Vitamin K Metabolism and Nutriture

M. J. Shearer

SUMMARY. Vitamin K functions as a co-factor for the post-translational carboxylation of specific glutamate residues to gamma-carboxyglutamate (Gla) residues in several blood coagulation factors (II, VII, IX and X) and coagulation inhibitors (proteins C and S) in the liver, as well as a variety of extrahepatic proteins such as the bone protein osteocalcin.

This review outlines some recent advances in our understanding of the metabolism of vitamin K and its role in human nutriture. The introduction of new methodologies to measure the low endogenous tissue concentrations of K vitamins and circulating plasma levels of des-gamma-carboxyprothrombin (PIVKA-II) have provided correspondingly more refined indices for the assessment of human vitamin K status. The assays for vitamin K have also been used to study the sources, intestinal absorption, plasma transport, storage and transplacental transfer of K vitamins and the importance of phylloquinone (vitamin K₁) versus menaquinones (vitamins K₂) to human needs.

The ability to biochemically monitor subclinical vitamin K deficiency has reaffirmed the precarious vitamin K status of the newborn and led to an increased appreciation of the risk factors leading to haemorrhagic disease of the newborn and how this may be prevented.

Biochemical studies are leading to an increased knowledge of the mode of action of traditional coumarin anticoagulants and how some unrelated compounds (e.g. antibiotics) may also antagonize vitamin K and cause bleeding. There is also an awareness of the possible deleterious effects of vitamin K antagonism or deficiency on non-hepatic Gla-proteins which may play some subtle role in calcium homeostasis.

It is more than 50 years since the antihaemorrhagic or Koagulation factor now known as vitamin K was discovered by Henrik Dam and the two major forms phylloquinone (vitamin K₁) and menaquinones (vitamins K₂) isolated and characterized from alfalfa (lucerne) and putrefied fish meal respectively.¹ ² Since then, progress in determining the biochemical function and in understanding the metabolism and nutritional role of the vitamin has been erratic. Several phases may be briefly noted.

The early phase of intensive study in the 1930’s and 1940’s saw the introduction of vitamin K into clinical practice, initially for the correction of the clotting defect in obstructive jaundice and other biliary diseases and then for the prophylaxis and treatment of the bleeding syndrome in the newborn known as the haemorrhagic disease of the newborn.¹ ²

Again, although the relationship between vitamin K deficiency and a decrease in the plasma concentration of prothrombin was established ¹ as early as 1936, it was not until the early to mid 1950’s that the three other procoagulant factors VII, IX and X were discovered and subsequently shown to require vitamin K for their biological activity and a further 20–25 years before the vitamin K-dependent coagulation inhibitors proteins C and S were discovered.³ ⁴ The role of all these vitamin K-dependent factors in
haemostasis has been discussed in a recent review in this journal.5  
The elucidation of the biochemical function of vitamin K came relatively late with the isolation in 1974 of a previously unknown acidic amino acid, gamma-carboxyglutamic acid (Gla) from bovine prothrombin and the parallel discovery that this was missing from the abnormal, functionally inactive molecules of prothrombin which circulate in the plasma of coumarin anticoagulated animals.6s7 It was equally realized that the Gla residues of prothrombin could provide the already well known calcium binding sites of the protein, that the abnormal prothrombin molecules might be similar to an already postulated liver precursor and that the role of vitamin K could be to act as an essential co-factor for a post-translational modification of prothrombin in which certain glutamic acid residues were transformed to gamma-carboxyglutamic acid (Gla) residues. All these postulates were subsequently shown to be true (for reviews see refs8s9).  
The discovery of an amino acid with a strict requirement for vitamin K for its synthesis has provided a ready means of probing for vitamin K-dependent proteins in other tissues, either by analysing the tissue or isolated protein for its Gla content or by demonstrating the associated enzyme activity (vitamin K-dependent carboxylase) responsible for this amino acid modification.10 These avenues have revealed the existence of many vitamin K-dependent proteins (or Gla-proteins) in a variety of calcified and non-calcified tissues but very few have been characterized as far as their amino acid sequence. The major exceptions are the two Gla-proteins osteocalcin (or bone Gla-protein) and matrix Gla-protein. It has been postulated that this class of non-hepatic vitamin K-dependent proteins may play a role, hereto undiscovered, in calcium homeostasis.10–12  
A detailed body of knowledge and understanding of the metabolism and nutritional role of vitamin K, such as exists for the other fat-soluble vitamins, has been held back firstly by the lack of sufficiently sensitive assays for vitamin K and its metabolites, and secondly, by the lack of sensitive criteria for monitoring subclinical states of vitamin K deficiency. These shortcomings are now being overcome by technological advances which have resulted in the development of chromatographic techniques13,14 to measure K vitamins at tissue concentrations and more sensitive coagulation or immunochemical methods15 for the detection of circulating species of abnormal prothrombin (des-gamma-carboxyprothrombin or PIVKA-II). These new methods offer new approaches to the study of several aspects of vitamin K metabolism and nutrure. Examples of outstanding problems are the nutritional origin and bioavailability of the various K vitamins (including the question of the utilization of enterically produced menaquiones), the mechanism of their intestinal absorption, plasma transport and tissue distribution at cellular and subcellular levels, and the utilization of different K vitamers by the vitamin K-dependent carboxylase. Finally, the application of these methods should provide a better understanding of vitamin K status in health and disease and the opportunity to re-examine the difficult question of dietary requirements and how these may be met by dietary intakes in individuals and population groups. Some of these questions will be addressed in this review.

