

## Theme Issue Article

## Metabolism and cell biology of vitamin K

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## Summary

Naturally occurring vitamin K compounds comprise a plant form, phyloquinone (vitamin K<sub>1</sub>) and a series of bacterial menaquinones (MKs) (vitamin K<sub>2</sub>). Structural differences in the isoprenoid side chain govern many facets of metabolism of K vitamins including the way they are transported, taken up by target tissues, and subsequently excreted. In the post-prandial state, phyloquinone is transported mainly by triglyceride-rich lipoproteins (TRL) and long-chain MKs mainly by low-density lipoproteins (LDL). TRL-borne phyloquinone uptake by osteoblasts is an apoE-mediated process with the LRPI receptor playing a predominant role. One K<sub>2</sub> form, MK-4, has a highly specific tissue distribution suggestive of local synthesis from phyloquinone in which menadione is an intermediate. Both phyloquinone and MKs activate the steroid and xenobiotic receptor (SXR) that

initiates their catabolism, but MK-4 specifically upregulates two genes suggesting a novel MK-4 signalling pathway. Many studies have shown specific clinical benefits of MK-4 at pharmacological doses for osteoporosis and cancer although the mechanism(s) are poorly understood. Other putative non-cofactor functions of vitamin K include the suppression of inflammation, prevention of brain oxidative damage and a role in sphingolipid synthesis. Anticoagulant drugs block vitamin K recycling and thereby the availability of reduced vitamin K. Under extreme blockade, vitamin K can bypass the inhibition of Gla synthesis in the liver but not in the bone and the vessel wall. In humans, MK-7 has a greater efficacy than phyloquinone in carboxylating both liver and bone Gla proteins. A daily supplement of phyloquinone has shown potential for improving anticoagulation control.

## Keywords

Vitamin K, phyloquinone, menaquinones, metabolism, cell biology, Gla-proteins, oral anticoagulants

Thromb Haemost 2008; 100: 530–547

## Introduction

Had this review been written just 25 years ago, the metabolism of vitamin K would have been considered just in the context of its role in haemostasis. Since that time vitamin K has been shown to have multiple functions. Many of these functions are mediated by old and new Gla proteins, some localised to specific tissues and some widely distributed, but other vitamin K functions seem to be independent of the classical co-factor function. Most of the metabolic processes we have covered are central to our understanding of the progression of vitamin K deficiency of which bleeding is still potentially the most serious known consequence. This review does not cover all aspects of vitamin K metabolism with equal weight. Rather, in the spirit of this Theme Issue we have selected certain aspects of vitamin K metabolism that we consider particularly relevant to functions of vitamin K and Gla proteins that lie outside of haemostasis, although in the case of

the inhibitory role in arterial calcification, vitamin K has found a new role in vascular biology. For these new Gla proteins, it is axiomatic that they require vitamin K for  $\gamma$ -carboxylation and that this modification is essential to their proper function, although the latter precept has not been proven formally for all. An understanding of how tissues that synthesize Gla proteins derive, utilize and catabolise vitamin K is therefore crucial to our understanding of how vitamin K deficiency may arise locally and lead to pathological changes. There is already evidence on this front that local deficiencies of vitamin K in bone and in the arterial tree may have deleterious health consequences.

An important aspect when considering vitamin K metabolism is that the several molecular forms have different co-factor activities and behave differently in processes such as absorption, transport, cellular uptake, tissue distribution and turnover. We have given special consideration to the properties of one molecular form, menaquinone-4 (MK-4). Firstly, we have reviewed the

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Received March 7, 2008  
Accepted after minor revision July 17, 2008

Prepublished online September 5, 2008  
doi:10.1160/TH08-03-0147

still perplexing data that MK-4 can be synthesised from phylloquinone in the body and has a highly unusual tissue distribution. Secondly, we have considered the large body of work on the molecular cell biology of MK-4 and the evidence that this isoprenologue has effects in bone biology and on cancer cells that are independent of  $\gamma$ -glutamyl carboxylation. Some of these effects of MK-4 on cells depend on higher than physiological concentrations. Finally, we have considered some rather unusual functions of vitamin K.

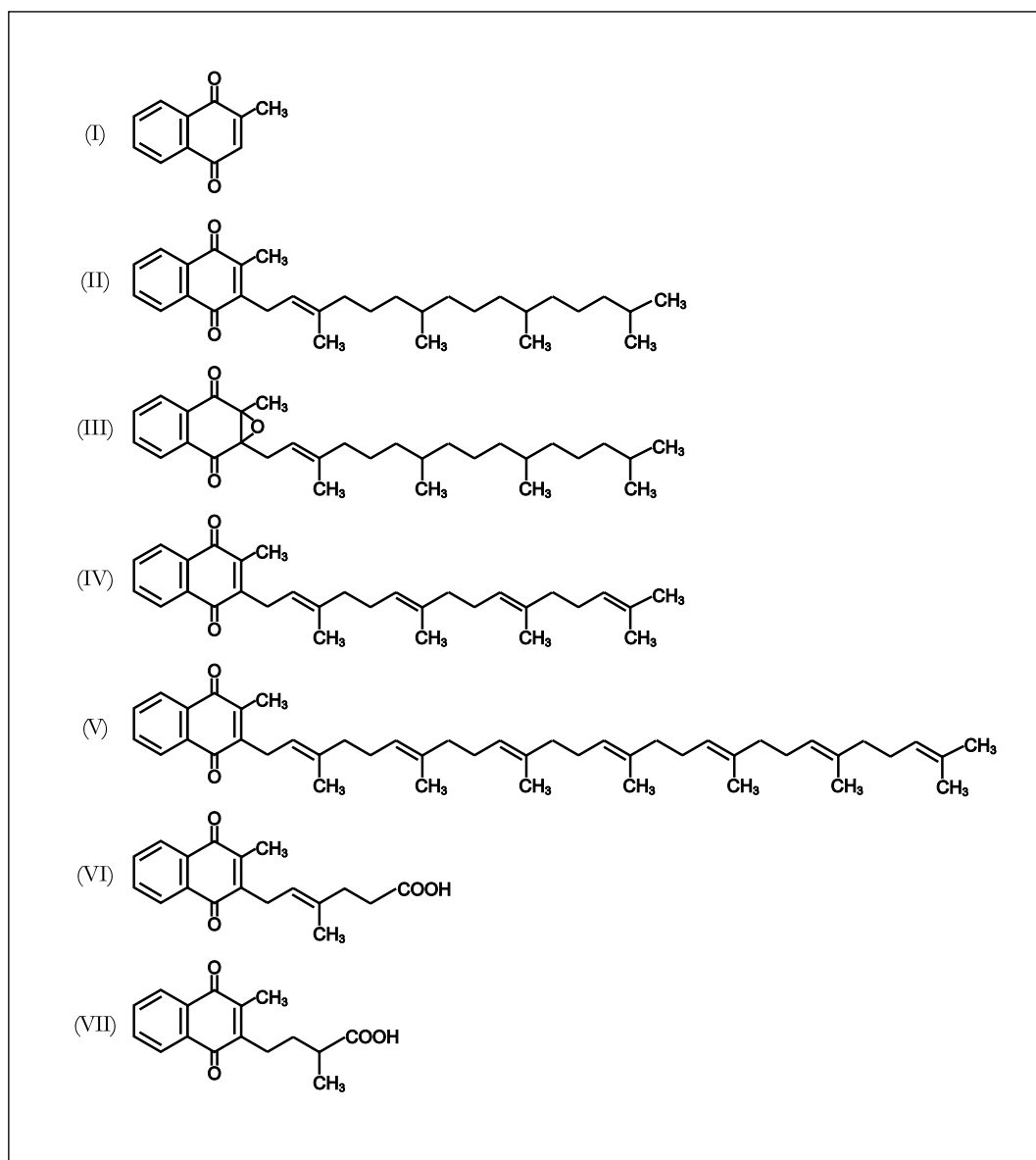
Throughout the history of vitamin K, researchers have been fortunate to have to hand specific vitamin K antagonists, of which the coumarin anticoagulants are the most notable. These continue to offer a powerful tool to investigate vitamin K-dependent processes while their clinical use as antithrombotic agents continues to thrive. In this review we consider how drugs like warfarin affect the availability and metabolism of vitamin K at the tissue and whole body level and how they have been used to investigate extrahepatic vitamin K functions. We also briefly

show how the use of vitamin K supplements may help to provide a greater stability of oral anticoagulant therapy.

## Chemical structures and nomenclature of K vitamins

Compounds that possess the classical vitamin K cofactor activity for the conversion of specific peptide-bound glutamate (Glu) residues to  $\gamma$ -carboxyglutamate (Gla) all possess a common 2-methyl-1,4-naphthoquinone structure, called menadione (Fig. 1; I), and a side chain at the 3-position. In nature, this 3-substituent has an isoprenoid structure with varying lengths and degrees of saturation depending on the organism by which it is synthesised. Plants and cyanobacteria, almost invariably, synthesise only one chemical form called phylloquinone (old and clinical nomenclature vitamin K<sub>1</sub>) which has the same phytyl side chain as in chlorophyll (Fig. 1; II). All other bacteria that possess the

**Figure 1: Chemical structures of some K vitamins and metabolites.** Nomenclature: chemical name and IUPAC name and abbreviation in brackets: (I) 2-methyl-1,4-naphthoquinone (menadione; K<sub>3</sub>), (II) 2-methyl-3-phytyl-1,4-naphthoquinone (phylloquinone; K<sub>1</sub>), (III) 2-methyl-3-phytyl-1,4-naphthoquinone-2,3-epoxide (phylloquinone epoxide; K<sub>1</sub>O), (IV) 2-methyl-3-geranylgeranyl-1,4-naphthoquinone (menaquinone-4; MK-4), (V) 2-methyl-3-farnesylgeranylgeranyl-1,4-naphthoquinone (menaquinone-7; MK-7), (VI) 2-methyl-3-(5'-carboxy-3'-methyl-2'-pentenyl)-1,4-naphthoquinone, (VII) 2-methyl-3-(3'-carboxy-3'-methyl propyl)-1,4-naphthoquinone.



machinery for vitamin K synthesis produce a plethora of isoprenologues called menaquinones (old nomenclature vitamins K<sub>2</sub>) (1). These differ from phylloquinone in that the 3-side chain comprises, for the most part, a polymer of repeating prenyl units rather than the phytyl chain synthesised by the plant kingdom, which has only one unsaturated bond. Bacterially synthesised menaquinones (Fig. 1; IV and V) that may contribute to human vitamin K requirements, either by being synthesised by the gut flora or being present in foods, generally have side chains with 4–12 prenyl units (2–5). For purposes of nomenclature, the menaquinones are classified according to the number of prenyl units; this number being given as a suffix (i.e. menaquinone-n, abbreviated MK-n). The picture is complicated by the fact that some bacteria produce isoprenologues in which one or more of the prenyl units is saturated; the additional hydrogen atoms being indicated by the prefix dihydro-, tetrahydro-, and so forth and are abbreviated MK-n(H<sub>2</sub>), MK-n(H<sub>4</sub>) etc.

## Sources of vitamin K

### Dietary sources

The major dietary source of vitamin K in most diets is phylloquinone from plant sources. Modern analyses of foods by HPLC have provided accurate food content data, enabling the compilation of national food databases for phylloquinone. To date, comprehensive food databases for phylloquinone have been compiled for the USA (6), UK (7) and the Netherlands (5). Highest concentrations of phylloquinone are found in green leafy vegetables but significant concentrations are also present in non-leafy green vegetables, several vegetable oils, fruits, grains and dairy produce. The last 10 years has seen a surge of studies in which dietary intakes of phylloquinone have been assessed in metabolic and epidemiological studies, and also in national population surveys. In some studies, intakes have been related to functional indices of vitamin K status such as the degree of carboxylation of osteocalcin. In Europe and the USA 60%, or more, of total phylloquinone intakes are provided by vegetables, the majority by green leafy vegetables (8–10). National surveys reveal that phylloquinone intakes are very wide (8–10). For example in mainland Britain there is typically a 10-fold difference for the inner 95% of phylloquinone intakes in both younger (9) and older people (8). Intakes determined by weighed dietary intakes are similar in mainland Britain to the USA with average intakes of around 70–80 µg/d.

