MECHANISM OF ACTION OF VITAMIN K: SYNTHESIS OF γ-CARBOXYGLUTAMIC ACID

Author: J. W. Suttie
Department of Biochemistry
College of Agricultural and Life Sciences
University of Wisconsin-Madison
Madison, Wisconsin

Referee: Gary L. Nelsestuen
Department of Biochemistry
College of Biological Sciences
University of Minnesota
St. Paul, Minnesota

INTRODUCTION

The dietary essentiality of vitamin K was discovered by Henrik Dam in the late 1930s as an outgrowth of his investigation into the possible essentiality of cholesterol for the chick. The chemical nature of the antihemorrhagic agent which he found in alfalfa was soon elucidated, and it was shown that, in addition to phylloquinone (2-methyl-3-phytyl-1,4-naphthoquinone), a number of similar compounds with an unsaturated multiprenyl side chain at the 3-position, or even the parent compound menadione (2-methyl-1,4-naphthoquinone), had biological activity (Figure 1). A decreased concentration of plasma prothrombin in vitamin K-deficient animals was apparent in early investigations, and as soon as they were recognized as unique proteins in the blood coagulation scheme, clotting factors VII, IX, and X were also shown to require vitamin K for their synthesis. The historical background of the discovery of vitamin K has recently been reviewed.[1-3] During this same period, the coumarin anticoagulants were discovered,[4] and their use as vitamin K antagonists was exploited both clinically, for the control of thrombogenic episodes, and as a rodenticide. Only recently has the biochemical role of the vitamin, a cofactor for a unique microsomal carboxylase reaction, been apparent. A number of other possible roles for the action of the vitamin have, however, been postulated during the roughly 40 years since its discovery.

Dam originally proposed that the vitamin, or at least a portion of it, was a part of the prothrombin molecule, but this observation could never be confirmed. Martius subsequently postulated that the vitamin had a function in mammalian electron transport and cited evidence of a defect in oxidative phosphorylation and low cellular ATP levels in vitamin K-deficient chicks.[5] He postulated that this would decrease the plasma concentration of those proteins with a rapid turnover rate such as prothrombin. Other investigators[6,7] could not reproduce these effects on cellular ATP concentrations. In the mid-1960s, Olson[8] suggested that the rate of prothrombin production is regulated by an effect of vitamin K on DNA transcription. This hypothesis was refuted by other investigators,[9-12] and subsequent investigations centered around two alternate hypotheses: (1) that the vitamin acts at a ribosomal site to regulate the *de novo* rate of prothrombin synthesis, or (2) that it functions postribosomally in a metabolic step which converts a precursor protein, which can be produced in the absence of the vitamin, to active prothrombin. Subsequent investigations demonstrated that the latter hypothesis was correct and that the vitamin participated in a reaction which results in the carboxylation of glutamyl residues of precursor proteins to form γ-carboxyglutamyl residues in completed proteins. Much of the evidence leading to the present general

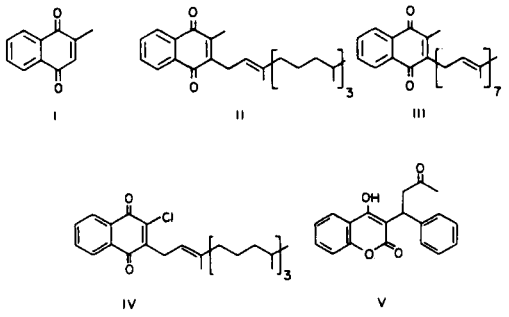

FIGURE 1. Structure of 2-methyl-1,4-naphthoquinone or menadione (I), the parent compound of the vitamin K series, and two naturally occurring active forms of the vitamin: (2-methyl-3-phytyl-1,4-naphthoquinone) phylloquinone or vitamin K_1 (II) and (2-methyl-3-farnesylgeranylgeranyl-1,4-naphthoquinone) menaquinone-7 or MK-7 (III). The two commonly used antagonists of the vitamin shown are (2-chloro-3-phytyl-1,4-naphthoquinone) chloro-K (IV) and (3-(α-acetonylbenzyl)-4-hydroxycoumarin) Warfarin (V).

state of knowledge has been recently reviewed[1,13-18] and is not extensively covered in this review. Rather, these findings will be briefly summarized, and detailed consideration will be given to the in vitro systems which are now used to study the action of the vitamin. Recent publications which are peripheral to this basic reaction but which have appeared since the last major review in this area will also be cited.

DISCOVERY AND PROPERTIES OF γ-CARBOXYGLUTAMIC ACID

Indirect Evidence for a Prothrombin Precursor Protein

The possibility that a precursor protein was involved in the formation of prothrombin was clearly stated by Hemker et al.[19] who noted a clotting time abnormality in plasma from patients receiving anticoagulant therapy and postulated that it was due to the presence of an inactive form of prothrombin in these patients. A number of investigators[11,20-22] noted that when a large dose of vitamin K was administered to severely hypoprothrombinemic rats, there was a delay of from 30 to 60 min in the appearance of plasma prothrombin followed by a burst of prothrombin which rapidly replaced about half of the steady-state level of the protein. Subsequent restoration of normal prothrombin concentration proceeded at a lower rate. It was clear from these data that the rate of prothrombin synthesis observed during this initial period exceeded the theoretical induction curve based on the experimentally determined half-life of prothrombin. It was later shown[23] that the appearance of plasma prothrombin in these rats was preceded by a transient increase of prothrombin in rat liver microsomal fractions which peaked soon after vitamin K administration and then fell as prothrombin appeared in the plasma. These indirect studies strongly suggested that a liver precursor in the hypoprothrombinemic rat was being converted to prothrombin in a vitamin-dependent step and that, following depletion of this pool, the rate of prothrombin synthesis slowed and became dependent on the rate of synthesis of the precursor.

This hypothesis was strengthened by the demonstration[11,21,22] that the vitamin K-stimulated initial burst of prothrombin in the hypoprothrombinemic intact rat was decreased only slightly by prior administration of the protein synthesis inhibitor cy-

cloheximide. These studies suffered from the usual ambiguities associated with the use of inhibitors of protein synthesis in intact animals, and direct evidence of the presence of a liver precursor protein was obtained when Shah and Suttie[24] demonstrated that prothrombin produced when hypoprothrombinemic rats were given vitamin K and cycloheximide was not radio-labeled if radioactive amino acids were administered at the same time as the vitamin. This study further indicated that administration of radioactive amino acids to hypoprothrombinemic vitamin K-deficient rats prior to cycloheximide and vitamin K administration resulted in the formation of radioactive plasma prothrombin. These observations were consistent with the presence of a precursor protein pool in the hypoprothrombinemic rat that was rapidly being synthesized and which could be converted to prothrombin in a vitamin K-dependent step which did not require protein synthesis.

Plasma Abnormal Prothrombin and the Discovery of γ-Carboxyglutamic Acid

The hypothesis that there was a liver precursor to prothrombin was strengthened by direct observations that the plasma of man or animals treated with coumarin anticoagulants contained a protein which was in many ways similar to prothrombin. A protein antigenically similar to prothrombin but lacking biological activity was first demonstrated in the plasma of patients receiving anticoagulant therapy by Ganrot and Nilehn,[25] and an inactive prothrombin species in these patients was detected by the use of staphylocoagulase by Josso et al.[26] This finding was confirmed in a number of laboratories, and evidence was also obtained that nonfunctional forms of the other vitamin K-dependent clotting factors were present in these patients.[16] The presence of this abnormal, anticoagulant-induced form of prothrombin was first demonstrated in bovine plasma by Stenflo.[27] Evidence for the existence of this protein in plasma of other species has been sought by both immunochemical methods and by the assay of thrombin generation by nonphysiological activators. The general consensus at the present time[28,29] is that there is little or none of these proteins present in the plasma of the hypoprothrombinemic rat, the species most often used for vitamin K studies. Some abnormal prothrombin has been observed in the plasma of the chick, but it has been reported[28] that this protein is missing in plasma from anticoagulant-treated mice, hamsters, guinea pigs, rabbits, and dogs. The basis for the species variations which have been observed is not clear. It is possible that the protein is excreted in an asialo form that is rapidly degraded and, therefore, not detected, or it is possible that it does not leave the liver. No experiments to differentiate these two possibilities have been performed.

It was the discovery of these abnormal proteins that provided the approach that eventually led to an understanding of the nature of the vitamin K-dependent alteration of the prothrombin molecule. The initial characterization of the bovine abnormal prothrombin indicated that it appeared to have the same molecular weight, amino acid composition, and carbohydrate composition as normal prothrombin. The abnormal prothrombin did not adsorb to insoluble barium salts as did normal prothrombin. The lack of barium salt adsorption and the calcium-dependent electrophoretic and immunochemical properties suggested a difference in calcium-binding properties of these two proteins which was subsequently directly demonstrated[33,34] and shown[35] to be a property of an amino-terminal peptide (prothrombin fragment-1) which could be derived from the two proteins. The observation[32] that the abnormal prothrombin would yield thrombin when treated with trypsin or snake venom-derived prothrombin activators indicated that this portion of the molecule was normal and that the critical difference in the two proteins was the inability of the abnormal protein to interact with calcium ions which are needed for the phospholipid-stimulated activation by fac-

tor X_a. This alteration of the calcium ion-mediated phospholipid interaction with prothrombin has now been directly demonstrated.[36]

Although the initial studies of the abnormal bovine plasma prothrombin clearly implicated the calcium-binding region of prothrombin as the vitamin K-dependent region, they provided no evidence as to the chemical nature of this region. Nelsestuen and Suttie[37] isolated an acidic tryptic peptide from the prothrombin fragment-1 region of normal bovine prothrombin which would adsorb to insoluble barium salts and which bound calcium ions in solution. Stenflo[38] later isolated two acidic peptides from the fragment-1 region by different methods, and both groups postulated the existence of some unknown acidic, nonpeptide, prosthetic group attached to this portion of the molecule. Similar acidic peptides were isolated in other laboratories.[39-41] These peptides could not be obtained when similar isolation procedures were applied to preparations of abnormal prothrombin. Stenflo et al.[42] succeeded in isolating a tetrapeptide (residues 6 to 9 of prothrombin) which had an apparent sequence of Leu-Glu-Glu-Val. They demonstrated by a combination of mass fragmentation, NMR spectra, and chemical synthesis that the glutamic acid residues of this peptide were modified so that they were present as γ-carboxyglutamic acid (3-amino-1,1,3-propanetricarboxylic acid) residues. Independently, Nelsestuen et al.,[43] by rather similar methods, characterized γ-carboxyglutamic acid (Gla) from a dipeptide (residues 33 and 34 of prothrombin) which appeared originally to be Glu-Ser. These characterizations of the modified glutamic acid residues in prothrombin were confirmed by Magnusson et al.[44] who have shown that all 10 of the first 33 Glu residues in prothrombin are modified in this fashion. Subsequent studies[45-47] have provided additional data to support the characterization of Gla residues in prothrombin. The structure of this amino acid and the location of the Gla residues in prothrombin are shown in Figure 2. The failure to identify this amino acid, which comprises almost 2% of the residues in prothrombin, in a protein which had been isolated for years and repeatedly subjected to amino acid analysis is, of course, due to its acid lability. This malonic acid derivative is quantitatively decarboxylated to glutamic acid by the standard conditions of 6 *N* HCl hydrolysis, and can only be quantitated by basic hydrolysis.

