitamin K_1 was used to initiate protein carboxylation, it was necessary to exclude the tritium from the carbon-14 window of the liquid scintillation spectrometer since the vitamin precipitated along with the proteins.

Cytochrome P450 was measured by the method of Omuro and Sato (1964), and microsomal protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

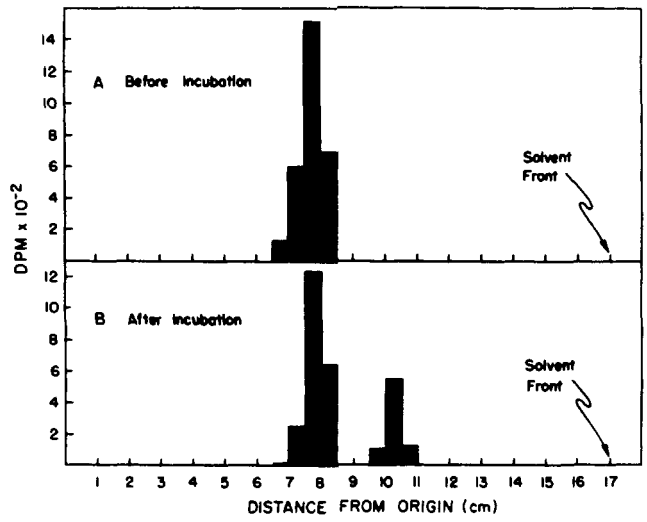

FIGURE 1: Partition thin-layer chromatography of hexane extractable metabolites of vitamin K_1 after in vitro prothrombin synthesis. The standard postmitochondrial supernatant incubation system containing 4 μg/mL of [^3H]vitamin K_1 was assayed for vitamin K metabolites as described in Materials and Methods. Authentic vitamin K epoxide was shown to cochromatograph with the leading radioactive peak of incubated chromatogram. (A) Before incubation; (B) following incubation at 27 °C for 30 min.

Analysis for Metabolites of Vitamin K_1. Aliquots (1.0–5.0 mL) from each incubation were extracted once with 2 volumes of isopropyl alcohol:hexane (3:2), and the upper hexane phase was collected, concentrated to dryness under a stream of nitrogen, and reconstituted in 0.5 mL of hexane containing 1 mg/mL each of unlabeled vitamin K_1 and vitamin K_1 2,3-epoxide. A 0.01-mL sample of each extract was analyzed by reversed-phase partition chromatography as described by Matschiner et al. (1970) on precoated plastic sheets (5 × 20 cm) of silica gel G with fluorescence indicator (Macherey-Nagel & Co.) using mineral oil (American white oil No. 31) as the stationary phase and acetone:water (92:8) as the mobile phase. After chromatography the vitamin and epoxide spots were visualized by fluorescence under ultraviolet light, cut away from the plate, placed in separate counting vials, mixed with 10.0 mL of organic scintillation fluid, and the radioactivity was determined in a liquid scintillation spectrometer.

Isolation of Metabolites for Isotope Incorporation Studies. When $^{18}O_2$ incorporation was studied, liver microsomes were prepared as usual and resuspended in 50 mL of nitrogen-purged buffer B in a 100-mL flask. The resuspended microsomes were evacuated and flushed three times with 95% N_2–5% CO_2. After the third evacuation, 30 mL of $^{16}O_2$ or $^{18}O_2$ (99%, Bio-Rad Laboratories) was added, and the pressure was adjusted to atmospheric with 95% N_2–5% CO_2. NADH was added in nitrogen-purged buffer B containing the ATP-generating system. Tritium-labeled vitamin K_1 was added by means of a syringe (0.2 mL, 200 μg) to give a final concentration in the incubation mixture of 4 μg/mL. After incubation at 27 °C for 30 min, the entire mixture was extracted with 125 mL of isopropyl alcohol:hexane (3:2). The hexane phase was collected, dried with anhydrous sodium sulfate, filtered, and concentrated to an oil which was applied to a 25-g silicic acid column (2.0 × 28 cm) as described by Matschiner et al. (1967). The radioactive fractions were concentrated and vitamin and epoxide separated by reversed-phase partition thin-layer chromatography as described above. The epoxide zone was extracted into chloroform, concentrated, and rechromatographed on silicic acid. Fractions containing the epoxide were

