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THE FUNCTION AND METABOLISM OF VITAMIN K

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CONTENTS

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INTRODUCTION

Vitamin K was discovered by Henrik Dam in 1929 (43) in studies of chicks fed fat-free diets. He observed quite unexpectedly that some of the chicks developed hemorrhages under the skin, in muscle, and in other tissues and that blood, taken for laboratory examinations, showed delayed coagulation (42). This delayed coagulation was discovered to be due to an absence of prothrombin activity in plasma (223). Similar observations were made in 1931 by McFarlane and his co-workers (165) in Canada and shortly afterward by Holst & Halbrook (107) of the University of California. The early work on the isolation and structural determination of both vitamin K and dicumarol (51) ushered in a period of study of prothrombin and other vitamin K–dependent factors. Over the period from 1950–1974, various hypotheses about the action of vitamin K included a role in mitochondrial electron transport (157), a possible function as an inducer of protein synthesis (185), and finally a role as an agent for posttranslational modification of a prothrombin precursor protein (240).

The discovery of γ-carboxyglutamic acid (Gla) in bovine prothrombin independently by Stenflo (240), Nelsestuen et al (179), and Magnusson et al (149) revolutionized ideas about the function of vitamin K. Since this modified amino acid was absent from prothrombin that circulated in the blood of animals given the anticoagulant dicumarol (Stenflo 240) it was concluded that vitamin K was involved in the γ-carboxylation of glutamate in a prothrombin precursor. Gla residues were first identified in a tryptic peptide containing residues 4–10 of prothrombin. Further enzymatic degradation of this peptide yielded a tetrapeptide containing residues 6–9 [Leu-Glx-Glx-Val] (243). Proton nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) showed that both glutamic acid residues in this peptide had one extra carboxyl group on their γ-carbon atoms (73). The corresponding peptide from dicumarol-induced prothrombin had unmodified glutamic acid residues in these positions. As shown in Figure 1, this new amino acid is a malonic acid derivative that is stable in base but labile in hot acid. This property may explain why it was not detected earlier in amino acid sequencing studies by the Edman technique.

The presence of Gla in another peptide from fragment 1 of prothrombin was reported by Nelsestuen et al (179), who identified a Gla • Ser dipeptide, representing residues 33 and 34 of prothrombin. Magnusson (147), who had been studying the amino acid sequence of bovine prothrombin for many years, also noted the abnormal anodal mobility of tryptic peptides representing prothrombin residues 4–10 and 4–11. In 1974 Magnusson et al (149) presented the amino acid sequence of the first 42 residues of bovine prothrombin. Gla residues were found at positions 7, 8, 15, 17, 20, 21, 26, 27, 30, and 33. Thus the first 10 glutamic acid residues in the N-terminal sequence of prothrombin

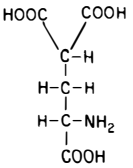

Figure 1 Structure of γ-carboxyglutamic acid. Molecular weight: 191; Ip: ~3.0.

are substituted with a γ-carboxyl group (111, 171), and none is carboxylated in the remainder of the molecule.

In addition to the four classical vitamin K–dependent coagulation proenzymes, factors II, VII, IX, and X, shown in Table 1, four additional γ-carboxy containing coagulation factors have been isolated over the past four years. These are protein C (239), protein S (50), protein M (226), and protein Z (207). All have an amino acid sequence homologous to that of prothrombin for residues 1–40 (106), and all require calcium ions for activity. Protein C contains two chains, a light chain of 155 amino acids and a heavy chain of 260 amino acids containing three carbohydrate side chains (72, 242). In addition to 11 Gla residues in positions 6, 7, 14, 16, 19, 20, 23, 25, 126, 29, and 35 of the light chain of protein C, a β-hydroxyaspartate was identified at position 71 (52). The heavy chain is the serine hydrolase portion of the molecule and is homologous with factors IX and X.

Protein C inhibits coagulation and promotes fibrinolysis (30). Activated protein C rapidly and selectively inactivates both normal and thrombin-modified factors V and VIII in vitro (154). The plasma of subjects with the rare heritable combined deficiency of factors V and VIII completely lacks an inhibitor of activated protein C (155). The rate of factor V_a inactivation by activated protein C is enhanced by protein S in the presence of phospholipid (274).

Proteins S and Z seem to be homologous with prothrombin in the N-terminal vitamin K–dependent portions of the peptide but possibly not in the C-terminal portion where serine hydrolase activity has not yet been detected. Protein S contains 10 and protein Z 13 Gla residues. Protein M, which appears to

Table 1 Vitamin K–dependent coagulation proenzymes

	Factor							
Characteristic	II	X	IX	VII	C	S	M	Z
Plasma concentration (μg/ml)	100	20	3	1	10	1	<1	<1
Molecular weight	72,000	55,000	55,000	46,000	57,000	69,000	50,000	55,000
Carbohydrate %	8	13	26	13	(8)	(+)	(+)	(+)
Number of chains	one	two	one	one	two	one	one	one
Number of Gla residues	10	12	12	10	11	10	(+)	13

promote prothrombin conversion to thrombin, has not been as extensively studied.

Vitamin K function and metabolism have been reviewed extensively since the landmark papers appeared in 1974 describing the isolation of γ-carboxyglutamate from bovine prothrombin (149, 179, 243). These reviews have sought to interpret the crescendo of papers that appeared in the wake of the discovery of Gla (87, 117, 151, 193, 246, 248, 254).

It is the purpose of the present review to bring biomedical students and investigators up to date on recent discoveries and concepts that characterize this fast-moving field. The nomenclature for the K vitamins used in this review is that of the IUPAC-IUB Commission on Biochemical Nomenclature (114).

BIOSYNTHESIS OF PROTHROMBIN

From Gene to mRNA

Expression of the biological information coded in eukaryotic genes is made possible by synthesis of a messenger RNA (mRNA) from the DNA by enzymatic transcription. This process involves generation of a complementary heterogeneous RNA transcript followed by extensive splicing and modification to yield a mRNA with a 5' cap of the general structure 7-methylguanosine-(5')PPP-(5')XpYp, where X and Y are the first two nucleotides of the coding sequence, followed by the other nucleotides in the coding sequence, and eventually by a 3'-poly(A) tail of 40–200 residues. The mRNA is then secreted into the cytoplasm where it is taken up by ribosomes, either free or attached to the endoplasmic reticulum, and translated into a protein with a specific amino acid sequence. The mRNA of proteins destined for secretion is attracted to ribosomes that become attached to the rough endoplasmic reticulum (RER) via a leader sequence of amino acids that permits internalization of nascent peptide in the RER (23, 24). These events permit further posttranslational modification of the peptide during the trip down the lumen of the endoplasmic reticulum to the Golgi apparatus, which, by exocytosis, secretes the protein into the plasma.

These tenets of molecular biology have been shown to be pertinent to the biosynthesis of prothrombin and the other vitamin K–dependent proteins, which undergo posttranslational carboxylation of selected glutamic acid residues in the N-terminal portion of the nascent chain followed by glycosylation at three asparagine residues located at positions further down the chain. Although prothrombin is one of the most abundant coagulation glycoproteins present in plasma (at a level of about 100 μg/ml), its mRNA constitutes only about 1% of the total mRNA in bovine liver compared to 10% for bovine serum albumin (144).

The level of RNA in liver made it difficult to purify prothrombin mRNA to the degree of homogeneity required for many biochemical investigations.

MacGillivray et al (145), however, increased the percentage of mRNA for prothrombin by specific immunoprecipitation of bovine liver polysomes to enhance the concentration of the desired mRNA by 8–20-fold with a 30% yield. Complementary DNA (cDNA) was then synthesized from the enriched mRNA fraction by reverse transcriptase and an oligoDT primer. A double-stranded cDNA was made in two steps, trimmed with appropriate enzymes, and 9 dCMP residues were added to the 3' terminus. It was then annealed to pBR322 DNA that had been cleaved at a Ps+I site and similarly tailed with dGTP. The resulting plasmids were used to transform *E coli* strain RR1.

Several recombinant plasmids were found to contain cDNA inserts coding for prothrombin. The DNA sequence of the longest contained a 700 base-pair insert that coded for the carboxyl-terminal sequence of 160 amino acid residues from prothrombin. The sequence from residue 423–583 corresponded closely to that determined by Magnusson and his co-workers (148) for bovine prothrombin. The stop codon UAG was followed by a noncoding region of 119 base-pairs and a poly(A)-tail of 60 base-pairs.

In a subsequent investigation, Degen et al (49) characterized a cDNA of 2005 base-pairs coding for human prothrombin isolated from a cDNA library prepared from human liver mRNA by methods similar to those described above for bovine prothrombin. This cDNA clone coded for a leader sequence of 36 amino acids, for the 579 amino acids in mature prothrombin, a stop codon, a noncoding region of 97 base-pairs, and a poly(A) tail of 27 base-pairs. The leader sequence of the cDNA studied corresponded to an incomplete hydrophobic signal sequence of 27 amino acids without an N-terminal methionine start site, followed by a 9 amino acid basic pro-segment containing 4 arginines, which was joined to the bonafide N-terminal alanine of mature human prothrombin. When MacGillivray et al (143) had earlier translated an enriched mRNA for bovine prothrombin in a rabbit reticulocyte lysate in the presence of radioactive amino acids, they found a sequence of 23 amino acids preceding the N-terminal alanine of mature bovine prothrombin. Residues -16 to -6 of this bovine mRNA translation product were identical to residues -36 to -26 of the human leader sequence. It is likely, therefore, that the human signal sequence is 7 amino acids longer than the cDNA insert provides information for and that the pro-sequence in the bovine cDNA is shorter.

The pro-segment of human prothrombin mRNA is compared with that of rat serum albumin in Table 2. The 6-residue pro-segments for rat and bovine serum albumin are identical in length and sequence (214, 247). The pro-segment for human prothrombin is longer (9 residues) and more basic, and the pro-segment for bovine prothrombin is shorter but may be homologous. Although mRNA for rat prothrombin has been enriched and translated in a reticulocyte lysate system to yield an immunochemically detectible prothrombin of mol wt 75,000 on SDS-polyacrylamide gels, the sequencing of the N-terminal portion is still incomplete (175).

Table 2 Amino acid sequences of signal portions specified by mRNA for prothrombin and albumin

mRNA	Pre-region NR residues	Pro-region sequence
Rat albumin	18	Arg Gly Val Phe Arg Arg
Bovine albumin	18	Arg Gly Val Phe Arg Arg
Human prothrombin	34	Arg Ser Leu Leu Glu Arg Val Arg Arg
Bovine prothrombin	18	Phe X Leu Val X

Degen et al (49) also isolated a λ-phage bearing a human DNA insert of 5 kilobases by cDNA hybridization with a human fetal liver genomic DNA library. By subcloning and sequencing, it was determined that this fragment contained half the gene for human prothrombin, which included six introns and five exons coding for amino acids 144–448 of human prothrombin. Thus the human gene for prothrombin, like those for many other mammalian proteins, contains interrupted noncoding sequences that, when transcribed as heterogeneous mRNA, must undergo considerable excision and splicing before a translatable mRNA is obtained (1).

Processing of Nascent Peptide to Mature Prothrombin

METABOLISM OF THE SIGNAL SEQUENCE The translation of the mRNA for prothrombin results in the formation of a nascent peptide that consists of a signal pre-segment of 18–34 amino acid residues, a basic pro-segment of 5–9 residues, a coding segment of 579–584 residues, and a stop codon, followed by a nontranslated tail-segment, as shown in Figure 2. Until the stop codon is reached the peptide remains attached to the ribosome and is considered to be a nascent peptide. The posttranslational modifications, namely γ-glutamyl carboxylation and glycosylation, occur normally on the nascent chain (113).

A protein with an N-terminal extended amino acid sequence preceding the amino acid sequence expressed in the mature protein is generally called a preprotein. This leader sequence, which permits entry of the nascent peptide into the membrane, is promptly cleaved by a signal peptidase on the lumenal side of the RER. If, in addition, the nascent peptide contains another adjacent N-terminal sequence necessary for proper folding (proinsulin) or some other physiological need (proalbumin), the original translation product is called a preproprotein. With prothrombin we have a problem in nomenclature because the mature protein is called a "proprotein" because of its role as a precursor for thrombin. Logically, the mRNA translation product for prothrombin should be called a pre-pro-pre^1-pre^2-prothrombin in which the pre^1 and pre^2 states denote prothrombin precursors uncarboxylated and unglycosylated. For simplicity I suggest that the initial translation product for prothrombin be called preproprothrombin with the acarboxy- and aglycosyl-intermediates called simply

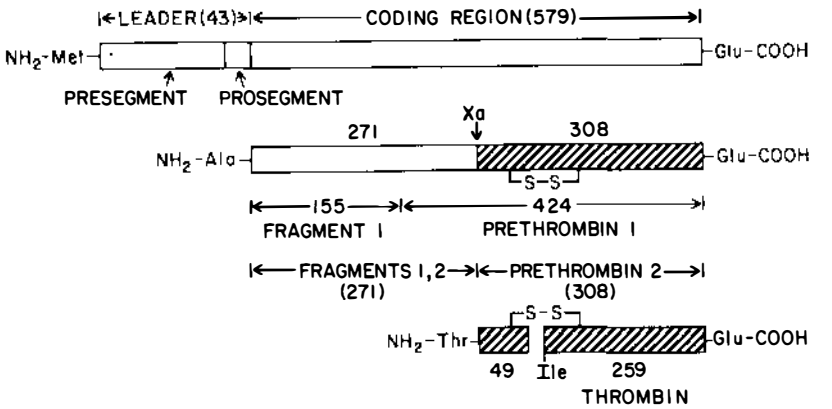

Figure 2 Structure of human prothrombin. A linear model of the nascent peptide is shown at the top. It has three components, a pre-segment of 34 amino acids, a basic pro-segment of 9 amino acids, and a coding portion of 579 amino acids. The pre-segment is cleaved by a signal peptidase on the lumenal side of the RER. The pro-segment is cleaved (in part) in the SER and totally in the Golgi apparatus. The second linear drawing shows the mature prothrombin secreted into plasma, which has an N-terminal alanine and a C-terminal glutamic acid. The thrombin portion is crosshatched. Prothrombin is split at arginine 271 by activated factor X to generate prethrombin-2, which is converted into an active 2-chain, disulfide-linked thrombin by a second factor X_a clip at arginine 320. The thrombin formed autocatalytically splits prothrombin and fragment 1,2 at arginine 156 to yield prethrombin-1 and fragments 1 and 2. The 10 Gla residues in fragment 1 are at positions 7, 8, 15, 17, 20, 21, 26, 27, 30, and 33.

"precursor forms". In fact, multiple intermediate forms of immature prothrombin have been found in the endoplasmic reticulum of vitamin K–deficient rats (59, 94, 174).

γ-CARBOXYLATION Scheinbuks (221) and Olson et al (187) observed that when rat liver microsomes from vitamin K–deficient rats were incubated with reduced menaquinone-2, cycloheximide, and $NaH^{14}CO_3$ for 10 min at 37°C, followed by fractionation of the RER, 2% of the incorporated $^{14}CO_2$ was in the heavy polysome fraction. This radioactive $^{14}CO_2$, furthermore, sedimented with the polysomes in CsCl gradients. Thirty percent of this radioactivity could be released from the ribosomes by incubation with low concentrations of puromycin, elongation factor 2 (EF-2), and GTP. Analysis of these ribosomes for prothrombin by radioimmunoassay revealed that one molecule of prothrombin was present per 1000 ribosomes. Eighty percent of the radioactivity in this prothrombin migrated on Dowex columns with Gla after basic hydrolysis, and about one half of that was found in glutamic acid after acid hydrolysis. Van Buskirk & Kirsch (270) also reported the presence of Gla in ribosomes from animals and wheat germ.