γ-Carboxyglutamic Acid in Other Proteins and Tissues

Coagulative factors VII, IX, and X also depend on vitamin K for their synthesis and would be expected to contain Gla residues. The amino-terminal region of factor IX and of the light chain of factor X are homologous with the amino-terminal region of prothrombin, and they have been shown[49-52] to contain these modified glutamic acid residues. Factor VII is present in very small amounts in plasma, and although sequence homology would indicate the presence of Glu residues in this protein, it has not been unambiguously sequenced at these positions.

It had been assumed for years that the biosynthesis of these few proteins represented the only role of vitamin K in animal tissues. Through the use of immunochemical methods, Stenflo[53] demonstrated that there was a fifth vitamin K-dependent protein (protein C) in bovine plasma. This protein has regions of homology with the other vitamin K-dependent clotting factors, binds to insoluble barium salts, and contains γ-carboxyglutamic acid. The physiological function of this protein is unknown. Current evidence[50,54-56] would suggest that it is not required in either the extrinsic or intrinsic pathways of blood coagulation as they are currently understood. Further work will be required to establish what role, if any, this protein has in normal hemostatic control. A sixth γ-carboxyglutamic acid-containing plasma protein (protein S) which, like protein C, shows considerable structural homology to the long-recognized vitamin K-dependent clotting factors has been identified in human plasma.[57] Prowse and Esnouf[58]

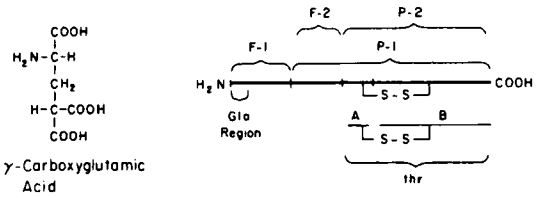

FIGURE 2. Structure of γ-carboxyglutamic acid (Gla) and a diagrammatic representation of the prothrombin molecule. Specific proteolysis of prothrombin by thrombin and factor X_a will cleave prothrombin into the specific large peptides shown: fragment-1 (F-1), fragment-2 (F-2), prethrombin-1 (P-1), prethrombin-2 (P-2), and thrombin (thr). For details of the activation of prothrombin to thrombin, see Reference 16. The Gla residues in bovine prothrombin are located at residues 7, 8, 15, 17, 20, 21, 26, 27, 30, and 33, and they occupy homologous positions in the other vitamin K-dependent plasma proteins.

have also described the isolation of a bovine plasma protein (protein Z) which contains Gla residues and which is probably different than the known coagulation factors or proteins C or S.

The demonstration by Hauschka et al.[59] that a Gla-containing protein could be isolated from chick bone raised the possibility that vitamin K-dependent proteins might serve important physiological functions in a number of tissues. A similar protein was independently isolated from bovine skeletal tissue by Price et al.,[60] and the amino acid sequences of this protein and of the swordfish protein have now been reported.[61] Three of the 49 residues of this small protein have been identified as γ-carboxyglutamyl residues, but there is no apparent structural homology between the bovine protein and the vitamin K-dependent plasma proteins. The function of this skeletal protein, which binds tightly to hydroxyapatite crystals, is not known, but its location and calcium-binding properties[62,63] suggest that it plays some role in either bone calcification or demineralization.[64-66] The presence of this amino acid in skeletal tissue[66a] has also raised the possibility[67] that it might be used as an index of organic matter leached from fossil bones.

The discovery of a protein(s) in bone, as well as plasma, which contained γ-carboxyglutamic acid indicated that the distribution of vitamin K-dependent proteins is much more widespread than was once realized. Analyses of a number of calcium-binding proteins from various sources and of barium citrate adsorbing proteins from various plasma and extracts[68] have not revealed a widespread distribution of the amino acid. A number of individual proteins have, however, been reported to contain Gla residues. These include a coagulating enzyme from Limulus crab,[69] which is involved in gelation of a clottable protein in the amoebocyte lysate, and proteins found in calcium-containing kidney stones.[70] A number of pathological states result in an ectopic calcification of various tissues, and protein-bound Gla has been found in these lesions.[71] Kidney cortex contains nearly one residue of Gla for each 10^4 amino acid residues,[72] and these appear to be associated with a kidney protein rather than with fragments of clotting factors being cleared from the plasma. All of these proteins may be associated in some way with calcium metabolism or binding, as is a protein in the chick choreoallantoic membrane which is vitamin K-dependent and which has also been reported to contain Gla.[73-76] One of the more intriguing recent reports is that of the presence of Gla residues in both eukaryotic and prokaryotic ribosomal proteins.[77,77a] If confirmed, this

observation would expand the potential role of the vitamin in basic cellular physiology. It is possible that some of the reports of Gla-containing proteins will not be substantiated, but at the present time, it does appear that a large number of Gla-containing proteins will eventually be found. If the reasonably large number now known were identified in a year or so by a few labs, there seems little doubt that many such proteins await discovery. The vitamin K-dependent carboxylase system is found in close association with a vitamin K epoxidase activity, and the significant activity of this enzyme in placenta and spleen[78] would suggest that these tissues are likely sources of Gla-containing proteins. The presence of this activity in marine invertebrates[79] has also raised the possibility that these lower forms of life might contain carboxylated protein and require vitamin K for their normal physiological well-being.

Chemistry and Metabolism of γ-Carboxyglutamic Acid

The discovery of a previously unknown amino acid has, of course, led to studies of its chemistry and metabolism. A number of syntheses of DL-γ-carboxyglutamic acid have been reported,[45,80-83] and one asymmetric synthesis has been accomplished.[84] This synthesis has not been proved to be useful for the production of useful amounts of the amino acid, and resolution of the racemic mixture has been reported.[85,86] The interaction of Gla-containing proteins with metal ions has suggested the use of low-molecular weight Gla-containing peptides as models for this interaction, and synthesis of these peptides has now been reported.[86,87] Proteins containing Gla were not detected for years because of its acid lability, but this amino acid can be quantitatively determined following alkaline hydrolysis. Methods for Gla analysis based on slight modification of the standard amino acid analyzer programs[88] or by the use of a completely different column[89] have been published. A number of other acidic compounds elute very close to the Gla region on a standard amino acid analyzer program, and its presence should always be confirmed by the disappearance of the assumed Gla peak by acid hydrolysis of the sample. The presence of Gla residues in mass spectrographic analysis of the phenylthiohydantoin derivatives of the amino acid[46] should probably serve as the definitive test of the presence of this amino acid. Chemical methods for the detection of Gla residues have also been reported.[49,90,91] Crystallographic analysis of the free acid[92] and Ca^{++}[93] salts has been reported, and a preliminary report[94] of the crystal structure of the F-1 region of prothrombin is available.

The discovery of γ-carboxyglutamic acid in prothrombin also raised questions of its metabolism. Fernlund[95] identified free Gla in human urine and determined that the daily excretions in their nonfasting males ranged from 27 to 42 μmol and that, in six nonfasting females, the excretion ranged from 19 to 32 μmol. Lian et al.[96] found normal excretion to be in the same range and that urine from patients receiving coumadin therapy contained only about 20% as much free Gla. Lian et al.[70] have also demonstrated the presence of a Gla-containing protein in calcium oxalate kidney stones, and stone formers were found to excrete more Gla than normal controls. The Gla excretion was also enhanced in patients with ectopic calcification. These lesions have also been shown to contain a Gla-rich protein. Protein-bound Gla was present to an extent of only a few percent of the free Gla found in the urine.[96] Bezeaud et al.[97] have shown that some prothrombin F-1 fragment is present in urine, and it is not clear whether the free Gla in urine arises from the breakdown of urinary Gla-containing proteins or whether these proteins are degraded and the free Gla excreted by the kidney. If the excretion is as free Gla, the tissue that is responsible for the complete breakdown of these proteins has not yet been determined. The Gla-containing bone protein, osteocalcin, represents a significant portion of the body Gla pool, but definitive studies of the turnover of this protein are not yet available.

Although it might be expected that there would be an active enzymatic system to

decarboxylate free Gla and convert it to glutamic acid, this is apparently not the case. Shah et al.[98] studied the metabolism of radioactive Gla and found that the injected amino acid was quantitatively excreted in the urine. In nephrectomized rats, distribution of injected Gla into various tissues was enhanced, but the amino acid was still not metabolized. After 2 hr, the majority of the injected Gla was in the bone. Studies with tissue slides[98] showed that kidney contains an active transport system for the amino acid, but that it appears to be passively distributed in other tissues studied.