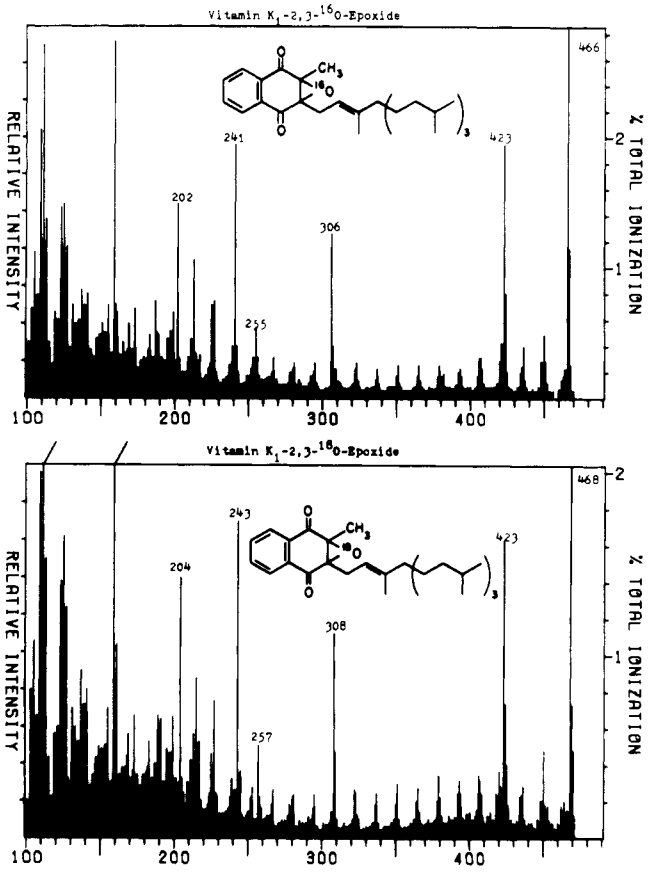

FIGURE 2: Mass spectra of vitamin K_1 2,3-epoxide. The epoxide was isolated as described in the text. Top: Incubation in a $^{16}O_2$ atmosphere. Bottom: Incubation in an $^{18}O_2$ atmosphere.

combined, concentrated, and examined by mass spectrometry. The conditions used to study D_2O incorporation were the same as those described for the $^{18}O_2$ experiments except that buffer B was made up in either deuterated water (99.8%, Sigma) or normal isotopic water, and the incubations were conducted open to the air.

Mass Spectrometry. Mass spectrometric determinations were carried out with an AEI MS-9 mass spectrometer, using a direct inlet probe at temperatures of 80 to 120 °C above ambient. All solvents used were of reagent grade, and those utilized for chromatography in the later stages of isolation of metabolites were spectral quality or redistilled before use.

Vitamin K_1, Vitamin K_1 2,3-Epoxide, Chemicals, and Inhibitors. Vitamin K_1 was obtained from Sigma (St. Louis, Mo.) and purified by silicic acid chromatography before use (Matschiner et al., 1967). Vitamin K_1 epoxide was prepared by oxidation of the vitamin with hydrogen peroxide in alkaline aqueous ethanol as described by Fieser et al. (1941). Vitamin K_1 hydroquinone (unlabeled or labeled) was prepared as previously detailed elsewhere (Sadowski et al., 1976). 5,6,7,8-^{3}H-labeled vitamin K_1 was synthesized (Matschiner, 1970) from tetrasodium 2-methyl[5,6,7,8-^{3}H]naphtho-1,4-quinol diphosphate (80 Ci/mmol, Amersham/Searle) to a specific activity of 5.3 Ci/mmol. Vitamin K_1-[U-^{14}C]phytol (50 mCi/mmol) was a gift from Dr. J. T. Matschiner (University of Nebraska, Omaha, Neb.). 2-Chloro-3-phytyl-1,4-naphthoquinone (Chloro-K)[1] was synthesized (Lowenthal and Chowdhury, 1970) by the Wisconsin Alumni Research Foundation laboratory, purified on silicic acid before use, and added to the incubation mixture in ethanol. Sodium Warfarin (Endo) was dissolved in 0.9% NaCl before use. Tetrachloro-4-pyridinol (TCP) was a gift from F. Marshall (Dow Chemical Co., Zionsville, Ind.) and was dissolved in ethanol before use. Creatine phosphokinase, ATP, NAD, NADH, NADP, NADPH, and bovine serum albumin were purchased from Sigma (St. Louis, Mo.). Creatine phosphate was obtained from Pierce (Rockford, Ill.). Unless stated otherwise, all chemicals used throughout this study were of reagent grade or the highest purity available from local supplies.

[1] Abbreviations used: Chloro-K, 2-chloro-3-phytyl-1,4-naphthoquinone; TCP, tetrachloro-4-pyridinol.

TABLE I: Effect of Chloro-K_1 on Prothrombin Synthesis and Vitamin K_1 Epoxidation.[a]

Chloro-K added	Prothrombin (units/g of liver)	Epoxide formed (nmol/g of liver)
Control	4.7	3.5
5 μg/mL	1.6	1.5
10 μg/mL	0.8	1.1
20 μg/mL	0.5	1.0
100 μg/mL	0	0.7
200 μg/mL	0	0.4

[a] Incubations were conducted in postmitochondrial supernatants prepared from vitamin K deficient rat liver, and prothrombin and epoxide formed were determined as described in Materials and Methods. Vitamin K_1 was present at a final concentration of 4 μg/mL in the incubation mixture. Incubations were conducted at 27 °C for 30 min. Final incubation volume was 5.0 mL. The results are an average of duplicate incubations.