Nutritional Sources and Intestinal Absorption

Chemical Structures

Naturally occurring compounds with vitamin K activity possess a common 2-methyl 1,4-naphthoquinone ring structure but differ in the structure of their side chain at the 3-position (Fig. 1). The only form synthesized by plants and algae is phylloquinone (originally named vitamin K1) and this has the same phytol side chain as chlorophyll. A second series, the menaquiones (originally named vitamins K2), are all synthesized by bacteria; these comprise a spectrum of molecular forms with side chains based on a number of repeating unsaturated 5-carbon (prenyl) units. The major forms are designated menaquione-n (MK-n) according to the number of prenyl units; the most commonly found forms in animal tissues are menaquiones 6–13. Some bacteria synthesize menaquiones in which one or more of the prenyl units is saturated. The division between the plant vitamer phylloquinone and the bacterial menaquiones is somewhat artificial (since phylloquinone may be regarded as a partially saturated form of menaquione-4) but serves to emphasize their different origins.

Dietary Sources

The major dietary form of vitamin K is phylloquinone. Methods employing high-performance liquid
chromatography are now available for the accurate measurement of phylloquinone in plant and animal tissues, but comprehensive food values have not yet been published. There is a wide variation in the phylloquinone content of different foods. Approximate ranges are: green leafy vegetables (100–500 μg/100g); other vegetables and fruit (1–50 μg/100 g); oils, fats and margarines (1–100 μg/100 g); dairy produce (0.5–5 μg/100 g); bread and cereal products (0.1–10 μg/100 g).

The bioavailability of phylloquinone from the above foods is unknown but is likely to vary widely being least efficient from green vegetables (where phylloquinone is tightly associated with chloroplasts) and being most efficient from processed foods.

### Intestinal Absorption

The intestinal absorption of dietary phylloquinone (and probably menaquinones) is thought to be governed by the same principles established for other fat-soluble vitamins and highly lipid-soluble nutrients. In the intraluminal phase of absorption, this involves the solubilization of vitamin K into mixed micelles composed of bile salts and the products of pancreatic lipolysis. Consistent with this model is the striking impairment of absorption seen in patients with extrahepatic cholestasis (obstructive jaundice) and severe pancreatic insufficiency. Patients with biliary obstruction lack the detergent components of micelles, and the degree of absorption depends on the severity of the bile salt deficiency. In patients with chronic pancreatitis, the primary disturbance is a reduced generation of the solutes of mixed micelles, namely, 2-monoglycerides and fatty acids. This also impairs the absorption of phylloquinone, though this is usually less severe than in bile salt deficiency. The essentiality of bile salts to the absorption of vitamin K is reflected in the higher incidence of vitamin K deficiency in cholestatic disease compared to most other gastrointestinal diseases including chronic pancreatitis.