IN VITRO SYSTEM TO STUDY VITAMIN K ACTION

Early Systems Producing Clotting Activity

Because of the sensitivity of assays for clotting factor activity, particularly factors VII and X, a number of systems utilizing rat liver slices and isolated liver cells[16] have been used to study vitamin K action. These systems were dependent on pretreatment of donor animals with the vitamin and did not furnish definitive answers to questions concerning the mechanism of action of the vitamin.

A number of in vitro cell-free systems which showed an increase in factor VII or factor X activity upon incubation have also been described.[99-103] As with the liver slice systems, they depended on prior treatment of the animals, and only recently[104] have data been presented to show that any of these systems responded to the in vitro addition of vitamin K. They have, therefore, been of limited value in determining the metabolic role of vitamin K. An in vitro system that produces radioimmunologically detectable amounts of prothrombin upon incubation has also been described.[105,106] The amount of prothrombin produced in this system is dependent on the prior vitamin K status of the rats used, but it does not respond to the in vitro addition of the vitamin. More recently, the synthesis of an immune reactive prothrombin species has been demonstrated[104] in a heterologous system using rat liver mRNA and rabbit reticulocytes. The material produced can be degraded by *Echis carinatus* venom and appears to have a molecular weight of about 75,000. Presumably the vitamin K-dependent carboxylase system is not functional in this system, and the material produced corresponds to the primary gene product (precursor protein) rather than to prothrombin. However, the product has not yet been completely characterized.

The first vitamin K-dependent in vitro system which produced prothrombin, rather than one of the activating factors, was that described by Shah and Suttie.[108] In this system, postmitochondrial supernatants from vitamin K-deficient rats were shown to respond to the addition of vitamin K by producing a significant amount of prothrombin as assayed by the standard two-stage assay. Prothrombin formation was partially dependent on an energy supply and was inhibited by direct antagonists of vitamin K but not by inhibitors of polypeptide chain biosynthesis.

In Vitro Vitamin K-Dependent Carboxylation

After the vitamin K-dependent step in prothrombin synthesis was shown to be the formation of γ-carboxyglutamic acid residues, the postmitochondrial system of Shah and Suttie was used[109] to demonstrate that the addition of vitamin K and $H(^{14}C)O^-_3$ promoted the carboxylation of endogenous microsomal proteins. This carboxylation reaction (Figure 3) has essentially the same requirements as the in vitro prothrombin synthesizing system. It was possible to isolate radioactive prothrombin from this system following incubation and to show that substantially all of the incorporated radioactivity was present as γ-carboxyglutamic acid residues in the fragment-1 region of prothrombin. These observations appeared to offer final proof of the role of vitamin K in the biosynthetic process, and subsequent studies of the action of vitamin K have

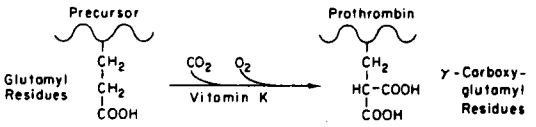

FIGURE 3. The vitamin K-dependent carboxylation reaction.

utilized this basic system or a modification of it. The vitamin K-dependent carboxylase, or in vitro prothrombin formation, has now been studied in postmitochondrial supernatants or washed microsomal preparations[109-114a] and in detergent-solubilized preparations.[115-119a] Most studies have utilized the endogenous microsomal protein precursors as substrates, but it has been demonstrated that low-molecular weight peptides which are homologous to the carboxylated regions of the prothrombin precursors will serve as substrates,[120] and these compounds are being increasingly used to study the action of the enzyme.[121-123] The currently accepted properties of this system will be considered in detail below. Vitamin K-dependent carboxylation has also been reported in kidney[72] and bone microsomes,[66,72a] but the majority of the available data deals with the liver system.

Other in Vitro Systems

Vermeer et al.[124] have described a bovine liver preparation which generates prothrombin when the purified abnormal prothrombin obtained from anticoagulant treated cattle is added. The preparation is obtained from Triton® X-100-treated microsomes, and the development of biological activity (two-stage prothrombin assay) is assayed. The dependence of this system on vitamin K and oxygen is less pronounced than in the carboxylase system used by others. The system is also more dependent on ATP than the solubilized or partially solubilized carboxylase preparations. In a subsequent publication[125] it has been reported that the enzyme (factor II synthetase) has been purified 10,000-fold from Triton® X-100 solubilized microsomes. This preparation has lost its requirement for ATP, and is now dependent on oxygen but not on NAD(P)H. In this system, it has been possible to demonstrate a fixation of $^{14}CO_2$ into $BaSO_4$ adsorbable proteins which parallels the increase in clotting activity. It has not been demonstrated that the ^{14}C is present in the abnormal prothrombin added. From 4 to 8% of the added abnormal (descarboxy) prothrombin is converted to prothrombin, and the system is inhibited by the addition of prothrombin. The relationship of the system to the rat liver microsomal carboxylase system studied by others is difficult to ascertain. It is not clear how many of the ten Gla residues in prothrombin must be present before it is active in the two-stage assay. Other workers have not found that crude preparations of descarboxy prothrombin are particularly good substrates for the rat liver carboxylase. Because of the differences which are apparent, and until further data on CO_2 incorporation become available, it may not be safe to assume that this system is the same as the rat liver carboxylase. In any event, it is a vitamin K-responsive system that in many respects may prove to have advantages over the rat liver system.

The other in vitro vitamin K-responsive system currently under study is a microsomal system reported by Lowenthal and Jaeger.[104] Incubation in the presence of vitamin K, but not the 2-ethyl or the normethyl derivative of phylloquinone, led to a threefold increase in factor VII activity. Based on a lack of a demonstrable energy requirement or a requirement for cofactors "usually" associated with carboxylation reactions, it was concluded that the carboxylation observed by others is not the vitamin K-dependent step. Experimental evidence to support this conclusion appears to be lacking.

VITAMIN K-DEPENDENT CARBOXYLASE

Substrates for the Carboxylase

The majority of studies dealing with the vitamin K-dependent carboxylase have utilized the endogenous rat liver microsomal precursors as substrates for the reaction. These proteins have been partially characterized. The existence of these precursors of the vitamin K-dependent clotting factors had been postulated on the basis of indirect evidence, and it was subsequently shown[126] that a thrombin-like activity could be generated in liver microsomal preparations obtained from vitamin K-deficient rats by limited treatment with *Echis carinatus* venom. Very little of this activity was present in preparations from normal rats. Further study[127] demonstrated that this precursor decreased rapidly when vitamin K was injected and, as its level fell, the amount of microsomal prothrombin increased and then fell as it moved out of the liver into the plasma.

Based on the *E. carinatus* assay, a protein was isolated[128] from the liver of Warfarin-treated rats which had the properties predicted for the prothrombin precursor. The purified precursor was a glycoprotein which was immunochemically similar to prothrombin and had a molecular weight indistinguishable from rat prothrombin. Electrophoretic and isoelectric focusing analyses indicated that the precursor was less negatively charged (pI = 5.8) than plasma prothrombin (pI = 5.0). Specific proteolysis of the precursor by thrombin, Taipan snake venom, or clotting factor X_a yielded fragments indistinguishable from those formed[129] by similar proteolysis of rat prothrombin. This protein did not adsorb to $BaSO_4$, and its rate of activation to thrombin by factor X_a and Ca^{++} was not stimulated by the addition of phospholipid. It differed from prothrombin in that it did not contain sialic acid residues nor γ-carboxyglutamic acid, and it differed from the bovine plasma abnormal (descarboxy) prothrombin in that it lacked the sialic acid residues which the plasma protein contains. A second protein with properties very similar but with an isoelectric point of 7.2, was subsequently isolated[130] from the same microsomal preparations. The increased basic nature of this protein is a property of the amino-terminal region of the molecule, but the chemical alteration responsible for the shift in pI has not been determined. Liver microsomal preparations from vitamin K-deficient or Warfarin-treated rats have also been investigated by the technique of crossed immunoelectrophoresis,[131,132] and two major proteins which react with prothrombin antibody and whose mobility is not altered by the presence of Ca^{++} in the buffers can be seen. The amount of both of these proteins decreases to a very low level when vitamin K is administered to the animals prior to preparation of the microsomes. One of these proteins has been shown[132] to be identical to the isolated pI 7.2 protein, but the other appears to be more basic than the pI 5.8 protein. Whether it is another major unspecified species in the microsomes or a complex of the pI 5.8 precursor and some other protein has not yet been determined.

There is now additional evidence of multiplicity of precursor forms in the liver. It has been shown[133] that a cultured rat liver hepatoma cell line will respond to vitamin K addition by increasing the concentration of both intracellular and media prothrombin, and the intracellular prothrombin precursor forms in this preparation have now been investigated. Precursors have been isolated[134] from cell homogenates by absorption and elution from a prothrombin antibody column followed by isoelectrofocusing. This technique has demonstrated the presence of three major prothrombin precursor species having isoelectric points of 7.2, 6.7, and 5.8. Alterations in the level of these proteins have been shown following vitamin K administration, but detailed understanding of the precursor-product relationship between the various species is still lacking.

Which of these species is (are) the substrate for the carboxylase in the in vitro system

TABLE 1

Activity of Various Peptide Substrates for the Vitamin K-Dependent Carboxylase

Peptide	Relative activity
Phe-Leu-Glu-Glu-Leu (I)	100
Phe-Ala-Glu-Glu-Leu (II)	72
Phe-Gly-Glu-Glu-Leu (III)	4
Phe-Glu-Leu-Glu-Leu (IV)	<1
Phe-Glu-Ala-Leu-Glu-Ser-Leu (V)	6
Val-His-Leu-Ser-Ala-Glu-Glu-Lys-Glu-Ala (VI)	26

Note: All peptides were assayed at a concentration of 1 m*M*, and the data are expressed relative to the activity of peptide I. Peptide V is analogous to the region of the bovine prothrombin precursor which would contain residues 29 to 35 of bovine prothrombin, and peptide VI is homologous to a portion of the amino-terminal region of the β-chain of hemoglobin. For details, see Reference 123.

is not clear. Although it has been demonstrated that CO_2 is incorporated into a fragment which appears to be the prothrombin fragment-1 region,[109] there are very few data in the in vitro system to indicate what is being carboxylated. The standard assay merely measures vitamin K-dependent TCA precipitable radioactivity. There is a small amount of preliminary data[135] to suggest that the pI 7.2 precursor can be carboxylated to a pI 6.8 form, but the data are not conclusive. The pI 7.2 form is more tightly associated with the membrane,[130] and it is an intriguing possibility that it is carboxylated, then subjected to a metabolic transformation to remove a basic prosthetic group before it appears as prothrombin.