Results

Characterization and Requirements of the System. When ^{3}H-labeled vitamin K_1 was added to postmitochondrial supernatants prepared from vitamin K deficient rat liver and incubated under conditions previously shown to result in the synthesis of prothrombin, a hexane-extractable metabolite more polar than vitamin K_1 was formed. This metabolite cochromatographed with unlabeled vitamin K_1 2,3-epoxide during reversed-phase partition thin-layer chromatography (Figure 1), and its structure was confirmed by mass spectrometry (see Figure 2). All of the radioactivity added to initiate the reactions was recovered in the hexane extract after incubation, and the data in Figure 1 indicate that all of the radioactivity applied to the thin-layer plates cochromatographed with either vitamin K or its epoxide. The concentration of vitamin K (20 μg/mL) routinely used in the original in vitro prothrombin synthesis system (Shah and Suttie, 1974) is in excess of that needed for maximum prothrombin synthesis or vitamin K dependent carboxylation (Sadowski et al., 1976). Preliminary experiments (data not shown) indicated that a concentration of 4 μg of vitamin K per mL supported nearly optimal prothrombin synthesis and resulted in sufficient conversion of the vitamin to the epoxide to allow an accurate determination of epoxidase activity.

Willingham et al. (1976) have reported that the vitamin K antagonist, Chloro-K, inhibits epoxidase activity both in vivo and in vitro, and the data in Table I indicate that incubation in the presence of increasing concentrations of Chloro-K inhibited both prothrombin synthesis and epoxide formation. Both of these activities were also abolished by the inclusion of 400 μg/mL of TCP in the standard incubation mixture.

Preincubation of the postmitochondrial supernatant for 15 min at 37 °C prior to the addition of vitamin K and subsequent reincubation at 27 °C for 30 min resulted in an almost complete inhibition of prothrombin synthesis and vitamin K epoxidation (Table II). The preincubation effect was not in-

TABLE II: Effect of Preincubation and Pyridine Nucleotides on Prothrombin Synthesis and Vitamin K_1 Metabolism.[a]

Incubation conditions	Prothrombin (units/g of liver)	Epoxide formed (nmol/g of liver)
No preincubation	4.9	3.3
Preincubation	0.6	0.2
Preincubation + NAD^+	4.1	2.9
Preincubation + $NADP^+$	3.9	2.3
Preincubation + NADH	3.8	2.7
Preincubation + NADPH	3.9	3.2

[a] Incubations were conducted in postmitochondrial supernatants prepared from vitamin K deficient rat liver, and prothrombin and epoxide formed were determined as described in Materials and Methods. Samples (5.0 mL) were preincubated at 37 °C for 15 min where indicated and then incubated at 27 °C for 30 additional min after the addition of the pyridine nucleotide (1 mg/mL) in the energy mix and vitamin K_1 (4 μg/mL). The results represent the average of duplicate incubations.

TABLE III: Subcellular and Molecular Requirements for Vitamin K_1 Epoxidase.[a]

Incubation conditions	Epoxide formed (nmol/g of liver) −NADH	+NADH
Microsomes + cytosol + vit. K_1	5.62 ± 0.08	8.83 ± 0.17
Cytosol + vit. K_1	0.19 ± 0.05	0.24 ± 0.09
Microsomes + vit. K_1	0.37 ± 0.03	7.42 ± 0.05
Microsomes + vit. K_1H_2	6.90 ± 0.21	9.18 ± 0.08

[a] Microsomes were prepared from vitamin K deficient rat liver, and epoxide formed was determined as described in Materials and Methods. Vitamin K_1 and vitamin K_1 hydroquinone (K_1H_2) were present at a concentration of 4 μg/mL. When present, NADH was at a final concentration of 1 mg/mL. Results are the average of triplicate incubations ± standard error of the mean.

TABLE IV: Comparison of the Requirements for Prothrombin Synthesis and Vitamin K_1 Epoxidation.[a]

Additions to microsomes	Prothrombin (units/g of liver)	Epoxide formed (nmol/g of liver)
Vit. K_1	0	0
Cytosol + vit. K_1	8.3	3.8
Cytosol + vit. K_1 + NADH	12.6	4.8
Vit. K_1 + NADH	4.5	3.1
Vit. K_1H_2	5.9	4.1

[a] Incubation conditions were identical with those described in Table III. Results are the average of duplicate incubations.

TABLE V: Substrate Specificity for Vitamin K_1 Epoxidation.[a]

Substrate	% of added ^3H as epoxide	% of added ^{14}C as epoxide
[^{3}H]Vit. K_1	0	
[^{14}C]Vit. K_1		0
[^{3}H]Vit. K_1 + NADH	33.3 ± 0.6	
[^{14}C]Vit. K_1 + NADH		34.7 ± 0.1
[^{3}H]Vit. K_1H_2	13.7 ± 0.7	
[^{3}H]Vit. K_1H_2 + NADH	27.6 ± 0.2	
[^{3}H]Vit. K_1H_2 + [^{14}C]Vit. K_1	13.1 ± 0.4	0
[^{3}H]Vit. K_1H_2 + [^{14}C]vit. K_1 + NADH	32.8 ± 1.0	32.3 ± 0.6

