

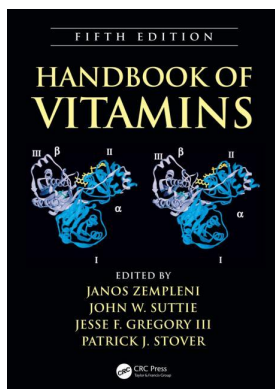
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## Handbook of Vitamins

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### 3 Vitamin K

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# 3 Vitamin K

*John W. Suttie*

## CONTENTS

3.1	History .....	90
3.2	Chemistry .....	90
3.2.1	Isolation .....	90
3.2.2	Structure and Nomenclature .....	90
3.2.3	Structures of Analogs, Commercial Forms, and Antagonists .....	91
3.2.3.1	Analogs and Their Biological Activity .....	91
3.2.3.2	Commercial Form of Vitamin K .....	91
3.2.3.3	Antagonists of Vitamin Action .....	92
3.2.4	Physical and Chemical Properties .....	94
3.3	Analytical Procedures and Vitamin K Content of Food .....	94
3.4	Metabolism .....	96
3.4.1	Absorption and Transport of Vitamin K .....	96
3.4.2	Plasma Concentrations of Vitamin K .....	97
3.4.3	Tissue Distribution of Vitamin K .....	98
3.4.4	Synthesis of MK-4 .....	99
3.4.5	Metabolic Degradation of Vitamin K .....	99
3.5	Vitamin K–Dependent Proteins .....	100
3.5.1	Plasma Clotting Factors .....	100
3.5.2	Calcified Tissue Proteins .....	102
3.5.3	Other Vitamin K–Dependent Proteins .....	103
3.6	Biochemical Role of Vitamin K .....	103
3.6.1	Discovery of $\gamma$ -Carboxyglutamic Acid .....	103
3.6.2	The Vitamin K–Dependent Carboxylase .....	104
3.6.3	The Vitamin K Epoxide Reductase .....	106
3.7	Health Impacts of Altered Vitamin K Status .....	107
3.7.1	Methodology .....	107
3.7.2	Adult Vitamin K Deficiency .....	108
3.7.3	Anticoagulant Therapy .....	108
3.7.4	Hemorrhagic Disease of the Newborn .....	109
3.7.5	Possible Role in Bone Health .....	109
3.7.6	Possible Role in Vascular Calcification .....	110
3.7.7	Other Factors Influencing Vitamin K Status .....	110
3.8	Vitamin K Requirements .....	111
3.8.1	Animals .....	111
3.8.2	Humans .....	112
3.9	Efficacy and Hazards of Pharmacological Doses of Vitamin K .....	113
	References .....	113

### 3.1 HISTORY

Vitamin K was discovered through a series of experiments in the early 1930s conducted by Henrik Dam who was investigating the possible essential role of cholesterol in the diet of the chick. Dam [1] noted that chicks ingesting diets that had been extracted with nonpolar solvents to remove the sterols developed hemorrhages and that blood taken from these animals clotted slowly. Subsequently, McFarlane et al. [2] and Holst and Halbrook [3] described clotting defects seen when chicks were fed ether-extracted fish or meat meal, or internal and external hemorrhages in chicks fed fish meal and yeast as a protein source. This disease could not be cured by the administration of any of the known vitamins. Dam continued to study the active component in vegetable and animal sources and in 1935 proposed [4,5] that the antihemorrhagic vitamin of the chick was a new fat-soluble vitamin, which he called vitamin K. Not only was K the first letter of the alphabet that was not used to describe an existing or postulated vitamin activity at that time, but it was also the first letter of the German word *koagulation*.

Numerous groups were involved in attempts to characterize this new vitamin, and Dam's collaboration with Karrer of the University of Zurich resulted in the isolation of the vitamin from alfalfa as a yellow oil. Subsequent studies soon established that the active principle was a quinone and vitamin K<sub>1</sub> was characterized as 2-methyl-3-phytyl-1,4-naphthoquinone and synthesized by Doisy's group in St. Louis [6]. The Doisy group also isolated a form of the vitamin from putrefied fish meal, which in contrast to the oil isolated from alfalfa was a crystalline product. This compound, called vitamin K<sub>2</sub>, contained an unsaturated side chain at the 3-position of the naphthoquinone ring. Sources of this form of the vitamin, such as putrefied fish meal, contained a number of different vitamins of the K<sub>2</sub> series with differing chain length polyprenyl groups at the 3-position. The 1943 Nobel Prize in Physiology and Medicine was awarded to Dam and Doisy, and much of the early history of the discovery of vitamin K has been reviewed by them [7,8] and by others [9–11].

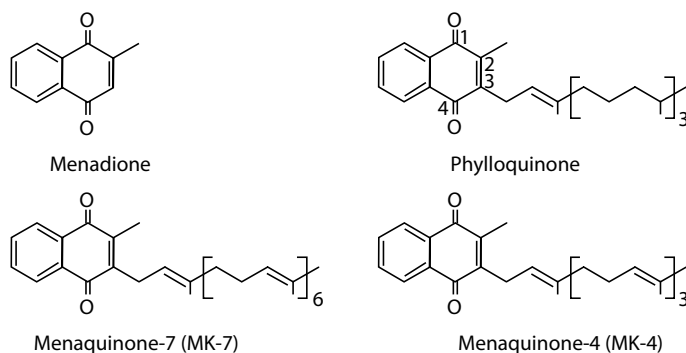
### 3.2 CHEMISTRY

#### 3.2.1 ISOLATION

The isolation of vitamin K from biological material is always complicated by the small amount of desired product in the initial extracts. Initial extractions are usually made with the use of some type of dehydrating conditions, such as chloroform–methanol, or by first grinding the wet tissue with anhydrous sodium sulfate and then extracting it with acetone followed by hexane or ether. Large samples (kilogram quantities) of tissues can be extracted with acetone alone, and this extract can be partitioned between water and hexane to obtain the crude vitamin. Methods for the efficient extraction of vitamin K from various food matrices have been developed [12]. Rather extensive databases of the vitamin K content of foods are now available, and following separation of the total vitamin K fraction from much of the contaminated lipid, the various forms of the vitamin can be separated by the procedures described in Section 3.3.

#### 3.2.2 STRUCTURE AND NOMENCLATURE

The nomenclature in general use at the present time is that of the most recently adopted International Union of Pure and Applied Chemistry–International Union of Biochemistry subcommittee report on Nomenclature of Quinones [13]. The term *vitamin K* is used as a generic descriptor of 2-methyl-1,4-naphthoquinone and all derivatives of this compound that exhibit an antihemorrhagic activity in animals fed a vitamin K–deficient diet. The compound 2-methyl-3-phytyl-1,4-naphthoquinone is produced in green plants and is generally called vitamin K<sub>1</sub>, but is preferably called phyloquinone. The United States Pharmacopeia (USP) nomenclature for phyloquinone is phytonadione. The compound first isolated from putrefied fish meal and called at that time vitamin K<sub>2</sub> is one of



**FIGURE 3.1** Structures of some compounds with vitamin K activity.

a series of vitamin K compounds with unsaturated side chains called multiprenyl-menaquinones that are synthesized by a number of facultative and obligate anaerobic bacteria [14]. The particular menaquinone shown in Figure 3.1 (2-methyl-3-farnesylgeranylgeranyl-1,4-naphthoquinone) has 7 isoprenoid units or 35 carbons in the side chain and was once called vitamin  $K_2$  but now is called menaquinone-7 (MK-7). Vitamins of the menaquinone series with up to 13 prenyl groups have been identified, as well as several partially saturated members of this series. The parent compound of the vitamin K series, 2-methyl-1,4-naphthoquinone, was at one time called vitamin  $K_3$  but is more commonly and correctly designated as menadione. Menaquinone-4 (MK-4) is a minor bacterial product but can be formed by animals by the alkylation of menadione or through the degradation of phylloquinone by a pathway not yet clearly defined (see Section 3.4.4).

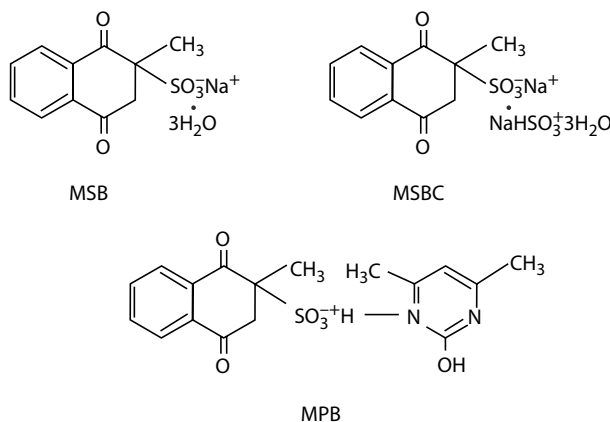
### 3.2.3 STRUCTURES OF ANALOGS, COMMERCIAL FORMS, AND ANTAGONISTS

#### 3.2.3.1 Analogs and Their Biological Activity

After the discovery of vitamin K, a number of related compounds were synthesized in various laboratories and their biological activity was compared with that of the isolated forms. Structural features found to be essential for significant biological activity included the following: a naphthoquinone ring, a 2-Me group on the ring, an unsaturated isoprenoid unit adjacent to the ring, and *trans* configuration of the polyisoprenoid side chain. The activity of various structural analogs of vitamin K in whole-animal assay systems were, of course, a summation of the relative absorption, transport, metabolism, and effectiveness of this compound at the active site as compared with that of the reference compound. When administered orally, isoprenalogs with three to five isoprenoid groups had maximum activity [15], and the lack of effectiveness of higher isoprenalogs in this type of assay may have been due to the relatively poor absorption of these compounds. When intracardial injection of vitamin K to deficient rats is used as a protocol [16], the very high molecular weight isoprenalogs of the menaquinone series are the most active.

#### 3.2.3.2 Commercial Form of Vitamin K

The major use of vitamin K in the animal industry is in poultry and to some extent in swine diets. Chicks are very sensitive to vitamin K restriction, and vitamin K is commonly added to poultry diets. Phylloquinone is too expensive for this purpose, and different forms of menadione have been used. Menadione itself possesses high biological activity in a deficient chick, but its effectiveness depends on the presence of lipids in the diet to promote absorption. There are also problems of its stability in feed products, and because of this, water-soluble forms are used. Menadione forms a water-soluble sodium bisulfite addition product, menadione sodium bisulfite (MSB) (Figure 3.2), which has been used commercially but which is also somewhat unstable in mixed feeds. In the presence of excess



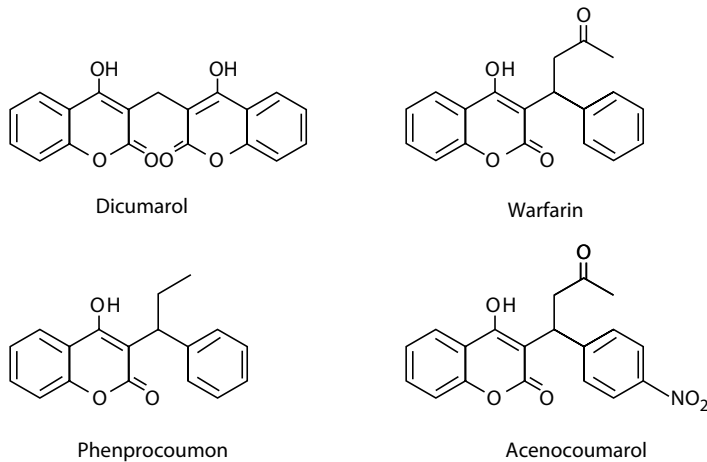
**FIGURE 3.2** Forms of vitamin K utilized in animal feeds.

sodium bisulfite, MSB crystallizes as a complex with an additional mole of sodium bisulfite; this complex, known as menadiol sodium bisulfite complex (MSBC), has increased stability, and it is widely used in the poultry industry. A third water-soluble compound is a salt formed by the addition of dimethylpyridinol to MSB; it is called menadiol pyridinol bisulfite [17].