The difficulty of manipulating the concentration of the endogenous substrate has prompted a search for a simpler substrate, and Suttie et al.[120] have shown that the pentapeptide Phe-Leu-Glu-Glu-Val, which is analogous to residues 5 to 9 of the presumed bovine precursor, is a substrate for the carboxylase. Houser et al.[121] subsequently demonstrated that similar pentapeptides with a carboxyterminal Leu and Ile residue were also substrates, and Suttie et al.[123] have prepared a number of low-molecular-weight peptides which serve as substrates for the reaction. The activities of some of these are shown in Table 1. Although detailed kinetic analyses have not been carried out, a reasonable degree of substrate specificity is apparent. It is unlikely that the sequence around the carboxylated residue is a major factor in the normal control of this carboxylation event. There is no great degree of homology in the amino acid sequence around the various carboxylated residues in prothrombin, and a small peptide containing a Glu-Glu sequence from the β-chain of hemoglobin is a substrate for the rat liver carboxylase.[123] It has also been shown[66] that bone microsomes will carboxylate Phe-Leu-Glu-Glu-Val, which is related to bovine prothrombin precursor. These observations suggest that, although many proteins with Glu-Glu or other carboxylated sequences must pass through the endoplasmic reticulum, only those with the correct tertiary structure have these residues readily available to the carboxylase.

The properties associated with peptide carboxylase activity have been found[123] to be similar to that of the carboxylase of the endogenous precursors. The most important difference is in the extent and rate of the reaction. Even at low temperatures, the carboxylation of the precursor proteins is very rapid, and the substrate is soon depleted. The peptide substrate most studied, Phe-Leu-Glu-Glu-Leu, has an apparent K_m of about 4 m*M*, and a linear rate of reaction can be observed for extended periods. These responses are shown in Figure 4. Peptide carboxylation appears to be more dependent on inhibitors and stimulators of carboxylation in the media, and it is strongly

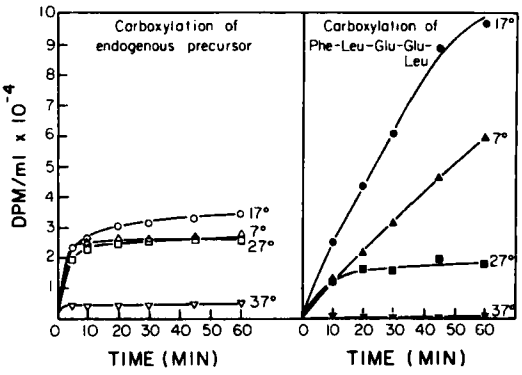

FIGURE 4. Relative rates of carboxylation of endogenous microsomal precursors and a peptide substrate, Phe-Leu-Glu-Glu-Leu, by a Triton® X-100 solubilized rat liver microsomal preparation. The microsomes were obtained from vitamin K-deficient rats, and the peptide concentration was 0.5 m*M*. See Reference 123 for details.

stimulated by the presence of pyridoxal phosphate which has no effect on the endogenous carboxylation. The presence of peptide substrates, even at concentrations which result in much more carboxylation of the peptide than of the endogenous substrate, has no effect on the extent of carboxylation of the endogenous substrate. Effects of these compounds on the rate of the endogenous substrate carboxylation rather than on its extent have not been reported.

The products of the carboxylation have not been definitvely characterized, and there are no data to indicate the extent of carboxylation of the endogenous precursors. The major product of the carboxylation of the pentapeptide Phe-Leu-Glu-Glu-Leu appears[136] to be Phe-Leu-Gla-Glu-Leu, with only a small amount of the dicarboxylated product being formed. It is apparent from in vivo studies that the precursor carboxylation is not an "all or none" event. The abnormal prothrombin studied by Stenflo and Ganrot[30] and by Nelsestuen and Suttie[32] which was isolated from bovine plasma following anticoagulant treatment apparently contained few, if any, Gla residues and would not bind to insoluble barium salts. A series of publications from Malhotra[137-146] and a report of Prowse et al.[141] describe a form of prothrombin obtained from similar plasma which, to some degree, maintains its ability to bind to insoluble barium salts and which is presumably partially carboxylated. These partially carboxylated forms have now been subjected to Gla analyses,[142,143] and it has been demonstrated that fractions with 6 to 7 or 3 to 4 Gla residues per mole of prothrombin can be obtained. Normal prothrombin contains ten residues of Gla per mole. It has been suggested[142] that the partially carboxylated forms contain a number of (Gla-Glu) pairs, but as these preparations are not homogeneous,[143] an unambiguous residue distribution cannot be assigned at this time. These data would suggest that the in vitro systems are also forming incompletely carboxylated proteins, but no data on the extent or distribution of the carboxylated residues are available. Further development of the system described by Vermeer et al.[124,125] might provide the most direct approach to this problem.

Active Forms of Vitamin K and Concentration Dependence

As with all vitamins, there is considerable literature dealing with the biological activity of different forms of vitamin K in various species. These studies have recently been

reviewed.[1,2] Relative activities measured in an intact animal are, of course, dependent on absorption and metabolism of the vitamin as well as on the interaction of the vitamin at its active site. The development of in vitro systems therefore opened the possibility that the true structural requirements for vitamin action would be established. Some information which is directed toward this ultimate goal is available; unfortunately, the published studies have been done on differing systems and are difficult to directly compare.

Friedman and Shia[113] utilized microsomes, suspended in buffer, and measured the carboxylation of endogenous precursors. They found that MK-2 (3-geranyl) had 10 times the relative activity of K_1, and MK-3 (3-farnesyl) had 80 times the activity. The *cis* isomer of vitamin K_1, the 2-demethyl derivative of vitamin K_1, MK-1 (methyllapachol), or menadione itself had little or no activity. The compounds were compared on the basis of the molar concentration of the compound which would be needed to give the same extent of carboxylation in a fixed time assay as vitamin K_1. Compounds which were not active were also tested in the hydroquinone form to assure that it was not the specificity of the vitamin K reductase which was being assayed. Jones et al.[111] studied prothrombin production rather than carboxylation in postmitochondrial supernatants from vitamin K-deficient rats and found that at a concentration of 4.4×10^{-5} *M*, vitamin K_1, MK-2, MK-3, MK-4, MK-5, MK-6, and MK-7 had roughly similar activity. Menadione or 2,3-dimethyl-1,4-naphthoquinone was inactive, and MK-1 had about 30% of the activity of vitamin K_1. The response of various compounds was temperature dependent, and the lower homologs of the vitamin were relatively much better forms of the vitamin at low temperature. The higher molecular weight homologs (MK-4 and above) were relatively inactive below 30°C, the melting point of the microsomal membrane. In a detergent-solubilized system,[144] vitamin K_1, MK-1, MK-2, MK-3, and MK-4 at a concentration of 10^{-4} *M* have been reported to have nearly comparable activities. Menadione or 2,3-dimethyl-1,4-naphthoquinone had minimal activity. Although a number of early studies demonstrated that menadione was not an active form of the vitamin, it was subsequently shown[18,144] that, in the presence of dithiothreitol (DTT), menadione is alkylated to the DTT adduct, and this compound has activity. A number of other 3-thioethers, and 3-*O*-ethers of menadione have been prepared,[145] and many have appreciable activity. In general, hydrophobic or noncharged hydrophilic thioethers are active, but derivatives with charged thioether side chains, such as a cysteinyl group, are inactive.[145]

In the intact animal, only 2-methyl-3-polyisoprenoidyl-1,4-naphthoquinones appear to have significant activity.[1,2] The *cis* isomer of vitamin K_1 has little if any activity, and compounds with hydrophobic groups other than polyisoprenoid chains at the 3-position are poor forms of the vitamin. The data from the in vitro systems suggest that much of this specificity is due to varying rates of metabolism of different forms of the vitamin and to membrane interactions at the site of the carboxylase. Only low activity has been reported for the desmethyl form of the vitamin or for other derivatives at the 2-position. Effective vitamin K activity at the active site, therefore, appears to be related to 2-methyl-1,4-naphthoquinone with some degree of nonpolar substitution on the 3-position. All of the comparisons of activity in the in vitro system which have been reported should be considered to be qualitative only. All measurements have involved the incorporation of radioactivity into the endogenous precursor proteins or production of biological prothrombin activity at a fixed time point. As these activities may have reached completion during the incubation, alterations in these measurements may bear little or no relationship to rates of reaction. It is possible that the many compounds which have been found to have roughly the same activity as vitamin K_1 differ appreciably in the rate at which they drive the reaction.

An accurate comparison of the vitamin K dependence of different in vitro systems is also difficult to obtain because of alterations in the systems used by various investigators. In general, the requirement appears to be significantly lower in a system containing cytosolic proteins (vitamin K-binding protein?) and is increased as the systems become more purified. In a postmitochondrial system, where prothrombin formation was measured, half maximal activity was observed[111] at 10^{-7} *M* vitamin K_1. In this system, no reducing agent is required. When $^{14}CO_2$ incorporation into endogenous protein was studied in washed microsomes suspended in buffer, and supplemented with NADH, half-maximal activity was seen[110] at about 10^{-5} *M* vitamin K_1. This requirement was reduced to about 5×10^{-7} when a cytosolic fraction was added back to the system. Two separate studies[112,115] have reported a requirement of about 5×10^{-6} *M* vitamin K for half-maximal activity in a detergent-solubilized system when $^{14}CO_2$ incorporation into endogenous protein was measured. In these systems, the requirement for [vitamin K_1 + NADH] expressed in this manner was about the same as that of vitamin KH_2. In a solubilized system, the vitamin K requirement for the carboxylation of a synthetic peptide has been found[123] to be about five to ten times higher (half-maximal activity) than for carboxylation of the endogenous proteins in the same system.