[a] Microsomes were prepared from vitamin K deficient rat liver, and epoxide formed was determined as described in Materials and Methods. All incubations contained either 2.5 μg/mL of each labeled vitamin as indicated, or 2.5 μg/mL labeled vitamin and 2.5 μg/mL unlabeled vitamin K_1 (quinone) so that the total amount of the vitamin was 5 μg/mL. The quinone of vitamin K_1 or the unlabeled form of the vitamin was always added before the labeled substrate or the hydroquinone. The results are the average of triplicate incubations ± standard error of the mean.

fluenced by the presence or absence of oxygen, Warfarin, or the ATP-generating system. Destruction of the microsomal membranes by lipid peroxidation was apparently not involved since anaerobic preincubation had the same inhibitory effect. Table II shows that both epoxidase activity and prothrombin synthesis were restored after addition of a pyridine nucleotide to the preincubated system. No preference for any of the four pyridine nucleotides could be demonstrated.

The requirement of various subcellular fractions for the epoxidation of vitamin K was examined, and the results are presented in Table III. When microsomes were resuspended in cytosol and incubated with vitamin K, epoxidation was stimulated about 40% by the addition of reduced pyridine nucleotides. If the microsomes were resuspended in buffer B and incubated with vitamin K, no epoxidation of vitamin K occurred unless either NADH or NADPH were included. Both NAD^+ and $NADP^+$ (data not shown) were inactive in the absence of the cytosolic components. Cytosol alone was inactive in the presence or absence of reduced pyridine nucleotides. These results suggest that the enzyme(s) catalyzing the epoxidation of vitamin K are microsomal and that the requirement for cytosolic components may be eliminated by the addition of reduced pyridine nucleotides.

Table IV shows that similar requirements apply to the synthesis of prothrombin from microsomal prothrombin precursor(s). When microsomes were resuspended in cytosol, there was no apparent NADH requirement for either prothrombin synthesis or epoxidation, but the addition of NADH did stimulate both reactions. The addition of vitamin K_1 to microsomes resuspended without cytosol did not support prothrombin synthesis or epoxidation unless NADH was added. These results are in agreement with our previous observations (Sadowski et al., 1976) that the vitamin K dependent carboxylase requires reduced pyridine nucleotides in the absence of cytosolic components.

The Role of Reduced Pyridine Nucleotide in the Synthesis of Prothrombin and the Epoxidation of Vitamin K_1. The specific requirement for reduced pyridine nucleotides in the synthesis of prothrombin, carboxylation of glutamic acid residues, and epoxidation of vitamin K suggested that the reducing equivalents were being utilized to form the hydroquinone of vitamin K_1. The results presented in Tables III and IV clearly show that the requirement for reduced pyridine nucleotide for vitamin K epoxidation and prothrombin synthesis is eliminated if the vitamin is supplied to the incubation mixture as the chemically reduced hydroquinone.

If the hydroquinone of vitamin K is the substrate utilized for the epoxidation reaction, it should preferentially be epoxidized in the presence of vitamin K quinone and in the absence of NADH. This hypothesis was tested by adding ^{3}H-labeled vitamin K_1 hydroquinone and ^{14}C-labeled vitamin K_1 quinone to the same incubation mixture and analyzing the epoxide formed for ^{3}H and ^{14}C. The results of this experiment are shown in Table V. When either ^{3}H- or ^{14}C-labeled vitamin K (quinone form) were added to resuspended microsomes, no epoxide was formed unless NADH was included. When ^{3}H-labeled vitamin K_1 hydroquinone was added in the presence of ^{14}C-labeled quinone, only ^{3}H could be detected in the iso-

TABLE VI: Effect of Nitrogen on Vitamin K Dependent Prothrombin Synthesis and Epoxidation of Vitamin K_1.[a]

Incubation conditions	Gas phase	Prothrombin (units/g of liver)	Epoxide formed (nmol/g of liver)
Vit. K_1 + NADH	Air	4.0 ± 0.2	3.42 ± 0.11
Vit. K_1 + NADH	$95N_2$:$5CO_2$	0.3 ± 0.1	0.75 ± 0.17
Vit. K_1H_2	Air	4.3 ± 0.1	4.03 ± 0.30
Vit. K_1H_2	$95N_2$:$5CO_2$	0.6 ± 0.1	0.66 ± 0.17

[a] Microsomes were prepared from vitamin K deficient rat liver and prothrombin and epoxide formed were determined as described in Materials and Methods. The $95N_2$:$5CO_2$ gas phase was established by blowing the gas over the resuspended microsomes for 20 min prior to the addition of the vitamin and allowing the gas flow to continue throughout the incubation. The results are an average of triplicate incubations ± standard error of the mean.