Dietary intakes of menaquinones have been less well studied except in the Netherlands (5, 11) but it is known that apart from animal livers, the richest dietary source of long-chain menaquinones are fermented foods (from bacteria not moulds or yeasts) typically represented by cheeses (MK-8, MK-9) in Western diets and natto (MK-7) in Japan. Food frequency questionnaire-derived estimates of relative intakes of phylloquinone and menaquinones in the Netherlands suggest that ~90% of total vitamin K intakes are provided by phylloquinone, ~7.5 % by MK-5 through to MK-9 and ~ 2.5 % by MK-4 (11). Further work is needed in this area. Most food assays measure only fully unsaturated menaquinones; accordingly cheeses have been found to contain MK-8 at 10–20 µg/100g and MK-9 at 35–55 µg/100 g (5). However, propionibacteria produce MK-9(H<sub>4</sub>) as a major

form and recent work has shown that Norwegian Jarlsberg and Swiss Emmental cheeses contain 20–65 µg/100g of MK-9(H<sub>4</sub>) that may go unrecorded in current food databases (12).

### Non-dietary sources

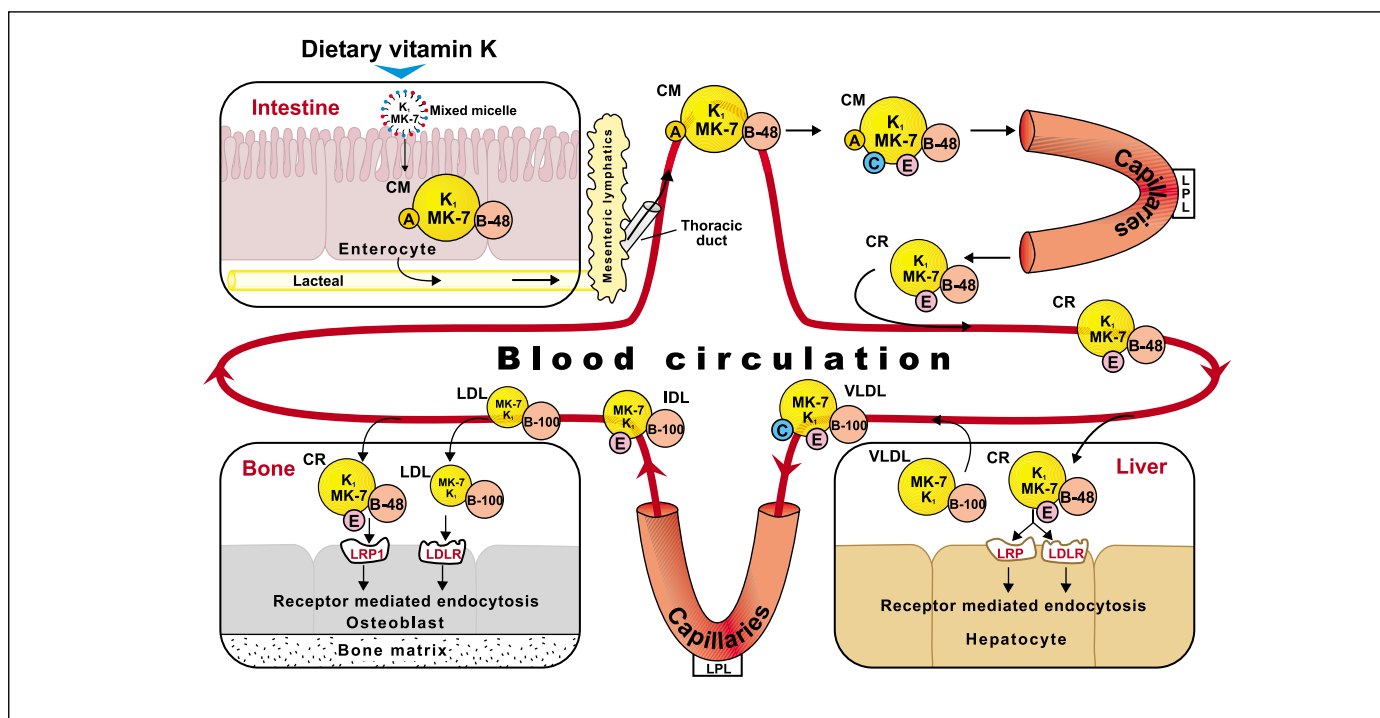
Many bacteria that populate the microbial ecosystem of the human intestine synthesise menaquinones, which they utilise as redox reagents in electron transport and oxidative phosphorylation. Bacterial numbers and composition vary considerably along the human gastrointestinal tract. In the small intestine, growth is limited by the rapid transit times and secretion of bile and pancreatic juice with numbers ranging from ~10<sup>4</sup> per ml in the proximal region to ~10<sup>6</sup>–10<sup>8</sup> per ml in the ileocaecal region (13). In humans, by far the largest reservoir of bacteria occupies the large intestine where the majority of species are strict anaerobes with typical numbers of ~10<sup>11</sup>–10<sup>12</sup> per gram (13). Quantitatively, the most important genera of intestinal flora are the bacteroides and bifidobacteria, which together can account for over half of the total anaerobic bacterial population (13); they differ, however, in that only the bacteroides synthesise menaquinones (1–3). Of various species of the genera *Bacteroides*, most synthesise the very long chain forms, MK-10 and MK-11 as the main isoprenologues with minor amounts of MK-7, MK-8, MK-9 and MK-12 (1, 2, 14). It is beyond the scope of this review to consider in detail the evidence for and against the relevance of intestinal flora to the maintenance of vitamin K status but this has been considered elsewhere (4, 15). In an extensive review in 1995, Suttie (4) concluded that gut menaquinones do contribute to human nutrition but to a degree that is less important than previously thought. In the intervening years there has been little or no evidence to contradict this conclusion and in several human studies the importance of the diet as the major source of functionally available vitamin K has been reinforced (see also section on *Dietary deficiency in humans*).

## Vitamin K transport and cellular uptake mechanisms

A schematic illustration of known metabolic processes that lead from the intestinal absorption of dietary vitamin K to its entry into cells is shown in Figure 2. Most of our knowledge of intestinal absorption, transport, cellular uptake and metabolic fate relates to phylloquinone with less known about menaquinones. For illustrative purposes, the scheme in Figure 2 shows the metabolism of phylloquinone and MK-7, the latter as a representative of the best studied dietary menaquinone. Not included in Figure 2 is MK-4 because the synthetic origin and metabolism of this vitamer is rather complicated and obscure (see section on *Metabolic transformation of phylloquinone to menaquinone-4*).

### Phylloquinone in the circulation: Carriage and clearance

Early studies using [<sup>3</sup>H] radiolabeled phylloquinone established that, after intestinal absorption, phylloquinone is incorporated into chylomicrons (CM), which are secreted into lymph lacteals and enter the blood via the thoracic duct (16). Once in the circulation, phylloquinone is rapidly cleared at a rate consistent with



**Figure 2: Schematic representation of the absorption, transport and cellular uptake of dietary phyloquinone ( $K_1$ ) and menaquinone-7 (MK-7).** Key processes shown are: *Intestinal absorption and entry into circulation:* After digestion, dietary vitamin K and the products of pancreatic hydrolysis of triglycerides (TG) are emulsified by bile salts to form mixed micelles which are taken up by the enterocytes of the intestinal epithelium and processed into nascent CM which contain apoA and apoB-48. CM are then secreted into the lacteals within the intestinal villi. The lacteals drain into larger lymphatic vessels eventually emptying into the blood circulation via the thoracic duct. Once in the blood, CM acquire apoC and apoE from HDL. In the capillaries of muscle, adipose tissues etc., CM are stripped of their TG by the action of lipoprotein lipase (LPL) which lines the capillaries. The resultant smaller CR re-enter the circulation having lost much apoA and apoC but retaining vitamin K in the lipophilic core. *Uptake by liver:* In the liver CR

enter hepatocytes by binding to LDLR and LRP followed by receptor mediated endocytosis. Their lipids are repackaged into VLDL (containing apoB-100) and return to the circulation where they acquire apoC and apoE. Further TG is removed by LPL in the capillaries resulting in VLDL remnants called IDL. Subsequent metabolism and loss of apoC and apoE from IDL gives rise to smaller LDL particles containing almost exclusively apoB-100. Vitamin K is presumed to be still located in the lipophilic core. *Uptake by bone:* Circulating lipoproteins such as CR and LDL can deliver lipids to osteoblasts which are attached to the surfaces of bone matrix. Osteoblasts express lipoprotein receptors such as LDLR and LRP1 which can interact with CR and LDL allowing receptor mediated endocytosis of the particles and their cargoes of vitamin K. Evidence suggests that osteoblasts obtain most of their  $K_1$  via the CR pathway and most of their MK-7 via the LDL pathway.

the known pathway of metabolism of CM. Thus, as CM circulate they lose their triglyceride (TG) through the action of lipoprotein lipase found on endothelial surfaces and eventually become CM remnants (CR). Together with very low-density lipoproteins (VLDL) these constitute the triglyceride-rich lipoproteins (TRL) that are the major carriers of phyloquinone in the circulation, with lesser but significant amounts carried by low- and high-density lipoproteins (LDL and HDL) (17–19). CM and CR are derived exclusively from the intestinal mucosa while VLDL, LDL and HDL, including various intermediate forms, are derived from the liver and differ in terms of their lipid and protein composition.

Analysis of plasma and lipoprotein fractions at different time points after feeding subjects a pharmacological dose (50  $\mu\text{g}/\text{kg}$  body weight) of phyloquinone with a fat-rich meal showed that the percentage of phyloquinone found in TRL was always far greater than in LDL or HDL throughout and beyond the postprandial state (18). Comparisons between the 3 h and 12 h time-points showed that whereas the proportion of phyloquinone car-

ried by TRL decreased from 91% to 70%, the proportions carried by LDL and HDL increased progressively from 3% to 14% for LDL and from 4% to 11% for HDL (18). Although the fractionation methods used could not distinguish between CM, CR and VLDL, the progressive increase in phyloquinone in LDL particles is consistent with the notion that some phyloquinone taken up by the liver in CR re-emerges later incorporated into VLDL which after further lipolysis become LDL particles still retaining their phyloquinone cargo (18). Another possibility is that some of the phyloquinone carried by LDL and HDL derives from the transfer of phyloquinone from intestinally derived CM and CR (18). Although low circulating levels make it difficult to assess lipoprotein distribution in the fasting state, available data suggests that some 50–70% of phyloquinone is associated with TRL with the remainder being approximately equally distributed between LDL and HDL (17, 18). More recently, the intrinsic labelling of phyloquinone into a food was achieved by growing a green vegetable (collard greens) in deuterated water: this enabled stable-isotope labelled phyloquinone to be fed to human

volunteers in a completely natural form and at a physiological dose (400 µg) (20). Plasma concentrations of phylloquinone peaked at around 6–9 hours (h) after ingestion and returned to baseline within 24 h (20). The results confirmed that the TRL fraction was also the main carrier of phylloquinone after giving a physiological dose (20). Labelled phylloquinone appeared even later in the LDL fraction (20) suggesting that it was first being processed through VLDL by the liver as postulated earlier (18). In summary, although TRL carry the greatest amounts under physiological conditions, it is likely that both LDL and HDL also have important functions in phylloquinone transport.