The interpretation of these data is difficult. There are no data available to indicate to what extent the vitamin is recycled (quinone to hydroquinone or vitamin K-epoxide to hydroquinone) in these systems, nor any data to indicate to what extent the vitamin is free or lipoprotein-bound in these membranous or detergent micellar preparations. In any event, it is apparent that rather low concentrations of vitamin are sufficient to drive the reaction. Most experiments directed toward an investigation of the properties of the vitamin K-dependent carboxylase have utilized vitamin concentrations ranging from 5×10^{-5} to 5×10^{-4} *M*, and it is probably safe to assume that the concentration of vitamin was not limiting as other variables were studied.

Requirement for Reducing Equivalents

The initial report[109] of the vitamin K-dependent carboxylation reaction utilized a postmitochondrial supernatant preparation from vitamin K-deficient rats, and no reductant was added. Subsequent studies[110,113,116] utilizing washed microsomes demonstrated a requirement for cytosolic factors which appeared to be due primarily to the presence of reduced pyridine nucleotides and/or a reduced pyridine nucleotide-generating system in the cytosol. It was then demonstrated that the chemically reduced form of the vitamin, the hydroquinone, could substitute for the cytoplasmic factors. Although the majority of the vitamin K reductase activity of the cell is in the cytosol, there is appreciable activity in washed microsomes, and vitamin K and NADH will drive the carboxylation almost as well as vitamin KH_2.

The relationship of the vitamin K reductase active in the microsomal carboxylase system to the extensively studied DT-diaphorase (NAD(P)H dehydrogenase) of liver has been an open question. This has recently been investigated in detail by Wallin et al.[119] who have isolated the enzyme by affinity chromatography on a Sepharose 4 B-menadione column. The oxidoreductase isolated by this method appears to be the same enzyme that has been extensively studied by Ernster et al.[146-149] and, as has been previously shown, the majority of the enzyme is in the cytosol. However, repeated washing of the microsomal pellet in buffer leaves a minor fraction of the activity in the fraction, and this activity can be isolated from detergent-solubilized microsomes by the same affinity technique that was used to isolate the cytosolic protein. This enzyme has the same sensitivity to Warfarin and the same elution properties as the cytosolic protein, but a more rigorous comparison of the two proteins has not been carried out.

Detergent-solubilized microsomal preparations with this activity removed do not demonstrate a vitamin K-dependent carboxylase activity unless purified reductase is added back or vitamin KH_2 is added. The data are consistent with the view[150] that the physiologically important vitamin K reductase activity in the liver is identical to DT-diaphorase and that under most conditions sufficient amounts of the enzyme are present in normal microsomal preparations to drive the carboxylase.

The effect of dithiothreitol (DTT) on the carboxylase system has been variable. In intact microsomes it appears to be able to act as a reducing agent for the vitamin K reductase and to stimulate the NADH-driven reaction.[113,116] It is difficult from the data available to determine if DTT is directly reducing the quinone form of the vitamin or if it is merely recycling a small amount of vitamin K epoxide found in those preparations through the active epoxide reductase which is present.[151] In solubilized preparations where the DTT-driven vitamin K epoxide reductase is inactive, DTT has been reported to substantially stimulate[116] or to have essentially no effect[119] on the NADH-driven carboxylase or to have no effect on the vitamin KH_2-driven carboxylase. More recent data[123] would suggest that independent of the source of reducing equivalents, [vitamin K + NADH] or vitamin KH_2, the presence of DTT stimulated the carboxylase. This stimulation was more significant in the case where NADH was used, which suggests that both the microsomal vitamin K reductase activity and the carboxylase itself are sensitive to oxidation and are more active when some essential sulfhydryl group is protected.

ATP Requirement and Biotin Involvement

One of the more important questions regarding the vitamin dependent reaction has been the source of energy to drive the reaction. The postmitochondrial system from vitamin K-deficient rats that was first used to demonstrate vitamin K-dependent synthesis of prothrombin[108] was influenced by the presence of ATP and an ATP-generating system. When this system was used to demonstrate a vitamin K-dependent carboxylation reaction,[109] a stimulation of CO_2 fixation by the addition of ATP was still seen. When the permeability of the microsomal membrane to added metabolites was increased[110] by the addition of 0.15% Triton® X-100, ATP no longer stimulated the carboxylase, but the activity was inhibited by the ATP analog AMPP(NH)P. At low concentrations of the inhibitor, the inhibition could be partially reversed by the addition of ATP, and these data suggested some involvement of ATP in the reaction. The situation in a solubilized system appears clearer. The omission of ATP from a Triton® X-100 solubilized carboxylase preparation, even after gel filtration to remove any endogenous ATP, has no effect on the rate of carboxylation nor does the addition of AMPP(NH)P influence the rate.[115] The data suggest that the carboxylase itself has no requirement for ATP and that the stimulation that is seen in intact microsomal preparations is an indirect effect. It is possible that this is due to an energy-dependent translocation or modification of the endogenous precursor substrates within the microsomes, or it may be due to some energy-dependent influence on movement of components across the intact microsomal membrane. One fact that should be considered is that the microsomal endogenous precursor pool contains a basic (pI 7.2) prothrombin precursor form which has not been chemically characterized. There is the possibility that this protein has been "activated" in some way by an ATP-dependent reaction so that the carboxylation seen in the in vitro system is merely a utilization of this previously activated form of the substrate. The lack of any ATP dependence in the carboxylation of chemically synthesized low-molecular-weight peptide substrates[120] would, however, rule out this possibility and offer more convincing evidence that the energy needed for this carboxylation is in some manner derived from the oxidation (to the quinone or the epoxide) of the reduced form of the vitamin.

The lack of an ATP requirement, and the apparent utilization of CO_2 rather than bicarbonate, would also suggest that the carboxylation reaction does not involve biotin as a cofactor. Attempts in our laboratory to influence carboxylase activity by an alteration of biotin status of animals or by the addition of avidin to the incubation have been negative. Similar negative results have probably been obtained in most laboratories studying the reaction, but the report of Friedman and Shia[118] contains the only comprehensive data dealing with the studies of this type. They were unable to modify carboxylase activity by the development of a biotin deficiency or by addition of the biotin enzyme inhibitor avidin to an intact or detergent-solubilized preparation. It is possible that this carboxylase contains an extremely tightly bound form of the cofactor which is not available to the inhibitor, but the mass of evidence would suggest that biotin is not involved as a CO_2 carrier in the reaction.

Requirement for (CO_2/HCO_3^-) and O_2

Both the vitamin K-dependent carboxylase[117] and vitamin K-dependent in vitro prothrombin synthesis[110,111] have been shown to require the addition of HCO_3^- to the media. The apparent K_m for total CO_2 in the media in these experiments has been found to range from 0.2 to 1.0 m*M*. The lack of any evidence of the involvement of biotin in the reaction would suggest that CO_2 rather than HCO_3^- is the active species in the reaction. This question has been directly approached by Jones et al.[117] They have taken advantage of the low rate of CO_2/HCO_3^- equilibration at low temperatures in the absence of carbonic anhydrase reaction at 10°. At that temperature, phylloquinone is not a particularly effective form of the vitamin, and MK-2 was used. The data obtained are consistent with CO_2 being the active species for carboxylation. The majority of the data were obtained, however, with an intact microsomal system, and if, as is likely, CO_2 is the form which passes the microsomal membrane, no other result may be possible in this system. Some of the experiments were repeated in a Triton-solubilized microsomal preparation, and although the data are not as conclusive, they are still consistent with the hypothesis that CO_2 is the active form of (CO_2/HCO_3^-) utilized by the enzyme. It is possible that the active forms of the enzyme in the preparations used are part of a detergent micelle and that the same arguments regarding transport of CO_2 across a nonaqueous phase would still apply, which would again predispose the experiment to the result obtained. Assuming that this is not the case, these experiments as well as the apparent lack of involvement of biotin would suggest that CO_2 is the species directly involved in the carboxylation. However, the apparent K_m for CO_2 is considerably lower than that observed for many other carboxylases which use CO_2, and this may imply a considerably different mechanism.

Early studies of the vitamin K-dependent microsomal reaction established[110,113] that the system required oxygen. A report that the utilization of vitamin KH_2 in the system, rather than [vitamin K + NADH], eliminated the requirement for O_2[112] has not been substantiated, and the requirement for O_2 can be demonstrated in solubilized carboxylase preparations utilizing either endogenous proteins[115] or synthetic peptides[123] or abnormal plasma prothrombin[125] as CO_2 acceptors. The O_2 requirement for this system has not been studied in great detail. In a system where formation of biologically active prothrombin was measured,[111] the pO_2 measured for half-maximal conversion was about 10 mm Hg. This requirement is considerably higher than that of cytochrome oxidase but in the same range as that observed for a number of microsomal hydroxylase activities.[18]

Carboxylase Inhibitors or Stimulators

The vitamin K antagonists which are effective in vivo have also been studied as inhibitors of the microsomal vitamin K-dependent carboxylase in vitro. The direct an-

tagonist, 2-chloro-3-phytyl-1,4-naphthoquinone (chloro-K),[152,153] has been shown to be an effective antagonist of the carboxylase in both solubilized[115] and intact microsomal systems.[109] Its inhibition is readily reversed[110] by increasing the vitamin K concentration, and it appears to be functioning in vitro as it does in the intact animal, as a true competitive inhibitor of the vitamin. The compound 2,3,5,6-tetrachloro-4-pyridinol[154,155] is an anticoagulant which appears[156] to have the same effect in intact animals as chloro-K. This direct antagonist of the vitamin has also been shown to be an effective antagonist of the solubilized carboxylase.[112,119]

The action of the coumarin anticoagulants is more complicated. In intact microsomes, and with NADH as a reducing agent, Warfarin is a very poor antagonist of the carboxylase,[110] and high concentrations are needed for significant inhibition. In intact microsomes, with DTT as a reducing agent, the vitamin K epoxide reductase is active, and Warfarin is an extremely effective inhibitor of the carboxylase.[151] The Triton® X-100 solubilized preparation of the carboxylase requires high concentrations of Warfarin for significant inhibition,[123] which is consistent with the absence of a significant amount of epoxide reductase in this preparation. The solubilized epoxide reductase system[157] recently reported also has carboxylase activity; as expected, the system is now Warfarin-sensitive.