The vitamin K–dependent γ-glutamyl carboxylase, which accomplishes the γ-carboxylation of 10–12 glutamic acid residues in vitamin K–dependent

coagulation proenzymes, is an intrinsic membrane protein liberated only by detergents and is concentrated in the RER. Its properties and mechanism of action, to the extent they are known, are discussed in the next section. It acts to carboxylate all of the glutamic acid residues from 7 to 33 in prothrombin. After residue 33, none of the remaining 33 glutamic acid residues in the protein is carboxylated. The specificity of the enzyme for given glutamic acid residues may involve a macromolecular recognition phenomenon—i.e. the conformation and/or location of the precursor protein in the membrane, rather than a given amino acid sequence.

GLYCOSYLATION The sites of carbohydrate attachment to prothrombin appear to be aspargine residues 78, 100, and 373, all of which have the adjoining sequence of Asn-X-Thr, where X equals any other amino acid. This sequence is associated with the action of protein-oligosaccharyltransferase in the RER, the enzyme that transfers an N-acetylglucosamine-branched mannose-glucose-containing oligosaccharide from dolichol to Asn residues in the biosynthesis of glycoproteins. Further processing of the oligosaccharide by removal of glucose residues and addition of N-acetyl glucosamine residues occurs in the smooth endoplasmic reticulum. The terminal sialic acid residues of the carbohydrate side chains are added in the Golgi apparatus just prior to secretion (176). Meeks & Couri (166, 167) have claimed that warfarin blocks and vitamin K stimulates the carbohydrate attachment to prothrombin in rat liver. It is likely, however, that this is a secondary event in the rat, an animal that does not secrete a mature prothrombin in the presence of warfarin. Coumarin drugs do not block the attachment of carbohydrate to acarboxyprothrombin in animals that secrete an acarboxyprothrombin.

SECRETION Suttie reported in 1973 (249) that a precursor protein that did not bind calcium and did not yield thrombin with factor X_a accumulated in the microsomes of warfarin-treated or vitamin K–deficient rats. It could be liberated with Triton X-100 and would produce thrombin when treated with *Echis carinatus* viper venom. This accumulated microsomal precursor protein could be carboxylated by injection of vitamin K into anticoagulated rats resulting in its conversion to prothrombin and secretion into the plasma. This finding also proved that the vitamin K–dependent carboxylase could recognize detached nascent chains in the vitamin K–deficient reticulum.

Several proteins have now been isolated from the livers of warfarin-treated rats (59, 94, 172, 230) that have properties consistent with incompletely processed prothrombin. They are glycoproteins immunochemically similar to prothrombin with molecular weights indistinguishable from those of rat prothrombin on SDS-polyacrylamide gels. Both electrophoretic and isoelectric-focusing analyses indicate that these precursor proteins are less negatively

charged than prothrombin. They range from pI 5.8 to 7.2. Specific proteolysis of these precursors by *Echis carinatus* viper venom or thrombin yielded fragments indistinguishable from those formed by similar proteolysis of prothrombin. These proteins do not contain a full complement of Gla residues (usually less than 4 per mol) and do not absorb to $BaSO_4$. These proteins also lack sialic acid residues. The increased basic nature of these proteins is a property of the amino-terminal region of the molecule and probably reflects the extent to which the pro-segment of the nascent peptide is retained.

The fully carboxylated, glycosylated, and secreted prothrombin has a pI of 5.0. Desialylated prothrombin has a pI of 5.5 (178). At pH 8.0 the ten Gla carboxyl groups should provide ten negative charges on the protein, which should make the isoelectric point of the mature protein more acidic than its acarboxy-precursor. This, however, does not occur, and secreted acarboxy-prothrombin (usually called abnormal prothrombin) has the same isoelectric point and mobility on disc gel electrophoresis as does normal mature prothrombin. If Ca^{2+} is added, however, the anodal migration of normal prothrombin is slowed somewhat (176, 240, 245). This indicates that the extra γ-carboxyl groups of normal prothrombin are not available externally and are probably salt-linked to basic groups in the interior of the molecule.

Munns et al (174) observed that confluent monolayers of H-35 hepatoma cells respond to vitamin K addition by the synthesis and secretion of mature prothrombin. As demonstrated by radioimmunassay, selective barium salt adsorption, and two coagulation assays that discriminate between precursor and mature prothrombin, these cells retained their ability to synthesize precursor prothrombins in the absence of exogenous phylloquinone. When phylloquinone was added to the medium (100 ng/ml), the existing intracellular concentration of preprothrombin was reduced to 50% within 1 hr after exposure to the vitamin and slowly declined thereafter to approximately 30% of control levels by 36 hr. Concomitant with the rapid loss of intracellular preprothrombin was the appearance of mature prothrombin in the medium.

In a subsequent study, Graves et al (96) labeled the precursor prothrombins in H-35 rat hepatoma cells with [^{3}H]-leucine for 2 hr in the absence of vitamin K and then isolated them by immunospecific adsorption to rat prothrombin antibodies coupled to Sepharose. Five distinct [^{3}H]-leucine-labeled precursors of mature prothrombin were identified possessing pI values of 5.5, 5.8, 6.2, 6.7, and 7.2 as determined on isoelectric focussing gels. All of these precursors of mature prothrombin had (*a*) immunochemical similarity, (*b*) inability to be adsorbed by insoluble barium salts, (*c*) common molecular weights of ~75,000, and (*d*) vitamin K dependency. When cells were labeled with ^{3}[H]-leucine in the presence of vitamin K, there was a marked reduction (70%) of intracellular pI 7.2 and 6.7 species and a moderate reduction (35%) of pI 6.2, 5.8, and 5.5 forms with a concomitant appearance of mature prothrombin in the

cell culture medium (pI value of 5.0, mol wt 75,000). The results showed that vitamin K accelerated the rate of synthesis and/or processing of precursor prothrombins resulting in the secretion of mature prothrombin molecules into the medium.

Graves, Grabau, and Munns (95) showed that an analysis of the distribution of radioactivity incorporated into each precursor during a short (5–10 min) and long (120 min) radioactive pulse of cells maintained in the presence and absence of vitamin K provided an insight into the vitamin K–dependent processing step. These data indicated that the presence of vitamin K significantly altered the percentages of radioactivity incorporated in the 6.7 and 7.2 precursor species. During a 10-min pulse the presence of vitamin K increased by 50% the radioactivity associated with the 7.2 precursor while decreasing by 25% the radioactivity incorporated into the 6.7 species. Furthermore, the pI 6.7 species was associated with the RER, the pI 7.2 and 6.2 species with the SER, the pI 5.8 and 5.5 species with the Golgi apparatus, and the pI 5.0 with the secreted product. It appears that the pro-segment of the prothrombin precursor is not fully removed until the peptide reaches the Golgi body as is also the case with serum albumin (214).

Animals and birds vary in the extent to which the acarboxyprothrombin in vitamin K–deficient or warfarin-treated organisms is processed by enzymes of the endoplasmic reticulum and secreted into plasma as a biologically inactive prothrombin. In the human (88), the cow, (36) and to a lesser extent the chicken (36, 89,), fully glycosylated acarboxyprothrombin appears in the plasma. The corresponding precursor prothrombin in the chick has not yet been characterized, but it is likely that it bears a similar relationship to plasma prothrombin in the chick, which has only 30% of the biological activity of rat prothrombin and contains no sialic acid (281).

The finding that the vitamin K–deficient chick accumulates only 24% as much precursor per gram of liver as the rat explains the heretofore puzzling difference in the relationship between vitamin K action and prothrombin secretion in the two species. Vitamin K–deficient rats given vitamin K show a marked "burst" of prothrombin secretion in the first hour, followed by a slower rate of plasma enrichment (12, 191, 250). Vitamin K–deficient chicks, on the other hand, show a linear response to the vitamin (192). Second, the action of vitamin K in the deficient rat is only partially inhibited by cycloheximide and puromycin (12, 191, 250) whereas in the chick it is completely inhibited (184, 192, 282). Since the chick secretes an acarboxy form of plasma prothrombin, whereas the rat does not (36, 173), the chick accumulates much less precursor in its liver than the rat and hence is more dependent than the rat upon ribosomal synthesis of precursor to sustain prothrombin secretion in response to vitamin K. It appears that the human and the cow resemble the chick in this regard more than the rat (36, 116, 245).

Immunochemical Detection of Abnormal Prothrombins

Ganrot and Nilehn (88) first demonstrated the presence of a protein in the plasma of human subjects receiving dicumarol that reacted to antibodies against human prothrombin but did not demonstrate any biological activity in a standard clotting assay using factor X and thromboplastin. This protein has been given different names by various investigators: protein induced by vitamin K absence (PIVKA), abnormal prothrombin, isoprothrombin, paraprothrombin, dicumarol-induced prothrombin, atypical prothrombin, acarboxyprothrombin, or des-γ-carboxyprothrombin. Since the extent of carboxylation of this group of γ-carboxy glutamyl-deficient prothrombins varies under different conditions of anticoagulation, "abnormal prothrombin" is used here to denote plasma variants and "precursor prothrombin" is used to denote immature forms in microsomes.

The initial studies of abnormal prothrombin (176, 240, 245) indicated that it appeared to have the same molecular weight and amino acid composition as normal prothrombin, that it was not adsorbed by insoluble barium salts as was normal prothrombin, and that it lacked biological activity in the standard clotting assay. The lack of barium salt adsorption and calcium-independent mobility in electrophoresis (240, 245) suggested a difference in calcium-binding properties of these two proteins, which was demonstrated by Nelsestuen & Suttie (177). The difference in calcium binding was shown by Stenflo (241, 244) to be a property of the prothrombin fragment 1 (Figure 2), and the fact that this abnormal prothrombin yielded thrombin when treated with trypsin or snake venoms indicated that the C-terminal portion of the molecule was normal. It was shown (62) that the abnormal prothrombin will not bind to a phospholipid surface in the presence of calcium ions and that the addition of phospholipid, which drastically stimulates the Xa-Ca^{2+} activation of prothrombin, has no effect on the rate of activation of abnormal prothrombin. A study (16) of the conformation of these two proteins by optical rotatory dispersion and circular dichroism revealed that they were indistinguishable in the absence of calcium ion. On the addition of calcium ions, however, spectral changes occurred in normal prothrombin as a result of Gla residues that were not seen in the abnormal prothrombin.

The early studies (176, 240, 245) of abnormal prothrombin indicated that its carbohydrate content and structure were similar if not identical to those of normal prothrombin and provided refutation of earlier claims (119, 197) that the vitamin K–dependent step in the formation of prothrombin involved glycosylation of the protein.

Furie et al (84, 85) reported that antibodies directed against the γ-carboxyglutamic acid–rich region of bovine prothrombin could be isolated from antisera raised in rabbits immunized with native bovine prothrombin. By using sequential immunoabsorption on Sepharose-bound prothrombin frag-

ments, a population of antibodies was identified specific for the region of prothrombin from amino acid residue 12 to residue 44, which contains eight of the ten Gla residues present in prothrombin. These anti-$(12–44)_N$ antibodies form soluble complexes with prothrombin and inhibit the conversion of prothrombin to thrombin in plasma. The anti-$(12–44)_N$ antibodies are IgG immunoglobulins that are heterogeneous with regard to charge and affinity for prothrombin. A subpopulation of anti-$(12–44)_N$ is calcium-dependent and binds prothrombin strongly only in the presence of $CaCl_2$. These anti-$(12–44)_N$ antibodies appear to be specific for a metal-stabilized conformation of the region 12–44 in prothrombin and do not cross-react with the region 12-44 in abnormal prothrombin. These results suggest that the Ca(II)-dependent structural transition of prothrombin involves, in part, alteration of the tertiary structure of the region 12–44.

Blanchard et al (19) then sought to find specific antibodies against abnormal prothrombin by immunizing rabbits against abnormal prothrombin from warfarinized calves. By selective immunoabsorption on Sepharose-bound des-γ-carboxy-fragment 1 and prothrombin, respectively, a purified antibody subpopulation was found that bound abnormal prothrombin but did not cross-react with normal bovine prothrombin. The interaction of these antibodies with abnormal prothrombin was not affected by the presence of calcium ions. The interaction of these antibodies with ^{125}I-labeled abnormal prothrombin was inhibited by the plasma obtained from a calf treated with warfarin but was not significantly inhibited by normal bovine plasma, human plasma obtained from a patient treated with warfarin, or normal human plasma. These studies indicate that human and bovine abnormal prothrombins do not share antigenic determinants against which these antibodies are directed, nor is abnormal prothrombin a significant component of normal bovine plasma.

Blanchard et al (22) then developed similar conformation-specific antibodies against human prothrombin, abnormal human prothrombin, and human prethrombin 1. The anti-prethrombin bound prothrombin and abnormal prothrombin equally. Standard radioimmunoassays were developed with ^{125}I-prothrombin, ^{125}I-abnormal prothrombin, and ^{125}I-prethrombin 1 and applied to the assay of human plasma. The correlation between the concentration of mature prothrombin by immunoassay and prothrombin activity by clotting assay was 0.99. No abnormal prothrombin was detected in normal subjects. In patients treated with warfarin, abnormal prothrombin ranged from 20 to 90% of total prothrombin. Patients treated with warfarin have multiple populations of partially carboxylated forms of prothrombin circulating in their blood (64). In the plasma of patients receiving long-term warfarin therapy Blanchard et al (22) found small but consistent concentrations of abnormal prothrombin components exist that bind poorly to anti-abnormal prothrombin and anti-prothrombin:Ca(II).

The assays described offer new ways to diagnose disorders associated with abnormalities of prothrombin biosynthesis. As reported previously, low levels of abnormal prothrombin circulate in the plasma of patients with liver disease (20). Since this antigen is not detectable in normal plasma, it may serve as a useful antigenic marker of abnormal liver function or vitamin K deficiency. Furthermore, these immunoassays are a much more sensitive measure of clinical and subclinical vitamin K deficiency than the prothrombin time. These assays also show promise for monitoring oral anticoagulant therapy (21). For example, 89% of patients with a bleeding or thrombotic complication during warfarin therapy were identifiable with the assay of prothrombin antigen. Only 33% would have been predicted to be at risk on the basis of the prothrombin time. Finally, these investigators have shown that abnormal prothrombin is an antigenic serum marker for primary hepatic carcinoma, present in 93% of the 73 patients tested (138).

VITAMIN K–DEPENDENT γ-GLUTAMYL CARBOXYLASE

Distribution

Although it was originally assumed that Gla residues would be found only in vitamin K–dependent plasma coagulation proenzymes, a search for this new amino acid in other proteins has revealed a broader distribution than in the coagulation system. Likewise, a survey of tissues has indicated that the vitamin K–dependent γ-glutamyl carboxylase is distributed in tissues other than the liver. As already mentioned, in addition to the four classical vitamin K–dependent coagulation factors, four new Gla-containing plasma proteins with various roles in coagulation have been discovered since 1974, all of which appear to be synthesized in the liver (60). It is not known, however, whether a single vitamin K–dependent carboxylase is responsible for the posttranslational modification of each of these eight factors or whether a family of isozymes accomplishes these carboxylations. All of these proteins have a homologous sequence in the N-terminal region of the native protein and contain 10–13 Gla residues.