TABLE VII: The Metabolism of Vitamin K_1 2,3-Epoxide and the Synthesis of Prothrombin.[a]

Incubation conditions	Prothrombin (units/g of liver)	Vitamin K_1 (nmol/g of liver)	Epoxide (nmol/g of liver)
Vit. K_1 + Warfarin (air)	5.8	18.5	3.7
Vit. K_1 + Warfarin (N_2:CO_2)	0	22.2	0
Vit. K_1 epoxide + Warfarin (air)	0	0	53.4
Vit. K_1 epoxide − Warfarin (air)	5.6	3.0	50.4
Vit. K_2 epoxide − Warfarin (N_2:CO_2)	0.3	7.1	46.3

[a] Incubations were conducted on postmitochondrial supernatants prepared from vitamin K deficient rat liver, and prothrombin and epoxide formed were determined as described in Table VI. Additions were: [^{3}H]vitamin K_1 (4 μg/mL), [^{3}H]vitamin K_1 2,3-epoxide (10 μg/mL), and Warfarin (20 μg/mL). The reactions were stopped by adding 200 μg/mL Chloro-K_1 and 400 μg/mL TCP. The results are an average from duplicate incubations.

TABLE VIII: Effect of Bicarbonate Concentration on Prothrombin Synthesis and Vitamin K_1 Epoxidation.[a]

Bicarbonate added	Prothrombin (units/g of liver)	Epoxide formed (nmol/g of liver)
None	0.6	4.5
0.02 mM	1.0	4.4
0.20 mM	1.6	4.6
2.00 mM	3.1	4.1

[a] Incubations were conducted on microsomes prepared from vitamin K deficient rat liver and resuspended in nitrogen-purged buffer B. Prothrombin and epoxide formed were determined as described in Materials and Methods. Bicarbonate was added as 0.2 mL of concentrated bicarbonate solution to each incubation. Vitamin K_1 was present at a final concentration of 4 μg/mL and NADH was added. The results are an average of duplicate incubations.

lated epoxide in the absence of NADH. However, when NADH was added to the same incubation, then the epoxide spot contained both ^{3}H and ^{14}C. These results do not prove that the hydroquinone is the direct substrate for oxygen attack in the epoxidation reaction, but strongly suggest that reduction of the vitamin must occur before epoxidation takes place.

Oxygen Dependence and Incorporation into Epoxide. It has previously been demonstrated that molecular oxygen is required for the in vitro formation of prothrombin and for the epoxidation of vitamin K_1. When microsomes were resuspended in buffer B and incubated in a 95% N_2–5% CO_2 atmosphere, both prothrombin synthesis and vitamin K_1 epoxidation were inhibited (Table VI). This inhibition was seen both in the presence of vitamin K and NADH and in the presence of chemically reduced vitamin (KH_2). The small amounts of prothrombin and epoxide formed in the absence of O_2 may represent that which was formed upon exposure to the air during the preparation of microsomes after incubation.

To determine if molecular oxygen was being incorporated into the vitamin to form the epoxide, incubations were carried out in $^{18}O_2$. The radioactive epoxide formed during incubation with microsomes resuspended in buffer B in the presence of $^{16}O_2$ was isolated, and its mass spectrum is shown in Figure 2. The parent molecular ion is seen at *m/e* 466 and corresponds to the molecular weight of vitamin K_1 2,3-epoxide. The fragmentation pattern was similar to that previously reported for the epoxide, thus confirming that the more polar metabolite formed in these is indeed the epoxide of vitamin K_1. The radioactive product formed during incubation of the vitamin in an $^{18}O_2$ atmosphere produced the mass spectrum shown in Figure 2. The parent molecular ion peak was at *m/e* 468, indicating an increase in molecular weight expected if molecular $^{18}O_2$ was incorporated into the vitamin during epoxidation. The large peak at *m/e* 423 was the same for both the ^{16}O and ^{18}O epoxides, whereas the peaks at *m/e* 306, 255, 241, and 202 in the $^{16}O_2$ epoxide were all 2 *m/e* units higher in the ^{18}O-labeled epoxide spectrum.

Effect of Vitamin K_1 2,3-Epoxide on Prothrombin Synthesis. The activity of vitamin K 2,3-epoxide as an active form of vitamin K in the biosynthesis of prothrombin was also investigated (Table VII). The epoxide was active in initiating prothrombin synthesis only in the absence of Warfarin and under aerobic conditions. Warfarin has previously been shown to inhibit the conversion of the epoxide to the vitamin (Matschiner et al., 1974), and, in the presence of Warfarin, no prothrombin synthesis could be detected upon addition of the epoxide and none of the added epoxide was converted back to the vitamin. At the concentration used, Warfarin did not inhibit the apparent synthesis of prothrombin by vitamin K nor did it block the epoxidation of the vitamin. Under anaerobic conditions, neither vitamin K_1 nor the epoxide supported prothrombin synthesis, even though the epoxide was extensively converted to vitamin K during the incubation. The apparent difference between the amount of vitamin found at the end of the anerobic and aerobic incubation in the presence of the epoxide but in the absence of Warfarin (4.1 nmol) was almost identical with the amount of epoxide formed from the addition of vitamin K_1 (3.7 nmol). This suggests that, under aerobic conditions, much of the vitamin formed from the epoxide is again epoxidized and the requirement for molecular oxygen in the synthesis of prothrombin is not removed by the addition of the epoxide.