The clinical use of vitamin K is largely limited to various preparations of phylloquinone. A water-soluble form of menadiol, menadiol sodium diphosphate, which was sold as Kappadione or Synkayvite, was once used to prevent hemorrhagic disease of the newborn, but the danger of hyperbilirubinemia associated with menadiol usage (see Section 3.9) has led to the use of phylloquinone as the desired form of the vitamin. Phylloquinone (USP phytonadione) is sold as AquaMEPHYTON, Konakion, Mephyton, and Mono-Kay. These preparations are detergent-stabilized preparations of phylloquinone and are used as intramuscular injections to prevent hemorrhagic disease of the newborn. In some countries, oral prophylaxis of vitamin K has been promoted, and these preparations are not well absorbed. A lecithin and bile salt “mixed micelle” preparation, Konakion MM, is now available and has been shown [18] to be effective when administered orally. Although not currently used in the United States or Western Europe, pharmacological doses of MK-4, menatetrenone, are utilized as a treatment for osteoporosis in Japan and other Asian countries (see Section 3.7.4).

### 3.2.3.3 Antagonists of Vitamin Action

The history of the discovery of the first antagonists of vitamin K, the coumarin derivatives, has been well reviewed by Link [19]. A hemorrhagic disease of cattle, traced to the consumption of improperly cured sweet clover hay, was described in Canada and the US Midwest in the 1920s. The compound present in spoiled sweet clover that was responsible for this disease had been studied by a number of investigators but was finally isolated and characterized as 3',3'-methylbis-(4-hydroxycoumarin) by Link's group from 1933 to 1941 and was called dicumarol (Figure 3.3). Dicumarol was successfully used for anticoagulant therapy in some early studies, and numerous substituted 4-hydroxycoumarins were synthesized both in Link's laboratory and elsewhere. The most successful of these, both clinically for long-term lowering of the vitamin K-dependent clotting factors and subsequently as a rodenticide, has been warfarin, 3-( $\alpha$ -acetonylbenzyl)-4-hydroxycoumarin. Although warfarin is the most extensively used drug worldwide for oral anticoagulant therapy, other coumarin derivatives such as its 4'-nitro analog, acenocoumarol, and phenprocoumon have been used. These drugs differ in the degree to which they are absorbed from the intestine, in their plasma half-life, and presumably in their effectiveness as a vitamin K antagonist at the active site. The clinical use of these compounds and many of their pharmacodynamic interactions have been reviewed by O'Reilly [20]. Although warfarin, as a vitamin K antagonist, has been the most widely used anticoagulant for 60 years, it does require routine coagulation monitoring and is, to some

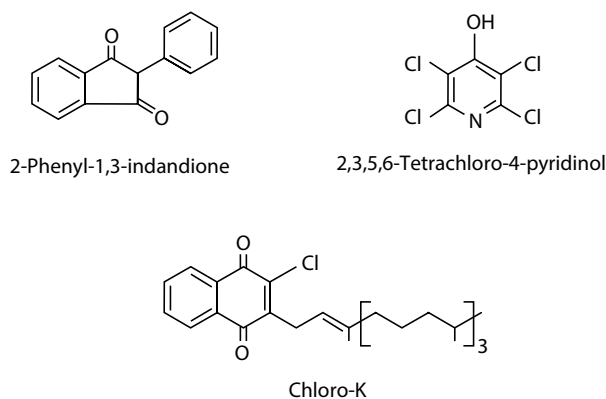


**FIGURE 3.3** Oral anticoagulants that antagonize vitamin K action.

extent, affected by diet. More recently, drugs that are direct inhibitors of the procoagulants, factor Xa and factor IIa, have become available as anticoagulants [21,22]. These drugs do not require the extent of coagulation monitoring that warfarin does, but the potential for excessive bleeding is still under consideration [23].

Warfarin has been widely used as a rodenticide, and its continual use has led to the development of anticoagulant-resistant populations [24]. More hydrophobic derivatives of 4-hydroxycoumarins are cleared from the body much more slowly and are effective rodenticides in warfarin-resistant rat strains. Compounds such as difenacoum and brodifacoum are now widely used for rodent control [25] but should be used with care as consumption of carcasses by birds or cats can lead to death.

Other compounds having anticoagulant activity that can be reversed by vitamin K administration are the 2-substituted 1,3-indandiones such as 2-phenyl-1,3-indandione (Figure 3.4). These compounds appear [26] to act by the same mechanism as the 4-hydroxycoumarins, and although they were administered as clinical anticoagulants and rodenticides at one time, they are currently seldom used. Some structural analogs of the vitamin have also been shown to antagonize its action. Early studies of the structural requirements for vitamin K activity [27] demonstrated that replacement of the 2-methyl group of phyloquinones by a chlorine atom to form 2-chloro-3-phytyl-1,4-naphthoquinone resulted in a potent antagonist of vitamin K. Chloro-K acts like a true competitive inhibitor of the vitamin at its active site, and it is an effective anticoagulant in coumarin anticoagulant-resistant rats [28]. Another structurally unrelated compound, 2,3,5,6-tetrachloro-4-pyridinol, has



**FIGURE 3.4** Other vitamin K antagonists.

anticoagulant activity [29], and on the basis of its action in warfarin-resistant rats, it would appear that it is functioning as a direct antagonist of the vitamin. Subsequent studies have demonstrated [30] that other polychlorinated phenols are also effective vitamin K antagonists.

### 3.2.4 PHYSICAL AND CHEMICAL PROPERTIES

Compounds with vitamin K activity are substituted 1,4-naphthoquinones and, therefore, have the general chemical properties expected of all quinones. The chemistry of quinoids and much of the data on the special and other physical characteristics of phyloquinone and the menaquinones are readily available [31–33]. The oxidized form of the K vitamins exhibits an ultraviolet (UV) spectrum that is characteristic of the naphthoquinone nucleus, with four distinct peaks between 240 and 280 nm and a less sharp absorption at around 320–330 nm. The molar extinction value  $\epsilon$  for both phyloquinone and the various menaquinones is approximately 19,000. The absorption spectrum changes drastically upon reduction to the hydroquinone, with an enhancement of the 245 nm peak and disappearance of the 270 nm peak. Vitamin K active compounds also exhibit characteristic infrared and nuclear magnetic resonance (NMR) absorption spectra that are largely those of the naphthoquinone ring. NMR analysis of phyloquinone has been used to firmly establish that natural phyloquinone is the *trans* isomer and can be used to establish the *cis*–*trans* ratio in synthetic mixtures of the vitamin. More recently, mass spectroscopy has been useful in determining the length of the side chain and the degree of saturation of vitamins of the menaquinone series isolated from natural sources. Phyloquinone is an oil at room temperature; the various menaquinones can easily be crystallized from organic solvents and have melting points from 35°C to 60°C, depending on the length of the isoprenoid chain.

### 3.3 ANALYTICAL PROCEDURES AND VITAMIN K CONTENT OF FOOD

Chemical reactivity of vitamin K is a function of the naphthoquinone nucleus, and as other quinones also react with many of the colorimetric assays that have been developed [32,33], they are of little analytical value. The number of interfering substances present in crude extracts is also such that a significant amount of separation is required before UV absorption spectra can be used to quantitate the vitamin. These simple methods are therefore not practical in the determination of the small amount of vitamin present in natural sources, and they have been superseded by high-performance liquid chromatography (HPLC) techniques. Analytical methods suitable for the small amounts of vitamin K present in tissues, serum, and most food sources are now available. All separations involving concentrated extracts of vitamin K should be carried out in subdued light to minimize UV decomposition of the vitamin. Compounds with vitamin K activity are also sensitive to alkali, but they are relatively stable to an oxidizing atmosphere and to heat and can be vacuum distilled with little decomposition. Interest in the quantitation of vitamin K in serum and animal tissues eventually led to the use of HPLC as an analytical tool to investigate vitamin K metabolism [34,35].

Satisfactory tables of the vitamin K content of various commonly consumed foods were not made available until the early 1990s. Many of the values previously quoted in various publications have apparently been recalculated in an unspecified way from data obtained by a chick bioassay that was not intended to be more than qualitative and should not be used to calculate intake. Tables of food vitamin K content in various older texts and reviews may also contain data from this source, as well as considerable amounts of unpublished data.

Current methodology utilizes HPLC analysis of lipid extracts and has been reported [12] to have a within-sample coefficient of variation for different foods in the range of 7%–14% and a between-sample coefficient of variation of 9%–45%. Although green leafy vegetables have been known for some time to be the major source of vitamin K in the diet, it is now apparent that cooking oils, particularly soybean oil and rapeseed oil [35], are major contributors. Human milk contains approximately 1 ng/ml of phyloquinone [36], which is only 20%–30% of that found in cow's milk.

Infant formulas are currently supplemented with vitamin K, providing a much higher intake than that provided by breast milk.

The data in Table 3.1 are taken from a survey of literature [37] that considered most of the reported HPLC-derived values for various food items and from analyses of the US Food and Drug Administration total diet study. An extensive updated US Department of Agriculture database containing the vitamin K content of a large number of foods can be accessed at <http://www.nal.usda.gov/fnic/foodcomp>. In general, green and leafy vegetables are the best sources of the vitamin, with cooking oils being the next major sources. In addition to the reports of the vitamin K content of common foods, databases of fast foods [38], mixed dishes [39], and baby food products [12] are available. The major source of vitamin K in foods, and the source usually reported, is phyloquinone. Along with the data from the United States, there are databases published from a number of other countries [40] that indicate that the phyloquinone intake of adults in Japan, China, and the Netherlands is substantially higher than that in the United States. Significant amounts of MK-4 are found in poultry meat and egg yolk as poultry rations are commonly supplemented with menadione, and some cheeses can have appreciable amounts of long-chain menaquinones [41] owing to the bacterial action during aging.