A bone Gla protein (BGP) was discovered independently by Hauschka et al (102) and Price et al (204). This protein is a matrix protein, is extractable from bone with EDTA, makes up 20% of EDTA extractable protein, and represents 1% of total bone protein. The Harvard group has called this protein osteocalcin. The amino acid composition and sequence have been determined by Price (206) in calf, swordfish, and human. The BGP for calf and human bone has a molecular weight of 5700 and contains 49 residues, of which residues 17, 21, and 24, clustered around a disulfide bridge, are Gla. The swordfish protein is homologous with 47 residues. None of these proteins is homologous with the

coagulation factors, but all bind Ca^{2+} and particularly hydroxyapatite. Its precise function in bone metabolism is not known.

Microsomes from embryonic chick bone contains a vitamin K–dependent γ-glutamyl carboxylase that can carboxylate endogenous bone protein and Glu-containing pentapeptides (136). The synthesis of BGP in embryonic chick and calf bones is inhibited by warfarin (87, 180). The synthesis of BGP, furthermore, is regulated by 1,25-dihydroxyvitamin D_3 in cultured osteosarcoma cells (203). This suggests that BGP may mediate some action of vitamin D on bone. While the present data show that a deficiency of BGP does not affect bone structure or fracture repair, plasma BGP is elevated in metabolic bone disease (205) and may stimulate bone modeling and mobilization of calcium.

A protein closely related to BGP has been found in calcified atherosclerotic plaques (atherocalcin) (134, 204) and in calcified aortic valves (135).

In 1976, Lian & Prien (137) reported the presence of another Gla-containing protein in the matrix of calcium-containing renal stones in humans. It was reported to have a molecular weight of 18,000 and to contain three to four residues of Gla. It may function to solubilize calcium salts in urine. In 1976 Hauschka et al (101) demonstrated that menaquinone-3 stimulated the synthesis of ^{14}C-Gla from $^{14}CO_2$ in kidney microsomes from dicumarol-treated animals, and Friedman et al (79) have localized this synthesis to the tubular cells. Gla biosynthesis has also been demonstrated in a mouse renal adenocarcinoma in tissue culture (266).

Bell (6) and Buchthal et al (32, 33) have reported that vitamin K–dependent γ-glutamyl carboxylase is present in microsomes from liver, lung, kidney, testes, spleen, and several tumors (melanoma, mammary gland, most cell and lymphomas) for rats and mice. Liver was the most active by a factor of 2–3. Vermeer et al (271, 273) found the vitamin K–dependent γ-glutamyl carboxylase in liver, testes, kidney, spleen, thyroid, pancreas, thymus, cartilage, and bone from horses and cows. No activity was found in muscle, heart, or lymphocytes.

Requirements

The requirements of crude systems for vitamin K–dependent carboxylation have been reviewed (193, 217, 248). Postmitochondrial supernatant suspensions and isolated microsomes from liver carboxylate their endogenous K–dependent factor precursors in the presence of oxygen, HCO_3^-, NAD(P)H or DTT and phylloquinone or a range of menaquinones from MK-2 to MK-10. Menaquinone-1 had a reduced activity and 2-methyl-1,4,-naphthoquinone and 2,3-dimethyl-1,4-naphthoquinone were ineffective (80, 120). Addition of ATP enhanced the biosynthesis of prothrombin in postmitochondrial supernatant suspensions but not in more purified systems (228).

It was soon discovered that the vitamin K–dependent γ-glutamyl carboxy-

lase system could be solubilized in dilute solutions of Triton X-100, which extracted both the precursor protein and the enzyme (61, 92, 109, 146, 190). The solubilized preparation retained many of the properties of the membrane-associated system requiring oxygen, bicarbonate, either NADH plus vitamin K or vitamin K hydroquinone, but had higher requirements for vitamin K and O_2, did not require ATP, and was unaffected by the inhibitory ATP analogs.

The requirements of this enzyme system as currently known are summarized in Table 3. The substrate can vary from a simple substituted glutamic acid (N-t-BOC-glutamic acid-α-benzylester) to precursor prothrombin containing 579 amino acids. Pentapeptides developed by Suttie et al (253) and by Houser et al (109), including Phe-Leu-Glu-Glu-Val, Phe-Leu-Glu-Glu-Leu, and Phe-Leu-Glu-Glu-Ile, have been used successfully. Rich et al (210) have studied a large number of related peptides and have observed a range of carboxylation activities, with none being significantly better than Phe-Leu-Glu-Glu-Leu. The K_m for these peptides is 4–6 mM. Oxygen is required for half-maximal activity at a pO_2 of about 60 mM Hg for the solubilized enzyme (256), but pO_2 is only 10 mM Hg for microsomes (120). This latter level is higher than that required for half-maximal activity of cytochrome oxidase but similar to that reported for cytochrome P-450-dependent cholesterol 7-α-hydroxylase (28). Carbon dioxide is required and appears to be the proximal reactant rather than bicarbonate (121). The K_m for bicarbonate was found to be 0.2–0.4 mM. ATP and biotin are not required, and avidin has no effect on the reaction. The apparent K_m for vitamin K in microsomes plus cytosol is about 0.1 μM (120), whereas in the solubilized system the apparent K_m for vitamin KH_2 is about 4 μM (98). This value is difficult to obtain because of the rapid fall in the concentration of added KH_2 due to autooxidation in vitro. Divalent cations, particularly Mn^{2+}, stimulate carboxylation as does 1 mM pyridoxal-5'-phosphate. Inhibitors of protein synthesis such as cycloheximide and actinomycin D have no effect on carboxy-

Table 3 Requirements for the vitamin K–dependent γ-glutamyl carboxylase

1. Vitamin KH_2
2. CO_2
3. O_2
4. Glu-peptide substrate (n = 1 to 582)

Optimum temperature = 17–20°C; pH 7.4.
Reaction is stimulated by Mn^{2+}, DTT, peptide substrate, and pyridoxal-5-phosphate.
Reaction is inhibited by P-chloromercuribenzoate, 2-chlorophylloquinone, and warfarin (variably).
Reaction is not inhibited by puromycin, cycloheximide, metyrapone, avidin, imido-ATP, FCCP, A-23187, X-537A.

lation, but inhibitors of sulfhydryl groups (PCMB or copper) have marked inhibitory effects (118, 186).

Purification

Attempts to purify the vitamin K–dependent γ-glutamyl carboxylase by a variety of methods have not been very fruitful. Ion exchange resins, chaotropic ions, sulfhydryl binding columns, thrombin affinity columns, hydrophobic affinity columns, and gel filtration in various combinations have provided some purification but not to a homogeneous protein. Affinity columns employing vitamin K homologs and analogs have not been useful because of the relatively low affinity of the enzyme for vitamin K or even vitamin KH_2. Attempts to purify the enzyme by reacting immunospecific adsorbants with its substrate preprothrombin have been partially successful since the enzyme and its substrate are closely associated and purify together. It has been extraordinarily difficult to remove the substrate from the enzyme when this enzyme-substrate complex is partially purified. In all these attempts the peptide carboxylation system was used as a guide to purification (186). Several methods for partial purification of the vitamin K–dependent carboxylase are described below.

CAPTURE OF ENZYME-SUBSTRATE COMPLEX In 1978 Houser et al (110) reported the partial purification of the vitamin K–dependent carboxylase from liver microsomes from vitamin K–deficient rats by using affinity columns for the substrate of the vitamin K–dependent carboxylase. This procedure involved the isolation of the rough reticulum from crude microsomes, followed by partial purification of the enzyme substrate complex on a heparin affinity column. Heparin binds the prothrombin precursors, which are tightly associated with the carboxylase. The enzyme-substrate complex was eluted with hypertonic salt in a single set of fractions. At this stage there was an approximate 100-fold purification of the enzyme from whole microsomes with a yield of 11%. When the heparin eluate was then rechromatographed on a prothrombin antibody affinity column, 50% of the enzyme now appeared in the void volume with some continuing retention of the enzyme substrate complex on the column. The increase in specific activity in this last step was about 3-fold to yield a product that was 285-fold purified from initial microsomes. This final fraction, however, represented only 2% of the original activity and was very unstable on storage. By subjecting the partially purified enzyme to isoelectric focusing it was possible to identify one peak that contained all the carboxylase activity and represented a further 3-fold purification, but unfortunately the isolation of this protein was not possible in the presence of ampholytes (186).

The vitamin K–dependent carboxylase-substrate complex was also purified about 400-fold from crude microsomes by Girardot et al (90, 91) using a different approach. In this preparation 3-([3-cholamidopropyl]dimethyl

ammoniol)-1-propane sulfonate detergent (CHAPS) was used to solubilize liver microsomes from vitamin K–deficient rats. The soluble mixture was precipitated by 55% ammonium sulfate and the proteins resuspended in buffer and applied to a column of ultragel AcA-34 previously equilibrated with buffer. Fractions able to carboxylate both added peptide and endogenous protein were eluted in a smooth peak, which was rechromatographed on carboxymethyl-Sepharose to yield fractions about 350-fold purified. The final step was chromatography on a hydrophobic column (Leu-Glu-Glu-Leu-Phe-ACH-48) to yield a fraction of carboxylase-substrate complex 400-fold purified. The KO:CO_2 ratio increased from 1.6 to 4.0 during the purification. Cytochrome P-450 activity was absent from this final product. Radioactivity from $^{55}FeSO_4$ given to rats prior to sacrifice did not parallel carboxylase activity in this purification.

REMOVAL OF MICROSOMAL IMPURITIES In 1980 Canfield et al (35) developed procedures to remove inactive proteins from Triton-solubilized microsomes in order to enhance the activity of the final material. Peripheral proteins were removed from the microsomes with 0.1% octylglycoside in buffer containing phenylmethylsulfonyl fluoride (PMSF) as an inhibitor of proteolysis. The pellet obtained after centrifugation in octyl glycoside was treated with the chaotropic ion perchlorate and with DTT to liberate inactive membrane proteins. The pellet was then extracted with 0.3% sodium cholate; the residue was suspended in 40 times the original microsomal volume of buffer, and saturated $(NH_4)_2SO_4$ was slowly added over a period of 5 min to give a final concentration of 33% saturation. The salted-out pellet was collected by centrifugation, and large volumes of buffer were used to remove the cholate. The pellet was resuspended in the original volume containing Triton X-100, DTT, and 0.5 molar KCL, dialyzed, and assayed. The resulting preparation, called complex A, was used immediately or stored at –70°C for use. The increase in specific activity from an initial value of 0.25 units/mg to 37.6 units/mg was due principally to the removal of inhibitors of the enzyme with enhancement of total activity by 40-fold. With loss of other membrane proteins, the specific activity of the final product was increased 150-fold from the initial microsomes.

The initial vitamin KH_2 concentration required for half-maximal activity of complex A was increased 4-fold, from 20 μg/ml to 80 μg/ml. The pH optimum was found to be 6.5, slightly lower than that of solubilized microsomes. The total carboxylase activity increased 30-fold, whereas the total epoxidase activity declined to 40%, a reduction in epoxidase:carboxylase ratio of 75-fold. Complex A was devoid of vitamin K reductase and hence required KH_2. Fifty percent of the phospholipid originally present in the microsomes was present, but microscopy showed no membranous material remaining in complex A. Most of the cytochrome P-450 system had been removed.

Attempts to further purify complex A by Wallin et al (275, 279) were unsuccessful. The use of Sepharose-desoxycholate and Sepharose-phosphatidyl choline affinity chromatography was effective in separating the protein to two peaks but with a loss of total activity. Endogenous carboxylase activity was present, suggesting that the endogenous substrates (one of high molecular weight) were tightly bound to the enzyme and were carried through the purification. The cytochrome P-450 activity of the preparation was diminished, and the carboxylase activity was enhanced 2-fold. This preparation was called complex B.

GEL FILTRATION The procedure of Vermeer et al (272) for the 10,000-fold purification by gel filtration of a vitamin K–dependent carboxylase from bovine liver microsomes for a decarboxy-prothrombin substrate could not be repeated by Uotila and Suttie (269). The latter investigators found the properties of the beef enzyme similar to but not identical with the rat liver vitamin K–dependent carboxylase. Pentapeptide was active but decarboxyprothrombin was inactive as a substrate for the bovine carboxylase.

Solid Phase Carboxylase

DeMetz et al (48) reported the partial purification of bovine liver vitamin K–dependent carboxylase by immunospecific adsorption onto Sepharose-bound antibodies to factor II, factor IX, and factor X. By analyzing carboxylated precursors from beef microsomes, preincubated with $^{14}CO_2$, with factor-specific Sepharose-bound antibodies, these investigators determined that 69% was factor X precursor, 21% prothrombin precursor, and 8% factor IX precursor. The immobilized factor X carboxylase containing factor X precursor was 97-fold purified from crude microsomes. When the solid-phase carboxylase was eluted with 2% SDS, in 6 M urea, their most purified preparations still contained eight proteins on SDS-polyacrylamide gels. They reported that 30% of the enzyme activity could be released into solution from the gel after incubation with KH_2, pentapeptide, $NaHCO_3$, and factor X. Unfortunately, the liberated enzyme was very unstable and could not be further purified.

In a subsequent paper, DeMetz et al (47) reported that Sepharose-bound vitamin–K dependent carboxylase (Sp-carboxylase) from bovine liver contained phosphatidylcholine, which is essential for its in vitro activity. The Sp-carboxylase could be depleted of phospholipids either by washing the enzyme with detergents or by treatment with phospholipase C. The enzyme could be reconstituted by adding mixed micelles of phosphatidylcholine and cholate (1:1) to the solid-phase enzyme. These investigators found the detergents Triton X-100, cholate, and deoxycholate, which are commonly used for the extraction of carboxylase from microsomal membranes, strongly inhibitory to Sp-carboxylase. This inhibition could be prevented by adding a crude extract

of microsomal phospholipids as a Sepharose-bound enzyme. DeMetz et al (45) further reported that acetone and dimethyl sulfoxide would stimulate carboxylation of peptapeptide and epoxidation of KH_2 by their Sp-carboxylase up to 5-fold. They speculated that these solvents might enhance the mobility of KH_2 in the lipid micelle and increase its availability as a substrate to the carboxylase.

Olson and colleagues (168, 186) also prepared a solid-phase rat liver carboxylase using Sepharose-bound antibodies to rat plasma prothrombin. Virtually all of the prothrombin precursor protein was bound by the antibody gel. The percentage of carboxylase enzyme activity bound by the antibody gel from four different preparations ranged from 40% to 69%, with a mean value of 50%. It seems possible from these results that the enzyme(s) are present in excess of the prothrombin precursor protein. It is known that about 50% of the carboxylase activity in solubilized rat liver microsomes precipitates with anti–factor X antibodies (285). Whether the enzyme that carboxylates factor X precursor is the same as the one that carboxylates preprothrombin is not known. As suggested earlier, the vitamin K–dependent carboxylases may be a family of isozymes.