### Microfloral Synthesis of Menaquinones

It has been commonly perceived that the enormous microfloral population of the large intestine provides an important nutritional source of vitamin K for human needs. This belief seems to stem, very largely, from early studies in rats and chicks which first demonstrated the presence of menaquinones in faeces and intestinal contents and then their synthesis by specific cultures of microorganisms including many gut inhabitants. This view is reinforced by the infrequency of primary dietary vitamin K deficiency in most mammalian species including man and the long established association of human vitamin K deficiency with antibiotic therapy. As argued cogently by Doisy and Matschner, this post hoc, ergo prompter hoc reasoning does not stand up to certain experimental findings especially those of Barnes and Fiala who showed that a ‘dietary’ deficiency of vitamin K in the rat (originally thought to be resistant to dietary deprivation) can be readily induced by preventing coprophagy. Such experiments illustrate that the rat can only usefully utilize enterically synthesized menaquinones by a second passage through the bowel and presumably absorption from the upper region of the small bowel. The lack of significant absorption of enterically synthesized menaquinones is supported by a recent study in which the menaquinone concentrations in the intestinal contents of rats fed different diets were measured directly by high-performance liquid chromatography; after 3 days the group fed a vitamin K-deficient diet developed signs of vitamin K deficiency but the amounts of menaquinones in the large intestine had actually increased compared to a control group of rats fed a normal diet. This study clearly showed that rats with a normal, or even increased, production of intestinal menaquinones could not maintain prothrombin synthesis from this source of vitamin K alone i.e. the diet must have been the major source of the vitamin. In the human too direct evidence of menaquinone absorption from the large intestine is presently lacking though it cannot be ruled out that small amounts are absorbed from this region. There is evidence that menaquinone absorption from the colon is possible by a passive diffusion process. However, given the evidence already cited, the amounts absorbed would seem to be insufficient to prevent the host from developing hypoprothrombinaemia under conditions of dietary deprivation.

A potential confounding factor to the above controversy is the diet which in rats has been shown to have a major influence on the intestinal synthesis of menaquinones. For example a low fibre diet based on boiled white rice consistently induced vitamin K deficiency in rats within 3 weeks, produced significantly lower faecal concentrations of several menaquinone-producing bacteria and lower hepatic levels of menaquinones. The above changes could be prevented by the dietary inclusion of black-eyed beans. Since hepatic concentrations of phylloquinone were also reduced it is difficult to pinpoint to what degree the development of deficiency was due to a primary dietary deficiency of phylloquinone, a secondary dietary deficiency of menaquinones (by coprophagy) or the reduced availability of menaquinones for direct enteric absorption.

Superficially the most persuasive evidence for the relevance of intestinal synthesis to human vitamin K nutriture comes from the finding of substantial concentrations of menaquinones in the liver. A more detailed consideration of the liver stores of menaquinones and their potential biological significance is given below. As to the argument of their origin, however, the clue may lie in the evidence that these highly lipophilic molecules are strongly retained by
the liver. If the turnover is prolonged then the menaquinone content of human liver may arise from a relatively slow assimilation whether this be an inefficient (passive) process from the large intestine of the potentially high concentrations produced by bacterial synthesis or a more efficient (bile salt-mediated) process from the small intestine of low concentrations present in certain foods.

Tissue Distribution and Storage

Assay Methods for Vitamin K

So far vitamin K, with its extreme lipophilic character, has proved resistant to immunochemical assay and the ability to measure tissue concentrations has only been possible since the advent of high-performance liquid chromatography leading particularly from the development of silica-based microparticulate packing materials, reliable solvent delivery systems and sensitive in-line detectors. Present methods are still time consuming and require a multi-stage purification procedure to removing interfering lipids. The most recent advances have centered on the introduction of more selective detection systems which by exploiting the reversible redox reaction of quinone and quinol forms (Fig. 2) allow sensitive electrochemical or fluorometric measurements.

Adult Plasma Concentrations

Plasma concentrations of vitamin K are considerably lower than other fat-soluble vitamins and comprise chiefly of phylloquinone. Detectable concentrations of menaquinone-7 and possibly menaquinone-8 have also been reported but longer chain menaquinones are beyond the limits of detection of current methodologies.

Despite the technological advances of the last decade reported values for the mean circulating concentration of phylloquinone in healthy adult populations show a 10-fold variation. This variation almost certainly reflects methodological differences rather than any great geographic or ethnic influences. Thus a number of laboratories on changing their original procedures have reported decreased values. As a result there has been a discernible downwards trend towards a normal fasting value of about 0.5 ng/ml (1.1 nmol/l).