Utilizing the available food composition data and food consumption data, it is possible to calculate average daily intakes of phyloquinone. Based on the Third National Health and Nutrition Examination Survey (NHANES III) data [42], the adult US male and female intakes were approximately 115 and 100  $\mu\text{g/day}$ . This is somewhat higher than previous estimates [43]. Mean phyloquinone intakes in Ireland for adult men and women have been reported to be 84 and 74  $\mu\text{g/day}$  [44]; in Scotland, 72 and 64  $\mu\text{g/day}$  [45]; in Britain, 70 and 61  $\mu\text{g/day}$  [46]; and in the Netherlands, 257

**TABLE 3.1**  
**Vitamin K Content of Ordinary Foods**

$\mu\text{g}$ Phyloquinone/100 g of Edible Portion					
Vegetables		Nuts, Oils, Seeds		Fruits	
Kale	817	Soybean oil	193	Avocado	40
Parsley	540	Rapeseed oil	141	Grapes	3
Spinach	400	Olive oil	49	Cantaloupe	1
Endive	231	Walnut oil	15	Bananas	0.5
Green onions	207	Safflower oil	11	Apples	0.1
Broccoli	205	Sunflower oil	9	Oranges	0.1
Brussels sprouts	177	Corn oil	3		
Cabbage	147	Dry soybeans	47	<b>Meats and Dairy</b>	
Lettuce	122	Dry kidney beans	19	Ground beef	0.5
Green beans	47	Sesame seeds	8	Chicken	0.1
Peas	36	Dry navy beans	2	Pork	<0.1
Cucumbers	19	Raw peanuts	0.2	Turkey	<0.1
Tomatoes	6			Tuna	<0.1
Carrots	5	<b>Grains</b>		Butter	7
Cauliflower	5	Bread	3	Cheddar cheese	3
Beets	3	Oatmeal	3	3.5% Milk	0.3
Onions	2	White rice	1	Yogurt	0.3
Potatoes	0.8	Wheat flour	0.6	Skim milk	<0.1
Sweet corn	0.5	Dry spaghetti	0.2	Mayonnaise	81
Mushrooms	<0.1	Shredded wheat	0.7	Egg yolk	2
		Corn flakes	<0.1	Egg white	<0.1

*Note:* Values are taken from a provisional table [37] and are median values from a compilation of reported assays.



and 244 µg/day [47]. The high consumption of cheese in the Netherlands also provides an intake of approximately 20 µg/day of long-chain menaquinones. As different databases are used, variations in the assumed vitamin K content of those few foods that contribute the most vitamins to the diet can result in large differences. In a study where four metabolic ward diets were directly analyzed to contain approximately 100 µg/day, the amount calculated by two different databases ranged from 84 to 160 µg/day [48]. Use of the current database information has, however, made it possible to formulate nutritionally adequate diets that contain only 10 µg/day of phylloquinone [49].

The conversion of liquid oils to solid margarines by commercial hydrogenation results in the formation of substantial amounts of 2',3'-dihydrophyloquinone, which, in the case of some of the harder margarines, can exceed the amount of unmodified phylloquinone [50,51]. Because of the large contribution of high-phyloquinone vegetable oils to many diets, the amount of the hydrogenated form represents around 20% of the total vitamin K in American diets [52]. Although this form of the vitamin has some biological activity, the degree of this response has not been well established in either human subjects or experimental animals.

### 3.4 METABOLISM

#### 3.4.1 ABSORPTION AND TRANSPORT OF VITAMIN K

The absorption of nonpolar lipids, such as vitamin K, into the lymphatic system requires incorporation into mixed micelles. Early studies demonstrated that these phylloquinone-containing micellar structures required the presence of both bile and pancreatic juice and that the absorption of radiolabeled phylloquinone was energy dependent and saturable. Normal human subjects were found [53] to excrete less than 20% of a large (1 mg) dose of phylloquinone in the feces, but as much as 70%–80% of the ingested phylloquinone was excreted unaltered in the feces of patients with impaired fat absorption caused by obstructive jaundice, pancreatic insufficiency, or adult celiac disease.

Early studies of the bioavailability of phylloquinone from different food sources have been somewhat variable. Human subject studies of phylloquinone absorption from green vegetables were found to be absorbed by 10%–20% when the diets contained substantial fat and much less in fat-free diets [54–56]. Some cheeses and a fermented soybean product, natto, consumed mainly in the Japanese market, do contain substantial amounts of long-chain menaquinones, and there are indications [41] that these forms may be more bioavailable than phylloquinone from vegetable sources. More recently, stable isotope-labeled phylloquinone has been used to assess the bioavailable absorption of vitamin K from foods [57–59]. Using this methodology, it has been found that the type of meal has a substantial impact on bioavailability [60] and that when this methodology was used to assess the absorption of vitamin K from kale, the amount of vitamin absorbed was approximately 5% with a large difference between the individuals studied [61].

Substantial amounts of vitamin K are present in the human gut in the form of long-chain menaquinones. Relatively few of the bacteria that comprise the normal intestinal flora are major producers of menaquinones, but obligate anaerobes of the *Bacteroides fragilis*, *Eubacterium*, *Propionibacterium*, and *Arachnia* groups are, as are facultatively anaerobic organisms such as *Escherichia coli*. The amount of vitamin K in the gut can be quite large, and the amounts found in total intestinal tract contents from five colonoscopy patients have been reported [62] to range from 0.3 to 5.1 mg with MK-9 and MK-10 being the major contributors. The total amount of long-chain menaquinones, mainly MK-6, MK-7, MK-10, and MK-11, present in human liver also greatly exceeds the phylloquinone concentration, which represents only approximately 10% of the total liver vitamin K [63]. There is some evidence [64] that the hepatic turnover of long-chain menaquinones is slower than that of phylloquinone, which would account for the increased concentration observed, but a major question that remains is how these very lipophilic compounds that are present as constituents of bacterial membranes are absorbed from the lower bowel. The oral administration of 1 mg mixed long-chain menaquinones to anticoagulated human subjects [65] effectively decreased the extent of

the acquired hypoprothrombinemia, demonstrating that the human digestive tract can absorb these forms of the vitamin from the small intestine but does not address their absorption from the large bowel. However, a small but nutritionally significant portion of the intestinal content of the vitamin is located not in the large bowel but in a region where bile acid-mediated absorption could occur [62]. The bioavailability of menaquinones present in food sources has not been carefully studied. It has been shown [66] that when equivalent oral doses of phylloquinone, MK-4, and MK-9 were given to healthy males, the peak concentration of the serum menaquinones was less than 20% of phylloquinone. The overall bioavailability of the menaquinones cannot be determined from these data, but it is likely to be less than that of phylloquinone. Menadione is widely used in poultry, swine, and laboratory animal diets as a source of vitamin K. It can be absorbed from both the small intestine and the colon by a passive process [67]. Menadione itself does not have biological activity, but after absorption, it can be alkylated to MK-4, a biologically active form of the vitamin.

Absorption of phylloquinone from the intestine is via the lymphatic system and has been recently reviewed in detail [59]. Phylloquinone in plasma is predominantly carried by the triglyceride-rich lipoprotein fraction containing very low density lipoproteins and chylomicrons, although significant amounts are located in the low-density lipoprotein fraction [68,69]. In a study [66] comparing the transport of different forms of vitamin K, significant amounts of MK-4 were found in the high-density lipoprotein fraction, and the half-life of MK-9 was found to be substantially greater than that of either phylloquinone or MK-4. As expected from lipoprotein transport, plasma phylloquinone concentrations are strongly correlated with plasma lipid levels [70]. The major route of entry of phylloquinone into tissues appears to be via clearance of chylomicron remnants by apolipoprotein E (apoE) receptors. The polymorphism of apoE has been found to influence the fasting plasma phylloquinone concentrations in patients undergoing hemodialysis therapy [70], and plasma phylloquinone concentrations have been shown to decrease according to apoE genotype: apoE2 > apoE3 > apoE4. This response is correlated to the hepatic clearance of chylomicron remnants from the circulation with apoE2 having the slowest rate of removal. Details of the secretion of phylloquinone from liver and the movement of the vitamin between organs are not available. The total human body pool of phylloquinone is very small, and early studies [53] using pharmacological doses of radioactive phylloquinone indicated that the turnover is rapid. There are only limited available data assessing the disappearance of small amounts (<1 µg) of infused <sup>3</sup>H-phylloquinone from human subjects, and these [71] are consistent with a body pool turnover of approximately 1.5 days and a body pool size of approximately 100 µg. Other data, based on liver biopsies of patients fed diets very low in vitamin K prior to surgery [72], indicated that approximately two-thirds of hepatic phylloquinone was lost in 3 days. These findings are also consistent with a small pool size of phylloquinone that turns over very rapidly.

### 3.4.2 PLASMA CONCENTRATIONS OF VITAMIN K

Early measurements of vitamin K by HPLC utilized UV detectors that lacked sensitivity; electrochemical detection or fluorescence detection of the vitamin after chemical or electrochemical reduction has replaced this methodology [73–75]. The most commonly used methodology at the present time involves fluorescence detection after zinc postcolumn reduction, and deuterium-labeled phylloquinone has been measured at lower concentrations through the use of HPLC/mass spectrometry [58,76,77].

Although earlier reports of plasma phylloquinone concentrations were somewhat higher, it now appears that normal fasting values are around 0.5 ng/ml (1.1 nmol/L). There is a strong positive correlation between plasma triglycerides and plasma phylloquinone [78], and the variation between samples measured at different days from the same subject is much higher than that for the other fat-soluble vitamins. Because of this, extreme caution should be used in attempts to determine the vitamin K status of an individual from a single day's sample of plasma. Circulating phylloquinone concentrations do respond to daily changes in intake and fall rather rapidly when intake is restricted

[79,80]. Within the United States, the vitamin K status does differ according to race and ethnicity [81]. Although there are very few foods containing appreciable amounts of long-chain menaquinones, they are detectable in plasma and, in some cases, have been reported to present at substantial levels [82,83]. One menaquinone, MK-7, is present in high concentrations in natto, a traditional food in eastern Japan. It is produced by growing *Bacillus natto* on cooked soybeans and is essentially eaten only in that region where it can provide more vitamin K than phyloquinone. When menadione is used in poultry rations, significant amounts of MK-4 are found in egg yolks and chicken meat, and a range of longer-chain menaquinones are found in both soft and hard cheeses. The predominant human source of vitamin K is, however, phyloquinone from green vegetables and oils [11].

### 3.4.3 TISSUE DISTRIBUTION OF VITAMIN K

The distribution of vitamin K in various body organs of the rat was first studied with radioactive forms of the vitamin, utilizing both excessive and more physiological amounts of phyloquinone. The liver was found to retain the majority of the vitamin at early time points, but as the half-life in the liver appears to be in the range of 10–15 h [84], it is rapidly lost. Studies utilizing radioactive phyloquinone [85,86] indicated that more than 50% of the liver radioactivity was recovered in the microsomal fraction, with substantial amounts found in the mitochondria and cellular debris fractions. A more detailed study [87] found the highest specific activity of radioactive phyloquinone to be in the Golgi and smooth microsomal membrane fractions. Only limited data on the distribution of menaquinones are available, and MK-9 has been reported [88] to be preferentially localized in a mitochondrial rather than a microsomal subcellular fraction.