With 5 mM DDT present, the carboxylase activity of the anti-prothrombin solid-phase enzyme toward exogenous pentapeptide was linear over a 2-hr period. This contrasts with the findings of DeMetz et al (47), who reported a 30-min lag in developing pentapeptide carboxylase activity with the beef enzyme precipitated with antibodies to factor X. The optimum temperature for peptide carboxylation for both the solid phase and soluble enzyme appeared to be around 18°C.

Phosphatidylcholine (0.2 mg) stimulated carboxylase activity approximately 25%. It also proved to be a stabilizing factor. $MnCl_2$ (10 mM) stimulated carboxylase activity 2-fold. Dithiothreitol (DDT) enhanced activity another 2-fold by maintaining the vitamin KH_2 in a reduced form for a longer period. Rat serum albumin (0.2 mg) stimulated activity, probably as a result of its free fatty acid binding properties. With all ligands present, the specific activity of the solid-phase enzyme was 240 times that of the soluble preparation.

One surprise was the total lack of stimulation of pentapeptide carboxylation by the solid-phase enzyme by 1 mM pyridoxal-5'-phosphate. Peptide carboxylation by the Triton-solubilized enzyme is stimulated 2–3-fold by pyridoxal-5'-phosphate (112, 255). It is likely that the PLP receptor site is blocked by antibodies in the solid-phase enzyme despite the availability of peptide to the active site.

$CuCl_2$ (2 mM), a blocker of -SH groups, inhibited the activity of the solid-phase enzyme. This inhibition was completely reversed with 5 mM DDT. This solid-phase enzyme exhibited a mixed "cytochrome type" difference spectrum in the presence of dithionite, showing a minimum at 410 nM and a

maximum at 426 nM. $^{59}FeSO_4$ given to rats prior to sacrifice was found in the solid-phase system at an increased specific activity (186).

Prothrombin precursor carboxylated by the solid state comigrated with prothrombin at an apparent molecular weight of 75,000. No other antibody-precipitated carboxylated protein was present except for a minor protein with an apparent molecular weight of 55,000. This carboxylation of the endogenous precursor occurred rapidly over a period of 1–2 min and then ceased. The enzyme-substrate complex was thus functional in the antibody-precipitated solid-phase state, as was also observed by Swanson & Suttie (259).

Kinetic Studies

PEPTIDE VS PROTEIN CARBOXYLATION One question that has intrigued investigators in this field is whether or not the liver microsomal vitamin K–dependent carboxylase that fixes CO_2 into synthetic Glu-containing peptides is the same enzyme that carboxylates the endogenous microsomal prothrombin precursor protein. As noted, peptide carboxylation in solubilized hepatic microsomes is stimulated 2–3-fold by Mn^{2+} and other divalent cations and by pyridoxal-5'-phosphate, and is inhibited 90% by chloro-K (1 μg/ml), 99% by warfarin (10 mM), and 93% by p-hydroxymercuribenzoate (0.5 mM). In contrast, the carboxylation of the endogenous protein is not stimulated or markedly inhibited by these ligands (112, 123, 255).

Kappel & Olson (122) undertook to study the mechanism of divalent cation activation of the solubilized rat liver vitamin K–dependent carboxylase from warfarinized rats. They also undertook a study of the effect of pentapeptide substrate on the initial velocity of endogenous microsomal protein carboxylation as well as the effect of the presence of endogenous protein substrate on the initial velocity of the pentapeptide carboxylation. The carboxylation rate of the pentapeptide substrate was stimulated 2–3-fold by 10 mM Mn^{2+}, Mg^{2+}, Ca^{2+}, and Sr^{2+}. The cation activation of the pentapeptide carboxylation is nonadditive and is probably a general action of divalent cations. These same cations exhibited a reproducible but much smaller activation (11–18%) of the endogenous protein carboxylation rate. The anions Cl^-, F^-, Br^-, and $H_2PO_4^-$ had no effect.

The mechanism responsible for the cation stimulation of the pentapeptide carboxylation was investigated in more detail. The data demonstrated that manganese chloride does not effect the V_{max} of the reaction but effectively lowers the apparent K_m of pentapeptide 3-fold, from 6.2 mM to 1.7 mM. Magnesium chloride exhibited identical effects on K_m and V_{max}.

The slight activation of the endogenous protein carboxylation rate by cations could be the result of the much lower K_m value for the natural endogenous substrate. Soute et al (238) have shown that the apparent K_m for added

decarboxylated plasma prothrombin and the decarboxylated NH_2-terminal residues 13–29 are 10-fold and 1000-fold lower, respectively, than the K_m for the pentapeptide. The concentration of precursor prothrombin in microsomes varies from 0.2 to 1.0 μM, so one would expect the K_m to be in the micromolar range for the endogenous substrate.

Kappel & Olson (122) found that the carboxylation of endogenous precursor was rapid at 20°C and was totally dependent on vitamin KH_2. The reaction exhibited a linear rate for only about 45 sec and was essentially complete within 5 min. The effect of pentapeptide substrate on the initial velocity of the endogenous protein carboxylation was investigated using 3 mM pentapeptide in the presence of 5 mM DTE, 10 mM manganese chloride at 20°C. A small but significant decrease in the initial rate of endogenous protein carboxylation of about 14% in the presence of 3 mM pentapeptide was observed. The final incorporation of $^{14}CO_2$ into the protein was equivalent in the presence and absence of peptide after 3 min. When peptide carboxylation was observed in the presence of uncarboxylated precursor, there was a 45-sec delay before the pentapeptide carboxylation became linear, indicating a competition with the endogenous protein for the vitamin K–dependent carboxylase. If the endogenous precursor was carboxylated prior to the addition of peptide, then no lag in the carboxylation of peptide was observed. These data plus identical thermal denaturation rates for the enzymes catalyzing protein and peptide carboxylation suggest strongly that a single carboxylase is responsible for the carboxylation of both endogenous precursor and added peptide. The initial carboxylation rate for endogenous protein is about 50 pmoles/min/mg protein, which is six times the peptide carboxylation rate in the presence of $MnCl_2$.

Shah & Suttie (227) approached this problem in a slightly different way by studying the comparative carboxylation activity for protein and peptide of partially solubilized (0.25% Triton X-100) and completely solubilized (1.0% Triton X-100) microsomes. The rate of vitamin K–dependent carboxylation of endogenous protein precursors was rapid in the completely solubilized liver microsomal preparation and was accompanied by the simultaneous carboxylation of the exogenous peptide Phe-Leu-Glu-Glu-Leu. In the partially solubilized liver microsomal preparation, however, the rate of protein carboxylation was reduced, and a lag in the carboxylation of exogenous substrate was observed. When the microsomes from vitamin K–deficient rats were depleted of endogenous precursor by prior vitamin K injections, the lag in peptide carboxylation was eliminated. They also concluded that both substrates utilized the same microsomal pool of carboxylase.

THE ROLE OF PYRIDOXAL-5'-PHOSPHATE It was first shown by Suttie et al (255) that pyridoxal-5'-phosphate (PLP) stimulated the carboxylation of pen-

tapeptide about 2-fold but had little, if any, effect on the carboxylation of the endogenous protein precursor. Dubin et al (53) showed that this effect was due not to Schiff-base formation between the peptide and the PLP but to the binding of the PLP to the microsomal carboxylase. Suttie et al (251) observed that the carboxylation of amino protected peptides that could not form Schiff-base with PLP are still stimulated with PLP.

Rikong-Adie et al (212) reported that the PLP adduct of Phe-Leu-Glu-Glu-Val reduced with $NaBH_4$ was a good substrate for the carboxylase but was not further stimulated by exogenous PLP. They concluded that the PLP effect was due to derivatization of peptide and not the enzyme.

Kappel & Olson (122) found that increasing concentrations of PLP enhanced the initial rate of vitamin K–dependent pentapeptide carboxylation 2–3-fold in the concentration range between 0.5 and 1.0 mM, whereas PLP concentrations of 2.0 mM or higher were less stimulatory. They observed that the K_m for pentapeptide was 7.1 mM in the absence of PLP and 2.4 mM in the presence of PLP. There was no effect on the V_{max} of the reaction.

The activation by PLP was found to be specific since pyridoxal, pyridoxine, pyridoxine-5'-P, pyridoxamine, pyridoxamine-5'-P, and 4-pyridoxic acid did not stimulate the rate of pentapeptide carboxylation. In fact, all six analogs inhibited the carboxylation reaction in either the presence or absence of added PLP. The activation of the carboxylase by PLP appears to be mediated by its direct binding to the enzyme via Schiff-base formation. Sodium borohydride reduction of solubilized microsomes in the presence of PLP, followed by dialysis to remove unbound material, resulted in a carboxylase preparation with a specific activity twice that of the untreated control microsomes when both were assayed in the absence of added PLP. The derivatized enzyme was not further stimulated by added PLP. The PLP-derivatized pentapeptide exhibited 150% of the carboxylase activity of the control pentapeptide, in agreement with Rikong-Adie (212); but in the presence of this substrate, exogenous PLP actually inhibited carboxylation.

It was concluded that PLP forms a covalent attachment to a lysine near the active center of the enzyme, which is stabilized by $NaBH_4$ reduction. Since the PLP-derivatized peptide is an active substrate that prevents further activation by external PLP, the peptide must bind in close proximity to the critical lysine, and this site is blocked from external PLP when the bulky PLP-derivatized peptide is the substrate.

Mechanism of Action

Martius (156) predicted that vitamin K would be found to be a member of a specialized electron transport chain in animals beginning with NADH and terminating with O_2. His prediction, though made for mitochondria, has been confirmed for a microsomal system. Vitamin K reduction may be initiated by

an NAD(P)H-dependent flavoprotein reductase (278) that converts vitamin K to the hydroquinone (KH_2) or by a dithiol-dependent reductase in rat liver microsomes (283) that accomplishes the same reaction.

The autooxidation of KH_2 results in the formation of K and H_2O_2 (170). Quinone-mediated electron transport in mitochondria produces small amounts of H_2O_2 in proportion to the content of reduced ubiquinone (27). The $\Delta G°$ for the oxidation $KH_2 + O_2 \rightarrow K + H_2O_2$ is about 35 kcal per mol, more than enough energy to accomplish the carboxylation of peptide-bound glutamate. The $\Delta G°$ for the oxidation of vitamin K hydroquinone to vitamin K-2,3-epoxide, namely $KH_2 + O_2 \rightarrow KO + H_2O$, is somewhat less but nonetheless sufficient to provide adequate energy for the carboxylation reaction. The main energy-requiring step is the removal of a proton or hydrogen from the γ-methylene carbon of peptide-bound glutamate. Since the system is membrane bound and does not require ATP, energy conservation is accomplished in some unknown way. Uncouplers of oxidative phosphorylation and two calcium ionophores (A-23187 and X-537A) do not inhibit the reaction (193). The system, furthermore, functions in the presence of detergent concentrations that would presumably destroy the membrane integrity needed to maintain an ion gradient.

The chemical possibilities to be considered as mechanisms for vitamin K–dependent γ-glutamyl carboxylation include (*a*) the activation of oxygen, (*b*) the activation of CO_2, and (*c*) the activation of glutamic acid.

As regards the activation of oxygen, the oxidation of hydroquinones results in the generation of superoxide anion ($O_2^{-}\bullet$) and H_2O_2. The evidence does not favor these reduced forms of oxygen as driving forces for the carboxylation reaction since superoxide dismutase and catalase do not inhibit the carboxylation significantly (63, 129).

As regards CO_2, Jones et al (121) investigated the active species of "CO_2" in the microsomal system by the low-temperature method of Filmer & Cooper (75). This was possible only because menaquinone-2 is quite active at 10°C in the microsomal system. The reactions were carried out with washed microsomes in the presence of cycloheximide, avidin, and acetazolamide to inhibit, respectively, protein synthesis, biotin-dependent reactions, and carbonic anhydrase. $^{14}CO_2$ or $H^{14}CO_3^{-}$ were generated preferentially, and the rate of uptake of isotope from each species into Gla residues in microsomal protein was measured at minute intervals for 5 minutes. The incorporation of radioactivity from $^{14}CO_2$ after 1 min was 5–10 times that from $H^{14}CO_3^{-}$. The presence of 1 mM ATP or the inhibitor ADP-NH-P had no effect on the rate of uptake of either species. When the carbonic anhydrase inhibitor was omitted, a linear $^{14}CO_2$ uptake curve, intermediate between those obtained in its presence, was obtained. The apparent K_m for $^{-}HCO_3$ in these experiments was 0.4 mM, which suggests that the K_m for CO_2 is the order of 2×10^{-5} M. The hydro-

phobic nature of the endoplasmic reticulum protects CO_2 against hydration and permits this tight binding of CO_2 to the vitamin K–dependent carboxylase.

No role for vitamin K hydroquinone as a CO_2 carrier via hemicarbonates [a role analogous to that proposed by Todd and his colleagues (264) for reduced napthoquinone hemiphosphates as intermediates in oxidative phosphorylation] was evident. The hemicarbonate ethylester of menaquinone-2 labeled with ^{14}C in the carbonate moiety proved inactive in the carboxylation assay (193).

The activation of CO_2 by a radical exchange reaction involving the formation of the semiquinone plus $\cdot CO_2^-$, namely

$$KH_2 + CO_2 \rightarrow \dot{K}H + \cdot COOH$$

as suggested by Gallop et al (86), has not yet been substantiated. Peroxy radicals of vitamin K have been produced by pulse radiolysis (209), but a radical-radical C-carboxylation reaction catalyzed by the vitamin K–dependent carboxylase has not been demonstrated.

The activation of the γ-methylene group of glutamyl residues to a γ-methylene carbanion or a γ-methylene radical is thus the only remaining possibility, and this mechanism has been intensively explored. Friedman et al (81) demonstrated with an acetone powder of microsomes from vitamin K–deficient rats that the release of tritium from the γ-position of glutamic acid in the peptide Phe-Leu-Glu*-Glu*-Leu (labeled in the γ and β protons of glutamic acid with ^3H) is dependent upon the concentration of vitamin KH_2 and O_2 but not CO_2. The ratio of tritium released to CO_2 fixed in these experiments varied from 21.6 at 0.2 mM HCO_3^-, to 1.9 at 4 mM HCO_3^-, as shown in Figure 3.

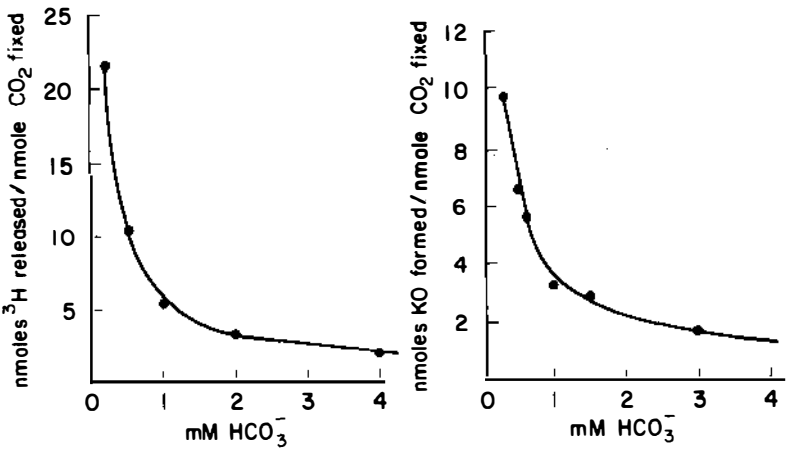

Figure 3 The ratios of the nanomoles of ^3H released from γ-^3H-glutamic acid containing pentapeptides per nanomole CO_2 fixed into Gla (left) and nanomoles KO formed per nanomole CO_2 fixed (right) are shown as a function of plasma HCO_3^- concentration. From (81) and (127).