Several groups have used radioisotopic techniques to follow the clearance kinetics of phylloquinone after giving intravenous doses ranging from 0.3 to 1,000 µg while in a recent study Jones et al. (21) used novel stable isotope methodology to analyse disposal kinetics of an intravenous dose of either 6 or 30 µg phylloquinone. All these studies show that phylloquinone disappears rapidly from the circulation. In general the disappearance kinetics over the first 8–10 h can be resolved into two exponentials, a rapid phase with a half-life of around 0.3 h and a slower phase with a half-life of around 2.5 h. The high clearance rates make it difficult to detect tracer phylloquinone after a few hours and to detect the terminal exponential that would allow accurate conclusions to be made about turnover and body pool sizes. It is likely that the two-compartmental model is too simplistic and kinetic analysis, including that from the latest stable-isotope study, points to the existence of other body pools with a slow rate of exchange (21). This is supported by human balance studies using radiolabeled phylloquinone which showed that the body retains some 35–40% of a tracer dose given intravenously (22) or about 30% of a physiological dietary dose given orally (23), thus suggesting that a fraction of the tracer phylloquinone has entered a pool with a very slow turnover rate. Candidate physiological organs for these slow-turnover pools of phylloquinone are bone and adipose tissue (22, 24).

A caveat to any conclusions made from intravenous data is that emulsified vitamin K preparations may not be cleared in the same way as the lipoproteins that are the physiological carriers after an oral dose. A completely different lipoprotein distribution of phylloquinone in plasma was seen when phylloquinone was injected via the intramuscular route when 10 h after a 5-mg dose some 49% was found in the LDL fraction and 36% in the HDL fraction (25). This is a high pharmacological dose and the major carriage of phylloquinone by LDL and HDL presumably reflects export from the muscle for liver catabolism and excretion.

### Menaquinone transport

All of the above studies addressed the question of how phylloquinone is transported in the circulation but when three different forms of vitamin K were ingested simultaneously they were found to behave differently (19). In a comparative study, healthy volunteers ingested a mixture of 2 µmol of phylloquinone, MK-4 and MK-9 with a test meal and the serum and lipoprotein profiles of each vitamer measured for up to 48 h. In agreement with previous studies, phylloquinone was found mainly in TRL and most was cleared from the circulation within 8 h. No more than 17% and 18% of circulating phylloquinone appeared in the LDL and HDL fractions respectively with the greatest proportions be-

tween 8–24 h. MK-4 concentrations were lower at all times than those of phylloquinone and were distributed almost equally between TRL, LDL and HDL up to 4 h. Thereafter, although circulating MK-4 was still being cleared quickly, the proportion in LDL increased sharply until at 8 h some 80% of that remaining was present in LDL. MK-9 had the lowest of all serum concentrations over the first 6 h; it was initially present only in TRL but began to appear in LDL by about 8 h after ingestion and remained there for at least 48 h. MK-9 was never found in HDL. The reasons why MK-4 and MK-9 were found at lower serum concentrations remain unclear though the authors speculate that it could be due to faster uptake by tissues relative to phylloquinone, at least for MK-4.

In a later study the same group compared the serum concentration-time profiles of phylloquinone with MK-7, derived from the Japanese food natto after healthy volunteers had ingested a mixture of the two vitamers (26). The results showed that MK-7 from the same bolus persisted in the circulation for up to 96 h. Although lipoprotein fractionation was not carried out, the data is consistent with the transport of MK-7 at later times with LDL, as previously shown for MK-9 (Fig. 2).

### Cellular uptake of vitamin K

All lipoproteins contain apolipoproteins which determine their interactions with LPL and cell-surface receptors, some of which can mediate endocytosis of the particles enabling their lipid contents, including vitamin K, to enter the cell. Much work has been done on cellular uptake of lipoproteins by hepatocytes and has shown the importance of apolipoproteins within lipoproteins and of heparan sulphate proteoglycans (HSPG) and high-affinity receptors on the cell-surface for this process (27). Several studies have suggested that the transport of vitamin K into different cell types is not uniform. Liver and bone are often cited as examples of tissues with differing functional vitamin K requirements (28–30) implying different modes, or different levels of efficiency, of vitamin K uptake.

To our knowledge, there have been no studies showing how vitamin K specifically is taken up by hepatocytes but based on the knowledge that newly absorbed vitamin K is largely carried by CR it seems safe to assume that vitamin K follows the well delineated pathway of CR uptake by the liver (27). On the other hand, the uptake of lipoproteins by bone has largely been neglected so that assumptions of how vitamin K is transported into bone cannot be made. For this reason, and given current interest in vitamin K and bone health, studies are now emerging of how vitamin K specifically is transported into bone cells. Newman et al. (31) added radiolabeled [<sup>3</sup>H]-phylloquinone to fractionated human lipoproteins and incubated them with cultured osteoblast cell lines to study mechanisms of vitamin K-lipoprotein uptake. Their findings demonstrated that phylloquinone uptake was reduced after enzymatic removal of cell-surface HSPG demonstrating that these molecules play a part in the process though the effect was more pronounced for phylloquinone carried by LDL than TRL which is the major carrier. Phylloquinone uptake was reduced by the presence of anti-apoE antiserum in the assay and was enhanced by prior enrichment of the lipoproteins with added apoE. Addition of apoE4 resulted in higher levels of phylloquinone uptake than E2 and E3 which agrees with other studies which

showed that CR are cleared more rapidly in individuals with the apoE4 genotype (32). Further evidence of the importance of apoE to osteoblastic uptake of vitamin K was provided by a group from Hamburg who demonstrated that the *ApoE* gene is strongly induced during the mineralization stage of primary mouse osteoblast cultures and that TRL uptake at this stage was lower in cells from *ApoE*-deficient mice than in controls (33). Osteocalcin from these *ApoE*-deficient animals was shown to have a greatly reduced hydroxyapatite binding capacity showing a high degree of undercarboxylation. This provides strong evidence at a functional level that a lack of apoE results in an impaired uptake of lipoprotein-borne vitamin K into osteoblasts (33).

In a further study from Hamburg, Niemeier et al. (34) characterised the receptors present in bone. Western blot analysis of cultured human osteoblast cell lines showed that low-density lipoprotein receptor-related protein 1 (LRP1) and low-density lipoprotein receptor (LDLR) were expressed at fairly high levels by all cell lines tested, but the VLDL receptor was only found in one of them. LRP1 expression on osteoblasts and osteoblast precursors was also detected by immunohistochemical staining of human bone sections. *In vitro*, cellular uptake assays of radio- and fluorescently labelled human CR using inhibitors specific for LRP1 and LDLR suggested that LRP1 is responsible for most of the CR uptake by osteoblasts (34). The specific uptake of vitamin K was studied by purifying CR from individuals who had received a 10-mg phylloquinone supplement, incubating the phylloquinone-enriched CR with osteoblasts and measuring cellular uptake of phylloquinone by HPLC (34). These experiments demonstrated that this phylloquinone uptake was stimulated by LPL and was sensitive to inhibition by lactoferrin; taken together the data is indicative of LRP1 making a substantial contribution to the uptake process (34). Very recently, Niemeier et al. (35) have provided the first direct evidence *in vivo* in a murine model that CR uptake by bone is quantitatively important for the delivery of dietary lipids to bone and, more specifically, that phylloquinone carried by CR is taken up by osteoblasts and is physiologically available for post-translational  $\gamma$ -carboxylation. To study these processes *in vivo*, Niemeier et al. injected mice intravenously with radio- or fluorescently labelled CR (35). They found that next to the liver, bone was the second most important organ for clearance of CR from the circulation, accounting for about 20% of the clearance attributable to the liver. The cells identified as taking up CR were sinusoidal endothelial cells, macrophages and osteoblasts. The authors speculated that the sinusoidal endothelial cells serve as a docking site to concentrate CR in bone marrow, and that CR are then transported through the endothelial fenestrae to the sub-endothelial space where CR are enriched with osteoblast-derived apoE before undergoing endocytosis by osteoblasts. In this sense apoE is postulated to serve the same secretion-capture role as described for uptake of CR by hepatocytes (27, 35). Finally and most importantly, Niemeier et al. (35) obtained evidence that the intravenous injection of phylloquinone-enriched CR resulted in a rapid and significant increase in the carboxylated form of osteocalcin in the mouse circulation. This represents the first demonstration *in vivo* that osteoblasts are able to rapidly take up CR and that phylloquinone carried by these particles can be utilised for  $\gamma$ -glutamyl carboxylation of osteocalcin.

The uptake of menaquinones has not been studied at the cellular level. Based on evidence that long-chain menaquinones such as MK-9 and MK-7 can remain circulating for extended times in LDL, it may be envisaged that they may be taken up by extrahepatic tissues by the ubiquitously expressed LDLR, as schematically postulated in Figure 2 for the uptake of MK-7 by bone. The ready uptake of MK-7 by bone has been shown indirectly by its greater efficacy than phylloquinone in carboxylating osteocalcin when taken as a daily supplement of 0.22  $\mu\text{mol/day}$  (26). This greater efficacy seems to be related to the greater residence time and higher serum concentrations of MK-7 achieved during prolonged intake of MK-7 (26).

## Tissue stores of vitamin K

The liver is the site of synthesis of the vitamin K-dependent coagulation factors and was originally thought to be the major site of storage of vitamin K. Vitamin K concentrations in liver vary widely; phylloquinone concentrations in post-mortem samples from British adults ranged from 2–47 pmol/g wet tissue with a median of 12 pmol/g and a median total liver pool of 18 nmol (36). Others have reported similar hepatic phylloquinone values in the Netherlands (37) and Japan (38).

In many species including humans, phylloquinone is a minor constituent of hepatic vitamin K content with the majority comprising long-chain menaquinones, MK-7 through to MK-13 (36–38). Typically the ratio is about 90% menaquinones and 10% phylloquinone (36, 38). A substantial proportion of the total menaquinone fraction comprises MK-10, 11 and 12 isoprenologues that are low constituents of most human diets (5) but are known to be produced by anaerobic *Bacteroides* species in the large intestine (2, 4, 14). From the concentration data, the average total liver pool of vitamin K in adults can be estimated to be in the range of 200 to 300 nmol but with wide inter-individual variations (36, 38).

With the widespread occurrence of extrahepatic Gla proteins there is a need for knowledge of the extrahepatic distribution of K vitamins. In a study of human tissues obtained at autopsy, Thijssen et al. found that only the heart and pancreas contained phylloquinone at comparable or higher levels than the 10 pmol/g found in the liver (37). Lower levels of phylloquinone (<2 pmol/g) were present in brain, kidney and lung. An intriguing finding was that MK-4 was ubiquitously present in extrahepatic tissues with particularly high levels, often exceeding phylloquinone, in the brain, kidney and pancreas. Absolute concentrations of MK-4 differed widely being relatively low in the heart and lung ( $\leq 1.0$  pmol/g) but about 10- to 20-fold higher in the brain, kidney and pancreas with the pancreas having a median concentration of MK-4 of 22 pmol/g (37). The origin of MK-4 is discussed in more detail in a later section. Notably, apart from the liver, the higher menaquinones (MK-6 to MK-11) were only found in some of the heart and pancreas samples and only at trace levels. In a previous study the same investigators had reported a similar pattern of distribution in the rat with MK-4 exceeding phylloquinone concentrations in the brain, pancreas, salivary gland and sternum (39).

What is missing is data from tissues with potentially large pools such as adipose tissue and skin (22). One study of bone-de-

rived lipid showed the presence of phyloquinone and three MK congeners (MK-6, MK-7 and MK-8) with phyloquinone generally predominating (24). However unpublished data from our laboratory using different methodology showed a different pattern with phyloquinone and MK-4 accounting for 60% and 22% of bone lipid, respectively, with the remaining 18% being equally divided between MK-5, MK-6 and MK-7; no higher isoprenologues were observed.