Because of the small amounts of vitamin K in animal tissues, it is difficult to determine which of the vitamers are present in tissue from different species. Only limited data are available, and these data indicate that phyloquinone is found in the liver of those species ingesting plant material and that, in addition to this, menaquinones containing 6–13 prenyl units in the alkyl chain are found in the liver of most species. More recently, analysis of a limited number of human liver specimens has shown that phyloquinone represents only approximately 10% of the total vitamin K pool and that a broad mixture (Table 3.2) of menaquinones is present [72]. The predominant forms appear to be MK-7, MK-8, MK-10, and MK-11. Although the long-chain menaquinones are potential sources

**TABLE 3.2**  
**Vitamin K Content of Human Liver**

Vitamin	pmol/g Liver <sup>a</sup>		
	Study A	Study B	Study C
Phylloquinone	22 ± 5	18 ± 4	28 ± 4
MK-5	12 ± 18	NR	NR
MK-6	12 ± 13	NR	NR
MK-7	57 ± 59	122 ± 61	34 ± 12
MK-8	95 ± 157	11 ± 2	9 ± 2
MK-9	2 ± 4	4 ± 2	2 ± 1
MK-10	67 ± 71	96 ± 16	75 ± 10
MK-11	90 ± 15	94 ± 36	99 ± 15
MK-12	15 ± 13	21 ± 6	14 ± 2
MK-13	5 ± 6	8 ± 3	5 ± 1

<sup>a</sup> Values are means ± SEM for 6 or 7 subjects in each study: study A [235], study B [236], and study C [72]. Values from studies A and B have been recalculated from data presented as ng/g liver. NR, not reported.

of vitamin K activity in liver, the extent to which they are utilized is not known. The utilization of MK-9 as a substrate for the vitamin K-dependent carboxylase has been shown [88] to be only approximately 20% as extensive as phylloquinone when the two compounds are present in the liver in equal concentrations. Recent data have suggested that MK-4 may play a role in satisfying unique, not-yet-defined vitamin K requirements of some tissues. Most analyses of liver from various species have not detected significant amounts of MK-4, but some nonhepatic tissues of the rat, particularly pancreas and salivary gland, have also been shown [89] to contain much more MK-4 than phylloquinone.

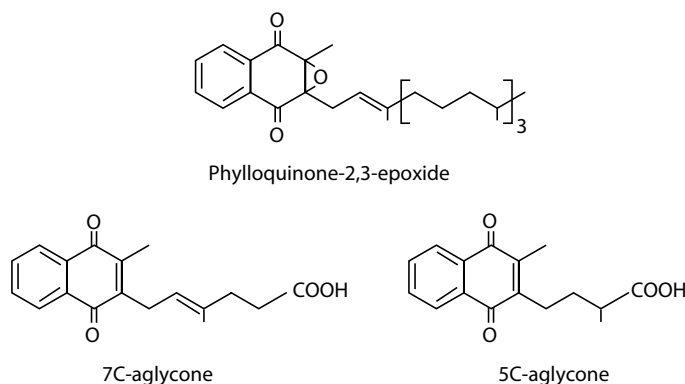
#### 3.4.4 SYNTHESIS OF MK-4

Long-chain menaquinones are synthesized by bacteria via pathways that have been well established [14]. However, MK-4 is not a major product of bacterial menaquinone biosynthesis, and it is formed by an alternate pathway. Menadione can be converted to MK-4 after oral administration or by *in vitro* incubation of liver homogenates with geranylgeranyl pyrophosphate [90]. Although MK-4 can be synthesized by direct prenylation of menadione, the majority of this menaquinone is formed from phylloquinone and other long-chain menaquinones after removal of the side chain. It was thought at one time that intestinal bacterial action might be required to remove the side chain that would allow prenylation of the resulting menadione. This has been found not to be the pathway used [91,92]. The phylloquinone-to-MK-4 conversion is very extensive in rat tissues such as brain, pancreas, and salivary gland, and the MK-4 concentrations in those tissues exceed that of phylloquinone. Similar distributions of MK-4 have been observed in human tissues [93], and it has been established that high tissue concentrations of MK-4 are more readily obtained in rats by phylloquinone supplementation than by administering MK-4 [94]. A more recent study has demonstrated that a human prenyltransferase (UBIAD1) is the enzyme that catalyzes the prenylation of menadione to MK-4 [95], and it has been suggested the initial side-chain cleavage of phylloquinone or other menaquinones is also a function of the same enzyme. Some recent data [96] suggest that UBIAD1 catalyzes only the prenylation step and a second enzyme may be involved in removing the initial side chain. However, additional studies will be needed to clarify this aspect of MK-4 synthesis.

#### 3.4.5 METABOLIC DEGRADATION OF VITAMIN K

Early studies of phylloquinone metabolism [97] demonstrated that the major route of excretion was in the feces and that very little unmetabolized phylloquinone was present. Subsequent studies [53] of the metabolism of radioactive phylloquinone in humans indicated that approximately 20% of an injected dose of either 1 or 45 mg of vitamin K was excreted in the urine in 3 days and that 40%–50% was excreted in the feces via the bile. In both rat and human studies, the metabolites of phylloquinone comprise the glucuronide conjugates of two carboxylic aglycones (Figure 3.5). One aglycone has a 7-C side chain with a 1',2' double bond, and the other has a 5-C side chain [59,98]. It is likely that the initial  $\omega$ -oxidation step to initiate the degradation of the side chain is carried out by cytochrome P450 in the same manner as seen in tocopherol degradation [59]. In a controlled study utilizing young adults consuming a control diet, a phylloquinone-restricted diet, and a repletion diet, it was found that approximately 75% of total urinary excretion was in the form of the 5-C-aglycone [99]. The amount of excretion was also related to changes in phylloquinone intake.

The most abundant metabolite of phylloquinone is its 2,3-epoxide (Figure 3.5) formed as a product of the action of the vitamin K-dependent  $\gamma$ -glutamyl carboxylation (see Section 3.6.3). This metabolite was discovered by Matschiner [100] who was investigating an observation [85] that warfarin treatment caused a buildup of radioactive vitamin K in the liver. Further studies of this compound revealed that approximately 10% of the vitamin K in the liver of a normal rat is present as the epoxide and that this amount can become the predominant form of the vitamin after treatment with coumarin anticoagulants. The distribution of the various urinary metabolites of



**FIGURE 3.5** Phylloquinone metabolites. The 7C-aglycone is 2-methyl-3-(5'-carboxy-3'-methyl-2'-pentenyl)-1,4-naphthoquinone and the 5C-aglycone is 2-methyl-3-(3'-carboxy-3'-methylpropyl)-1,4-naphthoquinone.

phylloquinone is also substantially altered by warfarin administration. The 7-carbon and 5-carbon side chain major urinary glucuronides (Figure 3.5) are decreased [101], and other uncharacterized metabolites, presumably arising from the epoxide, are increased. The major degradation products of vitamin K metabolism appear to have been identified and then are apparently formed either from phylloquinone or from menaquinones, but there may be a number of urinary and biliary products not yet characterized. Methodology that is useful for the routine analysis of the two major urinary aglycones of vitamin K has been developed [102], and it has been suggested that quantitation of these metabolites might be a useful noninvasive marker of vitamin K status.

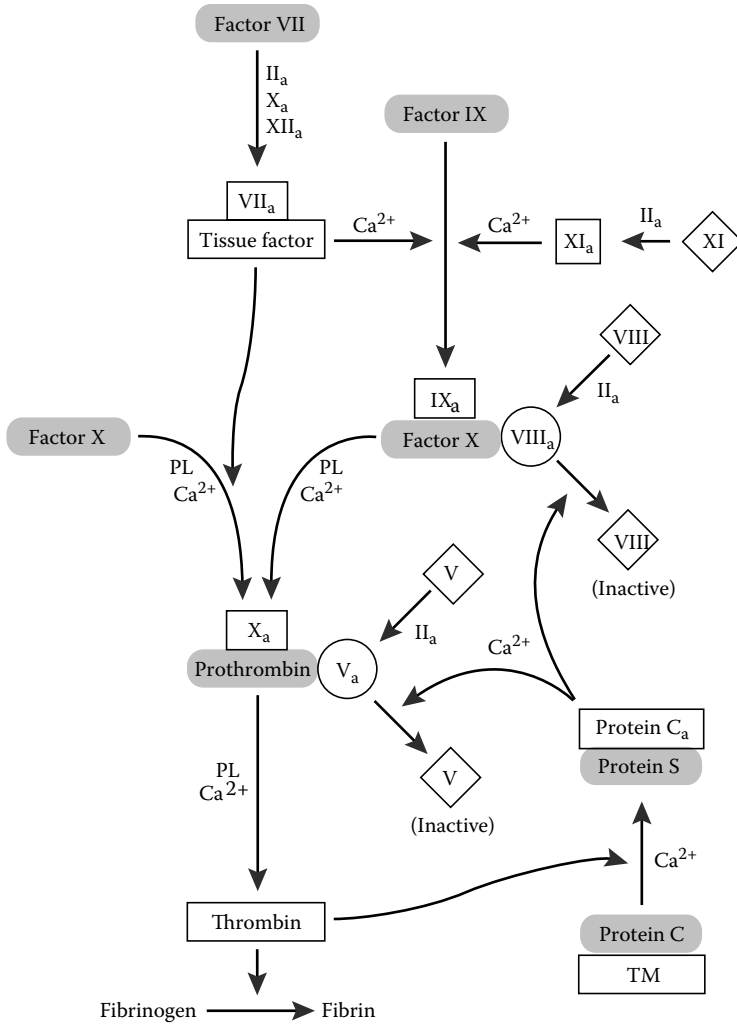
### 3.5 VITAMIN K-DEPENDENT PROTEINS

#### 3.5.1 PLASMA CLOTTING FACTORS

Soon after Dam's discovery of a hemorrhagic condition in chicks that could be cured by green plant extracts, it was demonstrated that the plasma of these chicks contained a decreased concentration of prothrombin. This protein (also called clotting factor II) was the first plasma protein clotting factor to be discovered. It is the most abundant of these proteins, and in the future, it was also the first protein demonstrated to contain  $\gamma$ -carboxyglutamic acid (Gla) residues. Plasma clotting factors VII, IX, and X were all initially identified because their activity was decreased in the plasma of a patient with a hereditary bleeding disorder [103] and were subsequently shown to depend on vitamin K for their synthesis. Until the mid-1970s, these four "vitamin K-dependent clotting factors" were the only proteins known to require this vitamin for their synthesis.

The process of blood coagulation is essential for hemostasis and, along with platelet activation, involves a complex series of events (Figure 3.6) that lead to the generation of thrombin by proteolytic activation of protease zymogens. The vitamin K-dependent clotting factors are involved in these activation and propagation events through membrane-associated complexes with each other and with accessory proteins. These proteins are characterized by an amino-terminal domain that contains a number of glutamic acid residues that have been posttranslationally converted to  $\gamma$ -carboxyglutamyl residues (see Section 3.6). The "Gla domain" of the four vitamin K-dependent procoagulants is very homologous, and the 10–13 Gla residues in each are in essentially the same position as in prothrombin.

After the discovery of Gla residues in vitamin K-dependent proteins, three more Gla-containing plasma proteins with similar homology were discovered. Protein C [104] and protein S [105] are involved in a thrombin-initiated inactivation of factor  $V_a$ , a clotting factor that is not vitamin K dependent and, therefore, plays an anticoagulant rather than a procoagulant role in normal hemostasis



**FIGURE 3.6** Vitamin K–dependent clotting factors. The vitamin K–dependent procoagulants (gray ovals) are zymogens of the serine proteases; prothrombin, F-VII, F-IX, and F-X. Coagulation is initiated when they are converted to their active (subscript a) forms. This process can be initiated by an “extrinsic” pathway when vascular injury exposes tissue factor to blood. The product of the activation of one factor can activate a second zymogen, and this cascade effect results in the rapid activation of prothrombin to thrombin and the subsequent conversion of soluble fibrinogen to the insoluble fibrin clot. A number of steps in this series of activations involve an active protease, a second vitamin K–dependent protein substrate, and an additional plasma protein cofactor (circles) to form a  $\text{Ca}^{2+}$ -mediated association with a phospholipid surface. The formation of activated F-X can also occur through an “intrinsic” pathway involving thrombin activation of F-XI and subsequently F-IX. Two other vitamin K–dependent proteins participate in hemostatic control as anticoagulants, not procoagulants. Protein C is activated by thrombin ( $\text{II}_a$ ) in the presence of an endothelial cell protein called thrombomodulin (TM). Protein C is then able to function in a complex with protein S to inactivate  $\text{V}_a$  and  $\text{VIII}_a$  and to limit clot formation.