Bicarbonate and Vitamin K Dependence of the Reactions. If the epoxidation and carboxylation reactions are closely coupled, it might be possible to demonstrate a dependence of the epoxidation reaction on bicarbonate concentration. The previously reported (Sadowski et al., 1976; Jones et al., 1976) effect of bicarbonate concentration on prothrombin synthesis was reinvestigated and contrasted to the effect on vitamin K epoxidation (Table VIII). Decreasing the amount of bicarbonate added to the in vitro incubations brought about a

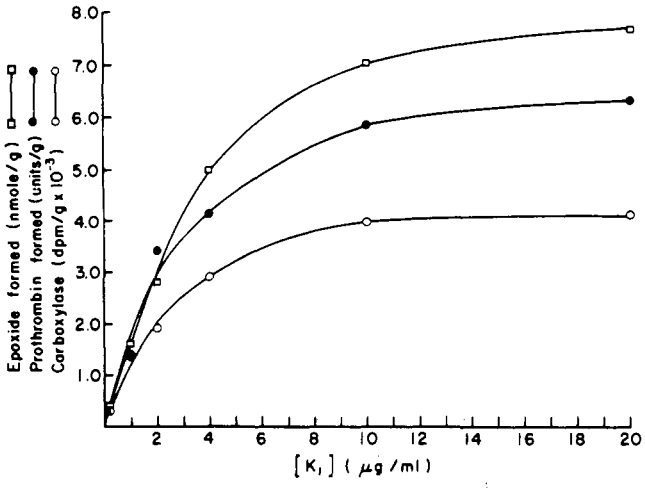

FIGURE 3: Effect of vitamin K_1 concentration on prothrombin synthesis, vitamin K dependent carboxylation, and vitamin K_1 epoxide formation. Microsomes were prepared from vitamin K deficient rats and prothrombin formed, epoxide formed, and vitamin K dependent $H^{14}CO_3^-$ incorporation into protein determined as described in Materials and Methods. All incubations contained 1 mg/mL NADH and 4.0×10^5 dpm of [^{3}H]vitamin K_1. The results are the average of triplicate incubations at each level of vitamin.

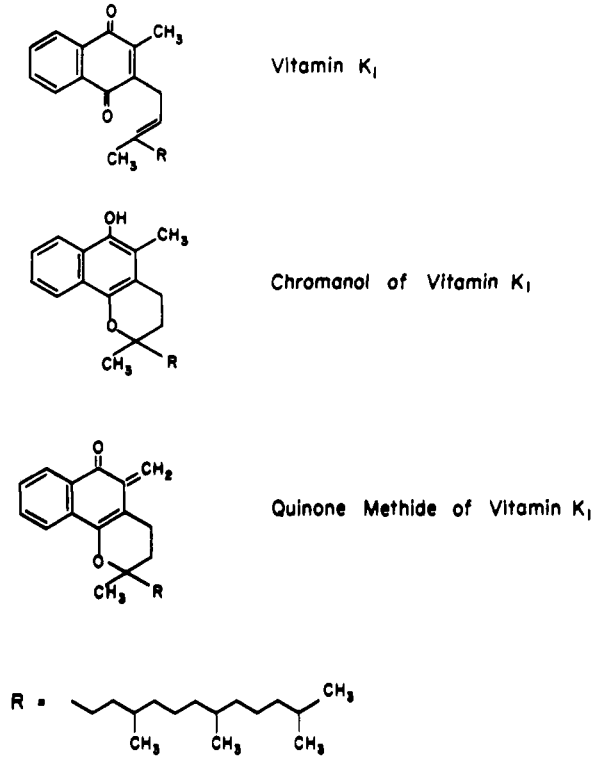

FIGURE 4: Possible intermediates of vitamin K_1 during epoxidation or prothrombin synthesis.

fivefold decrease in the amount of prothrombin formed but had no effect on vitamin K epoxidation.

Since the extent of the vitamin K dependent carboxylation reaction and in vitro prothrombin synthesis are both dependent upon the concentration of vitamin K added to the incubation, the epoxidation of vitamin K might show a similar dependence and be correlated to these other activities if it is essential for the synthesis of prothrombin. Figure 3 shows that prothrombin synthesis, vitamin K dependent carboxylation, and vitamin K epoxidation show a similar dependence on vitamin K concentration. The ratio of epoxide formed to prothrombin synthesized or $H^{14}CO_3^-$ incorporated into protein stayed relatively constant at the doses tested. In the numerous experiments performed in the course of this study, from 50 to 100 mol of epoxide were formed per mol of prothrombin synthesized. Prothrombin contains 10 modified glutamic acid residues (Magnusson et al., 1974) per mol, and it is probably not the only vitamin K dependent protein being synthesized during the incubation (Esmon and Suttie, 1976). However, the data would suggest that there is a considerable excess of epoxidation events compared with carboxylation events occurring in this system. If the epoxidation of the vitamin is coupled to prothrombin synthesis, it must be in a manner that allows some uncoupling of the two processes.