## Metabolic transformation of phyloquinone ( $K_1$ ) to menaquinone-4 (MK-4)

During the early 1960s, well before the discovery of  $\gamma$ -carboxyglutamic acid (Gla), a popular hypothesis of vitamin K action in animals (fitting with its quinone nature) was as an electron carrier in the respiratory chain and analogous to the function of the recently discovered ubiquinone (coenzyme Q) (40). The Swiss scientist Carl Martius had been a major proponent of this hypothesis from the 1950s and during the course of his studies had discovered a remarkable metabolic transformation aided by the use of isotopically labelled vitamin K compounds and what would seem to modern analysts a relatively crude separation procedure called countercurrent distribution (also called Craig partition), an early forerunner of reversed-phase chromatography (41, 42). In 1958 Martius (41) first showed that the organs of chicks and rats fed with 2- $^{14}$ C-methyl-1,4-naphthoquinone (menadione) (Fig. 1, I) contained a lipophilic compound indistinguishable by countercurrent distribution from 2- $^{14}$ C-methyl-3-geranylgeranyl-1,4-naphthoquinone, then known as vitamin  $K_{2(20)}$  but now renamed menaquinone-4 (MK-4) (Fig 1; IV). Martius postulated that since menadione is a precursor for MK-4, menadione may occur naturally in cells and be a degradation product of phyloquinone or other  $K_2$  vitamins. To study this, Martius fed doubly labelled phyloquinone (with  $^3$ H in the 2-methyl group of the naphthoquinone nucleus and  $^{14}$ C in the side chain) to chickens or pigeons and found that, apart from the unchanged starting material, several organs contained MK-4 labelled only in the nucleus (42, 43). The ratio of phyloquinone to MK-4 differed with organ and with time over the seven-day period of the experiment. Thus while unchanged doubly labelled phyloquinone was overwhelmingly present in the liver, with only a trace of MK-4, other organs like the heart, muscles, and kidney contained a much higher proportion of MK-4 than phyloquinone, even as early as 3 h after feeding the phyloquinone. In addressing the site of this metabolic transformation, Martius and co-workers found that when phyloquinone was administered parenterally to pigeons, there was no conversion of phyloquinone to MK-4 (44). They postulated that the first step in the conversion of phyloquinone to MK-4 takes place in the intestine but their experiments to show the side chain removal by bacteria were not convincing (44). Interestingly, using doubly labelled nucleus and side chain-labelled MK-4, they were also able to show that side-chain removal was not confined to phyloquinone and that the geranylgeranyl side chain of MK-4 can be split off and replaced by an unlabelled geranylgeranyl side chain (44).

The state of knowledge on this inter-conversion remained largely unchanged for about 30 years until John Suttie in Wiscon-

sin and Henk Thijssen in Maastricht took up the challenge using modern reversed-phase HPLC technology to separate and quantify phyloquinone and MK-4. In 1992 John Suttie's group confirmed the early work of Martius by showing that chicks could convert oral phyloquinone to MK-4 but in contrast to Martius, found that this conversion also occurred after intravenous injection of phyloquinone (45). Thijssen et al. (39) studied the tissue distribution of vitamin K in rats and like Martius found that the liver was high in phyloquinone and low in MK-4 while many extrahepatic tissues were rich in MK-4. To investigate the source of tissue MK-4, Thijssen analysed the tissue distribution after feeding rats a vitamin K-free diet followed by resupplementation with phyloquinone (39). These experiments clearly suggested that dietary phyloquinone was the natural source of vitamin K, since the tissue levels of both phyloquinone and MK-4 fell in deficient animals but rose again when the only dietary source was phyloquinone. Overall the same tissue selective pattern of phyloquinone-rich tissues (liver, heart) and MK-4-rich tissues (brain, kidney, pancreas, salivary gland) was maintained throughout this dietary manipulation. The fact that the diets used in these specific experiments contained no menadione (a normal component of commercial rat food) ruled out dietary menadione as a source of the tissue accumulation in these experiments. To investigate the possibility that the MK-4 was of bacterial (intestinal flora) origin, rats were also given phyloquinone by the intravenous route whereupon MK-4 rose to higher levels than after oral phyloquinone (39). Although Thijssen and Drijt-Reijnders concluded that dietary phyloquinone was the likely source of tissue MK-4, they thought that it may have originated via enterohepatic circulation of injected phyloquinone. However, this is unlikely because it has been shown, at least in humans, that phyloquinone is excreted in bile as polar metabolites which are excreted in the faeces without evidence of re-absorption (23). It is also unlikely that MK-4 is a product of bacteria because MK-4 is not a major bacterial form, especially of the intestinal flora (1, 4).

Final proof that the conversion of phyloquinone to MK-4 was not dependent on gut bacteria was provided independently by the groups of Thijssen (46) and Suttie (47) who showed that germ-free rats could carry out this conversion equally well as control animals. Having shown that animals have an intrinsic ability to transform phyloquinone to MK-4 the question arises as to whether the tissues that accumulate MK-4 do so through local biosynthesis or whether MK-4 is delivered to the target organ via the blood from a separate synthesising organ such as the liver. The findings that after oral phyloquinone administration to rats, MK-4 concentrations in target tissues rise much more slowly than that of phyloquinone (48) and MK-4 levels in plasma and liver are always low argues in favour of local synthesis (39, 48). If menadione is transported in blood it is likely to need a specific carrier because it is known that the reactive 3-position will bind non-specifically to thiols of large and small molecules; indeed this is partly the basis of its toxicity at large doses (49). The ability of various cell lines to carry out this conversion has been investigated, but the results have not been conclusive; some have not been able to demonstrate the conversion in any cell line (50), whereas others found evidence of the conversion in the kidney HEK-293 kidney cell line (47) and very recently by primary cultures of mouse cerebral hemispheres (51).

An unexplained phenomenon is the inconsistency of results on the dependence of the route of administration. Thus some studies failed to find the conversion after intravenous or parenteral administration of phylloquinone (44, 50, 51) while others have reported that MK-4 synthesis did occur after intravenous injection of phylloquinone (39, 45).

### Mechanism of phylloquinone to MK-4 transformation

Major questions still surround the biochemical pathway for the transformation of phylloquinone to MK-4. Two routes can be readily envisaged, either entire removal of the phytyl side chain followed by prenylation of the menadione or desaturation of the phytyl side chain. Martius favoured the former and in using phylloquinone specifically labelled at positions 1', 2' of the side chain produced evidence of the isolation of the phytyl side chain as an ester of phytanic acid suggesting complete side chain cleavage with oxidation of phytol to the acid and saturation of the 2',3' double bond (44). Side-chain removal also fits in with the ready ability of tissues to alkylate menadione from precursor pyrophosphates of polyisoprene alcohols. Liver homogenates alkylate pyrophosphates of geraniol, farnesol and geranyl-geraniol to produce menaquinones of the respective side chain lengths, although MK-4 with the C<sub>20</sub> side chain is the only major product seen *in vivo* (42, 52).

The measurement of the urinary output of menadione in humans in response to phylloquinone supplementation has lent support to the view that menadione is an intermediate in the phylloquinone to MK-4 conversion (50). Thus, menadione excretion in urine increased after single doses of oral phylloquinone and mirrored phylloquinone intakes in a 30-day depletion/repletion metabolic study (50). However, menadione excretion was not enhanced when phylloquinone was given subcutaneously or when subjects were supplemented with 2', 3' dihydrophylloquinone, a phylloquinone derivative that may occur in hydrogenated foods (50). Further evidence for the lower incorporation of 2', 3' dihydrophylloquinone into MK-4 was recently obtained in a comparative study in which rats fed dihydrophylloquinone showed significantly less accumulation of MK-4 in certain tissues (kidney, heart, testis and brain myelin) than rats fed phylloquinone (53).

A new study by Okano et al. (51) has employed NMR and LC-MS/MS technology to follow the transformation of stable isotope-labelled menadione and phylloquinone to MK-4 in mice with special reference to the accumulation of MK-4 in brain tissues. This study has provided unequivocal evidence that MK-4 is present in cerebra of mice and can be equally derived from the intestinal absorption of both menadione and phylloquinone. The conversion and cerebral accumulation of MK-4 from oral phylloquinone was dose-dependent over a physiological dose of 0.1  $\mu\text{mol/kg}$  body weight to a pharmacological dose of 10  $\mu\text{mol/kg}$  body weight. If phylloquinone was administered intravenously or by direct injection into the cerebra there was almost no detectable labelled conversion to MK-4. However, the phylloquinone to MK-4 conversion was readily detectable in cerebral slice cultures. Menaquinone-4 was also synthesised from phylloquinone by primary cultures of mouse cerebral hemispheres, albeit inefficiently, but not by cultures of neurons and astrocytes. On the other hand, MK-4 was readily synthesised by all these

cells when supplied with menadione (51). Using phylloquinone with deuterium labelling in both the ring and side-chain, Okano showed that the side chain of newly labelled MK-4 was unlabelled thus pointing to complete side chain removal of the phylloquinone substrate (51). Unfortunately, for methodological reasons, it was not possible to measure the putative menadione intermediate.

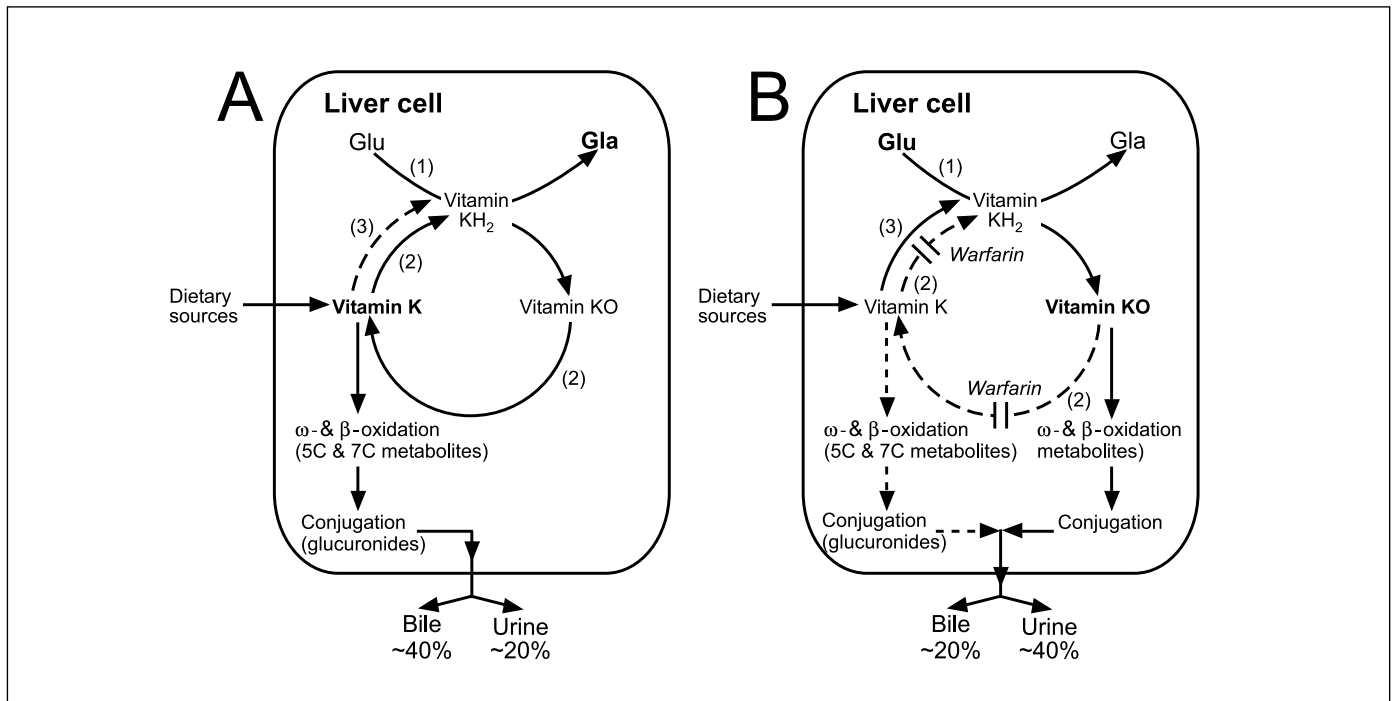
### Vitamin K catabolism

Most of our knowledge of the turnover and extent of metabolism of vitamin K in humans comes from studies with phylloquinone. The catabolism and ultimate loss of phylloquinone from the body via excretion is extensive, and accounts for the relatively low circulating and tissue stores compared to vitamins A, D and E. Based on early isotopic work in humans (23), it has been estimated that about 60–70% of a single dose of vitamin K is ultimately excreted as catabolic products in the bile and urine regardless of whether the administered dose was within the dietary range (45  $\mu\text{g}$ ) or in the low pharmacological range (1 mg) (Fig. 3).