[106]. In addition to the approximately 40-residue Gla domain, the vitamin K–dependent proteins have other common features. The Gla domain of prothrombin is followed by two “kringle” domains that are also found in plasminogen and a serine protease domain. Factors VII, IX, and X and protein C contain two “epidermal growth factor” domains and a serine protease domain, while protein S contains four epidermal growth factor domains but is not a serine protease. The function of protein

Z, the seventh Gla-containing plasma protein, which is also not a protease zymogen, was not known for some time, but has now been shown [107] to have an anticoagulant function under some conditions. As these proteins play a critical role in hemostasis, they have been extensively studied, the cDNA and genomic organization of each of them is well documented, and a large number of genetic variants of these proteins have been identified as risk factors in coagulation disorders [108].

### 3.5.2 CALCIFIED TISSUE PROTEINS

The first vitamin K-dependent protein discovered that was not located in plasma was isolated from bone [109,110]. This 49-residue protein contained three Gla residues, was called osteocalcin or bone Gla protein, and had little structural homology to the vitamin K-dependent plasma proteins. Although it is the second most abundant protein in bone, its function has been very difficult to define. Synthesis of the biologically active “Gla form” of osteocalcin can be blocked when rats are fed a diet containing the anticoagulant warfarin and also given large amounts of vitamin K to maintain plasma vitamin K-dependent protein production. Utilizing this protocol, no defects in bone were seen when bone osteocalcin was decreased to approximately 2% of normal after 2 months, and fusion of the proximal tibia growth plate was observed after 8 months [111,112]. These observations indicate that osteocalcin is involved in some manner in the control of tissue mineralization or skeletal turnover. However, osteocalcin gene “knockout” mice have been shown [113] to produce more dense bone rather than a defect in bone formation. Some of the osteocalcin produced in bone does appear in plasma at concentrations that are high in young children and approach adult levels at puberty.

The three Gla residues in the osteocalcin structure are critical for the role of this protein in the maturation of human bone mineral [114]. In most species, circulating osteocalcin is fully carboxylated, but in healthy humans, there are substantial amounts of undercarboxylated osteocalcin (ucOC) that respond to vitamin K depletion, repletion, and supplementation [115,116]. There have been a large number of randomized controlled clinical studies [117–120] that have tested the hypothesis that supplementation with vitamin K will slow the progression of bone loss in older adults and also some meta-analyses based on these and similar studies [121,122]. Although these efforts have had various results, they do not seem to be sufficient to promote supplemental vitamin K for the prevention of bone loss in the elderly. There are also some indications that osteocalcin may have a hormonal function. Initial studies of the osteocalcin knockout mice found elevated glucose and lipid concentrations and reduced insulin levels and glucose tolerance [123]. An unexpected finding was that the hormonal form of osteocalcin was the uncarboxylated form [124]. Human studies of the impact of osteocalcin on insulin resistance and glucose homeostasis [125,126] have resulted in variable responses, and an understanding of the hormonal impact of this vitamin K-dependent protein is not yet available.

A second low-molecular-weight (79-residue) protein with five Gla residues was also first isolated from bone [127] and is called the matrix Gla protein (MGP). This protein is structurally related to osteocalcin but is also present in other tissues and has been shown to be synthesized in cartilage and many other soft tissues. MGP has been difficult to study because of its hydrophobic nature, relative insolubility, and tendency to aggregate. The details of this physiological role are unclear, but it has been shown that MGP knockout mice die from spontaneous calcification of arteries and cartilage [128], and arterial calcification has been demonstrated in a warfarin-treated rat model [129]. More recent findings suggest that the major roles of MGP are its involvement in the regulation of soft tissue calcification and the prevention of arterial calcification [130,131]. MGP is not fully carboxylated and intervention studies that utilized phylloquinone supplementation have shown minor responses in the decrease of vascular calcification [132,133]. As the vitamin K status of individuals with chronic kidney disease is low, much of the current effort to define the role of MGP is directed to this problem.

A more recently discovered [134] vitamin K–dependent protein, the Gla-rich protein (GRP) is a small protein with 15–16 Gla residues that makes it the most densely  $\gamma$ -carboxylated protein identified to date. The protein was first found in sturgeons but has also been found in mice and humans [135]. The function of GRP has not yet been clearly defined, and no obvious phenotype alterations have been reported in null mice [136]. It may function as a modulator of calcium availability, cartilage stabilization, or skeletal development but definitive functions have not yet been identified.

### 3.5.3 OTHER VITAMIN K–DEPENDENT PROTEINS

A relatively small number of other mammalian proteins have now been shown to contain Gla residues [11] and are therefore dependent on vitamin K for their synthesis. The most extensively studied is Gas 6, a ligand for the tyrosine kinase Axl [137], which appears to be a growth factor for mesangial and epithelial cells. The physiological function of the protein is not clearly defined, but there are indications of its role in immunity, vasculature integrity, atherosclerosis, thrombosis, and cancer [138]. Two proline-rich Gla proteins (PRGP-1 and PRGP-2) were discovered [139] as integral membrane proteins with an extracellular amino-terminal domain that is rich in Gla residues. Subsequently, two other members of this transmembrane Gla protein family (TMG-3 and TMG-4) have been cloned [140]. The specifics of the role of these cell-surface receptors are not yet known. Another protein, periostin, had been a previously known protein before it was found to be a Gla-containing protein [141], and it appears to be extracellular matrix associated. Its major metabolic role is not known. The vitamin K–dependent carboxylase is a substrate for itself [142], and it is likely that, in time, other proteins will be shown to contain Gla residues.

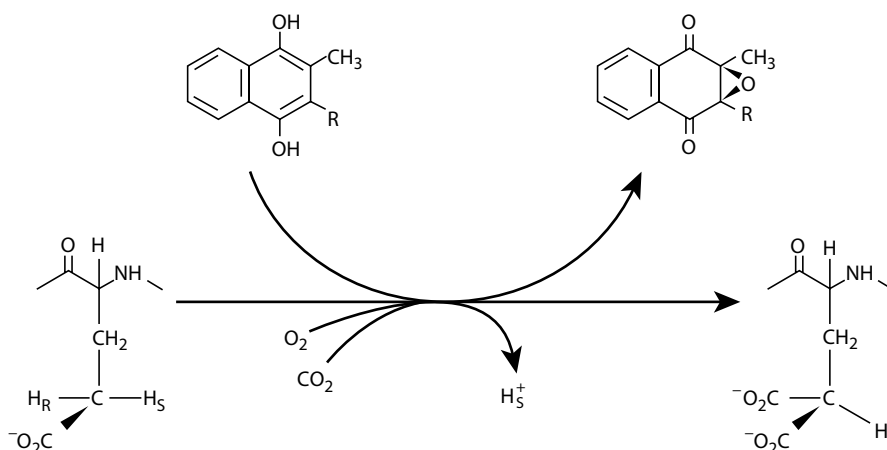
Vitamin K–dependent proteins are not confined to vertebrates. A large number of the toxic venom peptides secreted by marine *Conus* snails are rich in Gla residues [143], and vitamin K–dependent proteins have also been found in snake venoms [144,145]. The carboxylase has been cloned from a number of vertebrates, the *Conus* snail, a tunicate, zebrafish, and *Drosophila* [146–148], and has been identified in the genome of bacteria and archaea [149]. The strong sequence homology of the enzyme from these phylogenetic systems suggests that this posttranslational modification of glutamic acid is of ancient evolutionary origin and that numerous vitamin K–dependent proteins are yet to be discovered within the wide range of organisms capable of synthesizing this modified amino acid.

## 3.6 BIOCHEMICAL ROLE OF VITAMIN K

### 3.6.1 DISCOVERY OF $\gamma$ -CARBOXYGLUTAMIC ACID

A period of approximately 40 years elapsed between the discovery of vitamin K and the determination of its metabolic role. Beginning in the early 1960s, studies of prothrombin production in humans and experimental animals eventually led to an understanding of the metabolic role of vitamin K. Early theories that vitamin K controlled the production of specific proteins at a transcriptional level could not be proven, and alternate hypotheses were considered. Involvement of an intracellular precursor in the biosynthesis of prothrombin was first clearly stated by Hemker et al. [150] who postulated that an abnormal clotting time in anticoagulant-treated patients was due to a circulating inactive form of plasma prothrombin. It was subsequently demonstrated [151] that the plasma of patients treated with coumarin anticoagulants contained a protein that was antigenically similar to prothrombin but lacked biological activity. A circulating inactive form of prothrombin was first demonstrated in bovine plasma by Stenflo [152], and other observations [153] were consistent with the presence of a hepatic precursor protein pool in the hypoprothrombinemic rat that was rapidly being synthesized and that could be converted to prothrombin in a step that did not require protein synthesis.





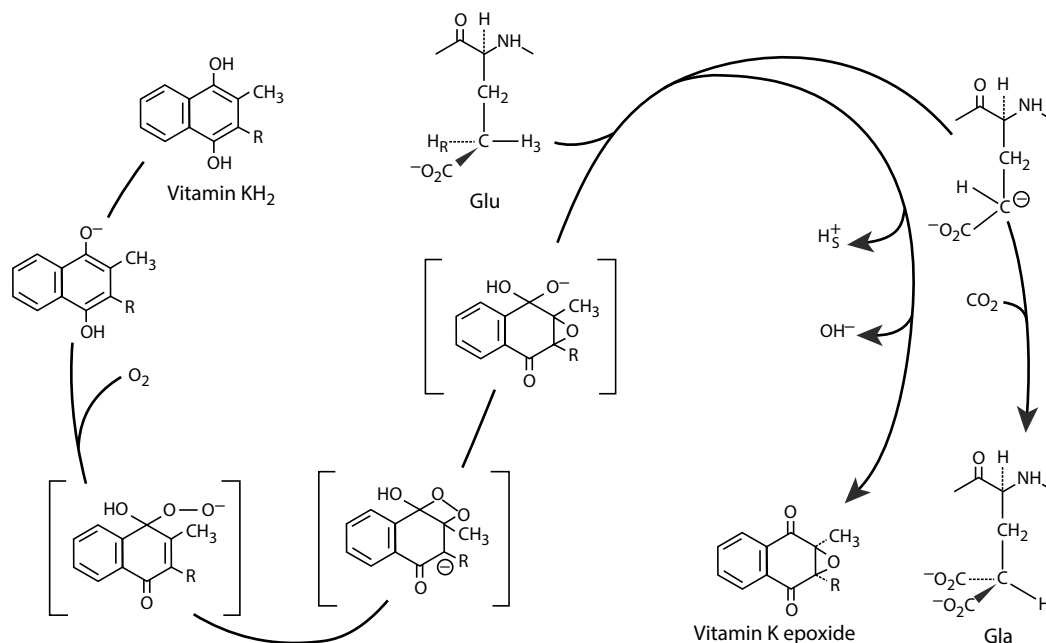
**FIGURE 3.7** The vitamin K–dependent carboxylase reaction. In the presence of reduced vitamin K,  $O_2$ , and  $CO_2$ , the enzyme converts a protein-bound Glu residue to a  $\gamma$ -carboxyglutamyl residue and generates vitamin K-2,3-epoxide.