Tritium release exceeded CO_2 uptake by a factor of two at the point of maximum coupling. In a subsequent paper these authors (127) showed that tritium release from Glu-labeled pentapeptide paralleled net vitamin K-2,3-epoxide (KO) formation in Triton X-100 solubilized rat liver microsomes. Under these conditions, however, the KO:CO_2 ratios varied from 16 to 27. In this study the ratio of KO:CO_2 formed varied with the $^{-}HCO_3$ concentration from 10:1 at 0.5 mM to 1:1 at 6 mM (Figure 3). These workers concluded that carboxylation of Glu-peptide and epoxidation are coupled 1:1 under physiological conditions. In other studies, the epoxidase:carboxylase ratio has varied from 0.8 to 5.0 in different preparations at the point of maximum coupling (46, 216).

Attempts to introduce tritium from ^3H_2O have thus far failed (78). It has been noted by Cram et al (40) that intramolecular proton transfers within diphenylbenzyl derivatives can occur without exchange of protons with relative nonpolar solvents. In order to determine whether the active form of peptide-bound glutamate is a carbonium on a carbon radical, Friedman et al (78) synthesized BOC-[γ-^{3}H] Glu-Glu-Leu-OMe with fluorine in the β-position of the Glu residues, expecting fluoride to be eliminated if a carbanion were formed. These fluorinated tripeptides, however, were not carboxylated when incubated with solubilized microsomes and $^{14}CO_2$, nor was fluoride eliminated. Other approaches are needed to determine the precise species of "active" glutamate.

Dubois et al (54) synthesized two fluoro-pentapeptides of general structure Phe-Leu-X-Glu-Val, where X is either the L-threo-γ-fluoroglutamic acid or the L-erythro-isomer, to determine the stereochemical course of the vitamin K–dependent carboxylation. Both peptides were carboxylated, but the reaction occurred exclusively on the glutamic acid of the L-threo-γ-fluoroglutamate-containing peptide, whereas both glutamic and fluoroglutamic residues of the L-erythro-γ-fluoroglutamate-containing peptide were carboxylated. A study of hydrolysis products of these carboxylated fluoropeptides revealed that no carboxylation of the L-threo-isomer of glutamate occurred, but excellent carboxylation of the L-erythro-isomer was observed. It was also shown that glutamic residue 4 in the pentapaptide can be carboxylated if a nonreactive residue occurs in position 3. In nonfluorinated pentapeptide, the preferential site of carboxylation in vitro is residue 3 (76, 212).

These results reveal that the enzymatic γ-hydrogen abstraction step is stereospecific and corresponds to the elimination of the pro-S hydrogen of glutamic acid. The precise reaction mechanism by which hydrogen is removed from the pro-S position on the γ-methylene carbon of peptide-bound glutamate by vitamin K–dependent carboxylase remains unsettled.

One hypothesis proposed for the removal of the γ-hydrogen from glutamate is via an intermediate in the oxidation of vitamin K hydroquinone (KH_2) to vitamin K-2,3-epoxide (KO). Willingham & Matschiner (286) were the first to associate enhanced epoxidase activity with the elevation of prothrombin pre-

cursor in liver microsomes from rats with vitamin K deficiency or warfarin treatment. In their experiments the administration of vitamin K to these anticoagulated rats stimulated prothrombin appearance in the plasma and caused a fall in vitamin K epoxidase activity. They postulated that "the enzymic interconversion of phylloquinone and its 2,3 epoxide is involved in the vitamin K–dependent production of prothrombin."

Later, Sadowski, Schnoes & Suttie (218) correlated carboxylation activity with epoxidase activity in rat liver microsomes under a variety of conditions, whereas Wallin et al (276) could dissociate the two activities completely by chromatographic fractionation of detergent-solubilized rat liver microsomes. Nonetheless, any hypothesis for the role of vitamin K in the carboxylation reaction must consider the relationship between these two activities. Both reactions are located in the same tissues (83), and both activities are enriched in the RER (37, 56). Both enzymatic activities utilize the reduced vitamin as a substrate (220), are inhibited by chloro-K (14), and are active with the same forms of the vitamin (82). It has been demonstrated, furthermore, that increasing the concentration of a peptide substrate for the carboxylase, which increases the number of carboxylation events, also increases epoxidase activity (252).

Although the KO:CO_2 ratio appears to be 1.0 under some conditions, the carboxylation and epoxidation rates can be uncoupled under a variety of conditions. The addition of 10 mM CN^- inhibits carboxylation 50–70% and increases epoxidation 150–200% above normal (46, 127, 131). Larson et al (127) noted under their conditions of KCN inhibition of carboxylation that ^3H release was inhibited 30% despite a doubling of the rate of epoxidation. Manganese ions at 10 mM stimulated carboxylation, epoxidation, and ^3H release (128). Various methods of extracting microsomes can also change the ratio of carboxylation and epoxidation (35).

These data lead to the general working hypothesis for the action of the vitamin K–dependent carboxylase depicted in Figure 4. The reaction is presented as an ordered mechanism. Basically, three coupling sites are proposed. The first is the combination of the enzyme with KH_2 and O_2 to yield a ternary enzyme-substrate couplex and possibly a vitamin KH_2-(O_2) compound. This step is inhibited competitively by Cl-phylloquinone and tetra-chloropyridinol (284). A ternary complex of KH_2 and O_2 with a metalloprotein has also been proposed (186). Some leakage will occur at this point to produce vitamin K and H_2O_2 as indicated.

The 2-hydroperoxide of vitamin K has been suggested as the active intermediate (44, 129). t-Butyl-hydroperoxide, proposed as a model for the vitamin K hydroperoxide by Larson & Suttie (129), is not an agonist of vitamin K in solubilized microsomes (99) nor is it a competitive inhibitor of the vitamin K–dependent carboxylase (98). The NAD(P)H-dependent carboxylated product with t-butyl-OOH is not Gla but an unidentified acid (99, 280).

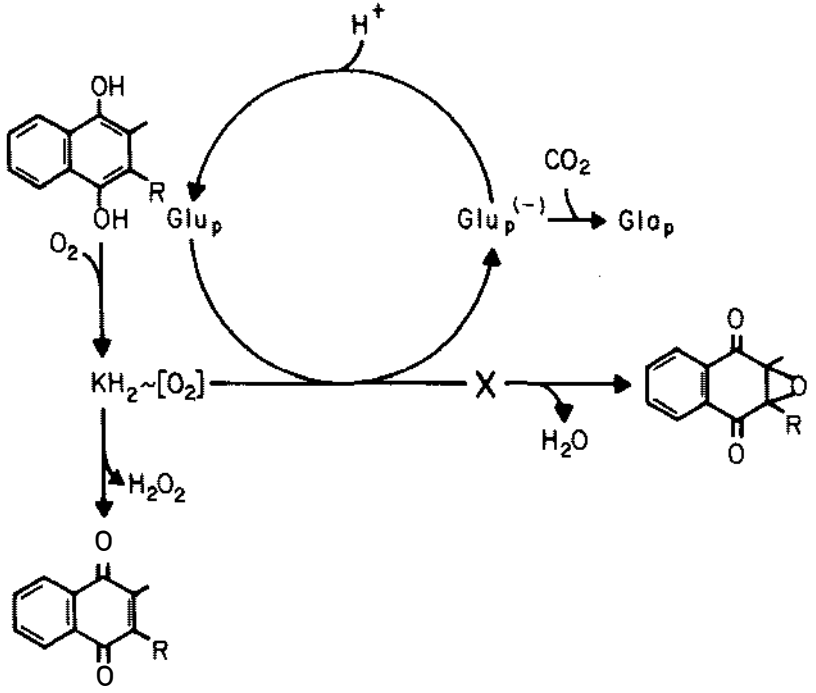

Figure 4 Mechanism of action of the vitamin K–dependent γ-glutamyl carboxylase. $KH_2\sim(O_2)$ is an unknown ternary complex of KH_2 and O_2 with the carboxylase. X is a chemical intermediate of KH_2 and O_2 that leads to vitamin K-2,3-epoxide. Glu_p is the glutamic acid containing peptide substrate and $Glu_p^{(-)}$ is the activated (presumably the γ-carbanion) form of Glu_p. Gla_p is the γ-carboxyglutamate containing peptide substrate. More epoxide is produced than Gla_p if the glutamate carbanion is protonated as shown in the $Glu_p \rightleftharpoons Glu_p^{(-)}$ cycle in mid-diagram. Modified from (127).

The second coupling site is between $KH_2\sim(O_2)$ metabolism and glutamyl peptide activation. The peptide (3rd substrate) is visualized as binding to the enzyme system after the addition of KH_2 and O_2. Epoxidation will occur in the absence of peptide, and this blank is subtracted in computing $KO:CO_2$ ratios. Peptide addition, however, usually enhances both carboxylation and epoxidation. CN^-, however, uncouples both ^3H release and carboxylation from epoxidation, whereas Mn^{2+} ions appear to stimulate all three events. X is put in series with $KH_2\sim[O_2]$ and KO because the intermediate steps are not known with certainty. Larson & Suttie (129) proposed that a hypothetical vitamin K-hydroperoxy anion might be sufficiently basic to remove the γ-methylene proton from peptide-bound glutamate. Olson et al (186) argued the contrary and suggested an induction mechanism for the labilization of the γ-methylene proton. They also reported (98) that in their hands pure glutathione peroxidase did not inhibit vitamin K–dependent carboxylation.

DeMetz et al (44) have suggested that heterolytic fission of the hypothetical hydroperoxide group could generate a hydroxyl ion basic enough to remove a

γ-proton from peptide-bound glutamate. They also claimed that HSO_3^- ions caused reduction of the hypothetical KH-OOH to hydroxyvitamin K and water, a claim that has not been confirmed (169).

Further work is needed on purer preparations of the carboxylase to advance understanding of this complicated mechanism.

ABSORPTION AND METABOLISM OF VITAMIN K

Absorption

The absorption of phylloquinone and the menaquinones requires bile and pancreatic juice for maximum effectiveness (150). Dietary vitamin K is absorbed in the small bowel, is incorporated into chylomicrons, and appears in the lymph (25). The efficiency of absorption has been measured at 40–80%, depending upon the vehicle in which the vitamin is administered and the extent of the enterohepatic circulation. When isotopically labeled phylloquinone was administered orally to animals (287) and humans (232) in doses ranging from the physiological to the pharmacological, the vitamin appeared in the plasma within 20 min and reached a peak in the plasma in two hours. When 1 mg of phylloquinie was given intravenously, peak plasma levels of about 100 ng/ml were observed. The concentration of the vitamin then declined exponentially to low values over a period of 48–72 hr to reach postabsorptive values of 1–5 ng/ml (38, 132, 231, 234). During this period, it appeared to be transferred from the chylomicron remnants to the liver, where it is incorporated into very low density lipoproteins (VLDL) and ultimately distributed to tissues via low density lipoproteins (LDL). This transfer system also applies to dietary sterols and carotenoids. Thus far a specific plasma carrier protein for vitamin K has not been identified.

Intermediary Metabolism

The liver appears to be the primary target of administered vitamin K in animals and humans. As much as 50% of a parenterally administered dose of vitamin K_1 may appear in the liver of rats within 1 hr (11). After oral administration, the liver may contain as much as 20% of the administered dose in 2 hr, which then declines to low values after 24 hr. The principal sites of uptake, after liver, were skin and muscle. Fractionation of liver tissue, after the administration of ^{3}H-phylloquinone to rats, showed the following relative distribution of radioactivity: nuclei, 13%, mitochondria, 9%, microsomes, 63%, and cytosol, 14% (11). In omnivorous animals like humans, both phylloquinone and the higher molecular weight menaquinones (MK-7 to MK-13) of bacterial origin are found in the liver (158, 211). Haroon & Hauschka (100) have found phylloquinone levels in rat liver to vary between 8 and 44 ng/g fresh weight (20–100 pmoles/g). Taggart & Matschiner (260) estimated that 10 pmoles/g (4.5 ng/g) of vitamin K is the minimum hepatic concentration to sustain normal prothrombin levels in the rat.

The turnover of vitamin K in the animal body is rapid, and the total body-pool size is surprisingly small. Bjornsson et al (17) infused 300 μg of ^{3}H-phylloquinone into human volunteers with or without previous drug loading with warfarin or clofibrate. The initial half-time ($t_{\frac{1}{2}\alpha}$) for the first exponential phase was 26 ± 8 min and the average terminal half-time was 166 ± 9 min under all conditions. Shearer et al (233) found similar results with a 1 mg intravenous dose of ^{3}H-phylloquinone, namely $t_{\frac{1}{2}\alpha}$ = 20–24 min and $t_{\frac{1}{2}\beta}$ = 120–150 min. From data on the volume of distribution and clearance rate, Bjornsson et al (18) calculated the fractional turnover rate to be 0.4/hr, suggesting that the body pool was turning once every 2.5 hr. From approximate daily intakes of vitamin K, body-pool sizes were estimated to be 50–100 μg, which is less than the body-pool size from vitamin B_{12} and extraordinarily low for a fat-soluble vitamin.

Vitamin K–Vitamin K-2,3-Epoxide Cycle

In 1970, Matschiner et al (160) reported the isolation and characterization of phylloquinone 2,3-epoxide as a new metabolite of phylloquinone in the rat. Although small amounts of this metabolite were found in normal rats, a marked accumulation of the epoxide was demonstrated in the presence of warfarin. Approximately 30 years earlier, Fieser and his colleagues (74) synthesized the 2,3-epoxide of phylloquinone and showed that it was rapidly converted to vitamin K in normal animals. Bell & Matschiner (10) then reported that phylloquinone epoxide had the same biological activity as phylloquinone in the vitamin K–deficient rat but was much less active in the warfarin-anticoagulated rat. They suggested that warfarin inhibited the reductase required for the normal conversion of phylloquinone oxide to phylloquinone. Bell & Matschiner (9) then suggested that vitamin K epoxide was not only a metabolite of vitamin K but in fact a competitive inhibitor of the vitamin at its active site on the carboxylase. They (13) administered ^{3}H-phylloquinone and ^{3}H-phylloquinone epoxide to normal and warfarin-treated rats. Vitamin K_1-epoxide:vitamin K_1 ratios were found to be elevated in animals receiving warfarin as opposed to controls, but the hepatic vitamin K epoxide:vitamin K ratios obtained in rats pretreated with warfarin receiving various doses of vitamin K from 5 to 100 μg varied over too small a range (0.9–1.9) to account for the changes in prothrombin response (0–60%). Further, Bell & Caldwell (8) showed that in warfarin-resistant rats where vitamin K_1 epoxide appeared to accumulate in the absence of warfarin, "inhibitory ratios" of vitamin K epoxide:vitamin K_1 were obtained at 100% plasma prothrombin levels.