Despite the therapeutic use of large pharmacological doses (45 mg/day) in Japan there appears little data on the absorption, and excretion of MK-4 in humans. In rats, a direct comparison of the autoradiographic body distribution and metabolism in rats of equimolar doses (1  $\mu\text{mol}$ ) of 2-<sup>14</sup>C-methyl labelled compounds showed that the removal of MK-4 from the liver is much faster than for phylloquinone and that 74.1% of the MK-4 was excreted in the bile within 12 h as compared to only 8.5% for phylloquinone (54). This large excretion difference was not accompanied by any detectable difference in the organ distribution of phylloquinone and MK-4 at 24 h (54). A much reduced liver retention of MK-4 compared to phylloquinone is also supported by the lower efficacy and shorter duration of effect in supporting coagulation factor synthesis when single doses were given orally or subcutaneously to vitamin K-deficient rats (55). Although animal data cannot be directly extrapolated to humans, the peak plasma concentration after oral administration of MK-4 was lower and earlier than for an equimolar dose (2  $\mu\text{mol}$ ) of phylloquinone (19). Coupled with knowledge of the low hepatic and circulating levels of MK-4, the data is at least compatible with the rat data of a more extensive catabolism of MK-4 than phylloquinone. This question could be resolved by stable-isotope studies of MK-4 metabolism in humans.

Human urinary metabolites of phylloquinone comprise glucuronide conjugates of two major carboxylic aglycone moieties (56). Their chemical structures have been characterized as side chain-shortened carboxylic acids, one of which has a 7C-side chain with the 1', 2' double bond retained (Fig. 1; VI) and the other of which has a saturated 5C-side chain (Fig. 1; VII) (23, 57). The same two metabolites are produced by animals (58).

By analogy to the metabolism of similar isoprenoid compounds (ubiquinone, vitamin E), as well as fatty acids, steroids and prostaglandins, it was proposed that side-chain degradation proceeds via an initial  $\omega$ -hydroxylation followed by  $\beta$ -oxidation (59). That the liver is central to the elimination of phylloquinone from the body was shown by the finding that hepatectomized rats given 2-methyl <sup>14</sup>C labelled phylloquinone excreted virtually no



**Figure 3: Scheme showing hepatic metabolism of vitamin K in absence (A) and presence of warfarin (B).** The conversion of peptide-bound glutamic acid (Glu) to  $\gamma$ -carboxy glutamic acid (Gla) in vitamin K-dependent proteins is linked to an enzymic cycle called the vitamin K-epoxide cycle which carries out both  $\gamma$ -glutamyl carboxylation and serves as a salvage pathway to recover vitamin K from its epoxide metabolite (KO) for reuse in carboxylation. Enzyme activities shown are (1)  $\gamma$ -glutamyl carboxylase; (2) vitamin K epoxide reductase (VKOR) and (3) NAD(P)H-dependent quinone reductase(s). The active form of vitamin K needed by the  $\gamma$ -glutamyl carboxylase is the reduced form vitamin K quinol (KH<sub>2</sub>). An obligatory metabolic consequence of  $\gamma$ -carboxylation is that KH<sub>2</sub> is oxidised to KO which in turn undergoes reductive recycling, first to the quinone and then to KH<sub>2</sub>. A) Under usual physiological conditions vitamin K is probably mainly recycled by VKOR. B) However, in the presence of vitamin K antagonists such as warfarin the activity of the VKOR (2) is blocked leading to a build up of KO in the cell. An alternative hepatic quinone reductase activity (3) can bypass the

warfarin inhibition of the VKOR to provide the KH<sub>2</sub> substrate for the carboxylase enzyme and overcome the inhibitory action of warfarin, even under extreme blockade. The liver is also the site of a catabolic pathway, common to phylloquinone and MKs, whereby their respective side chains undergo  $\omega$ -oxidation followed by  $\beta$ -oxidation leading to two major aglycone metabolites with side chain lengths of five and seven carbon atoms respectively (5C and 7C metabolites). After conjugation (mainly with glucuronic acid) these metabolites are excreted in the bile and urine. Phylloquinone, the major dietary form, is rapidly and extensively catabolised in humans with about 40% of a daily physiological dose being excreted via the bile and 20% via the urine (A). In the presence of warfarin (B) the excretion pathways are reversed such that urinary excretion predominates over biliary excretion. Evidence suggests that the major urinary metabolites during warfarin therapy originate from  $\omega$ - and  $\beta$ -oxidation metabolism of KO although the metabolites have not been formally characterized. There is no equivalent excretion data for MKs.

radioactivity in the urine within 24 h compared to 10% in control animals (60). This was in marked contrast to hepatectomized rats given 2-methyl <sup>14</sup>C menadione which excreted the same proportion of the dose (~70%) in urine as rats with an intact liver (60).

Although these carboxylic acid aglycone metabolites were characterized in the 1970s, a method that allows their routine measurement at physiological levels of excretion has only been developed recently (61). As expected from the proposed common metabolic pathway, the same 5C- and 7C-side chain aglycones were excreted and their levels rose after human volunteers took supplements of menaquinones (MK-4 and MK-7) in the same way as after phylloquinone supplementation (61). Supplementation with menadione also results in an increased output of these metabolites and this can be explained by the conversion of menadione to MK-4 and subsequent degradation of the side chain of MK-4. The existence of this common pathway means that the urinary excretion of these two metabolites is representative of the turnover and excretion of all body stores of vitamin

K. A subsequent metabolic study showed that urinary excretion of these two metabolites responds to dietary manipulations of phylloquinone and dihydrophylloquinone suggesting its utility as a marker of vitamin K dietary exposure and status (62).

The enzymic pathway for the catabolism of K vitamins has not been studied in detail. By comparison with the catabolic pathway for similar compounds, it seems likely that the initial  $\omega$ -oxidation step is carried out by cytochrome P450 (CYP). For example the  $\omega$ -oxidation of tocopherols is catalysed by CYP4F2 (63) but a role for the isoform CYP3A4 is also implicated in the metabolism of higher concentrations of tocopherols and also vitamin K (64, 65). This is because both vitamin E and vitamin K upregulate CYP3A isoforms by binding to and activating the steroid and xenobiotic receptor (SXR), also known as the pregnane X receptor (PXR) (65–67). The SXR is a nuclear receptor which acts as a sensor for the presence of many drugs such as phenobarbital, taxol, rifampicin and others and initiates the detoxification process. SXR is involved in the transcriptional regulation of

enzymes such as cytochrome P450s and transporter molecules which clear these substances from the body. The relative abilities of isoprenoid compounds to activate SXR have been studied in a HepG2 cell culture model transfected with the human SXR and the chloramphenicol acetyl transferase (CAT) gene linked to two SXR responsive elements (64). In this model MK-4 was a strong activator of gene expression, inducing SXR-mediated CAT activity by eight-fold (similar to rifampicin but slightly less than  $\gamma$ -tocotrienol) while phylloquinone gave a 2.8-fold increase (66). The efficacy of SXR activation does not necessarily reflect the degree to which various isoprenoids are metabolized (64) but isoprenoids with an unsaturated side chain like MK-4 tend to have greater SXR-activating properties than molecules with more saturated side chains (66). This induction of CYP3A by the substrate itself may be one mechanism for regulating the excretion of excess K vitamins. It also raises the possibility of detrimental interactions between vitamin K and E when either is given at pharmacological doses (65). An existing example of a probable drug-induced CYP interaction with vitamin K metabolism is the well known ability of anticonvulsants to induce a state of vitamin K deficiency in newborns exposed *in utero* to anticonvulsant drugs such as phenytoin and carbamazepine (68). This interaction has not been described in adults, but the human infant is born with precarious vitamin K stores so that any factor that may cause increased degradation of vitamin K may tip this balance towards a deficient state. The implications of SXR activation to the possible non-carboxylation functions of MK-4 are discussed in the section *Molecular cell biology of menaquinones*.

Although there is no direct proof, it has been presumed that the  $\beta$ -oxidative pathway of side-chain shortening is carried out in mitochondria, an organelle that together with the endoplasmic reticulum is known to accumulate vitamin K (69, 70). The fact that vitamin K-dependent carboxylation occurs in many cell types and tissues, raises the question of whether the primary degradative pathway to the carboxylic acid metabolites can occur outside of the liver. It is known that fibroblasts in cell culture rapidly metabolize radiolabelled phylloquinone and phylloquinone epoxide to unknown polar metabolites but their identity has not been investigated (71). Again it is presumed that the phase II conjugation with glucuronic acid occurs in the liver although glucuronidation of xenobiotics by the gastrointestinal mucosa and the kidney has been reported.

## Effect of dietary vitamin K deficiency on tissue distribution and metabolism of K vitamins

Little is known of how the tissue distribution and metabolism of K vitamins may change during the progression towards vitamin K deficiency and whether or not certain tissue stores can be mobilized to protect essential functions, the obvious critical one being maintenance of haemostasis. Among the factors that need to be taken into consideration are differences in bioavailability, transport, tissue distribution, metabolic handling and turnover of individual vitamin K isoprenologues. Then there is the age-old question of the extent to which intestinal synthesis of bacterial menaquinones can maintain hepatic and extra-hepatic synthesis

of Gla-proteins, both when nutritional supply is optimal but more relevantly when dietary intakes are compromised.

We are fortunate in having certain blood measures that act as surrogate markers of the  $\gamma$ -carboxylation status of Gla-proteins, some of which are tissue specific. While there are issues on how to interpret assays for undercarboxylated proteins, at best they are true functional markers and, together with plasma measurements of K vitamins, provide a good picture of the bioavailability of vitamin K at a tissue level. At the present time, assays for undercarboxylated Gla-proteins have been validated for hepatic prothrombin (PIVKA-II or ucFII) and bone osteocalcin (ucOC) while tests for MGP are coming on stream (see article by Schurgers et al. in this issue beginning on page 593). Although there are methodological uncertainties with the quantitative determination of the  $\gamma$ -carboxylation status of bone osteocalcin it is generally agreed that to avoid most of the influence of bone turnover effects, the values of ucOC that represent the component of vitamin K bioavailability are best expressed as a percentage or ratio to total osteocalcin and that a correction factor for total circulating osteocalcin is also advisable (72).