Studies of the inactive “abnormal” prothrombin [154] demonstrated that it contained normal thrombin, had the same molecular weight and amino acid composition, but did not adsorb to insoluble barium salts as did normal prothrombin. The critical difference in these two proteins was the inability of the abnormal protein to bind to calcium ions, which are needed for the phospholipid-stimulated activation of prothrombin by factor X. Acidic,  $Ca^{2+}$ -binding peptides could be isolated from a tryptic digest of the amino-terminal domain of normal bovine prothrombin but could not be obtained when similar isolation procedures were applied to preparations of abnormal prothrombin. Stenflo et al. [155] succeeded in isolating an acidic tetrapeptide (residues 6–9 of prothrombin) and demonstrated that the glutamic acid residues of this peptide were modified so that they were present as  $\gamma$ -carboxyglutamic acid (3-amino-1,1,3-propanetricarboxylic acid) residues (Figure 3.7). Nelsestuen et al. [156] independently characterized  $\gamma$ -carboxyglutamic acid (Gla) from a dipeptide (residues 33 and 34 of prothrombin), and these characterizations of the modified glutamic acid residues in prothrombin were confirmed by Magnusson et al. [157], who demonstrated that all the 10 Glu residues in the first 33 residues of prothrombin are modified in this fashion.

### 3.6.2 THE VITAMIN K–DEPENDENT CARBOXYLASE

The discovery of Gla residues in prothrombin led to the demonstration [158] that crude rat liver microsomal preparations contained an enzymatic activity (the vitamin K–dependent carboxylase) that promoted a vitamin K–dependent incorporation of  $H^{14}CO_3^-$  into endogenous precursors of vitamin K–dependent proteins present in these preparations. The same microsomal preparations and incubation conditions that fixed  $CO_2$  into Gla would convert vitamin K to its 2,3-epoxide [159] (Figure 3.8). Small peptides containing adjacent Glu–Glu sequences such as Phe–Leu–Glu–Glu–Val were found to be substrates for the enzyme [160], and they were used to study the properties of this unique carboxylase. The vitamin K–dependent carboxylation reaction does not require ATP, and the energy to drive this carboxylation reaction is derived from the oxidation of the reduced hydronaphthoquinone form of vitamin K (vitamin  $KH_2$ ) by  $O_2$  to form vitamin K-2,3-epoxide, and carbon dioxide rather than  $HCO_3^-$  is the active species in the carboxylation reaction. Although some differences in carboxylase activity can be measured, phyloquinone, MK-4, and the predominant intestinal forms of the vitamin, MK-6 and MK-8, are all effective substrates.

Normal functioning of the vitamin K–dependent carboxylase poses an interesting question in terms of enzyme–substrate recognition. This microsomal enzyme recognizes a small fraction of the



**FIGURE 3.8** The vitamin K–dependent  $\gamma$ -glutamyl carboxylase. An interaction of  $O_2$  with vitamin  $KH_2$ , the reduced (hydronaphthoquinone) form of vitamin K, generates intermediates eventually leading to an oxygenated metabolite that is sufficiently basic to abstract the  $\gamma$ -hydrogen of the glutamyl residue. The products of this reaction are vitamin K-2,3-epoxide and a glutamyl carbanion. Attack of  $CO_2$  on the carbanion leads to the formation of a  $\gamma$ -carboxyglutamyl residue (Gla). The bracketed peroxy, dioxetane, and alkoxide intermediates have not been identified in the enzyme-catalyzed reaction but are postulated on the basis of model organic reactions. The available data are consistent with their presence.

total secretory protein pool of the appropriate tissue and then carboxylates all the available Glu sites in these proteins. Cloning of the vitamin K–dependent proteins has revealed that the primary gene products of all these proteins contain a very homologous “propeptide” between the amino-terminus of the mature protein and the signal peptide. This region appears to be a “docking” or “recognition site” for the enzyme [161] and has also been shown [162,163] to modulate the activity of the enzyme by decreasing the apparent  $K_m$  of the Glu site substrate. As there are multiple Glu sites on the vitamin K–dependent protein substrates, they could bind and dissociate from the carboxylase many times in a distributive mechanism until all Glu sites are converted to Gla residues. However, full carboxylation is known to occur through a processive mechanism in which all Glu sites are carboxylated as the result of one binding event [164,165].

The role of vitamin K in the overall reaction catalyzed by the enzyme is to abstract the hydrogen on the  $\gamma$ -carbon of the glutamyl residue to allow attack of  $CO_2$  at this position coupled to conversion of the vitamin to its 2,3-epoxide. A number of studies that utilized substrates tritiated at the  $\gamma$ -carbon of each Glu residue provided the initial information of the action and the stoichiometry involved [166,167]. The enzyme catalyzes a vitamin  $KH_2$ – and  $O_2$ –dependent (but  $CO_2$ –independent) release of tritium from the substrate, and at saturating concentrations of  $CO_2$ , there is an apparent equivalent stoichiometry between vitamin K-2,3-epoxide formation and Gla formation. The mechanism by which epoxide formation is coupled to  $\gamma$ -hydrogen abstraction is key to a complete understanding of the role of vitamin K. The reaction efficiency defined as the ratio of Gla residues formed to  $\gamma$ -C–H bonds cleaved has been shown to be independent of Glu substrate concentrations and to approach unity at high  $CO_2$  concentrations.

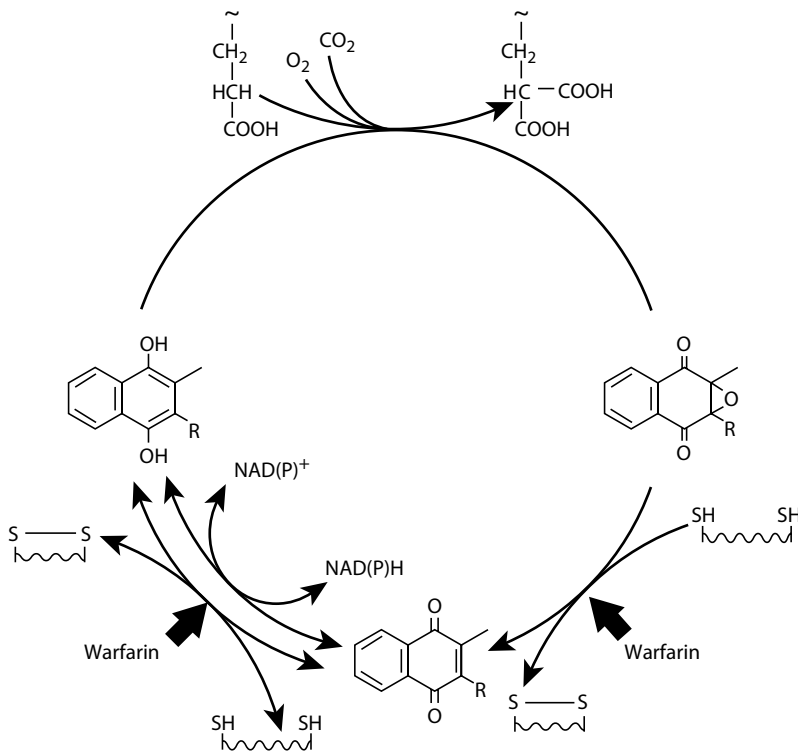
Experiments designed to identify an intermediate chemical form of vitamin K that is sufficiently basic to abstract the  $\gamma$ -hydrogen of the glutamyl residue have been a challenge. The most likely hypothesis is that first proposed by Dowd et al. [168,169] who suggested that an initial attack of  $O_2$  at the naphthoquinone carbonyl carbon adjacent to the methyl group results in the formation of a dioxetane ring that generates an alkoxide intermediate (Figure 3.8). This intermediate was hypothesized to be the strong base that abstracts the  $\gamma$ -methylene hydrogen and leaves a carbanion that can interact with  $CO_2$ . The active-site base that is needed to deprotonate the vitamin K hydroquinone was initially thought to be a cysteine residue, but more recent studies have identified it as a lysine residue [170,171]. It has also been demonstrated that when affinity-purified carboxylase rather than less purified microsomes is studied, a histidine residue is involved in the deprotonation process. A concerted mechanism allows the Glu deprotonation to be directly followed by  $CO_2$  to neutralize the negative charge that occurs when the proton is removed to allow the formation of the Gla residue and allow nothing else to interact with the negative charge [172,173].

Progress in purifying the carboxylase was slow, but the enzyme was eventually purified to near homogeneity and cloned [174]. The carboxylase is a unique 758-amino-acid residue protein with a sequence suggestive of an integral membrane protein with a number of membrane-spanning domains in the N-terminus and a C-terminal domain located in the lumen of the endoplasmic reticulum. The membrane topology of the carboxylase has not yet been firmly established. The amino acid sequence of the carboxylase indicates seven hydrophobic regions in the protein [175], and it has been proposed that the enzyme has five transmembrane regions spanning the endoplasmic reticulum. Alternative models of the topology [164,176,177] are also possible, and additional data are needed. Although the general scheme shown in Figure 3.8 is consistent with the available data, the mechanism is still an active area of research.

### 3.6.3 THE VITAMIN K EPOXIDE REDUCTASE

The degradation of vitamin K-dependent proteins generates Gla residues that are not metabolized but are excreted in the urine [178]. Human adult Gla excretion is in the range of 50  $\mu\text{mol/day}$ , indicating that a similar amount is formed each day. The average dietary intake of vitamin K is only approximately 0.2  $\mu\text{mol/day}$ , and a mole of vitamin is oxidized for each mole of Gla formed. It is clear that the vitamin K 2,3-epoxide generated by the carboxylase must be actively reduced and recycled, and the hepatic ratio of the epoxide relative to that of the vitamin was found to be increased in animals administered the 4-hydroxycoumarin anticoagulant warfarin [179]. This suggested that warfarin inhibition of vitamin K action was indirect through an inhibition of the enzyme called the vitamin K epoxide reductase (VKOR) [180]. Blocking of the reductase prevents the reduction of the epoxide to the quinone form of the vitamin and eventually to the carboxylase substrate, vitamin  $KH_2$ . Widespread use of warfarin as an anticoagulant rodenticide led to the appearance of strains of warfarin-resistant rats, and the study of the activity of the epoxide reductase in livers of these animals was key to an understanding [181,182] of the details of what is now referred to as the "Vitamin K cycle" (Figure 3.9). Three forms of vitamin K (the quinone [K], the hydronaphthoquinone [ $KH_2$ ], and the 2,3-epoxide [KO]) can feed into this liver vitamin K cycle. In normal liver, the ratio of vitamin K-2,3-epoxide to the less oxidized forms of the vitamin is approximately 1:10 but can increase to a majority of epoxide in an anticoagulated animal.