Neither Goodman et al (93) nor Sadowski & Suttie (219) could confirm the conclusion that vitamin K-2,3-epoxide was an inhibitor of the vitamin. Goodman et al (93) infused 1.6 mg of vitamin K_1 oxide (K_1O) per hour for 4 hr intravenously into warfarin-resistant rats and demonstrated that the KO:K ratio in the liver reached high values (7.2 ± 1.6 SEM) without any effect upon

plasma prothrombin levels (100 ± 10%). In a companion experiment in which warfarin was given in doses of 1 mg/kg simultaneously with physiological amounts of vitamin K (50 ng/hr intravenously), warfarin had its usual effect upon prothrombin level when the ratio of vitamin K_1 oxide:vitamin K_1 was in the physiological range of 0.13 ± 0.03. Sadowski & Suttie (219) conducted similar studies, which demonstrated that the conversion of microsomal precursors of prothrombin to the active zymogen was not blocked by a high level of administered vitamin K epoxide. Bell and colleagues (7) finally retracted this hypothesis.

Warfarin-resistance is observed in both animals and humans (182). Mutant strains of wild rats having a greatly increased resistance to warfarin and other 4-hydroxycoumarin anticoagulants were discovered in Wales (97). When these rats were crossbred to the warfarin-susceptible Sprague-Dawley (SD) rat, selecting for the trait of resistance (which is carried on an autosomal dominant gene), the resultant offspring required 50–200 times more warfarin to anticoagulate and 20 times more vitamin K to maintain hemostasis. Despite this, no marked changes in the overall metabolism of warfarin or vitamin K from that of the sensitive Sprague-Dawley rat could be demonstrated in early studies (103, 198, 265). Shah & Suttie (229) found that the 2-chloro-3-phytyl-1,4-naphthoquinone was as effective, if not more effective, in suppressing prothrombin synthesis in warfarin-resistant rats than in normal rats. Zimmerman & Matschiner (289) reported that the K-epoxide reductase of warfarin-resistant rats was 30 times more resistant to inhibition by warfarin in vitro than the reductase present in Sprague-Dawley control animals. They concluded that this defect was the molecular basis of the warfarin resistance.

The vitamin K cycle is shown in Figure 5. In essence it is a salvage pathway for vitamin K, a vitamin present in only nanomolar quantities in liver and other tissues. It postulates that the vitamin K epoxidase (which may be linked to, or identical with, the carboxylase) converts vitamin K to its 2,3-epoxide usually in excess of the carboxylation rate. The epoxide is then reduced to the quinone by an epoxide reductase. The regenerated vitamin K is now reduced to the vitamin K hydroquinone by several possible enzymes, at least one driven by a dithiol and several by NAD(P)H. The dithiol-dependent epoxide reductase and quinone reductase are strongly inhibited by warfarin, whereas the NAD(P)H dependent dehydrogenases are relatively insensitive (278).

Vitamin K Epoxidase

This enzyme was first studied by Willingham & Matschiner (286), who indicated that a phylloquinone "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 (284) indicated that the epoxidase

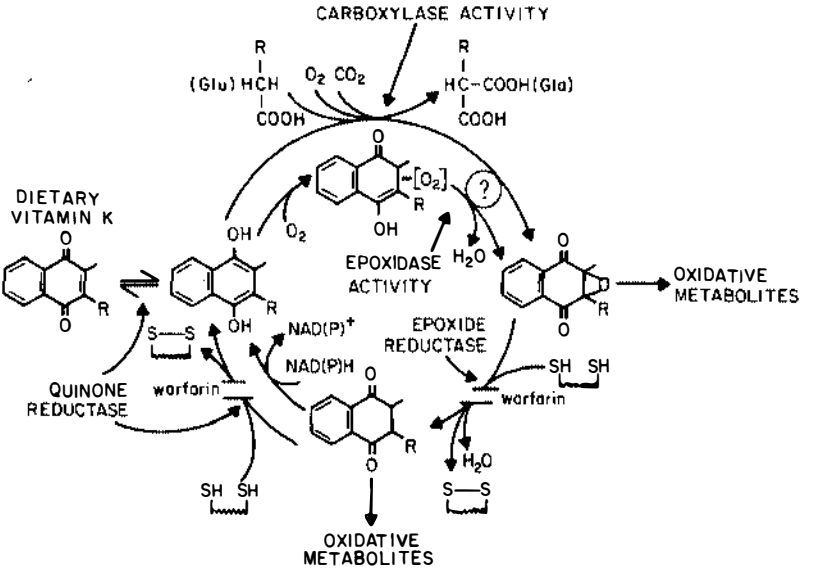

Figure 5 Vitamin K–vitamin K-2,3-epoxide (KO) cycle. The cycle is initiated by vitamin KH_2, which drives both carboxylase and epoxidase activities shown at the top. The variable degree of coupling of these two activities is indicated. The epoxide is reduced by a dithiol-driven KO reductase that yields vitamin K quinone. A number of reductases, shown at the left, are able to convert the regenerated quinone, as well as newly introduced dietary vitamin K, to the active KH_2 required to reinitiate the cycle.

activity was inhibited most effectively by direct antagonists of vitamin K action, such as tetrachloro-4-pyridinol.

A subsequent study by Sadowski et al (218) 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. With isotopically labeled oxygen, it was demonstrated that O_2 is the source of the epoxide oxygen. The overall reaction appears to be an atypical mixed-function oxidase with the required reducing equivalents coming from the quinol form of the vitamin. Although the epoxidase activity can be induced by phenobarbital treatment, the lack of sensitivity to CO (125) and the lack of effect of commonly used inhibitors of cytochrome P-450 (218) suggest that it is not a P-450 enzyme. The most intriguing aspect of microsomal vitamin K epoxidase activity is the possibility, discussed above, that it is part of the vitamin K–dependent γ-glutamyl carboxylase.

Preusch & Suttie (200) have shown that the epoxidase reaction generates a stereospecific (+)phylloquinone-2,3-epoxide, which has been assigned the 2S,3R configuration shown in Figure 6.

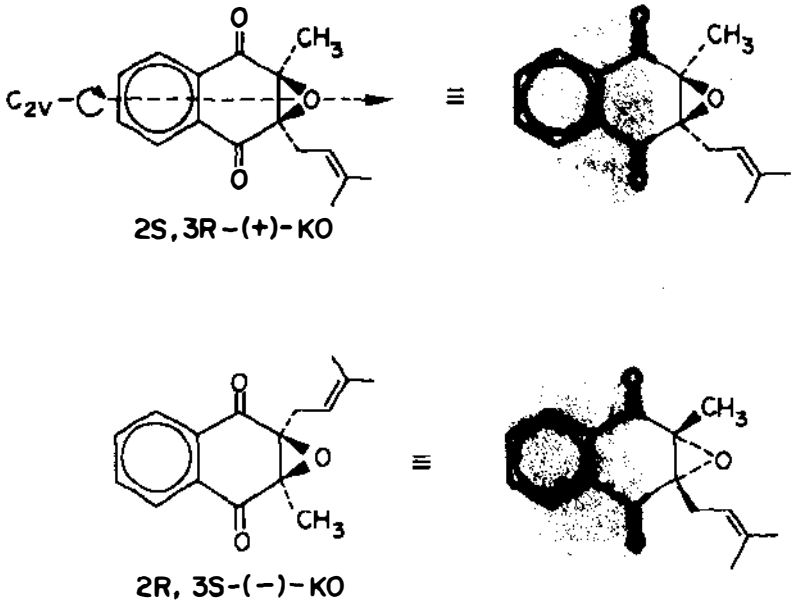

Figure 6 The two enantiomers of vitamin K epoxide are shown at the left. These enantiomers are presented in inverted orientations about the *symmetry axis*, which places the oxirane ring in the same position in the active site of the enzyme. Right, the planar portion of the molecule is shown interacting with the surface of vitamin K epoxide reductase. After (200).

Vitamin K-2,3-Epoxide Reductase

In addition to epoxidase activity, liver microsomes contain an epoxide reductase activity that can convert the vitamin K-2,3-epoxide back to the vitamin (163, 289). This enzyme is inhibited by warfarin. It has been postulated 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 (29, 142), and the epoxide reductase activity in these rats has been shown to be much less sensitive to warfarin than that of normal rats (289). More hydrophobic 4-hydroxy coumarin drugs like difenacoum inhibit the epoxide reductase in warfarin-resistant rats equally well (105).

Some of the properties of this enzyme have been described by Whitlon et al (283). The enzyme has little activity in the presence of NAD(P)H but is active when dithiothreitol is used as a reducing agent. The physiologically active reducing agent is not known. Lipoic acid at the same concentration was 53% as activity as DTT and, as is the case with DTT, the lipoate-driven reductase was warfarin sensitive. The vitamin K-2,3-epoxidediol is not an intermediate in the reaction (5). More recently, Siegfried (235) has reported success in solubilizing

the reductase in potassium cholate. The properties of the solubilized enzyme appear to be similar to those observed for the microsomal-bound enzyme. A protein factor present in the cytosol was observed to enhance the microsomal epoxide-reductase activity (235).

Preusch & Suttie (200) studied the stereoselectivity of vitamin K epoxide reductase for the oxirane ring configuration by recovering the partially resolved, unreacted substrate following incubations of racemic vitamin K epoxide with rat liver microsomes. After incubation, this substrate was enriched for the (−)-enantiomer, but selectivity for the biologically relevant (+)-enantiomer was low. This result was confirmed by direct comparison of the rates of reaction of the racemic substrate compared to the naturally occurring (+)vitamin K epoxide. The selectivity of vitamin K epoxide reductase for the *cis*- or *trans*-phytyl configuration of the vitamin K side chain was also low. These results suggest an enzyme active site that is open toward the 2,3 positions and is able to bind the substrate in two opposite orientations with respect to the positions of the methyl and phytyl side-chain substituents, as shown in Figure 6.

Silverman (236) suggested a mechanism for vitamin K epoxide reductase based on a chemical model that depicted a simultaneous acid-base catalysis involving protonation of the oxygen in the oxirane ring and an attack on carbon-2 of the epoxide by a sulfhydryl group to give a thioether of vitamin K with opening of the oxirane ring. An adjacent sulfhydryl in the enzyme was visualized as attacking the sulfur binding the 2-position with formation of a keto-enol tautomer at position 1 that stabilizes the substrate and permits the elimination of water and the reconstitution of vitamin K–quinone. This model (see Figure 7) has been restudied by Preusch & Suttie (201), who proposed the enol as the sole intermediate in the reductive step prior to the elimination of water.

Fasco et al (68) have identified 3-hydroxyvitamin K [(3)-hydroxy-2,3-dihydro-2-methyl,3-phytyl-1,4-naphthoquinone] as a product of vitamin K epoxide reduction in hepatic microsomes from warfarin-resistant rats, but not in those obtained from normal rats and not in detergent-solubilized microsomes from either strain of rat. The structure of 3-hydroxyvitamin K (3-KHOH) was determined by comparison of the HPLC behavior, UV, IR, CD, and mass spectra of the unknown with chemically synthesized standards. Alterations in the formation of hydroxyvitamin K occurred in parallel with alterations in total vitamin K epoxide reduction in warfarin-resistant rats with respect to reaction time, extent of reaction, effect of detergents, and inhibition by warfarin. Thus, 3-KHOH appears to be a product of the warfarin-resistant vitamin K epoxide reductase. It is neither a substrate nor an inhibitor of the epoxide reductase. KHOH is formed from both enantiomers of vitamin K-2,3-epoxide, but (+)vitamin K-2,3-epoxide yields (+)3-KHOH. In microsomes of warfarin-resistant rats the enolate (VI), shown in Figure 7, may be more loosely bound.

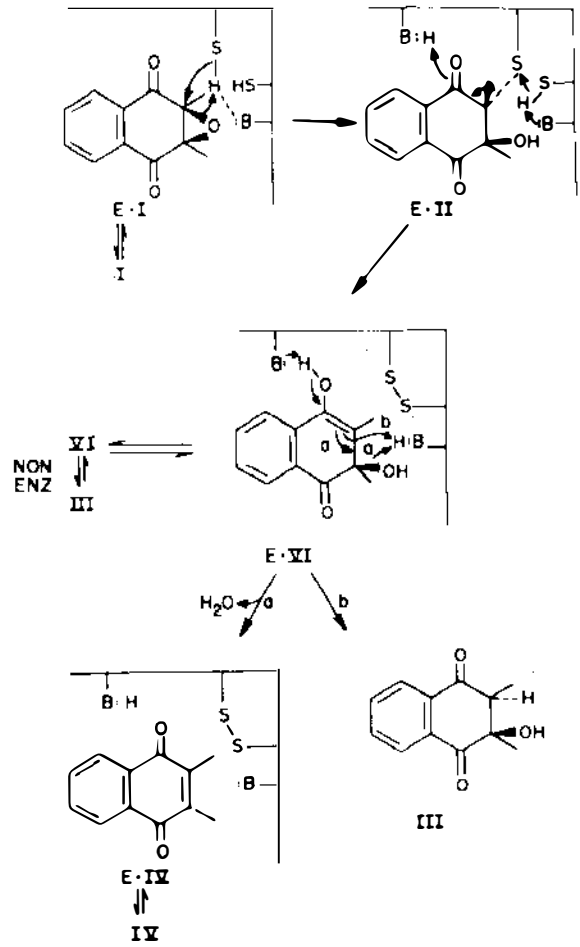

Figure 7 Mechanism of action of vitamin K-2,3-epoxide reductase. It is proposed that the vitamin K-epoxide (KO)(I) is attacked at position 2 by an -SH group of the enzyme to form a thioether adduct with opening of the oxirane ring (II). Attack by an adjacent -SH to form a disulfide enzyme forms an enolate of 3-hydroxy K. The enolate is then visualized as rearranging to form either the quinone (IV) and water (pathway a) or 3-hydroxy vitamin K (pathway b). A less tight binding of the enolate to the enzyme would favor pathway b. After (68).

This could permit dissociation and chemical rearrangement to the vitamin K hydroxide.

Vitamin K Reductases

The active form of vitamin K in the carboxylase system is KH_2. Early studies of washed liver microsomes revealed that a cytoplasmic factor plus NAD(P)H was required for carboxylation activity with vitamin K (217). The cytoplasmic

factor was a vitamin K reductase. Chemically prepared vitamin K hydroquinone can substitute for vitamin K plus NAD(P)H and a reductase.

Since vitamin KH_2 is rapidly oxidized by air to vitamin K in foods, the dietary intake of the vitamin is solely as the quinone, which appears to circulate in the blood as the quinone at levels of 1–5 ng/ml. One of the current questions about the metabolism of vitamin K and the action of the 4-hydroxycoumarin drugs is the identity of the reductase(s) that carry out the reduction of vitamin K in the endoplasmic reticulum.

There are several enzymes in tissues that can reduce vitamin K to its hydroquinone. Märki & Martius (153) described an NADH–vitamin K reductase from beef liver, which was found principally in the cytoplasm and to a lesser extent in mitochondria. At about the same time, Ernster et al (57, 58) described a flavoprotein enzyme from rat liver, which they named DT-diaphorase (E.C.1.6.99.2) because it catalyzed the oxidation of both NADH and NAD(P)H with various organic dyes and quinones, including vitamin K. The enzyme was found principally in the cytoplasm, was strongly inhibited by dicumarol, and was activated by serum albumin. The sensitivity to dicumarol raised the question whether the reduction of vitamin K was the 4-hydroxycoumarin-sensitive step in anticoagulation.