The vitamin K-dependent carboxylase system is also rather sensitive to sulfhydryl poisons and is effectively blocked by *p*-hydroxymercuribenzoate[113,116] but is apparently not particularly sensitive to iodoacetamide or *N*-ethylmaleiamide.[157] A recent preliminary report[158] has claimed that a number of naphthoquinones, such as menadione, which are not in themselves active forms of the vitamin in vitro, are in fact inhibitors of the vitamin K-dependent carboxylase. The compounds which are active appear to be those which can react with thiols, and they may be functioning to block the same essential sulfhydryl that is titrated with *p*-hydroxymercuribenzoate. Other inactive forms of the vitamin such as 2-hydroxy or 2-methoxy-1,4-naphthoquinone, which do not react with thiols, were not inhibitors. However, the hydroquinone forms of these compounds were inhibitors, and it was suggested that the compounds represent a new class of true direct antagonists of the hydroquinone form of the vitamin at its active site.

A large number of other compounds have been tested on a rather hit-or-miss basis as inhibitors or activators of the system. The spin-trapping agent 5,5-dimethyl-1-pyroline-*N*-oxide inhibits at high concentrations,[112] and the system is rather sensitive to ethanol,[123] which has sometimes been used as a vehicle for vitamin or potential inhibitor addition. The solubilized carboxylase system has also been reported to be inhibited by the enzymes glutathione peroxidase[159] and superoxide dismutase.[119a] Among the compounds reported not to inhibit the reaction are CN^-, azide, cytochrome P-450, ATPase, AMPP(NH)P, EDTA, dithionite, bisulfite, or avidin. Many of these compounds have not been tried at a wide range of concentration in the various possible systems, i.e., soluble or intact microsomes, [vitamin K + NADH] or vitamin KH_2, and it is possible that, under certain conditions, some of these compounds may be found to be inhibitors. One problem with the carboxylase system is that essentially all of the data available are measurements of CO_2 fixation into endogenous protein precursors at a fixed time, usually 20 or 30 min. The reaction is very sensitive to elevated temperatures, and at temperatures as low as 10 to 15°C where the system is stable, the reaction is essentially over at 10 min (Figure 4) and is limited by the amount of substrate available. It may be that a number of compounds which have been tested do have a highly significant effect on the rate of carboxylation but that they have not been detected by the assay conditions used. A study of the effect of a large number of alterations of the system on the carboxylation of both endogenous protein precursors and low-molecular-weight peptide substrates has recently been completed.[123] The data

suggest that, in most respects, the carboxylation of peptide substrates is more sensitive to almost any perturbation of the system. This may indicate some basic difference in the two systems, or it may simply reflect the increased ability of the peptide substrate assay to detect alterations in the rate of the reaction.

Other than the effect of reducing agents such as DTT in stabilizing and, in some circumstances, stimulating the carboxylase reaction, there have not been many compounds which have been shown to stimulate the carboxylase. Pyridoxal phosphate has been shown[123] to give a three- to fivefold stimulation in the rate of peptide carboxylase activity with little or no effect on endogenous protein carboxylation. The basis for this stimulation is not yet clear. The properties of the system are summarized in Table 2.

Solubilization, Induction, Localization, and Purification of the Carboxylase

Although early studies on the vitamin K-dependent carboxylase were carried out in intact microsomal preparations, it was soon demonstrated[115,116] that the activity could be readily solubilized. Concentrations of Triton® X-100 above 1% were found to be compatible with activity of the enzyme, and at these concentrations essentially all of the activity remained in the supernatant following centrifugation at 105,000 × g for 1 hr.[115] Although most studies of the carboxylase activity have utilized Triton® X-100 as a detergent, the system has also been solubilized in Lubrol-PX®,[116] Renex®,[160] deoxycholate,[116] and octylglucoside.[161] The system is rather sensitive to high concentrations of deoxycholate, and it must be removed for the activity to be completely assayed. A solubilization of the microsomal vitamin K epoxide reductase activity by treatment with potassium cholate has recently been reported,[157] and this preparation has been shown to also have carboxylase activity. It has also been possible[162] to prepare a stable acetone powder from the standard rat liver microsomal preparation.

The initial studies of peptide substrates for the enzyme[120] demonstrated that the enzyme activity was much higher in vitamin K-deficient rats than in normal rats. This apparent induction of enzyme activity has now been studied in more detail.[163] Feeding rats a vitamin K-deficient diet or the administration of Warfarin caused a two- to threefold increase in carboxylase activity. The increase in peptide carboxylase activity was not dependent on the presence of increased amounts of prothrombin precursors in the microsomes and was more closely correlated with the degree of plasma hypoprothrombinemia than with the level of these precursors in the liver. The ability of the microsomal carboxylase to carboxylate endogenous protein precursors appeared to reflect the level of these precursor substrates in the liver rather than the activity of the carboxylase. The apparent induction of peptide carboxylase activity was not due to an alteration in affinity of the enzyme for its peptide substrate. The carboxylase has a very short biological half-life, and this apparent rapid turnover of the enzyme complicated the interpretation of experiments which utilized the protein synthesis inhibitor cycloheximide. However, the data obtained were consistent with the hypothesis that the increased carboxylase activity measured represented a synthesis of an increased amount of some component enzyme rather than an activation of existing inhibited activity.

Helgeland[114] has studied the submicrosomal localization of the prothrombin precursors and the vitamin K-dependent carboxylase in rat liver. Her data indicate that precursor activity is present in both rough and smooth microsomes, but concentrated in the former. As indicated from studies of the isolation of these proteins,[128,130] much of the precursor activity was present in the lumen of the microsomal vesicles, but some was tightly complexed to the membrane. The carboxylase activity was found to be almost exclusively located in the rough endoplasmic reticulum and appeared to be predominately associated with the lumenal membrane surface. These studies, utilizing the

TABLE 2

Properties of the Vitamin Dependent Carboxylase

Absolute requirements
- Vitamin K and NAD(P)H or vitamin KH_2
- O_2
- CO_2 acceptor (endogenous precursors or synthetic peptides)

Known inhibitors
- Chloro-K
- Warfarin[a]
- *p*-Hydroxymercuribenzoate

Noninhibitory conditions
- ATP analog AMPP(NH)P[b]
- Avidin

Stimulatory conditions
- Dithiothreitol
- Additional substrate (peptide)

Note: There is not complete agreement in the published literature on all points; the properties assigned represent the author's evaluation of the consensus of the published literature, and only those properties confirmed by more than one laboratory are included.

[a] At low concentrations when epoxide reductase is active, at higher concentrations under other conditions.
[b] Some inhibition when intact microsomes are present.

endogenous precursors as substrates for the carboxylase, may have failed to detect carboxylase activity not associated with the presence of precursor, and the localization has recently been studied utilizing solubilized microsomal fractions and peptide substrates.[132,164] The rough microsomal fraction was again found to be highly enriched in carboxylase activity. Lower, but significant, levels were found in smooth microsomes. Mitochondria, nuclei, and cytosol had negligible activities. The addition of 0.2% Triton® X-100 to intact microsomes results in a 10- to 20-fold stimulation of peptide carboxylation. This marked latency suggested that the enzyme system may be accessible only from the lumen of the microsomal membrane. This finding was supported by the inaccessibility of any strongly rate-limiting component of the carboxylase to trypsin in the absence of Triton. As in Helgeland's studies, the data obtained are consistent with the hypothesis that carboxylation occurs primarily on the lumen side of the rough endoplasmic reticulum.

No substantial purification of the carboxylase has been published at this time. During attempts at purification, the enzyme acts as a typical integral membrane protein. It requires appreciable concentrations of detergent to solubilize and is not removed from the membrane by techniques which are able to remove many peripheral or lumenal microsomal proteins. A preliminary report[165] has indicated that a 270-fold purification of peptide carboxylase activity can be achieved by methods that depend largely on the interaction of adsorbants with the prothrombin precursor proteins within the membrane. Presumably, the product isolated was a precursor-carboxylase complex, and the yield in this method was only about 1% of the total activity. One major problem involving the purification appears to be the presence of an inhibitor of carboxylase activity[122] in the microsomal membrane. Many of the manipulations that appear to lead to purification of the carboxylase may well represent only a removal

of this factor. A purification of 10,000-fold has been reported for the somewhat different system (factor II synthetase) that has been studied by Vermeer et al.[124,125] How this bovine liver activity is related to the rat liver carboxylase has not yet been determined.

VITAMIN K METABOLISM

Vitamin K Epoxidase

A significant advance in an understanding of the metabolism of vitamin K came with the discovery by Matschiner et al.[166] that an appreciable amount of the total vitamin K in liver was present in the form of its 2,3-epoxide. Early studies by Willingham and Matschiner[167] indicated that an "epoxidase" activity could be demonstrated in isolated microsomes and that this activity required a soluble protein, a heat stable factor, and O_2. Subsequent studies[168] indicated that the epoxidase activity was inhibited by direct antagonists of vitamin K action, such as tetrachloro-4-pyridinol and chloro-K. Although they were not antagonists of this reaction at low concentrations, the coumarin anticoagulants were shown to be effective inhibitors[169] of the epoxidase at elevated concentrations.