Because of the wide interlaboratory differences little is yet known of how plasma concentrations vary in health and disease. Some comparative measurements from the author’s laboratory are shown in the Table. They reveal how both diet and metabolic disorders, particularly those affecting lipid metabolism, may influence circulating levels of the vitamin. The most powerful metabolic determinant are plasma lipids leading to significantly raised concentrations in primary hyperlipidaemia or in conditions associated with secondary hyperlipidaemia (e.g. diabetes and haemodialysis patients). Among these patient groups highly significant statistical correlations of circulating phylloquinone in the fasting state are seen with total plasma triglyceride but not with cholesterol even though patients with discrete hypercholesterolaemia (ie without hypertriglyceridaemia) do tend to have raised phylloquinone levels (Table).

A similar positive association of plasma phylloquinone with plasma triglycerides in the fasting state has been reported in a population survey of over 300 healthy (but not necessarily normolipaemic) adults.

Plasma Transport

Unlike vitamins A and D, no specific plasma carrier protein for vitamin K has been identified. Instead vitamin K is probably transported entirely by lipoproteins, a property it shares with vitamin E. Current evidence suggests that dietary K vitamins are incorporated chemically unchanged into chylomicrons in the intestinal mucosa, secreted into the lymph, and

Table Mean plasma phylloquinone concentrations in health and disease

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Plasma K₁ (ng/ml)</th>
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<tr>
<td></td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Healthy newborn (cords)</td>
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</tr>
<tr>
<td>Healthy adults</td>
<td>42</td>
<td>0.41</td>
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<tr>
<td>Healthy adults (non-fasting)</td>
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<tr>
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<tr>
<td>Diabetes (raised lipids)</td>
<td>38</td>
<td>0.88</td>
</tr>
<tr>
<td>Haemodialysis patients</td>
<td>20</td>
<td>0.74</td>
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* All subjects were fasting except where stated
* Untreated non-insulin dependent
eventually enter the liver cells via chylomicron remnant particles. Detailed knowledge of the inter-relationship of vitamin K with various lipoproteins remains to be established but phylloquinone has been located in each of the three major classes, namely very low density, low density and high density lipoproteins. Fractionation studies have also revealed that the excess phylloquinone in hyperlipidaemic patients with raised plasma concentrations of the vitamin is carried by the triglyceride rich very low density lipoproteins. This would explain the strong statistical association with triglycerides.

**Relationship of Plasma Levels to Vitamin K Status**

With the present inadequate knowledge of the mechanism of transport of vitamin K the question of whether plasma concentrations are an accurate indicator of tissue reserves or vitamin K 'status' cannot be convincingly answered at this time. One obvious limitation, already discussed, is the influence of plasma lipids. Two important consequences follow. The first is that plasma measurements should be made, wherever possible, in the fasting state; the second is that an improved correlation with vitamin status might be obtained by expressing plasma concentrations as a ratio of phylloquinone to triglycerides. The possible unmasking effect of the latter procedure is illustrated by the fact that although plasma concentrations of phylloquinone show a moderate correlation with age which is more pronounced in men than in women when plasma phylloquinone is corrected for triglycerides both elderly men and women (65–92 years) show significantly reduced values compared to a younger age range (20–49 years). This may indicate that the elderly have lower reserves of phylloquinone.

Even without correcting for triglycerides there is some evidence that plasma concentrations of phylloquinone may be a useful indicator of tissue reserves. Thus, low plasma concentrations have been found in hospitalized patients who had other evidence of a poor nutritional status or were being fed parenterally, in both studies the presentation of a low plasma phylloquinone was a risk factor for the subsequent development of antibiotic-induced hypoprothrombinaemia.

More direct evidence of the responsiveness of plasma levels to dietary deprivation of vitamin K has come from studies showing a rapid decline in plasma phylloquinone by dietary restriction either in normals or in patients undergoing surgery. In the surgical group a low vitamin K intake for only three days resulted in a 4-fold lowering of liver concentrations compared to a group on a normal diet. This rapid decline in both tissues suggests that in situations of reduced dietary intake, plasma concentrations mirror hepatic stores quite closely.

**Biological Significance of Hepatic Menaquinones**

The biological relevance of the relatively large hepatic stores of menaquinones is as mysterious as their origin. Early bioassay data in which various forms of vitamin K were given intracardially to deficient rats suggested that long chain menaquinones (7, 9 and 10) were more efficient in reversing hypoprothrombinaemia and up to 25 times as active as phylloquinone itself. It is also known that both phylloquinone and menaquinones may serve as a co-factor for the hepatic carboxylase in vitro.