VKOR has been shown to be a three-exon protein containing 163 amino acids with a molecular mass of 18,000 Da called VKORC1 [183–185]. Both the quinone and epoxide forms of vitamin K are reduced to the hydronaphthoquinone form through an electrical relay involving paired Cys residues within the VKORC1 structure to generate the vitamin K hydroquinone, which is needed to drive the carboxylase. Interaction of warfarin with the VKOR decreases the ability of VKOR to recycle the vitamin K epoxide to the quinone, which results in an increase of the amount of circulating epoxide. The presence of the identified VKOR gene in *Drosophila* and other insects [186] suggests that this activity may be as widespread as the carboxylase. The importance of the epoxide



**FIGURE 3.9** Tissue recycling of vitamin K. Vitamin K epoxide formed in the carboxylation reaction is reduced to the quinone form of the vitamin by a warfarin-sensitive enzyme, the VKOR. This reaction is driven by a reduced dithiol. The naphthoquinone form of the vitamin can be reduced to the hydronaphthoquinone form either by the same warfarin-sensitive dithiol-driven reductase or by one or more of the hepatic NADH or NADPH-linked quinone reductases that are less sensitive to warfarin.

reductase for the synthesis of vitamin K–dependent proteins is illustrated by observations [187] that the production of reduced vitamin K by this enzyme rather than the activity of the carboxylase is the rate-limiting step in the production of these important proteins.

### 3.7 HEALTH IMPACTS OF ALTERED VITAMIN K STATUS

#### 3.7.1 METHODOLOGY

The classical method used to define an inadequate intake of vitamin K was to measure the plasma concentration of one of the vitamin K–dependent clotting factors: prothrombin (factor II), factor VII, factor IX, or factor X. Standard tests currently in use measure the time it takes recalcified, citrated, or oxalated plasma to form a fibrin clot. The standard “prothrombin time” or PT (historically called a “quick prothrombin time”) assay measures clotting times in plasma after the addition of calcium and a lung or brain extract (thromboplastin) preparation to furnish phospholipids and tissue factors. Variations of this assay have been developed, and commercial reagent kits are available. The assay responds to the levels of prothrombin and factors VII and X, and as factor VII has the shortest half-life, it is likely that these one-stage prothrombin assays often measure the level of factor VII rather than prothrombin. As the vitamin K–dependent clotting factors are serine proteases, chromogenic substrates can also be used to assay their activity. These assays, when utilized to assay prothrombin activity, actually measure the concentration of thrombin that has been generated from

prothrombin by various methods [188]. Because of their relative lack of sensitivity, these historical clotting factor assays have had little value in determining vitamin K status.

Human vitamin K deficiency results in the secretion into the plasma of partially carboxylated species of vitamin K–dependent proteins. Because they lack the full complement of  $\gamma$ -carboxyglutamic acid residues, their calcium binding affinity is altered, and they can be separated from their normal form by alterations in their ability to bind to barium salts or by electrophoresis. Antibodies that are specific for these “abnormal” prothrombins have been developed and can also be used to detect a vitamin K deficiency. Vitamin K status is also reflected in alterations of circulating levels of the vitamin, but these values are subject to day-to-day variation on the basis of recent intake of the vitamin. The need for complete carboxylation is not as important as it once was thought. It has been shown [189] that undercarboxylated plasma MGP is associated with the intake of vitamin K, and the degree of the undercarboxylated form decreases as the vitamin K intake increases. However, the measurements of coronary artery calcium were not shown to decrease as carboxylation of MGP increased.

### 3.7.2 ADULT VITAMIN K DEFICIENCY

The human population normally consumes a diet containing an amount of vitamin K in excess of that needed to maintain normal hemostasis, but a vitamin K–responsive human hypoprothrombinemia can sometimes be observed. O’Reilly [20] has reviewed the potential problem areas and has pointed out the basic factors needed to prevent a vitamin K deficiency: (a) a normal diet containing the vitamin, (b) the presence of bile in the intestine, (c) a normal absorptive surface in the small intestine, and (d) a normal liver. Cases of an acquired vitamin K deficiency do, therefore, occur in the adult population and, though relatively rare, present a significant problem for some individuals. A relatively high percentage of an older adult hospital-admitted population has been shown to have a hypoprothrombinemia that responds to administration of oral vitamin K [190], but a more recent study [191] indicates that age or sex does not influence a response to alterations in dietary vitamin K. Vitamin K–responsive hemorrhagic events have frequently been reported in patients receiving antibiotics and have been extensively reviewed [192]. These episodes have usually been assumed to be caused by decreased menaquinone availability from the gut, but it is possible that many cases may represent low dietary intake alone and that the presumed effect on gut bacteria was not related to the hypoprothrombinemia. Some second- and third-generation cephalosporins have been implicated in a large number of hypoprothrombinemic episodes, and it is likely that they are exerting a coumarin-like response [193], which might be more important than a presumed influence on the gut bacterial population.

Experimentally induced vitamin K deficiencies that are sufficiently severe to reduce PT measurements have been rare. An often cited study [194] investigated the vitamin K requirement of starved intravenously fed debilitated patients given antibiotics to decrease intestinal vitamin K synthesis. A significant degree of vitamin K responsive hypoprothrombinemia was clearly established in these subjects. More recently, a number of controlled studies utilizing diets containing approximately 10  $\mu\text{g}/\text{day}$  or less of phylloquinone [80,116,195] have demonstrated alterations using more sensitive markers of vitamin K status, but a clinically significant decrease in PTs was not seen.

### 3.7.3 ANTICOAGULANT THERAPY

Inhibition of the VKOR by the oral anticoagulant warfarin results in the secretion to the plasma of vitamin K–dependent proteins lacking all or a portion of the normal number of Gla residues. The magnitude of the anticoagulant effect produced by a given dose of warfarin varies by as much as 20-fold between individuals and may vary substantially in an individual patient over time. Drug interactions have been found to be responsible for some of this variation, and drugs have been shown to alter the displacement of warfarin from its plasma albumin carrier, induce the hepatic

P450 that metabolizes warfarin, interfere with warfarin clearance, or bind to warfarin in the gut. Alterations of vitamin K intake or absorption can also alter warfarin efficacy [196], and genetic variability is also undoubtedly important. Polymorphisms of the reductase gene itself, VKOR1, or of the P450 variant CYP2C9 [197,198] appear to be responsible for most of the variation in effective warfarin doses. Supplemental amounts of vitamin E have been shown to increase bleeding and decrease vitamin K status by a mechanism that is not yet clearly defined [199].

The anticoagulant effect of warfarin therapy is monitored by measurement of the PT, a measure of combined procoagulant status rather than a true measure of prothrombin activity. As thromboplastin reagents vary widely in their sensitivity to depressed levels of various clotting factors, plasma from a warfarin-treated patient may yield very different PTs when tested with different thromboplastins. To overcome this problem, the international normalized ratio (INR) is now used as a standardized method for reporting PT results. The INR allows interconversion of PT ratios (patient PT/mean normal PT) by use of an international sensitivity index, which corrects for differences in thromboplastin sensitivities. The goal of anticoagulant therapy is to achieve steady-state levels of vitamin K–dependent procoagulants in the range of 20%–30% of normal, which would be an INR of 2–3 [200]. The most common complication of anticoagulant therapy, bleeding, is directly related to the INR with few bleeds at a stable INR of <4.0 and a relatively high incidence with INRs of >7.0. Overanticoagulation can be brought back to the desired level by lowering the warfarin dose, or if severely out of range by subcutaneous or even slow intravenous infusion of phyloquinone.

### 3.7.4 HEMORRHAGIC DISEASE OF THE NEWBORN

Hemorrhagic disease of the newborn or early vitamin K deficiency bleeding (VKDB) occurring during the first week of life in healthy-appearing neonates [201] is the classic example of a human vitamin K deficiency. The low vitamin K content of breast milk, low placental transfer of phyloquinone, low clotting factor levels, and a sterile gut all contribute to the disease. Although the incidence is low, the mortality rate from intracranial bleeding is high, and prevention by oral or intramuscular administration of vitamin K immediately after birth is the standard cure. Late VKDB is a syndrome occurring between 2 and 12 weeks of age predominantly in exclusively breastfed infants [202] or infants with severe intestinal malabsorption problems. Although oral administration of vitamin K appears to be as effective as parenteral administration to prevent early VKDB, it may not be as effective for preventing late VKDB. A report in the early 1990s [203] suggested that intramuscular injection of vitamin K to infants was associated with an increased incidence of certain childhood cancers. This led to a switch to oral administration of vitamin K in some countries and an increase in the incidence of late VKDB. Subsequent studies have failed to show a correlation between the use of intramuscular vitamin K and the incidence of childhood leukemia or other cancers [204]. The current recommendations of the American Academy of Pediatrics [205] advise that “vitamin K (phyloquinone) should be given to all newborns as a single, intramuscular dose of 0.5 to 1 mg.”

### 3.7.5 POSSIBLE ROLE IN BONE HEALTH

Osteocalcin, MGP, and protein S are all known to be synthesized in bone. Because of its relatively high concentration in bone, attention has been directed toward osteocalcin as a possible factor in bone health. Small amounts of this protein circulate in plasma at concentrations that are four- to fivefold higher in young children than in adults, and reach the adult levels at puberty. Much of the circulating osteocalcin in individuals within the normal population is not completely  $\gamma$ -carboxylated and the extent of undercarboxylation can be influenced by vitamin K status [59,115]. An immunochemical assay for the des- $\gamma$ -carboxylated form of osteocalcin is available, but most studies have defined under- $\gamma$ -carboxylated osteocalcin (ucOC) as that fraction that does not adsorb to hydroxyapatite under standard conditions. In most species, all three Gla sites are fully carboxylated, but the fraction of ucOC reported in normal healthy human populations ranges from 40% to 50%. These

data have established that the normal dietary intake of vitamin K is not sufficient to maximally  $\gamma$ -carboxylate osteocalcin, and it has been shown [206] that supplementation with 1 mg phylloquinone per day ( $\sim 10 \times$  the current recommended daily intake [RDI]) is required to achieve maximal  $\gamma$ -carboxylation. At the present time, there is no clear evidence to support a link between increased ucOC and decreased mineralization. When  $\gamma$ -carboxylation of osteocalcin is effectively blocked in a rat model [112], a mineralization disorder characterized by complete fusion of proximal tibia growth plate and cessation of longitudinal growth has been observed. These data suggest that a skeletal vitamin K-dependent protein, probably osteocalcin, is involved in regulating bone mineralization, but does not indicate that low vitamin K status would decrease mineralization. Studies utilizing transgenic mice lacking the osteocalcin gene [113] have demonstrated that the phenotype increased bone mineralization rather than decreased bone mass.

There have been numerous observational studies that have examined the association of ucOC with bone mineral density and hip fracture risk, and these studies have reported mixed outcomes after supplementation with vitamin K. More recently, a series of randomized controlled clinical trials [59,114] have been conducted to determine if supplementation of vitamin K will slow the progression of bone loss in older adults. Although all these studies [117–119,207] reported a reduction in ucOC as supplemental vitamin K was added to the diets, only one study [207] found an increase in hip bone mineral density. Although maximal carboxylation of osteocalcin does not appear to be needed for normal bone mineralization, supplementation with MK-4 is a common therapy for osteoporosis in Japan and other Asian countries. The standard therapy is 45 mg of MK-4 per day, a pharmacological rather than a nutritional approach. Recent meta-analyses and trials [121,122] have found that only a small decrease in the adult fracture rate, if any, was observed after this therapy.