A subsequent study by Sadowski et al.[170] demonstrated that the heat stable factor could be replaced by NADH and that, at least under these circumstances, no soluble cytoplasmic protein was required. The NADH requirement was abolished if the reduced form of vitamin K was used as the substrate, and it was shown that the hydroquinone form of the vitamin was the species that was converted to the epoxide. If the molecular mechanism involves attack of O_2 on the quinone form, the data would suggest that quinone formed by the enzyme must remain bound to the surface of the enzyme and not equilibrate with quinone formed in the media. Isotopically labeled oxygen was utilized to demonstrate that O_2 is the source of the epoxide oxygen, and the entire reaction appears to be an internal mixed function oxidase with the required reducing equivalents coming from the reduced form of the vitamin itself. Although the epoxidase activity can be induced by phenobarbital treatment, the lack of sensitivity to CO,[67] and the lack of effect of commonly used inhibitors of cytochrome P-450[160,170] suggest that it is not a function of this microsomal oxidase activity. The most intriguing aspect of the microsomal epoxidase activity is the possibility that it is somehow coupled to the microsomal vitamin K-dependent carboxylase activity. This was suggested soon after the discovery of the epoxidase activity by Willingham and Matschiner[167] and by Ernster et al.[149] Clear evidence on this point has not been forthcoming, but a number of observations have tended to support the hypothesis that there is some relationship between these activities.

Relationship Between Epoxidation and Carboxylation

The first indication that there might be some interrelationship between the microsomal vitamin K epoxidase activity and the vitamin K-dependent carboxylase activity came from early observations that the level of epoxidase assayed in vitro varied in the same manner as the level of the liver prothrombin precursors.[167] Both activities were increased in livers from hypoprothrombinemic animals and decreased when the vitamin was administered. Continued studies of these responses led to the postulation[149,167] that the formation of the epoxide was an obligatory step in the action of the vitamin in promoting prothrombin biosynthesis.

Subsequent studies in a number of laboratories have recently been reviewed[122] and have established that the requirements for in vitro prothrombin synthesis and epoxidation are similar and, in many respects, agents which stimulate or inhibit one of these reactions have a similar effect on the other. Both reactions require O_2 and a reduced

pyridine nucleotide, are inhibited by the vitamin K analog chloro-K, and can be induced by phenobarbital treatment. At sufficiently high Warfarin concentrations, both prothrombin synthesis and epoxidation are Warfarin sensitive. One difference in the two systems is the requirement of HCO_3^- for prothrombin synthesis and not for epoxidation. Although little evidence is available, it would appear that the pO_2 for prothrombin synthesis is considerably higher than that needed for epoxide formation.

There is no doubt that if the carboxylation event is coupled to the formation of the epoxide, it is not on a one-to-one basis, and it must be postulated that they can be uncoupled. A calculation of moles of CO_2 fixed per mole of epoxide formed is experimentally difficult due to problems associated with equilibration of the gas phase with added $H(^{14}C)O_3$ over the course of incubation. If reactions are carried out in intact microsomes, it is difficult to insure that some epoxide has not been reduced back to vitamin, and even though the vitamin K epoxide reductase does not appear to be active in most solubilized microsomes, it is not possible to rigorously prove that some formed epoxide has not been recycled. Calculation of moles of CO_2 fixed per mole of epoxide formed are, therefore, subject to considerable error. In a soluble carboxylase system using synthetic peptide substrates, values on the order of 5 mol of epoxide formed per mole of CO_2 fixed can be seen.[136] In a pyridoxal phosphate stimulated system, when the amount of CO_2 fixed into both peptide and endogenous protein is considered, it appears that an almost equal amount of carboxylation and epoxidation can be measured. The available data would suggest that under most conditions that have been employed there are many more epoxidation events than carboxylation events. However, unless a significant amount of undetected recycling of epoxide is occurring, it also appears that conditions may have been obtained where the ratio of epoxide formed to CO_2 fixed is near unity, or possibly less than one. The data to support any claim that active carboxylating preparations lack epoxidase activity should be carefully considered. The standard epoxidase assay[170] has a rather high blank, and unless the total carboxylation in the system is extensive, even a stoichiometric amount of epoxidation may not be detected.

A study of the localization of various vitamin K-metabolizing enzymes in rat liver microsomes[132,164] has indicated that the epoxidase activity is located precisely in the same subcellular area, the lumenal side of the rough endoplasmic membrane, as is the carboxylase (Figure 5). Crude fractionation of the vitamin K-dependent carboxylase activity by ammonium salt fractionation of a Triton®-solubilized microsomal fraction and chromatography on QAE Sephadex results in a roughly fivefold enrichment in specific activity of both fractions.[122] Further purification of this ammonium sulfate fraction by various methods[161] has yielded preparations of high but variable specific activity with a considerable loss of total carboxylase activity. These fractions have not been routinely assayed for epoxidase activity, but some preparations which contain a very small proportion of the total protein are enriched over 100-fold in both activities. However, other partially purified carboxylase preparations have been reported[165] to lack epoxidase activity.

The original observation that epoxidase activity was stimulated in a hypoprothrombinemic animal could have been due to an increase in epoxidase activity in these animals (more enzyme) or to an increased epoxidation associated with the carboxylation of an increased amount of precursor in these animals. This response can now be investigated by the addition of an exogenous peptide substrate to the system. The apparent K_m for the peptide Phe-Leu-Glu-Glu-Leu is about 4 m*M*[123], and at low concentrations of peptide, there is an increase in the total number of carboxylation events as peptide concentration is increased. Peptide addition also increases the amount of epoxide which is formed in the system,[122] and a weak stimulation of epoxidation has been

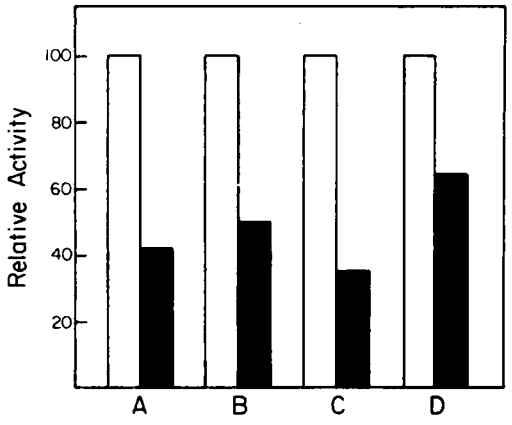

FIGURE 5. Distribution of vitamin K-dependent activities in rough and smooth microsomal preparations. The data are expressed as relative specific activities (units per milligrams of protein) with the activity of the rough microsome fraction set at 100. The activities are (A) vitamin K-dependent carboxylation of endogenous microsomal protein substrates, (B) vitamin K-dependent carboxylation of synthetic peptide substrate, (C) vitamin K epoxidase activity, and (D) vitamin K epoxide reductase activity. The open bars are activity of the rough microsomal fractions, and the shaded bars are the activity of the smooth microsomal fraction. For details, see References 132 and 164.

observed[160] upon the addition of crude preparations of rat liver prothrombin precursor to a detergent-solubilized carboxylase preparation. This stimulation appears to represent rather strong evidence for some type of interrelationship between these two events. The observation[171] that the *cis* isomer of vitamin K, which has little biological activity, is not a good substrate for the epoxidase would also support some type of relationship between the two activities.

Further evidence to link these two reactions in some manner is the recent observation[122] that they may involve a common oxygenated intermediate. Glutathione peroxidase (GSH-Px) has been shown to act on and reduce a number of hydroperoxides to the corresponding alcohols, and the activity of both the detergent-solubilized microsomal epoxidase and carboxylase can be decreased by the addition of increasing amounts of GSH-Px. These data suggest that a hydroperoxide of the vitamin, which would be a logical intermediate in epoxide formation, might also be involved in the carboxylase reaction.

The possibility that the presumably unstable hydroperoxide intermediate of the vitamin is used to drive the carboxylase suggested that other, more stable, hydroperoxides might serve as analogs for the vitamin. It has been shown[122] that the addition of *t*-butyl-OOH to a vitamin K-free incubation weakly stimulates CO_2 fixation and that this stimulation is blocked by the inclusion of GSH-Px in the incubation. The fixed CO_2 has been shown to be present in γ-carboxyglutamic acid, and *t*-butyl-OOH has been shown to be effective as a vitamin K analog in the absence of O_2. It has also been shown[159] that in the presence of vitamin K, *t*-butyl-OOH acts as an apparent competitive inhibitor of both the epoxidation and carboxylation reactions. The *t*-butyl-OOH-driven carboxylation requires the presence of NADPH. Why NADPH is required is not yet clear, but it is possible that the carboxylation event involves transfer of elec-

trons from the reduced vitamin to a microsomal component. If a hydroperoxide intermediate of the vitamin were replaced by another organic hydroperoxide, reducing equivalents not available from the vitamin would have to be furnished from some other source.

The available data here are consistent with some type of close association between the carboxylation and epoxidation events, but they do not prove any direct coupling of the two events. It may be that some intermediate of the vitamin, such as the hydroperoxide, is used to drive the carboxylation event, and at the same time, it is converted to the epoxide. There is no reason to believe that these two phases of the reaction would need to function at 100% efficiency, and it seems possible that the lack of stoichiometry between these two reactions that has been observed in in vitro systems could easily be obtained.

Vitamin K Epoxide Reductase

In addition to epoxidase activity, liver microsomes contain an epoxide reductase activity which can convert this 2,3-epoxide back to the vitamin.[172,173] This enzyme is inhibited by Warfarin, and it has been postulated (see below) that the cyclic interconversion of the vitamin to its epoxide and back is required for its action and that Warfarin exerts its effect on prothrombin synthesis through its action on this enzyme. Warfarin-resistant strains of rats were first identified in northern Europe,[174,175] and the epoxide reductase activity in these rats has been shown to be much less sensitive to Warfarin than that obtained from normal rats.[172]

The activity of this enzyme in in vitro preparations has been subjected to less study than the epoxidase, but some of its properties have been described by Whitlon et al.[151] The enzyme has very little activity in the presence of NADH or NADPH, but is active when dithiothreitol is used as a reducing agent. The physiologically active reducing agent is not known. At a concentration of 1 m*M*, NADH, NADPH, cysteine, ascorbate, or glutathione all have less than 15% of the activity of 1 m*M* DTT in a standard reductase assay. Lipoic acid at the same concentration has 53% of the activity of DTT and, as is the case with DTT, the lipoate-driven reductase was Warfarin sensitive. Whether lipoate may be the physiologically active reductant or whether, in this case, it is acting as an analog of DTT is not clear. More recently, Siegfried[157] has reported success in solubilizing the reductase activity in potassium cholate. The properties of the solubilized enzyme appear to be similar to those observed for the microsomal-bound enzyme. There is also a protein factor present in the cytosol[157] which enhances the microsomal epoxide-reductase activity. Whether this protein has a catalytic effect on the activity or whether it is a binding protein for this rather hydrophobic substrate has not been determined.