## Dietary deficiency in rats

Studies of vitamin K deficiency in rats are complicated by the fact that rats may derive a significant proportion of their dietary vitamin K requirements from coprophagy. A useful experimental animal model for studying the effects of vitamin K deficiency arose out of the serendipitous findings that rats given a low-fibre diet based on boiled white rice developed severe vitamin K deficiency within 23 days (14). Besides providing negligible dietary vitamin K, the rice diet reduced intestinal synthesis of menaquinones (14). When combined with tail cups to prevent coprophagy a protocol was developed in which rats became severely vitamin K-deficient in one week (55). Using this rat model, Sato et al. (29) studied the differential effects of vitamin K deficiency on the tissue levels and Gla-protein synthesis in liver and bone. A period of 17 days of the vitamin K-deficient diet was sufficient to deplete liver stores of phylloquinone to 4% of the initial level and to reduce the originally low hepatic MK-4 to undetectable levels. Over the same period the levels of both phylloquinone and MK-4 in bone femur only declined to 40% of the initial levels. It should be emphasized that MK-4 was by far the most predominant vitamer in bone (double the phylloquinone levels and declining in parallel to phylloquinone on the vitamin K-deficient diet) whereas in liver MK-4 only accounted for some 5% of the phylloquinone level in the vitamin K-repleted state. The different response of liver and bone to acute vitamin K deficiency was reflected in Gla-protein synthesis in the respective organs with the decline in liver levels being reflected by a doubling of the prothrombin time (PT) but no change in %ucOC. These results suggested that the turnover of vitamin K in the femur was slower than that of the liver. In the second part of the same study Sato et al. (29) administered an equimolar single dose (250 nmol/kg) of either phylloquinone, MK-4 or MK-7 separately to three groups of vitamin K-deficient rats and over the following three days showed that the level of all three vitamers markedly increased in the liver but not in the femur. This dose was sufficient to give a rapid correction of the PT but liver levels of MK-7 declined much more slowly than phylloquinone or MK-4. The slower hepatic metab-

olism of MK-7 is common to all long-chain menaquinones and is in agreement with previous findings showing that MK-9 also had a slower hepatic turnover than phylloquinone in rats (73) and maintained prothrombin synthesis for a longer period than phylloquinone or MK-4 (55).

The majority of studies into dietary vitamin K deficiency in rats, like the Sato study above (29), have tried to induce an acute vitamin K-deficient state. The question arises does vitamin K metabolism adapt when a less severe deficiency is induced over a longer time period? To study the chronic effects on bone of a less severe dietary deficiency, Binkley et al. fed rats a low vitamin K dietary intake (20 µg/kg diet) for 80 days (74). In contrast to the acute deficiency model of Sato, the rats in this chronic deficiency study maintained a normal PT but the %OC rose from a value of 25% in rats fed the control diet to nearly 50% in rats fed the deficient diet. Although no vitamin K measurements were made in this study, the functional status markers clearly suggest that a chronic dietary deficit of phylloquinone had no haemostatically relevant effect on the synthesis of vitamin K-dependent coagulation factors but did significantly impair  $\gamma$ -carboxylation of bone Gla-proteins such as OC. One possible design difference in the Binkley study was that although attempts were made to minimize coprophagy, it may not have been fully prevented as in the Sato study. It is possible that small amounts of long-chain menaquinones obtained by coprophagy may have been absorbed and sequestered by the liver. There is evidence that such bacterial menaquinones are much less available to bone than phylloquinone. Thus, concentrations of long-chain menaquinones are very much higher in liver than in bone. In two comparable studies (29, 39), significant concentrations of MK-6 through to MK-9 were detected in rat liver but apart from a very small concentration of MK-7 in one study (29), the same menaquinones were undetectable in bone.

### Dietary deficiency in humans

To our knowledge there is only one human study by Usui et al. (38) that has been able to directly address the question of how sudden and severe dietary vitamin K depletion may affect tissue stores. This study investigated changes in plasma and liver-biopsy vitamin K concentrations in patients pre- and post-gastrointestinal surgery. The patients were divided into two groups according to whether they received a standard or low vitamin K diet before the operation. After the operation all patients received only electrolytes, water-soluble vitamins and antibiotics for three days. In the group fed preoperatively with the low vitamin K diet (5 µg K<sub>1</sub>/day) for three days, plasma phylloquinone fell by over 50%. In the group fed the standard diet (150–450 µg K<sub>1</sub>/day), plasma phylloquinone fell precipitously by about 70% during the first 24 h of the three-day post-operative fasting period; thereafter the levels declined only slightly and had plateaued by three days. Although fasting induced a fall in MK-7 levels, the slope of disappearance was much more gradual than for phylloquinone. Perhaps the most significant finding came from the comparison of the liver biopsy analyses for the standard and low vitamin K diets. This data showed that whereas after three days of the low phylloquinone diet the liver contained only 25% of the phylloquinone content of patients on the standard diet, the hepatic menaquinone concentrations remained essentially un-

changed. This study showed for the first time by direct vitamin K measurement that phylloquinone stores in the liver are very labile and readily depleted after only a few days of dietary depletion. Importantly, it also showed a slower decline of MK-7 in plasma and a negligible decline of all long-chain menaquinones in the liver. This is in keeping with the prediction of Duello and Matschiner (75) that the turnover of long-chain menaquinones would be slower because their highly lipophilic nature confers a greater affinity for membranes but represented the first definitive proof in humans that long-chain menaquinones have a slower metabolic turnover. Although there was a non-significant trend for the PT to increase in both groups post-operatively, the PT values always remained within the reference range (38). Unfortunately no sensitive functional marker of vitamin K status such as PIVKA-II was employed to see if subtle changes in  $\gamma$ -carboxylation of the vitamin K-dependent coagulation factors were completely preventable by the substantial hepatic concentrations of menaquinones.

Using such sensitive biomarkers in a depletion-repletion study, Booth et al. (76) established that a vitamin K-restricted diet taken by young adults for 15 days resulted in a sufficient lowering of tissue stores to cause an increase in PIVKA-II and %OC. The depletion-diet of 10 µg/day of phylloquinone represented about one-tenth of average habitual intakes but intakes as low as this have been recorded in some healthy people (8). The increase in PIVKA-II represents only a small fraction of native, carboxylated prothrombin and there was only an average 0.3-second increase in the PT, which though statistically significant, was not clinically relevant. The decreased  $\gamma$ -carboxylation of osteocalcin was gradual but substantial and the value of %OC rose by 67% (28% to 47%) after 15 days of dietary depletion (76). The changes in both PIVKA-II and %OC were reversed by a subsequent 10-day repletion phase with phylloquinone at a dose of 200 µg/day (76). Analyses of the archived 24-h urine samples from this same depletion-repletion study revealed that urinary excretion fell progressively to ~70% of that at study entry during the 15-day depletion phase (62). However, evidence from the same study suggested that 7C-aglycone excretion was still falling, at approximately the same rate, after 25 days of the restricted diet containing only 10 µg/day of phylloquinone. Making certain assumptions, it was also predicted that after the period of dietary restriction some 85% of the daily metabolite excretion might have originated from non-dietary sources. This would include dietary menaquinones (not likely to be more than 20 µg/day [11]) and mobilized tissue stores of K vitamins. The lack of a plateau in the excretion rate after nearly a month on a very low phylloquinone intake suggests the existence of a pool(s) of vitamin K with a slow turnover rate. The fact that the slow loss of vitamin K via excretion was accompanied by the aforementioned increases in both PIVKA-II and %OC shows that some of these losses represented losses of functional stores of vitamin K from the liver and bone, respectively (62).

The dietary restriction study (76) described above and others of similar design (77) show that low intakes of dietary phylloquinone can, within only two weeks, result in measurable decreases in the  $\gamma$ -carboxylation of both hepatic and bone Gla proteins. In the case of the coagulation proteins, the changes after 13–15 days of depletion were clinically insignificant (76, 77), showing

that there is a considerable buffer of liver vitamin K stores that prevents any major effects on coagulation during short-term dietary depletion of healthy subjects. However, as reviewed by Suttie (4), significant lowering of vitamin K-dependent coagulation proteins, sufficient to be detected by conventional clotting tests, has been achieved (in healthy subjects and patients) over about one month by using more drastic measures to reduce vitamin K intakes, including giving antibiotics to parenterally fed patients. Such studies have provided evidence that intestinal menaquinones cannot provide enough vitamin K to maintain hepatic stores of vitamin K under conditions of zero or negligible vitamin K intakes. The more recent data using more sensitive functional status indices in subjects whose intakes were reduced to about 10 µg/day (76, 77) also support the concept (4, 15) that the potential source of intestinal menaquinones is less important than sometimes presumed. The study by Usui et al. (38) clearly highlights the importance of pre-existing liver stores of long-chain menaquinones with slow turnover in providing a buffer against overt deficiency if phylloquinone intakes are low. On the other hand human bone tissue, unlike liver, lacks the same reservoir of long-chain menaquinones, and this may account for the greater absolute changes in the degree of carboxylation of osteocalcin compared to that of prothrombin seen in phylloquinone depletion studies (76).

## Effect of oral anticoagulants on the metabolism of K vitamins

The discovery that warfarin induces the accumulation in tissues of a stable metabolite, vitamin K 2,3 epoxide (Fig. 1; III) was made by Matschiner et al. (78) before the  $\gamma$ -carboxylation function of vitamin K itself was known. This metabolite led to the discovery of enzyme vitamin K epoxide reductase (VKOR) as the target of coumarin action and to the discovery of the vitamin K-epoxide cycle (Fig. 3). The finding in Matschiner's experiments that more than 50% of administered phylloquinone was recovered in the liver as phylloquinone epoxide (78) shows the extent to which warfarin diverts normal vitamin K metabolism. No known adverse consequences from the excess epoxide are known in humans. During therapeutic anticoagulation there appears to be a shift in metabolite excretion of phylloquinone from the usual major biliary route to the urinary route (Fig. 3) and only about 10% of the urinary metabolites are identifiable as the normal 7C- and 5C-side-chain metabolites (23). The warfarin-induced metabolites have not been characterized but they appear to be analogous to side-chain shortened metabolites of phylloquinone epoxide (23).

The ability to dissociate the vitamin K requirements of Gla proteins in hepatic and extrahepatic tissues is best illustrated by the warfarin rat model developed by Paul Price (30). This model arose out of the desire to study the effects of warfarin on the  $\gamma$ -carboxylation of osteocalcin in bone (and later  $\gamma$ -carboxylation of MGP in vascular tissues) without compromising synthesis of vitamin K-dependent coagulation factors which in usual protocols led to animals bleeding. Price found that provided the animals had received an appropriate high-dose phylloquinone treatment by subcutaneous injection prior to and concurrent with

warfarin administration, daily doses as high as 7.7 mg warfarin per 100 g body weight could be administered without affecting coagulation. This warfarin dose was approximately 150 times higher than the 50 µg per 100 g body weight required to double the prothrombin time in rats which were not given extra vitamin K. In contrast the warfarin dose of 30 µg per 100 g required to reduce the  $\gamma$ -carboxylation status of osteocalcin by about 50% of normal was essentially unaffected by the high-dose vitamin K regimen (30). This warfarinized animal model indicates that vitamin K can overcome warfarin antagonism in liver but not in bone. One factor considered by Price to account for these findings is that the liver takes up phylloquinone more avidly than bone. However, this cannot be divorced from considerations of the metabolism of warfarin which is known to be metabolized in the liver by cytochrome P450 enzymes that are presumably absent or of low activity in bone.

Price's high-dose warfarin model applies not just to bone but to other extrahepatic tissues. In a modified model using more frequent warfarin administration to lessen plasma fluctuations and higher vitamin K doses to prevent bleeding, Price was able to induce rapid calcification of rat arteries and heart valves (79). Apart from the possibility of poor uptake of vitamin K by vessel walls another reason for the poor antidotal effect of vitamin K is that NAD(P)H-dependent quinone reductase in vascular smooth muscle cells (VSMC) is 100-fold less active than in the liver (80). In the liver, NAD(P)H-dependent quinone reductase serves as a rescue enzyme to bypass the warfarin-inhibited VKOR and so facilitate reduction of vitamin K quinones to their hydroquinone cofactors (Fig. 3). The low activity of this quinone reductase in the vessel wall shows that its carboxylation system is similar to bone and that the antidotal pathway cannot maintain  $\gamma$ -carboxylation of MGP during high-dose warfarin treatment (80). Surprisingly, the VKOR activity in VSMC was three-fold higher than the liver activity raising questions about additional functions of the vitamin K cycle in the vessel wall beyond  $\gamma$ -carboxylation (80).