### 3.7.6 POSSIBLE ROLE IN VASCULAR CALCIFICATION

Studies of the MGP knockout mouse indicated that these animals died from massive calcifications of the large arteries within 8 weeks of birth [128], and a rapid calcification of the elastic lamellae of arteries and heart valves has been seen in a rat model when MGP carboxylation was blocked [129]. It has been found [208] that mutations in the MGP are associated with the phenotype of the Keutel syndrome, a rare autosomal recessive condition characterized by abnormal cartilage calcification. The action of MGP requires the  $\gamma$ -carboxylated form, and studies of the relationship between aortic calcification and phylloquinone [209] or total vitamin K [210] intake have shown no relationship or slightly lower aortic calcification in subjects with a lower vitamin K intake. A much larger epidemiological study has shown, however, an inverse relationship between dietary menaquinone and aortic calcification, myocardial infarction, and sudden cardiovascular death [211]. There are, however, no data to link a low intake of menaquinone to under- $\gamma$ -carboxylation. Current interest in this area of research has focused on the relationship between various types of aortic calcification and the dietary intake of vitamin K and menaquinones [130,131]. It is clear that recent studies [212–214] indicate that chronic coumarin treatment, which would substantially reduce the degree of carboxylation of vitamin K-dependent proteins, is associated with increased levels of coronary calcification, but the health impact of these changes is not clearly defined.

### 3.7.7 OTHER FACTORS INFLUENCING VITAMIN K STATUS

Although it is easily demonstrated that the vitamin K requirement of the rat is greatly increased under germ-free conditions [215], the significance of the utilization of gut menaquinones by humans has been difficult to quantitate. Phenobarbital and diphenylhydantoin administration to pregnant women has been reported [216] to produce a vitamin K-responsive hemorrhage in the newborn. Early studies of vitamin K requirements indicated that female rats had a lower vitamin K requirement and that nutritional deficiencies are much more readily produced in male rats. Castration of both sexes unifies this vitamin K response, and in the castrated rat, prothrombin concentrations can

be increased with estrogens and decreased with androgens [217]. The available evidence suggests that the influence of estrogens on rate of synthesis is reflected in a higher rate of synthesis and accumulation of prothrombin precursors in the microsomes [218]. Hypothyroidism in humans results in a decrease in both the rate of synthesis and destruction of the vitamin K–dependent clotting factors [219], and it is likely that these hormonal effects are related to rates of synthesis of the proteins involved rather than to any effect on vitamin K metabolism.

Early studies of vitamin K function established that the inclusion of mineral oil in diets prevented its absorption, and mineral oil has often been used in vitamin K–deficient diets. High dietary vitamin A has also been recognized for some time to adversely influence vitamin K action [220]. Whether this is a general effect on nonpolar lipid absorption or a specific vitamin K antagonism is not clear, but it can be observed at relatively low dietary levels of retinol acetate and retinoic acid. The addition of vitamin E to the diet of a patient on coumarin anticoagulation therapy was reported to cause a hemorrhagic episode years ago [221], and the relationship between these two vitamins has been under study for 50 years [222]. The basis for this interaction has been thought to be related to the similar degradation of both vitamins, and there are some indications that  $\alpha$ -tocopherol may be the causative agent [223]. A more recent study [199] suggests that alteration of transporters for these vitamins may also be involved in this interaction.

Vitamin K is present in the brain mainly in the form of MK-4, and it is implicated in modulating the activity of key enzymes of sphingolipid biosynthesis [223]. More recent studies have found that brain sulfatides and sphingomyelin do increase in response to similar increases in dietary vitamin K [224,225]. On the basis of the available data [226], a relationship between vitamin K status and human cognitive ability should be further investigated, and studies utilizing lifelong low phyloquinone fed rats have found that vitamin K intake is associated with cognitive impairments in old rats.

Although the only known function of vitamin K has been its ability to drive the carboxylase, it is possible that there are numerous but not yet identified functions. It has recently been shown [227] that a *Drosophila* mitochondrial dysfunction can be rescued by MK-4, which serves as a mitochondrial electron carrier to maintain normal ATP production. Whether MK-4 exerts a similar carrier function in eukaryotic cells is not yet known.

## 3.8 VITAMIN K REQUIREMENTS

### 3.8.1 ANIMALS

The establishment of a dietary vitamin K requirement for various species has been difficult because of the varying degrees to which they utilize the large amount of vitamin K synthesized by intestinal bacteria and the degree to which different species practice coprophagy. A spontaneous deficiency of vitamin K was first noted in chicks, and poultry are much more likely to develop symptoms of a dietary deficiency than any other species. This has usually been assumed to be due to the rapid transit rate of material through the relatively short intestinal tract of the chick or to limited synthesis of menaquinones in this species. A more recent study [228] suggests that limited recycling of vitamin K because of low epoxide reductase activity may be the cause of the increased requirement.

Ruminal microorganisms synthesize large amounts of vitamin K, and ruminants do not appear to need a source of vitamin in the diet. Deficiencies have, however, been produced in most monogastric species. The majority of the data are old, different forms of the vitamin were used, and different methods were employed to establish the requirement. Phyloquinone has been used for most experimental nutrition studies, whereas other forms of vitamin K are usually used in practical rations. Menadione is usually considered to be from 20% to 40% as effective as phyloquinone on a molar basis, but this depends a great deal on the type of assay that is used. It is rather ineffective in a curative assay, where the rate of its alkylation to MK-4 is probably the rate-limiting factor, but often shows activity nearly equal to phyloquinone in a long-term preventive assay. Commercial livestock rations usually utilize a water-soluble form of menadione, such as MSBC. This compound



**TABLE 3.3**  
**Vitamin K Requirements of Various Species**

Species	Daily Intake ( $\mu\text{g}/\text{kg}$ per day)	Dietary Concentration ( $\mu\text{g}/\text{kg}$ diet)
Dog	1.25	60
Pig	5	50
Rhesus monkey	2	60
Rat, male	11–16	100–150
Chicken	80–120	530
Turkey poult	180–270	1200

*Note:* Data have been summarized from a more extensive table [231] and are presented as the amount of vitamin needed to prevent the development of a deficiency. No correction for differences in potency of equal weights of different forms of the vitamin has been made.

appears to be about as active on a molar basis as phyloquinone in poultry rations, and at least in this species, the activities of menadione, MSBC, and phyloquinone are roughly equal on a weight basis.

The available data indicate that the vitamin K requirement for most species falls in a range of 2–200  $\mu\text{g}$  of vitamin K per kilogram of body weight per day [220,229,230]. The data in Table 3.3, which have been adapted from a table presented by Griminger [231], give an indication of the magnitude of the requirement for various species. This requirement can be altered by age, sex, or strain, and any condition influencing lipid absorption or conditions altering intestinal flora will have an influence of these values. A considerably higher level of dietary vitamin K has been recommended for most laboratory animals by the National Academy of Sciences [229]. Recommendations for most species are in the range of 3000  $\mu\text{g}/\text{kg}$  of diet, but the rat requirement has been set at 50  $\mu\text{g}/\text{kg}$ . Although this level is sufficient in most cases, it did not prevent all signs of deficiency [84], and the American Institute of Nutrition [232] has now recommended that purified diets for laboratory rodents should have 750  $\mu\text{g}$  of phyloquinone added to each kilogram of diet.

### 3.8.2 HUMANS

The most recent values for vitamin K intake were established in 2001 as part of the comprehensive Dietary Reference Intakes project of the Food and Nutrition Board/Institute of Medicine and have been published by the National Academy of Sciences [42]. There are ample data to establish that very few, if any, individuals consume sufficient vitamin K to maximally  $\gamma$ -carboxylate their circulating osteocalcin and MGP and that supplementation with approximately 1 mg/day of phyloquinone is needed to achieve this response. As there appeared to be no clinical significance of this apparent deficiency, this index of adequacy was not used to set a reference value.

Currently, the only indicator of vitamin K status with clinical significance is the PT, and alterations in the PT by changes in dietary intake alone are uncommon to nonexistent. As circulating phyloquinone concentration is very dependent on previous day intakes, it is also not a satisfactory indicator of an adequate intake. Intakes of vitamin K that are in the range of 10% of normal have been demonstrated under controlled conditions to result in decreases in urinary Gla excretion and increases in under- $\gamma$ -carboxylated prothrombin, which can be measured by a commercially available immunoassay. However, no studies utilizing a range of intakes that would allow the calculation of an estimated average requirement (EAR) on the basis of these markers are available. Reports that might implicate bone or vascular health (see Sections 3.7.5 and 3.7.6) to alterations in vitamin K status also fail to provide the data needed to establish an EAR. If available data allow the determination of an EAR, the historical term used to indicate nutrient requirements, the recommended dietary allowance, can be calculated. As sufficient data to determine an EAR are not available, the

**TABLE 3.4**  
**AIs of Vitamin K**

Population	Vitamin K ( $\mu\text{g/day}$ )
0- to 6-month-old infants	2.0
7- to 12-month-old infants	2.5
1- to 3-year-old children	30
4- to 8-year-old children	55
9- to 13-year-old boys and girls	60
14- to 18-year-old boys and girls <sup>a</sup>	75
19- to >70-year-old men	120
19- to >70-year-old women <sup>a</sup>	90

*Note:* Dietary reference intakes [42].

<sup>a</sup> No alteration of intake for pregnancy or lactation.

RDI currently in use is the adequate intake (AI) for different age groups shown in Table 3.4. The value is defined as “the recommended average daily intake level based on observed or experimentally determined approximations or estimates of nutrient intake by a group or groups of apparently healthy people that are assumed to be adequate.” AIs of infants are based on the phyloquinone content of human milk and assume that infants also receive prophylactic vitamin K at birth. AIs for children, adolescents, and adults are based on the highest median intake for each age group reported by the NHANES III. On the basis of those data, the intakes of pregnant or lactating women do not differ from those of the general population.

### 3.9 EFFICACY AND HAZARDS OF PHARMACOLOGICAL DOSES OF VITAMIN K

No hazards attributed to the long-term ingestion of elevated amounts of the natural forms of vitamin K have been reported [233,234]. For treatment of prolonged clotting times when hemorrhage is not a problem, vitamin K can be given orally or parenterally. If given orally to patients with impaired biliary function, bile salts should also be administered. Vitamin K<sub>1</sub> is available as the pure compound or as an aqueous colloidal solution that can be given intramuscularly or intravenously. Some adverse reactions have been noted after intravenous administration, and unless a severe hemorrhagic episode is present, intramuscular injection is the recommended route of therapy. Effective therapy requires synthesis of normal clotting factors, and a number of hours may be necessary before a substantial decrease in clotting times is apparent.

The relative safety of phyloquinone and, presumably, menaquinones does not hold for menadione or its water-soluble derivatives. These compounds can be safely used at low levels to prevent the development of a deficiency but should not be used as a pharmacological treatment for a hemorrhagic condition. Although once prescribed for treatment of the hemorrhagic disease of the newborn, these compounds are known to react with free sulfhydryl groups of various tissues and to cause hemolytic anemia, hyperbilirubinemia, and kernicterus. This marked increase in conjugated bilirubin is extremely toxic to the neonatal brain and has caused death in some instances [233].

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