Other Possible Intermediates of Vitamin K in the Reaction. Attempts to detect the formation of any stable or unstable vitamin K dependent ^{14}C-labeled lipid in organic solvent extracts of vitamin K deficient microsomes incubated with vitamin K and $H^{14}CO_3^-$ were unsuccessful. Although ^{14}C-labeled material was routinely found in the lipid extracts, its presence or amount was unaffected by the addition of vitamin K_1. The possible formation of a transitional six-membered chromanyl ring that was not stable during isolation or did not accumulate was also considered. Some of the possible chromanyl derivatives of vitamin K_1 which have been hypothesized as intermediates in oxidative phosphorylation (Gutnick and Brodie, 1965; Asano et al., 1962) are shown in Figure 4. The formation of these compounds and their subsequent conversion to either the vitamin or the epoxide would require protonation of the phytyl side chain for chromanyl formation and of the 2-methyl group for quinone methide formation and can, therefore, be detected by the use of hydrogen isotopes (Di Mari et al., 1968). Thus, incorporation of deuterium into the vitamin and its 2,3-epoxide was studied in a microsomal system from rat liver under conditions which allowed for both prothrombin synthesis and epoxidation. An analysis of the mass spectrum obtained from vitamin K_1 and vitamin K_1 2,3-epoxide isolated after incubation with microsomes resuspended in buffer B containing greater than 95% deuterium oxide revealed that neither the vitamin nor the epoxide contained any detectable deuterium. This conclusion was drawn from the observation that there was no shift in the parent *m/e* peak for either the vitamin or the epoxide. Furthermore, the parent + 1 peaks were of the same height relative to the parent peak as the P + 1 peaks obtained from the spectra of vitamin K_1 and vitamin K_1 epoxide isolated from incubations conducted in normal isotopic water. These data appear to rule out the possibility that these intermediates are on the pathway to epoxide formation. Further, they suggest that, if they are intermediates required for prothrombin synthesis, only a small pool of added vitamin must be involved.

Possible Involvement of Cytochrome P450 in the Epoxidase Activity. Willingham and Matschiner (1974) found that the microsomal vitamin K epoxidase was not inhibited by incubation in the presence of CO, while the cytochrome P450 dependent α-hydroxylase activity in the same preparation was inhibited by 50–75%. The possible relationship of the epoxidase activity to cytochrome P450 activity was studied in additional experiments. Phenobarbital treatment (Table IX) of vitamin K deficient rats resulted in the anticipated proliferation of microsomal protein and an increase in cytochrome P450 content. The total amount of prothrombin precursors in the liver was increased, but was unchanged on a per-gram-of-liver basis. The activity (per-gram-of-liver basis) of the vitamin K epoxidase, vitamin K dependent carboxylation, and in vitro prothrombin synthesis were all substantially increased by phenobarbital treatment. A number of compounds that have been

TABLE IX: Effect of Phenobarbital on Prothrombin Synthesis, Vitamin K_1 Epoxidase Activity, and Protein Carboxylation.[a]

Variable measured	Normal rats	Vitamin K deficient rats	Phenobarbital-induced, vitamin K deficient rats
Wet liver weight (g)	7.0 ± 0.4	7.0 ± 0.3	10.2 ± 0.6
Microsomal protein (mg/g of liver)	40.3 ± 1.3	37.7 ± 0.3	54.4 ± 1.4
Cytochrome P450 content (nmol/g of liver)	14.5 ± 1.8	11.6 ± 1.1	61.9 ± 2.2
Vitamin K epoxidase act. (nmol of epoxide/g of liver)	2.5 ± 0.9	4.4 ± 0.7	9.6 ± 0.6
Microsomal precursor (units/g of liver)	13.6 ± 3.9	36.9 ± 4.2	37.4 ± 3.8
Prothrombin synthesized (units/g of liver)	1.2 ± 0.6	2.8 ± 0.6	5.3 ± 0.8
[^{14}C]Protein carboxylation (dpm/g of liver × 10^{-3})	2.5 ± 0.6	5.3 ± 0.4	9.2 ± 0.9

[a] Phenobarbital was administered by adding 1 mg/mL sodium phenobarbital in the drinking water for 10 days. Microsomes were prepared, incubations performed, and prothrombin, precursor, protein carboxylation, vitamin K epoxidase, cytochrome P450, and microsomal protein were measured as described in Materials and Methods. Vitamin K_1 was present at a final concentration of 4 μg/mL. NADH was present at 1 mg/mL. The results presented represent the average value obtained from four individual animals in each group ± standard error of the mean.

reported to inhibit cytochrome P450 linked reactions were tested for their effect on the vitamin K sensitive systems. Metyrapone (0.1 and 1 mM), aminoglutethimide (0.1 and 1 mM), and glutethimide (0.1 and 1 mM) had no effect on vitamin K epoxidation or prothrombin synthesis, and Benzphetamine and Norbenzphetamine (200 μg/mL) were shown to have no effect on epoxide formation. Since no inhibition of vitamin K epoxidation has been demonstrated by the addition of any of the cytochrome P450 inhibitors tested, it seems reasonable to conclude that the epoxidation of vitamin K is not mediated by cytochrome P450. The increase in vitamin K epoxidase activity seen after the induction of cytochrome P450 by phenobarbital is presumably the result of a general increase in microsomal enzymes due to the proliferation of the endoplasmic reticulum.