Using the same warfarin/vitamin K model, Schurgers et al. showed that warfarin-induced calcification in rats could be partially reversed by high-dose phylloquinone or MK-4 within six weeks of stopping warfarin (81). Measurements of vitamin K in rat aortas showed that whereas control rats had twice as much MK-4 as phylloquinone, no MK-4 was detectable in the warfarin treated rats although the aortas accumulated large amounts of phylloquinone and phylloquinone epoxide. The high amounts of epoxide suggest that the VKOR cycle was active but could not prevent calcification. The absence of MK-4 in the arteries of the combined warfarin/vitamin K-treated rats is in agreement with older data showing that prenylation of menadione to MK-4 is inhibited by coumarin antagonists (82). The site of this blockade on the conversion to MK-4 has yet to be determined. On stopping warfarin, high-doses of phylloquinone or MK-4 led to a similar high aortal accumulation of MK-4 suggesting that this form may be important in vessel wall function.

## Vitamin K-oral anticoagulant interactions in humans

Given the widespread clinical use of oral anticoagulants, surprisingly little attention has been paid to the interaction between different levels of dietary intakes of K vitamins with anticoagulant-

induced effects on coagulation and extrahepatic functions. In one extensive study, healthy volunteers were stably anticoagulated (international normalised ratio [INR] 2.0) with acenocoumarol and maintained on the same dose so that the response to weekly incremental doses of dietary phylloquinone (ranging from 50–500 µg) could be measured (83). The threshold dose causing a statistically significant lowering of the INR was 150 µg/day. Using more sensitive functional markers (PIVKA-II and ucOC) it was shown that whereas hepatic carboxylation of FII was significantly increased at a dose of 100 µg, a dose of 300 µg phylloquinone was needed to affect the carboxylation status of osteocalcin (83). These findings are similar to the warfarin/vitamin K animal model and presumably reflect the better uptake of phylloquinone by the liver than the bone. In a parallel study with a similar design, the effects of weekly incremental doses of MK-7 were examined in anticoagulated volunteers (26). The results showed that MK-7 is much more effective than phylloquinone in lowering the INR in stably anticoagulated individuals, thus having the potential for greater interference if patients take high doses of MK-7 as nutritional supplements during anticoagulant therapy. Together with results showing that MK-7 is better than phylloquinone in increasing osteocalcin carboxylation in non-anticoagulated individuals (26), the data confirms the greater all-round efficacy of MK-7 for carboxylation of liver and bone Gla proteins. This can at least be partly explained by higher blood levels of MK-7 resulting from its different transport pathway compared to phylloquinone (19). The greater efficacy of MK-7 may be beneficial in correction of chronic nutritional vitamin K deficiency states, but great care needs to be taken if MK-7 supplements are used by patients on oral anticoagulant therapy.

There is evidence that patients taking oral anticoagulants who have a low dietary intake of vitamin K are more likely to suffer from unstable anticoagulant control (84). This implies that INR stability may be improved by giving regular daily supplement of vitamin K and evidence for this was recently obtained in the first randomized, controlled trial of vitamin K supplementation (85). Seventy patients with atrial fibrillation (AF) in the UK were selected as having unstable anticoagulant control (defined as standard deviation [SD] of INR >0.5) and were randomly assigned to receive either a daily dose of 150 µg phylloquinone or a placebo orally for six months. Vitamin K supplementation resulted in improved stability as evidenced by more than a two-fold decrease in the SD of INR and nearly a two-fold increase in the percentage time spent in range (85). Anticoagulation control improved in 33/35 patients taking vitamin K (with 19 now classifiable as stable with SD of INR <0.5) compared to 24/33 patients taking the placebo (only 7 of whom were stable). Another randomized controlled trial in the Netherlands used a lower daily dose of 100 µg phylloquinone and showed only a relatively modest 4% improvement of time in the therapeutic range for the vitamin K group over the placebo group (86). It seems likely that this Dutch study was underpowered and that factors other than vitamin K dose or the anticoagulant (UK warfarin, Holland phenprocoumon) accounted for the modest effect. Factors which may have contributed to the different outcomes could have been firstly, population differences such that UK patients were pre-selected, unstable patients, all with AF whereas

Dutch patients had already been anticoagulated for one year; secondly, baseline differences such that 59–63% patients were in range in the UK compared to 79–80% in the Netherlands; thirdly, target range difference which was narrower in the UK (all 2.0–3.0) than in the Netherlands (either 2.0–3.5 or 2.5–4.0). Nevertheless these appear promising results. The pros and cons of whether it would be better to use MK-7 rather than phylloquinone have been debated (87, 88). The advantage of phylloquinone is that it has already been tested in a patient population. The criticism that use of extra phylloquinone increases the warfarin dose (by 16% in the Sconce study), and therefore increases susceptibility to arterial calcification needs to be considered in the context that the calcification model developed by Price (79) translated to an average patient would mean giving a twice daily dose of 10 grams warfarin and a daily dose of 1 gram phylloquinone administered subcutaneously. This is not to deny evidence that long-term warfarin therapy may increase calcification of the aortic valves (89).

## Molecular cell biology of menaquinones

Phylloquinone and menaquinones differ only in the structure of their 3-side chains; however, in addition to the well-known function of K vitamins as cofactors for  $\gamma$ -glutamyl carboxylase a considerable body of evidence now shows that they have additional properties in certain cell and tissue types, particularly in bone and certain cells of the immune system. There is a wealth of literature on cell biological aspects of vitamin K in several different fields, and the findings are not all consistent. The diversity of the literature is partly due to the multiple actions that appear to be mediated by vitamin K which are as yet, far from well understood. What follows is an attempt to review the relevant literature, much of which concerns the menaquinones, particularly MK-4.

### Menaquinones as cofactors for $\gamma$ -glutamyl carboxylase

Menaquinones are capable of acting as cofactors for  $\gamma$ -glutamyl carboxylase in the same way as phylloquinone although the efficacy of this function has at times been controversial (reviewed by Suttie [4]). Measurements of prothrombin time in vitamin K-deficient chicks that were then orally administered with various forms of vitamin K seemed to suggest that menaquinones with chain lengths greater than eight prenyl units were less active in reducing prothrombin time than phylloquinone (90). However, subsequent work in rats injected intracardially with vitamin K showed the opposite result, i.e. that long-chain menaquinones were more active (91), which suggests the results were due to differences in bioavailability between the two administrative routes rather than to differences in carboxylase cofactor activity *per se*. Buienhuis et al. (92) measured the relative ability of phylloquinone and several menaquinones to act as cofactors for  $\gamma$ -glutamyl carboxylase in a partially purified hepatic microsomal enzyme preparation. Their results showed that phylloquinone and MK-4 had roughly similar cofactor activity with MK-3 having the highest of all those tested and menadione had none at all. The activity of the menaquinones varied with 3-side chain length and decreased as the length increased.

### Menaquinone-4 effects in bone

Several vitamin K-dependent proteins are found in bone including osteocalcin, matrix-Gla-protein, protein S and gas6 and their properties will be described in detail by other authors in this Theme Issue. However, vitamin K has properties in bone that are not attributable to carboxylation. Much of the evidence, though not all, relates specifically to MK-4 which was first thought to have special importance to bone health in the early 1990s. It was observed that low circulating levels of menaquinones were associated with osteoporotic fractures in the elderly (93), and this was followed by a clinical evaluation of MK-4 given to osteoporotic women in Japan (94). In a six-month double-blind placebo-controlled study, bone mineral density in metacarpal bone was elevated in the group given 45 mg MK-4 a day but decreased in the placebo group. MK-4 was subsequently developed as a treatment for osteoporosis in Japan and is widely used today.

Soon after these findings, investigators began to probe the mechanism by which MK-4 acts on bone and a combination of *in vivo* and cell culture experiments found that it can reduce bone losses caused by either oestrogen withdrawal or corticosteroid treatment. Rats experience a reduction in bone mass after ovariectomy, but the loss of bone in terms of dry weight, calcium and hydroxyproline content and mechanical strength was reduced in animals treated with MK-4 (95). Similarly, bone loss in rats was induced by prednisolone treatment, but this effect was also lessened by administration of MK-4 (96). These findings were backed by studies *in vitro* which showed that MK-4 inhibits the synthesis of prostaglandin E2 (PGE2), a bone resorption-inducing agent, in cultured osteoblasts (97) and in calvaria organ culture (96).

Other studies on vitamin K focused on the formation of osteoclasts which are recruited from monocytic progenitor cells in the bone marrow. Akiyama et al. (98) demonstrated that MK-4 inhibits the formation of osteoclast-like cells in bone marrow-derived cultures. The system they employed required the presence of  $1,25(\text{OH})_2\text{D}_3$  in the final stage of multinucleate cell formation and it was this stage that was inhibited by MK-4 suggesting that the compound suppresses the differentiation of monocytic progenitor cells into mature osteoclasts. Hence, MK-4 was found to be active on both osteoclasts and osteoblasts and in intact calvaria raising the possibility that more than one mechanism is involved in its action.

Hara et al. (99) suggested that the 3 polyprenyl side-chain of MK-4 alone could mediate at least some of the effects of the whole molecule since equal concentrations of MK-4 or geranylgeraniol (GG), the alcohol derivative of the side chain, were able to inhibit the release of calcium from mouse calvariae induced by either  $1,25(\text{OH})_2\text{D}_3$  or PGE2. A related compound, geranylgeranylacetone inhibited osteoclastogenesis from monocytes treated with receptor activator of NF- $\kappa$ B ligand (RANKL) and also prevented bone loss in two different animal models, ovariectomy and hindlimb unloading (100).

It is worth mentioning at this point that a major axis of control in bone metabolism was being elucidated in the 1990s at around the time that research into the actions of MK-4 in bone was first being investigated. Very briefly, a trio of proteins exert control over the generation of osteoclasts. Firstly, receptor activator of NF- $\kappa$ B ligand (RANKL) is a cytokine essential for the formation

of osteoclasts and is expressed by osteoblasts and stromal cells of bone marrow. Secondly, receptor activator of NF- $\kappa$ B (RANK) is the receptor of RANKL and is expressed by osteoclasts and their precursors, and thirdly osteoprotegerin (OPG) is a soluble decoy receptor which is produced by osteoblasts and stromal cells and can interfere with the interaction between RANKL and RANK (reviewed by Kearns [101]). These proteins turn out to feature in several significant publications on the action of MK-4 in bone.

Hiruma et al. (102) obtained evidence that MK-4 and GG acted independently of each other by using a murine co-culture system in which stromal cells from bone marrow were grown with primary spleen cells. Two different clones were derived from the stromal cells which behaved differently when co-cultured with spleen cells and induced with  $1,25(\text{OH})_2\text{D}_3$ . In one case both MK-4 and GG inhibited osteoclast-like cell formation but in the other, only MK-4 had this effect suggesting that the two compounds were acting through different mechanisms. The stromal cells were found to secrete PGE2, a potent inducer of osteoclast formation, and MK-4 was able to inhibit this by reducing the expression of cyclooxygenase-2 (COX-2), an enzyme required to synthesise PGE2, while GG was not able to do this. However, when osteoclast formation was induced directly by the addition of PGE2, GG was able to inhibit osteoclastogenesis while MK-4 was not, showing that GG was interacting with an event further downstream from the PGE2 signal. This appears to be via control of RANKL, since the expression of  $1,25(\text{OH})_2\text{D}_3$ -induced RANKL mRNA was reduced by GG and the addition of soluble RANKL could restore osteoclast-like cell formation in co-cultures in which GG had been added.