Wallin et al (277) purified DT-diaphorase from rat liver by affinity chromatography and showed that this enzyme was also present in solubilized microsomes. They removed the enzyme from solubilized microsomes by affinity chromatography and showed that all carboxylation activity due to vitamin K was lost. The carboxylase activity of this inactive preparation could be completely restored by the addition of the purified DT-diaphorase plus NAD(P)H. In this experiment dithiothreitol (DTT) could not replace the DT-diaphorase. It was concluded that DT-diaphorase, though principally cytosolic, is also present in microsomes and is the physiologically relevant vitamin K–reductase.

More recently, Wallin & Hutson (278) raised antibodies to pure DT-diaphorase and showed that only 45% of the NAD(P)H activity of Triton X-100 solubilized microsomes (the warfarin-sensitive fraction) could be neutralized with this antibody. They concluded that about one half of the NAD(P)H-dependent reductase activity in microsomes is due to a warfarin-insensitive reductase different from DT-diaphorase. This warfarin-insensitive enzyme appears to be the one relevant to γ-glutamyl carboxylation that is reduced in activity by riboflavin deficiency (202).

Fasco & Principe (71) identified vitamin KH_2 as a product of DTT reduction of vitamin K in intact rat liver microsomes. This enzyme is sensitive to warfarin inhibition (ca 1 μM). This system also produced KH_2 from K-2,3-epoxide in accord with the observations of Whitlon et al (283), who found in intact liver microsomes from vitamin K–deficient rats that DTT would support protein carboxylation better than NAD(P)H, particularly at low levels of vitamin K. In

their studies, warfarin (0.16 mM) totally inhibited these reductases, whereas the NAD(P)H reductases were not inhibited (70).

Kinetic studies by Fasco & Principe (69) then revealed that the DTT-driven reduction of vitamin K-2,3-epoxide in microsomes was an ordered reaction resulting in the formation first of vitamin K and later, after attaining a certain steady-state level of the quinone, vitamin KH_2. The amounts of warfarin required for inhibition of the two reductase activities were, for susceptible and warfarin-resistant rats, different by 2–3-fold. Fasco & Lee (67) have suggested that this ordered reaction by the DTT-driven vitamin K-2,3-epoxide reductase may be explained by assuming that KO and K reductions occur at separate catalytic sites and that an essential disulfide, reducible by DTT, supplies reducing equivalents for both reductions.

Terminal Oxidation Sequence

Wiss & Gloor (287) observed that the principal excretory form of vitamin K in rat urine was a metabolite resembling the lactone of vitamin E first described by Simon & colleagues (237). It was identified as a chain-shortened and oxidized derivative of vitamin K that forms a γ-lactone and is probably excreted as a glucuronide. Similar studies have been carried out by Shearer and colleagues in humans (233). This confirms the oxidation pathway for degradation of vitamin K involving ω-oxidation followed by β-oxidation of the side chain toward the ring to generate the 6'-carboxylic acid (V) and γ-lactone (VI) (Figure 8). In addition, they showed further that a new, additionally foreshortened 4'-carboxylic acid (IV) was present. These phylloquinone metabolites were reduced to 15% of the total in subjects treated with warfarin in whom analogous epoxide metabolites (III and VII) were found (164). These metabolites are usually conjugated with glucuronic acid and participate in an enterohepatic circulation.

ACTION OF VITAMIN K ANTAGONISTS

4-Hydroxycoumarin Drugs

A hemorrhagic disease in cattle that had consumed spoiled clover was described by Schofield in 1922 (222) and attributed to a depressed prothrombin level by Roderick in 1931 (213). In 1941, Campbell and co-workers (34) demonstrated that the active agent in spoiled clover was bishydroxycoumarin (dicumarol). A variety of related compounds, either derivates of 4-hydroxycoumarin or phenindandione, have been synthesized and tested for anticoagulant activity in animals and humans. One of the more popular ones in the United States is warfarin (3-[α-acetonyl benzyl]-4-hydroxy coumarin), a more soluble compound than dicumarol. Longer acting, more hydrophobic

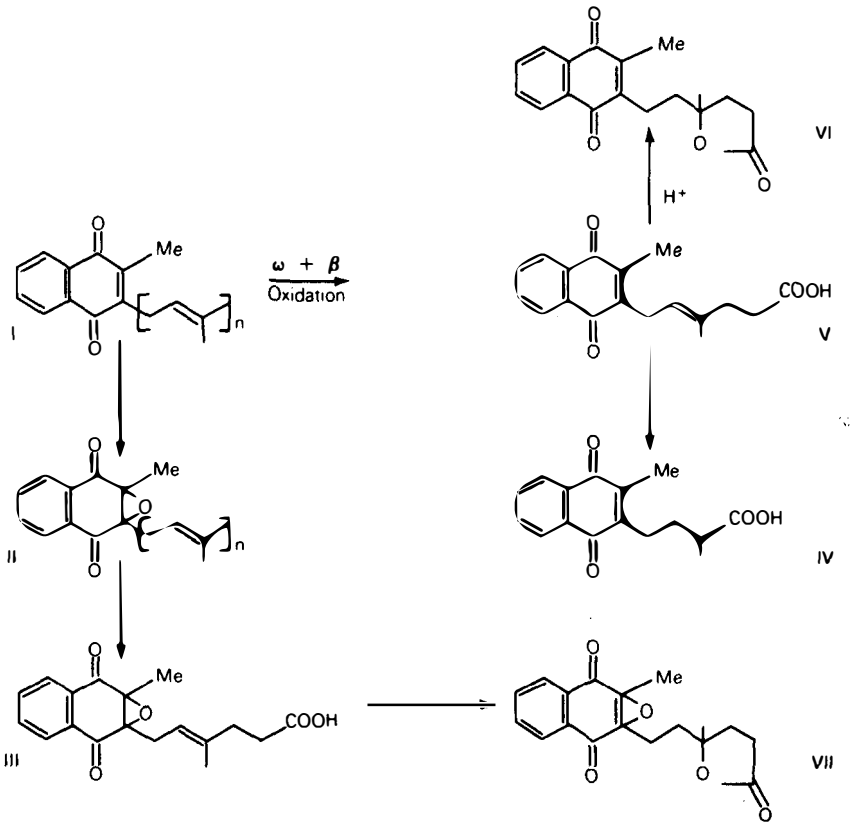

Figure 8 Terminal oxidation of vitamin K. The shortening of the isoprenyl side chain of vitamin K by ω- and β-oxidation yields a 6'-carboxylic acid (V) that can form a γ-lactone (VI) or a further-shortened 4'-carboxylic acid (IV). Vitamin K-2,3-epoxides (II) that accumulate after warfarin treatment undergo similar side-chain oxidations to yield compounds III and VII.

4-hydroxy coumarins being used in animals as rodenticides include difenacoum (3-[3-p-diphenyl-1,2,3,4-tetrahydronaphth-1-yl]-4-hydroxy coumarin) and brodifacoum (3-[3-(4'-bromodiphenyl-4-yl)-1,2,3,4-tetrahydronaphth-1-yl]-4-hydroxycoumarin).

The oral anticoagulant agents regulate the biosynthesis of prothrombin (factor II) and other vitamin K–dependent factors in the liver. They also induce hypoprothrombinemia and other factor deficiences at specific rates when given in saturating doses, even though the half-life of various drugs varies from hours to days in different animals and in humans. As soon as there is an effective concentration of the drug, vitamin K–dependent factor biosynthesis by liver is shut off and the factors then decay in plasma at their specific half-lives (181). Hydroxylated products of these drugs, generated by the enzymes in the liver microsomes, are inactive.

The mechanism of action of the 4-hydroxycoumarin drugs is still under

investigation, although it seems fairly clear that these agents do not directly block the vitamin K–dependent carboxylase. As noted earlier, Bell & Matschiner (10) proposed that the primary action of warfarin was to inhibit vitamin K-2,3-epoxide reductase and prevent the recycling of the epoxide to vitamin K, thus reducing the concentration of KH_2 in vivo to ineffective levels. This view has been strongly supported by others (248). More recently, Fasco et al (66) have shown that the DTT-driven vitamin K reductase is more sensitive to warfarin than the vitamin K epoxide reductase in both normal and warfarin-resistant rats and that the vitamin K-KO cycle is blocked at both reduction steps by warfarin. Fasco et al (69) have also observed that warfarin and vitamin K-2,3-epoxide are not competitive with respect to each other, and that warfarin binding occurs exclusively to the disulfide (oxidized) form of the epoxide reductase. They speculated that DTT antagonizes warfarin by maintaining the epoxide reductase in the reduced state. The extent of KO/K elevation in warfarin-treated rabbits does not correlate well with the dose required for anticoagulant activity (196).

The kinetics of 4-hydroxycoumarin action in whole animals are not competitive with vitamin K (129, 193). In vitro, warfarin inhibits vitamin K–dependent carboxylation of prothrombin precursor in rat liver microsomes but has little effect on the solubilized carboxylase system (193). The amount of vitamin K required by microsomes for carboxylation activity is lower by a factor of 100 than that required in detergent-solubilized preparations. The apparent K_m for intact microsomes is about 100 pmoles/g (120, 283) and corresponds to the amount actually present in mammalian liver. It is possible that the DTT-driven vitamin K reductase has a very low K_m when compared to the NAD(P)H-dependent vitamin K reductase, and hence under physiological conditions the DTT-powered warfarin-sensitive enzyme is the pathway used. In order to overcome the warfarin block, large amounts of vitamin K are necessary. At these high levels, the relatively warfarin-insensitive NAD(P)H reductases are used (278), restoring carboxylation as long as KH_2 can be formed.

Warfarin-binding studies have shown that normal microsomes bind warfarin more tightly and in larger amounts than those from warfarin-resistant rats (141, 263). Searcey and colleagues (225) reported the isolation of warfarin-binding proteins (WBP) for microsomes of normal and warfarin-resistant rats. These appear to be intrinsic proteins of the endoplasmic reticulum since they are liberated only by detergent. The warfarin-binding protein isolated from Sprague-Dawley rats was half-saturated at 8 μM warfarin and bound 0.7 mol of warfarin per mol of protein at saturation. The corresponding protein from warfarin-resistant rats did not saturate at levels of 30 μM warfarin and bound only 0.1 mol of warfarin per mol of protein. The molecular weight of the binding protein from both normal and warfarin-resistant rats was 32,000 by SDS-gel electrophoresis. Maximum warfarin binding by the protein from

normal rats occurred at 37°C at pH 7.3 in the presence of 200 mM KCl. [^{14}C]Warfarin binding was inhibited by unlabeled warfarin, phylloquinone, and tryptic digestion. The (S)-enantiomer of warfarin was the preferred ligand. On the basis of these data it is likely that this protein is the warfarin receptor protein that mediates the effect of the drug on prothrombin synthesis. The isolated protein showed no enzymatic activity, which could have been lost during the isolation procedure.

Difenacoum and brodifacoum are more potent and persistent antagonists to vitamin K than warfarin (130, 194). They appear to have the same general mode of action—i.e. to inhibit vitamin K or vitamin K epoxide reductases with elevation of the KO:K ratio in plasma in liver—but have a much higher affinity for these enzymes and a longer duration of action. These agents were equally effective in the susceptible and the warfarin-resistant rats (105).

Salicylate and Other Drugs

Salicylate in doses greater than 6 g per day can induce hypoprothrombinemia in humans. The mechanism appears to be different from that of the 4-hydroxycoumarin drugs even though plasma KO:K ratios were found to rise in rabbits and the effect of salicylate could be overcome by high doses of vitamin K (195). Hildebrandt & Suttie (104) observed that although hepatic KO:K ratios increased with salicylate treatment of rats, there was no effect in vitro on the vitamin K-2,3-epoxide reductase. There was a marked effect, however, on DT-diaphorase and vitamin-K+NADH-driven carboxylation. These authors concluded that the pharmacologic effect of salicylate is on vitamin K reductase and that the effects on the epoxide reductase and the KO:K ratio are indirect.

Ticrynafen [2,3-dichloro-4-(2-thienyl-carbonyl)-phenoxy acetic acid] a nonsulfonamide diuretic, uricosuric, and antihypertensive agent, which causes hypoprothrombinemia in humans, has the same effect as salicylate in the rat. It markedly inhibits DT-diaphorase and tends to cause a rise in hepatic vitamin KO:K ratios (199).

Broad spectrum cephalosporin antibiotics (Moxam®, Cefamandol®) have been observed to cause vitamin K–reversible hemorrhage in humans (108). Lipsky (140) reported that the N-methylthiotetrazole moiety of these antibiotics could block vitamin K-dependent peptide carboxylation in Triton-solubilized rat liver microsomes in a dose-dependent manner. Uotila & Suttie (268) verified the effect of these antibiotics on the vitamin K–dependent carboxylase in vitro. This inhibition was noncompetitive with respect to pentapeptide and KH_2 and was partially reversed by pyridoxal-5'-phosphate.

Diphenylhydantoin, an anticonvulsant drug, has been observed to reduce prothrombin levels in adults but particularly to increase the susceptibility of infants born of epileptic mothers to vitamin K deficiency (183).

Butylated Hydroxytoluene

Recently it has been reported that toxic doses of butylated hydroxytoluene (BHT) cause a hemorrhagic death in rats (262). The mechanism of this effect involves an antagonism to vitamin K as indicated by a fall in the vitamin K–dependent coagulation proenzymes and correction of the defect by added phylloquinone (258). In a recent paper, Suzuki and co-workers (257) reported that the vitamin K content of the livers of rats fed 0.25% BHT was reduced, and fecal excretion of vitamin K metabolites was increased compared to controls. These authors concluded that BHT was not acting as a coumarin-like antagonist, but may be inhibiting the uptake of vitamin K by intestinal or liver cells.

Takahashi & Hiraga (261) have recently reported that the quinone methide of BHT (2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone) a metabolite of BHT, inhibits vitamin K epoxide reductase in rats 20–30%. No effect of this compound was noted on vitamin KH_2 or vitamin-K+NADH-driven carboxylation. Strangely, vitamin K epoxide given to rats with BHT-induced hypothrombinemia restored prothrombin levels to normal. Further studies of BHT poisoning are indicated.

Vitamins A and E

The fat-soluble vitamins A and E are known to antagonize vitamin K. It has been recognized since 1944 (139) that hypervitaminosis A in the rat leads to hemorrhages and hypoprothrombinemia that can be prevented by the administration of vitamin K. Matschiner & Doisy (161) found there was a close relationship between the level of dietary vitamin A (above normal) and the resulting deficiency of vitamin K as expressed by depressed prothrombin levels in rats. The effect of vitamin A was more severe in males than in females, and retinoic acid was more effective than vitamin A acetate. In a subsequent paper in which the anti-coagulant effects of squalene and retinoic acid were studied in adult male rats, Matschiner and colleagues (159) observed that 0.5% squalene or 50 I.U. retinoic acid per day increased the vitamin K requirement to 10 times that seen in germ-free rats. No effect of parenterally administered retinoic acid was observed. These investigators concluded that the action of excess vitamin A was to prevent the absorption of vitamin K.