Discussion

Since the initial report (Esmon et al., 1976) of a vitamin K dependent carboxylase in rat liver microsomes, this reaction has been studied by a number of groups. The available data have, however, not been sufficient to determine the biochemical mechanism of action of vitamin K in this new carboxylation reaction. One interesting hypothesis (Willingham and Matschiner, 1974) has been that the formation of the 2,3-epoxide of the vitamin is coupled in an obligatory way to the carboxylation reaction. This report has been an attempt to more completely characterize this reaction and to ascertain to what extent prothrombin synthesis is correlated with epoxidation.

It is clear from these studies that vitamin K 2,3-epoxide is the major product which accumulates during the in vitro synthesis of prothrombin. All of the radioactivity initially present as vitamin K could be accounted for at the end of incubation as either vitamin K or vitamin K epoxide. The formation of vitamin K hydroquinone was inferred, since chemically reduced vitamin K hydroquinone could substitute for NADH for epoxidation, prothrombin synthesis, and carboxylation. The extent of hydroquinone formation was not established. The density of the microsomal suspension used precluded direct spectrophotometric measurement of the extent of hydroquinone formation. If there are minor metabolites of the vitamin formed under conditions where prothrombin is being synthesized, they must either cochromatograph with the two species identified, or be present in very small amounts. The lack of deuterium incorporation indicates that the isoprenoid side chain and 2-methyl group of vitamin K were not chemically modified in either the epoxidation or carboxylation reaction, or that an extremely small pool of the vitamin participated in such a reaction.

Both prothrombin synthesis and epoxidation were shown to be dependent on molecular oxygen, and the $^{18}O_2$ incorporation study clearly indicates that the oxygen of the epoxide ring comes from molecular oxygen rather than from water. The requirement of O_2 for prothrombin synthesis when vitamin K hydroquinone is used is in disagreement with the report of Girardot et al. (1976) that the requirement for molecular oxygen is eliminated when the hydroquinone is used to drive the carboxylation. It is possible that their incubations were not completely anaerobic, as vitamin K quinone mediated carboxylation was inhibited less than 50% by nitrogen, whereas we have previously reported (Sadowski et al., 1976) over a 90% inhibition in a N_2 atmosphere. The data presented here have demonstrated that the hydroquinone is the form of the vitamin which is the substrate for the epoxidase. Whether it, or the quinone, is the molecular species which is attacked to yield the epoxide cannot be determined from these data, and it is possible that the hydroquinone is oxidized and then epoxidized without leaving the surface of the enzyme. The effectiveness of the hydroquinone as a substrate in the absence of a reduced pyridine nucleotide suggests that the reducing equivalents needed to drive the epoxidation can come from the vitamin itself.

The epoxidase activity appears to be associated with the microsomes, and the requirement of both soluble proteins and a heat-stable factor (Willingham and Matschiner, 1974) appear to be requirements for a pyridine nucleotide and an enzyme system that can generate reduced pyridine nucleotides during the incubation. Whether either NADH or NAD(P)H can reduce the vitamin, or if there is sufficient transhydrogenase activity to interconvert them cannot be determined from these data. The epoxidase activity was strongly inhibited by tetrachloropyridinol or Chloro-K, but not by inhibitors of cytochrome P450. Warfarin at low concentrations did not inhibit the reaction, but Bell and Stark (1976) have reported that, at high concentrations, Warfarin and other coumarins are able to inhibit epoxidation. The concentration used in their studies would suggest that Warfarin does not inhibit epoxidation of vitamin K in vivo at doses used to block prothrombin synthesis.

This study further illustrates that the requirements for the in vitro synthesis of prothrombin and for the vitamin K dependent carboxylation of the glutamyl residues in microsomal prothrombin precursor(s) are similar to the requirements for the epoxidation of vitamin K_1. Under a variety of experimental conditions, carboxylation or prothrombin synthesis was never observed without simultaneous epoxidation of vitamin K_1. In addition, stimulation or inhibition of carboxylase resulted in a similar effect on epoxidation. However, one major difference between the carboxylation and epoxidation of vitamin K_1 must

be emphasized. The synthesis of prothrombin can be shown to be dependent upon the concentration of bicarbonate while the epoxidation was not affected. If epoxidation is an obligatory molecular event for carboxylation, then either the vitamin does not act directly to transfer the carboxyl group or the mechanism may be uncoupled in the absence of bicarbonate in an analogous manner to the uncoupling of oxidative phosphorylation from electron transport in mitochondria. It is, of course, possible that the epoxidation reaction has nothing to do with the vitamin K dependent carboxylation, and that the similar requirements are fortuitous. Purification of these activities from the microsomal membrane should make it possible to answer these questions.

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