Koshihara et al. (103) also used a complex cell culture system based on human bone marrow in which the cells were grown in the presence of  $1,25(\text{OH})_2\text{D}_3$  for up to 16 days. The authors claim that this culture system produced both osteoclasts and stromal cells which could be separated by enzymatic digest after a period of incubation with individual vitamin K compounds, allowing their effects to be studied on both cell types. Their results showed that both phylloquinone and MK-4 had a dose-dependent inhibitory effect on osteoclast formation. At the same time, MK-4 enhanced the formation of stromal cells and this correlated with the simultaneous dose-dependent reduction of RANKL and elevation of OPG in the stromal cultures. In a modified assay, the effects of vitamins K were studied on osteoblastogenesis, and both phylloquinone and MK-4 were seen to enhance the formation of alkaline phosphatase positive cells which also expressed the osteoblastic transcription factor Cbfa-1. This was one of the few publications which showed an effect of phylloquinone on osteoclastogenesis, possibly because a culture of whole bone marrow was used. It is worth noting that the concentrations of vitamin K used in these studies on bone are all in the micromolar range, far higher than physiological levels found in the circulation.

Osteoclasts are short-lived cells whose activity is often terminated by apoptosis. Kameda et al. (104) have claimed that MK-4, though not phylloquinone is capable of inducing apoptosis of osteoclasts in a cycloheximide-inhibited manner but this does not seem to have been supported by further reports. However, there is much literature concerning the apoptotic effects of MK-4 and related compounds on leukaemia cells (see next section) so it

is plausible that the monocytic progenitors of osteoclasts may be sensitive to micromolar concentrations of MK-4 in this way.

### Vitamin K and cancer

Several studies have shown that MK-4 can inhibit the growth of certain types of cancer cells including those from liver and gut tumours and some types of leukaemia cells often by causing the cells to differentiate or by inducing apoptosis. Human myeloid leukaemia cell lines were induced to differentiate by MK-4 (105) or undergo apoptosis (106) while GG can induce apoptosis in U937 cells, a human leukaemia cell line (107). In a study on gastric cancer cell lines, MK-4 dose-dependently inhibited cell growth and caused the cells to undergo cell cycle arrest in G<sub>0</sub>/G<sub>1</sub> phase (108). MK-4, menadione and a menadione-related analogue vitamin K<sub>5</sub>, all reduced cell proliferation in colorectal cancer cell lines and also exhibited anti-tumour effects on colorectal tumours *in vivo* (109).

To date, the mechanism of vitamin K action in tumour cells has been studied to the greatest depth in hepatocellular carcinoma (HCC). MK-4 has been reported to suppress the development of HCC in a small study of cirrhotic patients (110) and to reduce the recurrence of HCC after resection (111). In the second of these studies all patients had had primary tumours either removed by surgical resection or by local ablation therapy and were required to be clear of tumours at the start of the study. One patient group was given an oral dose of 45 mg of MK-4 daily, and the control group was given nothing as it was not a placebo-controlled trial. The MK-4-treated group showed a significantly reduced rate of HCC recurrence and a significant increase in the cumulative survival rate during the 36-month study. Although only a small study of 61 patients in total, the results do seem to justify a larger-scale controlled trial.

Animal studies showed that MK-4 was somehow affecting the cell cycle because the cyclin-dependent kinase cdk4 was reduced in HCC by treating the animals with either MK-4, menadione or vitamin K<sub>5</sub> (112). Recently Ozaki et al. (113) demonstrated that MK-4 slowed the growth of three HCC cell lines and reduced DNA synthesis as assayed by bromodeoxyuridine incorporation. Flow cytometry showed an increase in the number of cells which arrested in the G<sub>1</sub> phase of the cell cycle, which corresponded strongly with a decrease in cyclin D1 protein and mRNA. At the same time an increase in message and protein was seen for cdk inhibitors p21 and p27. The effect of MK-4 on cyclin D1 expression was investigated further and found to be caused by a decrease in binding of the transcription factor NF- $\kappa$ B to the cyclin D1 promoter, caused in turn by a reduction in phosphorylation of its inhibitor protein I $\kappa$ B $\alpha$ . However, levels of I $\kappa$ B kinase (IKK) were unaltered implying that MK-4 was modulating IKK activity through an unknown pathway and the molecular targets of MK-4 in HCC have yet to be identified.

### A receptor for vitamin K?

Some of the properties of MK-4 suggest that there may be a receptor for this compound since it appears to elicit a number of diverse functions in a variety of cell types and to modulate the expression patterns of many genes. Several research groups have looked for vitamin K-binding proteins and have found a candidate protein in the nucleus of osteoblasts which binds MK-4 and

with lower affinity, phylloquinone as well (114). This protein has a molecular weight of approximately 40,000 and an N-terminal peptide was largely homologous to the enzyme glyceraldehyde-3-phosphate dehydrogenase and was also precipitated with antibodies to this protein. There appear to have been no further reports concerning this protein.

MK-4 was shown to bind to SXR/PXR (see *Vitamin K catabolism* section) in a sensitive ligand binding assay and to induce osteoblastic markers in several human osteosarcoma-derived cell lines and calvarial bone cells (67). Micro-array analysis revealed that a number of genes were up-regulated by both MK-4 and rifampicin, a prototypic SXR ligand, which are involved in the accumulation of extracellular matrix in MG-63 osteoblast-like cells (115). SXR/PXR was found to be required for the osteoblastic differentiation of a murine cell line MC3T3-E1 upon treatment with MK-4 (116). This receptor forms a heterodimer with retinoid X receptor which activates expression of an osteoblastic transcription factor Msx2 by interacting with an MK-4 response element in its promoter.

The affinity of SXR/PXR to such a variety of ligands is somewhat problematic in explaining the observations of the effects of MK-4 in bone. However, recently it was demonstrated by Ichikawa et al., again using micro-array analysis, that some genes respond specifically to MK-4 but not to other SXR ligands (117). Their studies showed that at least two genes, GDF15 and STC2 are regulated by MK-4 but not by phylloquinone, other menaquinones, GG or rifampicin. These genes are not regulated by processes dependent on carboxylation but are thought to be regulated through a mechanism involving protein kinase A. One interpretation of the results could be the existence of another receptor specific for MK-4.

## Miscellaneous functions of K vitamins

### Vitamin K and thrombosis

While the effects of vitamin K deficiency on blood coagulation are well characterised and manifested by an extended prothrombin time, little work has been done on the effects of very high doses of vitamin K on haemostasis and thrombosis. Ronden et al. (118) used a measurement of arterial thrombosis tendency in which a polyethylene loop is inserted into the aorta of a rat enabling visual inspection of the blood inside. An occlusive thrombosis is indicated by a change in blood colouration. Very high doses had no effect on blood coagulation but did affect the mean loop obstruction time, which indicates arterial thrombotic tendency, MK-4 lengthening the time and phylloquinone shortening it. When the experiment was repeated with the alcohol forms of the 3-side chains of the two molecules, the same trends were observed though phytol was closer to control values than phylloquinone was. The mechanism for this effect remains elusive although the authors suggest an effect on the differentiation of monocytes to macrophages may be responsible, possibly similar to the effects of MK-4 and GG on osteoclast differentiation in Kameda's experiments (see above).

### Vitamin K and inflammation

A small number of investigations have claimed to demonstrate an inverse relationship between vitamin K and an inflammatory

response. Cell culture studies showed that lipopolysaccharide (LPS)-treated fibroblasts secrete interleukin (IL-6) but this is reduced if the cells are first treated with vitamin K (119). Experiments on rats demonstrated that animals fed on vitamin K-deficient diets had an enhanced expression of genes involved in an acute inflammatory response compared to those on normal or phylloquinone-supplemented diets and also that the supplemented diet suppressed the inflammatory response induced with LPS (120). Recently a human population study using the Framingham Offspring Study cohort, examined cross-sectional associations between both status and intake of vitamins K and D (which is also believed to lower inflammatory responses in certain disease states, though not in healthy individuals) and a number of systemic proinflammatory biomarkers (121). The results showed that vitamin K status whether measured as plasma concentration or as phylloquinone intake was inversely and significantly related to individual inflammatory markers and to the overall inflammatory process.

### Vitamin K and oxidative damage

Another recently reported activity of vitamin K is the prevention of oxidative damage to oligodendrocyte precursor cells and foetal cortical neurons (122). This is thought to be an important cause of cell death in brain disorders such as perinatal hypoxia/ischemia in which premyelinating oligodendrocytes become damaged by reactive oxygen species. Li et al. used a primary cultured rat oligodendrocyte system in which oxidative stress was induced by switching cells to cystine-free culture medium which results in glutathione deficiency and subsequent cell death. Phylloquinone and MK-4 both abolished cystine-depletion-induced cell death and could do so at very low concentrations in the nanomolar range or roughly equal to that in the normal adult circulation. This effect was independent of  $\gamma$ -glutamyl carboxylase activity and required the intact vitamin K molecules including the 3-side chains. Vitamin K has previously been reported to possess a free-radical scavenging activity when assayed in non-aqueous solvents (123) and could suppress lipid peroxidation in a rat liver microsomal preparation (124). However, in the oligodendrocyte experiments it did not appear to be protecting the cells directly through an anti-oxidant action because two separate assays failed to detect any anti-oxidant activity associated with vitamin(s) K. At the time of writing, the mechanism of this protective function of vitamin K remains unknown, but its low toxicity makes it a potentially useful therapeutic agent for preventing ischemic injury in premature infants.

### Vitamin K and sphingolipid synthesis

Vitamin K is involved in the synthesis of sphingolipids, a group of lipids found in large amounts in the brain. Vitamin K was first

linked with sphingolipid synthesis through work done on bacteria over 35 years ago (125) in which it was found that when *Bacterioides melaninogenicus*, a vitamin K-requiring bacteria, was grown in K-depleted medium it had defective cell envelopes and appeared to require the vitamin for synthesis of ceramides. This was caused by a reduction in the activity of 3-ketodihydro-sphingosine synthase, the first enzyme in the sphingolipid pathway. The same enzyme is depleted in microsomes prepared from the brains of young mice treated with warfarin (126), which led to a substantial reduction in sulfatides after a two week period of treatment which phylloquinone was able to restore. Further investigation demonstrated that another brain enzyme, galactocerebroside sulfotransferase was also sensitive to vitamin K depletion (127) and that either phylloquinone or MK-4 could restore activity (128). MK-4 is known to be the principal form of vitamin K found in the brain of rats (39) and humans (37) and it has been found to occur in highest concentrations in regions where large amounts of sphingolipids are located (129). Thus MK-4 is the physiologically active form most heavily implicated in the synthesis of these structural lipids *in vivo*. The mechanisms by which vitamin K mediates these effects are still unknown, but it is clearly an important nutrient in the brain and its requirement for synthesis of lipids required for myelinated cells may perhaps explain some behavioural disorders that have been attributed to vitamin K deficiency or warfarin treatment in animals (130).

### MK-4 in the exocrine pancreas

The tissue distribution of phylloquinone and the menaquinones may give some clue to their relative importance in different organs. As already described, MK-4 is the most abundant form in several organs. One such organ, the pancreas, has been found to secrete MK-4 upon stimulation with secretagogues cholecystokinin-8 and secretin which act upon the pancreatic acinar cells (131). MK-4 secretion was concurrent with the secretion of phospholipase and the membrane trafficking protein caveolin-1, possibly suggesting some function in exocrine cells, though such a function remains undefined. Carboxylation does not appear to be the reason for the presence of MK-4 in this tissue as little staining was observed in acinar cells when pancreatic tissue was stained with an antibody directed against Gla residues (132), most positive staining being seen in glucagon-secreting  $\alpha$ -cells.

### Acknowledgements

We would like to thank Renata Gorska for her skilful artwork and preparation of the illustrations.

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