Large amounts of vitamin E may also antagonize the action of vitamin K. March et al (152) observed that when diets containing 2200 I.U. vitamin E per kilogram were fed to chicks, prothrombin times were lengthened. This prothrombin deficiency was responsive to vitamin K. Prolongation of prothrombin times as a result of megavitamin E therapy has also been reported in humans (126). This conditioned vitamin K deficiency in human subjects taking megadoses of vitamin E has also been expressed as a hypersensitivity to the coumarin anticoagulant drugs. Corrigan & Marcus (39) reported clinical evidence of vitamin K deficiency in a patient taking 5 mg warfarin, 2.0 g of clofibrate, and 1200 I.U. of vitamin E each day. Upon discontinuation of his

megavitamin E therapy, the bleeding tendency and prolonged prothrombin time normalized. Lesser doses of vitamin E in humans (42 I.U. per day for 30 days) do not potentiate the anticoagulant effects of warfarin (224).

The question of whether this antagonism between α-tocopherol and phylloquinone is exercised at the level of absorption or metabolism remains unsettled. Woolley (288) first observed in rats that α-tocopherolquinone in large doses caused hemorrhages that could be prevented by menadione. These observations were confirmed in pregnant rats by Rao & Mason (208).

Olson & Jones (188, 189) observed that high dietary intakes of vitamin E in rats antagonize vitamin K activity and increase the vitamin K requirement. Vitamin E does not appear to affect vitamin K uptake or distribution from the gut, nor does vitamin E directly affect the vitamin K–dependent carboxylase in vitro.

Although the tocopherolquinone can be produced in vitro by $FeCl_3$ or other oxidative agents, it is not clear what amounts of quinone and hydroquinone are formed after ingestion of vitamin E in various animals. Apparently, the amounts normally formed in vivo are small (41) and probably vary with the amount of α-tocopherol administered. It has been reported that there is a marked increase of tocopherylquinone in the livers of mice after intraperitoneal administration of large amounts of α-tocopheryl acetate (4).

Bettger, Jones & Olson (15) found that d-α-tocopherolquinone (d-α-TQ) is a more potent antagonist of vitamin K activity in the rat than d-α-tocopherol. Neither compound appeared to affect the absorption of distribution of vitamin K. Feeding high levels of vitamin E to rats (2 g/kg diet) resulted in an increase in d-α-TQ in liver microsomes from 2.5 nmol/g to 9.4 nmol/g. The microsomal concentration of d-α-TQ appears to be of physiological importance in its role as a vitamin K antagonist, since d-α-TQ inhibits vitamin K–dependent carboxylase activity in vitro at these concentrations. A variety of antioxidants also antagonize vitamin K activity in vivo; however, only n-propyl gallate is a potent inhibitor of the vitamin K–dependent carboxylase in vitro.

NUTRITIONAL REQUIREMENTS FOR VITAMIN K IN HUMANS

Sources of Vitamin K

Vitamin K is derived both from foods and from the microflora of the gut. The vitamin K content of common foods have been determined by bioassay in chicks and by HPLC chromatography (183). Much of the older work using bioassay techniques must be repeated to develop better values. In general, green leafy vegetables are high, fruits and cereals low, and meats and dairy products intermediate in concentration. In fact, the intestinal absorption of vitamin K from plant sources ranges from 30% to 70% of the actual content determined by extraction. In human liver, Rietz and associates (211) have

demonstrated the presence of menaquinone-7, menaquinone-8, menaquinone-9 (2H), menaquinone-9 (4H), menaquinone-10, and menaquinone-11 in addition to phylloquinone. It is clear that the distribution of vitamin K homologs in mammalian liver reflects both dietary and bacterial sources of the vitamin present in their intestinal tracts. Tobacco is one of the richest sources of phylloquinone known, containing about 5 mg/100 g. A small percentage of this vitamin K is volatilized in smoking and is absorbed through the mucous membranes of the nasal pharynx and bronchi.

Vitamin K Deficiency

Primary vitamin K deficiency is uncommon in both animals and humans. This is due to widespread distribution of vitamin K in plant and animal tissues and to the microbiological flora of the normal gut, which synthesize the menaquinones in amounts that may supply the bulk of the requirement for vitamin K. The causes of deficiencies in the vitamin K–dependent coagulation factors in humans are largely secondary to disease or drug therapy and are presented in Table 4.

HEMORRHAGIC DISEASE OF THE NEWBORN Newborn infants represent a special case of vitamin K nutrition because (*a*) the placenta is a relatively poor organ for the transmission of lipids, and (*b*) the gut is sterile during the first few days after birth. In normal infants, the plasma prothrombin concentration, and that of the other vitamin K–dependent factors, may decrease to a level as low as 30% in the second and the third days of life. As food is taken, the levels gradually climb to normal adult values over a period of weeks. Human milk is a poorer source of vitamin K (2 μg/liter) than cow's milk (10 μg/liter) (231). If prothrombin values fall below 10%, hemorrhagic disease of the newborn appears (31). Premature infants are even more susceptible to vitamin K deficiency than are full-term infants.

Table 4 Causes of deficiencies of vitamin K–dependent coagulation factors

1. Hemorrhagic disease of the newborn
2. Dietary inadequacy (low-fat diets, protein-calorie malnutrition)
3. Total parenteral nutrition
4. Biliary obstruction (gallstones, stricture, fistulas)
5. Malabsorption syndromes (cystic fibrosis, sprue, celiac disease, ulcerative colitis, regional ileitis, short-bowel syndrome)
6. Liver disease
7. Drug therapy:
Coumarin anticoagulants and related drugs (warfarin, indanediones, phenprocoumon, hydantoins, salicylates)
Antibiotics including cephalosporins
Typhenacryn
Megadoses of vitamin E

Infants of mothers on hydantoin anticonvulsants should have prophylactic vitamin K because diphenylhydantoin is an antagonist to vitamin K (65). Neonatal complications such as diarrhea, malabsorption, cystic fibrosis, idiopathic cholestasis, atresia of the bile duct, and prolonged parenteral nutrition are indications for intramuscular vitamin K administration.

DIETARY INADEQUACY Healthy adult subjects fed low–vitamin K diets (10 μg per day) for periods of several weeks show minimal signs of vitamin K deficiency (i.e. plasma prothrombin values of 60–90%) unless they are also given bowel-sterilizing antibiotics such as neomycin (77, 267). In one study of apoplectic patients, intravenous nutrition plus neomycin was required to lower the vitamin K–dependent clotting factors to below 20% of normal (77) in four weeks. The intravenous administration of vitamin K in various doses (0.03–1.5 μg/k) to these patients caused a proportional rise in the concentration of these depressed values to normal levels. In unusual cases, self-imposed dietary restriction induces hypoprothrombinemia with hemorrhage responsive to oral vitamin K (2, 124). Dietary deficiency of vitamin K becomes manifest more quickly in debilitated patients with or without antibiotics (3).

TOTAL PARENTERAL NUTRITION With the advent of subclavian-vein catheterization in 1968 for long-term parenteral nutrition of both surgical and medical patients unable to eat, new nutritional deficiency syndromes have been reported (55), among which is hemorrhage due to vitamin K deficiency (215). Physiologic amounts of the fat-soluble vitamins, particularly vitamins E and K, are not metabolized normally when introduced into the central venous circulation. It is advisable to give doses of 1 mg phylloquinone per week (equivalent to about 150 μg vitamin K per day) in cases of prolonged total parenteral nutrition.

BILIARY OBSTRUCTION Prior to the discovery of vitamin K and the recognition of its deficiency in obstructive jaundice, bleeding commonly occurred after surgical correction of biliary obstruction (26). Because the secretion of bile salts is essential for the absorption of fats and fat-soluble vitamins, it is not surprising that biliary obstruction was early identified as a cause of vitamin K deficiency. All patients with obstructive jaundice should receive parenteral vitamin K_1 (5 mg per day) for three days prior to surgery.

MALABSORPTION SYNDROME Depression of the vitamin K–dependent coagulation factors is frequently found in the malabsorption syndromes and in other gastrointestinal disorders (e.g. cystic fibrosis, sprue, celiac disease, ulcerative colitis, regional ileitis, ascaris infection, and short-bowel syndrome).

LIVER DISEASE Patients with parenchymal liver disease may have hypoprothrombinemia and an elevation in plasma des-γ-carboxyprothrombin. They are unable to utilize vitamin K in the biosynthesis of vitamin K–dependent clotting factors, usually as a result of destruction of the rough reticulum in the hepatocyte. Patients with liver disease, however, should be challenged with vitamin K to determine the extent of the organic disorder preventing biosynthesis of prothrombin. To do this, a parenteral dose of 10 mg daily for three days should be administered.

DRUG THERAPY

Coumarin and related anticoagulants The coumarin anticoagulant drugs can induce serious hypoprothrombinemia. Some of the contributory causes are reduction in dietary intake of vitamin K, ingestion of interfering drugs, and the inadvertent alteration of the anticoagulant dosage schedule. Salicylates in large doses may also depress vitamin K–dependent factors.

Broad-spectrum antibiotics Intestinal bacteria comprise an important source of vitamin K in humans. As has been mentioned, the gastrointestinal flora may supply an individual's entire requirement. However, vitamin K is not well absorbed from the colon. Udall (267) showed that large amounts of vitamin K (of the order to 500 mg per day) instilled into the cecum did not elevate depressed coagulation factors in anticoagulated patients, whereas the same dose given orally gave a prompt response. It appears that the microorganisms synthesizing vitamin K in the gut must reside in the ileum, where absorption of vitamin K is possible.

Sulfa drugs, neomycin, and other broad-spectrum antibiotics are capable of sterilizing the bowel and inducing hemorrhagic syndrome. Cephalosporin antibiotics, in addition, inhibit DT-diaphorase and further compromise the supply of KH_2 for carboxylation.

Ticrynafen As discussed earlier this diuretic can cause hypoprothrombinemia.

Fat-soluble vitamins As mentioned in a previous section, large amounts of vitamins A and E may antagonize the action of vitamin K.

Vitamin K Requirements

The vitamin K requirement of mammals is met by a combination of dietary intake and microbiologic biosynthesis in the gut. Furthermore, genetic factors influence the vitamin K requirement in both animals and humans. In conventional rats, the vitamin K requirement is about 10 μg/kg/day supplied in 0.1 μg/g food, whereas in germ-free rats the requirement is more than doubled to

about 25 μg/kg/day (133). Chicks have a higher requirement of 50 μg/kg/day, equivalent to 0.5 μg/g food.

In human subjects, the vitamin K homologs stored in the liver suggest that about 40–50% of the daily requirement is derived from plant sources (that is, vitamin K_1) and the remainder from microbiologic biosynthesis. Both phylloquinone and menaquinone are present in human plasma (38, 133, 234). Phylloquinone is present in the range of 0.5–5.0 ng/ml with an average of 2 ng/ml in healthy persons. Menaquinone is present in lower amounts. By assuming that the intravenous dose of vitamin K required to raise depressed levels of vitamin K to normal for 1 day is 1 μg/kg (77), and that 50% of the vitamin K appearing in the lumen of the gut each day is absorbed, the total daily requirement for the vitamin would be 2 μg/kg/day. If, however, one assumes that 50% of the requirement is derived from intestinal microorganisms, then the dietary requirement would be reduced again to 1 μg/kg/day. These assumptions need further experimental verification.

With the information at hand, this is a rough estimate, particularly because there is controversy concerning the relative activity of phylloquinone and the menaquinones in stimulating prothrombin synthesis (115, 162). A "normal mixed diet" in the United States contains 300–500 μg vitamin K per day, an amount more than adequate to supply the dietary requirement of vitamin K.

SUMMARY AND CONCLUSIONS

Since the discovery of γ-carboxyglutamic acid a decade ago, great progress has been made in advancing our knowledge of the function and metabolism of vitamin K. The distribution of this new amino acid in proteins of diverse origin and the presence of the vitamin K–dependent carboxylase in diverse tissues have emphasized the widespread significance in biology of a new triad: vitamin K, Gla, and calcium.

New knowledge has been obtained on the importance of the utilization and reutilization of vitamin K, whose body pools are extremely low for a fat-soluble vitamin, for the posttranslational carboxylation of peptide-bound glutamate residues in the vitamin K–dependent proteins. The regulation of the activation of the vitamin K–vitamin K-epoxide cycle by drugs and nutrients appears to be the key to controlling the synthesis of vitamin K–dependent proteins, eight of which are involved in blood coagulation. The purification of the vitamin K–dependent γ-glutamyl carboxylase has turned out to be a more formidable task than anyone had imagined. Many of the questions about its complicated mechanism, utilizing as it does four substrates (KH_2, O_2, CO_2, and a Glu-containing peptide), cannot be answered until the enzyme is homogeneous.

Basically, the vitamin K–dependent carboxylase system consists of a specialized microsomal electron transport system coupled to a carbon dioxide

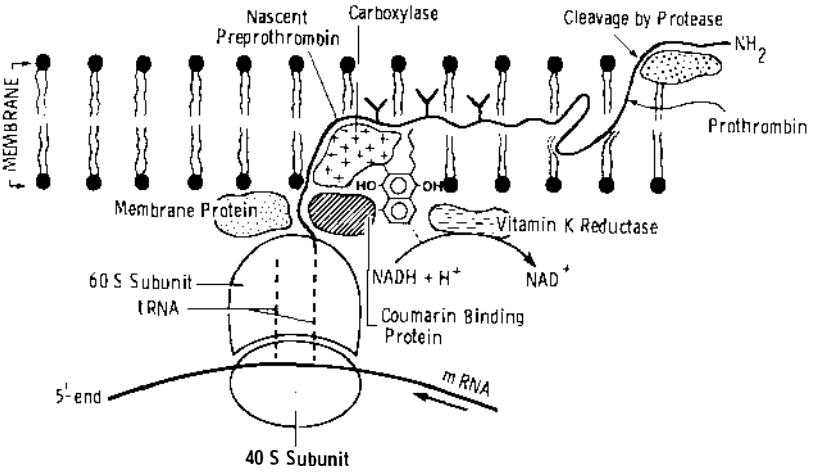

Figure 9 Rough reticulum of a hepatocyte synthesizing prothrombin. One leaf of the endoplasmic membrane is shown. Vitamin K is depicted as being inserted on the cytoplasmic side, whereas the signal peptide cleavage enzyme is shown on the lumenal side. The carboxylase may be a transmembrane protein, although it is shown as a central (intrinsic) membrane protein. Adjacent to the carboxylase is presented a coumarin-binding protein, which could be a regulatory subunit or a relevant KO or K reductase. The vitamin K reductase shown could be driven by either NAD(P)H or dithiol.

fixation. The reaction does not require ATP but apparently utilizes the energy of vitamin KH_2 oxidation to perform the chemical work required in Gla synthesis. Why a quinone is employed in this system when other mechanisms exist for CO_2 fixation is still mysterious unless the whole process goes by one electron transport. Whether the final CO_2 addition to the γ-methylene group of glutamic acid is a radical reaction is unsettled. Since this enzyme is an intrinsic membrane-bound protein, the scientific attack on its structure and function is at one of the present frontiers of molecular biology. A view of the synthesis of vitamin K–dependent proteins in the RER is shown in Figure 9.

Finally, the nutritional requirements for vitamin K in humans are unknown. An unknown fraction of vitamin K in humans is derived from menaquinone biosynthesis in the intestinal flora. Contributions from diet and biosynthesis have not yet been quantitated. Sensitive HPLC methods for measuring plasma phylloquinone are now available, and related methods for measuring long-chain menaquinones can be developed.

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