Mechanism of Coumarin Action

The availability of in vitro systems for the study of the metabolism and action of vitamin K has provided a further insight into the mechanism of action of the coumarin anticoagulants. It is usually assumed that the coumarins are not direct antagonists of vitamin K but act in some indirect manner. When increasing amounts of an inhibitory ratio of vitamin K and a coumarin are given to rats, the response seen is not the continual inhibition that would be expected from an agonist and its competitive antagonist, but rather the coumarin inhibition is overcome. This noncompetitive nature of the antagonism has been extensively studied by Lowenthal.[153] There have been a number of theories proposed to explain the indirect action of these chemically important drugs, and these theories have been recently reviewed.[1,177,178]

One theory of coumarin action[179-181] has been that coumarins act by blocking a nor-

mal transport route for vitamin K, while direct antagonists such as chloro-K act as competitive inhibitors of the vitamin at its physiologically active site. At what membrane this coumarin-sensitive site functions or what cellular pools of the vitamin it separates is not defined by this theory. It is postulated that high doses of the vitamin overcome the action of coumarins because the vitamin can bypass the specific transport site and reach its receptor protein by an alternate, non-Warfarin-sensitive route after the cellular concentration of the vitamin reaches a certain level. This theory assumes that the coumarin anticoagulants have no effect on whatever metabolic step in the system actually requires the vitamin. The evidence to support the theory is indirect and, at the present time, other theories appear more attractive.

A second theory of coumarin anticoagulant action that received considerable attention was that proposed by Matschiner and Bell who noted[182-184] that the ratio of vitamin K-oxide to vitamin K increased when rats were given Warfarin; they postulated that the epoxide acted as a competitive inhibitor of vitamin K at its site of action and that coumarins were inhibitors of vitamin K action only to the extent that they increased the cellular ratio of oxide to vitamin. Much of this theory rested on the observation that when the K-oxide/K ratio in the liver was high, prothrombin production was blocked. Observations[156,185,186] that rats which have a genetic resistance to Warfarin did not respond to Warfarin administration by increasing the concentration of the epoxide were also consistent with this theory. The data in these studies were complicated by the fact that Warfarin was always administered to keep the epoxide level high, and it was difficult to determine if the high epoxide level was responsible for the block in prothrombin production or if it was merely occurring at the same time. Further studies of the relationship between epoxide levels and prothrombin synthesis[187-189] established that synthesis could proceed in the presence of high liver concentrations of vitamin K epoxide and appeared to rule out a possible role of the epoxide as an inhibitor of vitamin action. These studies did not, however, invalidate the hypothesis that the inhibition of the epoxide reductase might be involved in the anticoagulant action of Warfarin.

Although it has been convincingly demonstrated that an elevated concentration of vitamin K epoxide does not inhibit the action of vitamin K, much of the available data suggests that the effect of coumarins on the epoxide reductase is the biologically important action of the drug. If the cyclic interconversion of the vitamin to its epoxide and back is required to maintain an effective concentration of the vitamin, an inhibition of this cycle would result in an inhibition of the action of the vitamin. Recent studies of the DTT-dependent vitamin K epoxide reductase[151,157] provide strong evidence that Warfarin inhibition of in vitro carboxylation systems is mediated through its effect on this enzyme. These data, and studies of vitamin K epoxide metabolism and prothrombin production in both man[190-192] and animals,[193,194] strongly suggest, but certainly do not prove, that the in vivo site of action involves the same enzyme. A major difficulty in assigning a specific in vivo site of action to the coumarin drugs is the fact that, at sufficiently high concentrations, they inhibit almost all enzymes involved with vitamin K metabolism. Menadione alkylation is inhibited in vivo by Warfarin;[195] coumarin administration in vivo increases the conversion of vitamin K to metabolites more polar han the epoxide;[187,188] coumarins inhibit DT-diaphorase (menadione-NADH reductase activity) as well as other vitamin K reductases;[196] and, at relatively high concentrations, coumarins and indandiones inhibit the in vitro epoxidation of vitamin K.[169] Whether the vitamin K-dependent carboxylase is also directly inhibited by high concentrations of coumarins is difficult to determine because of the possible association between epoxidation and carboxylation. Although many vitamin K-metabolizing enzymes do appear to be sensitive to coumarins, it also appears that

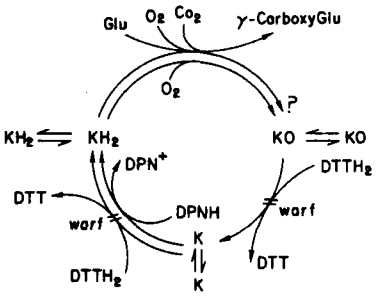

FIGURE 6. Vitamin K-related metabolic activities in rat liver microsomes. The pathways crossed and indicted warf are those that are sensitive to the action of the coumarin anticoagulants. The ? in the figure indicates that the product of the involvement of vitamin K hydroquinone (KH_2) in the carboxylation reaction is not known. It may be vitamin K-2,3-epoxide (KO), vitamin K quinone (K), or some unidentified intermediate. For details, see Reference 151.

the enzymatic alteration in the Warfarin-resistant rat is a decreased sensitivity of the vitamin K epoxide reductase to Warfarin.[172] Difenacoum, a coumarin anticoagulant effective in Warfarin-resistant rats,[197,198] is also an effective inhibitor in vitro of the epoxide reductase from Warfarin-resistant rats.[151] The relationships of the various vitamin K-metabolizing activities in the microsomal membrane to each other are shown in Figure 6.

It has also been possible to demonstrate an altered Warfarin-binding protein in microsomal preparations from Warfarin-resistant rats. Enhanced Warfarin binding to a crude ribosomal fraction from normal as compared to Warfarin-resistant rat liver was demonstrated by Thierry et al.,[199] and subsequent studies[200] established that the protein involved was a microsomal membrane component. This protein has now been isolated in a highly purified state.[201] At this time, it has not been possible to demonstrate that this protein is a component of any of the known vitamin K-associated enzyme activities. It does, however, have a significantly altered Warfarin-binding affinity when isolated from Warfarin-resistant rats, and if it can be shown to be a component of the epoxide reductase or some other enzyme, it should greatly aid in establishing the physiological role of the coumarin anticoagulants.

MOLECULAR ROLE OF VITAMIN K

The discovery of a new carboxylase system has, of course, raised considerable speculation regarding the mechanism of action of this enzyme and, in particular, the molecular role of vitamin K. Analogy with other carboxylases has suggested one of two general roles for the vitamin: (1) as a carrier for CO_2, i.e., a lipid-soluble biotin function or (2) as a cofactor for a reaction which labilizes the hydrogen on the γ-position of the glutamyl residue so that CO_2 (free or bound) may attack.

There are essentially no experimental data to support the idea that the vitamin is involved in any type of a complex with CO_2. Attempts in various laboratories to isolate such a compound or to demonstrate transfer of CO_2 from a stabilized CO_2 hydroqui-

none ester of the vitamin to a glutamyl acceptor have not been successful. If such an intermediate does exist, it might be very unstable, and these negative data do not rule out the possibility of such a role for the vitamin. The localization of the carboxylase in the hydrophobic environment of the microsomal membrane suggests that a compound with the physical properties of the vitamin might be effective as a CO_2 carrier, but numerous other lipoidal compounds could also serve this purpose.

Most speculation on the action of the vitamin has centered on the possibility that the vitamin is in some manner utilized to labilize the hydrogen on the γ-position of the glutamyl residue to allow CO_2 attack at this position. Data that would allow the construction of a detailed molecular mechanism are lacking at the present time, and such an exercise contributes little to an understanding of the mechanism. One major question, whether or not the formation of the vitamin K epoxide is in some manner coupled to the carboxylation event, is still unsettled. However, the available evidence would strongly suggest that they are at least related through a common intermediate which is formed subsequent to the hydroquinone. This intermediate would then be used to drive the carboxylation event, and during this step, the epoxide may or may not be formed, depending on the conditions of the incubation. It is also clear that vitamin K epoxide can be formed without a carboxylation event, which appears to rule out the hypothesis that these two reactions are coupled in some obligatory fashion.

Although there is evidence to suggest that the active form of the vitamin may be some oxygenated compound such as a hydroperoxide, unequivocal evidence of this is lacking. Any detailed mechanism of how such an oxygenated form could be used to drive the carboxylation is, at the present time, purely speculative. There is no real evidence to determine if the hydrogen removal is an abstraction of a proton to leave a formal carbanion on the glutamyl residue, or if the reaction is a radical-mediated sequence of events. The possibility that the only involvement of the vitamin is to furnish reducing equivalents for the activation of oxygen and that a form of activated oxygen is used to drive the carboxylase cannot be ruled out by the available data. It should be emphasized that the oxygen involvement in the reaction is the least studied parameter of the system, and there are no data to indicate how the reducing equivalents that presumably are obtained from vitamin K hydroquinone to reduce dioxygen are transferred. Whether this transfer is direct, mediated through a flavoprotein, a heme center, or if some other intermediate is involved has not been determined.

The data reviewed here indicate that, although some progress has been made in describing this unique oxygen-dependent carboxylase, the critical data needed to establish a detailed mechanism of the reaction are lacking. Increased activity in the field, progress toward more clearly defined systems, and progress toward purification of the enzymes involved should lead to a much clearer understanding of the system in the near future.

NOTE ADDED IN PROOF

This review was completed in the fall of 1978 and a significant amount of new material is therefore not included. Much of this recent information can be obtained in the proceedings of a recent Symposium on Vitamin K Metabolism and Vitamin K-Dependent Proteins.[202] (per JWS, February 1980)

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