The ability of humans to efficiently utilize the large...
endogenous hepatic menaquinone pool has been put into some doubt by the study of Suttie et al.\textsuperscript{51} who found that biochemical and coagulation abnormalities consistent with signs of a mild deficiency could be rapidly induced in human volunteers by a fairly modest dietary restriction of phylloquinone-rich foods. On the other hand overt vitamin K deficiency with bleeding is rarely seen, even in severe malnutrition,\textsuperscript{19} which does suggest that the ability to utilize menaquinones may prevent the appearance of severe deficiency symptoms. A key question is whether there are mechanisms by which the menaquinones which undoubtedly are present in many membranous organelles may be transported to and utilized by the microsomal carboxylase in situations of dietary lack. This question is made more relevant by the first direct evidence that whereas phylloquinone hepatic stores are rapidly depleted by dietary restriction, menaquinone stores are not.\textsuperscript{37}

Another key to the above inconsistencies may lie in the differences in disposition and rates of metabolism of various molecular forms of vitamin K. For example the relative bioassay data in rats\textsuperscript{55} were made by comparing the response in prothrombin levels at 18 h. As Matschner\textsuperscript{56} later pointed out the much higher activity of long chain menaquinones in these experiments could be partly explained by their sustained response. Thus the response to phylloquinone peaked at 5 h and by 18 h had declined substantially. These results and the more direct evidence from human liver measurements\textsuperscript{37} discussed above suggest that phylloquinone is utilized and metabolized at a faster rate than long chain menaquinones. This slower turnover of higher molecular weight members of the menaquinone series is presumably due to their high degree of lipophilicity and greater affinity for membranes.

**Vitamin K Metabolism in the Newborn**

Interest in the metabolism and nutriture of vitamin K in the perinatal period stems from the increased risk that babies have of developing vitamin K deficiency which at its most extreme manifests as the bleeding syndrome known as haemorrhagic disease of the newborn. Some recent research using the new sensitive assays for K vitamins and des-gamma-carboxyprothrombin has sought to pinpoint the metabolic/nutritional reasons for this susceptibility. The progress made in this area is reviewed below.

**Maternal-cord Gradient.** The first direct evidence for the existence of a placental barrier to the transport of vitamin K to the human fetus came from early high-performance liquid chromatographic measurements which showed that although phylloquinone was readily detectable in maternal plasma, the concentrations in cord plasma were undetectable.\textsuperscript{57} These findings of very low endogenous cord plasma concentrations have been disputed by some\textsuperscript{40,41,58} but confirmed by others.\textsuperscript{52,59–61} This variation in literature values, which at their most extreme differ by several 1000-fold, suggests serious methodological discrepancies. As with adult values, however, there has been a trend towards lower values, several groups now agreeing that cord concentrations are below 50 pg/ml (110 pmol/l) and that the average maternal/fetal concentration gradient of phylloquinone is within the range of 20:1 to 40:1.\textsuperscript{35,42,43,61} This is by far the highest placental blood gradient of any of the fat-soluble vitamins.

Further evidence that phylloquinone does not readily cross the placenta is shown by the similar high maternal/fetal concentration gradient when the vitamin is administered to mothers shortly before delivery.\textsuperscript{42,57,61} The extent to which cord blood concentrations were raised depended on the dose, route and time interval between maternal administration and cord sampling, ranging from 2 to 5-fold when single doses of 1–5 mg were given intravenously or intramuscularly\textsuperscript{57,61} to about 40-fold when mothers received 20 mg/day orally for at least 3 days.\textsuperscript{42}

Despite the fact that supplementation procedures do raise cord blood levels significantly, in one study\textsuperscript{42} to the endogenous maternal range, there was no corresponding effect on the vitamin K-dependent clotting factor activities.\textsuperscript{42,61} These results offer strong evidence that the well known low concentrations of vitamin K-dependent clotting factors in the newborn are not the consequence of vitamin K deficiency but rather the result of the reduced synthesis of their precursor proteins possibly due to reduced mRNA transcription or translation.\textsuperscript{52,62} This does not of course discount the possibility of vitamin K deficiency occurring in individual babies at birth (see under haemorrhagic disease of the newborn).

One group of babies who may benefit from the maternal route of vitamin K supplementation are those born to mothers on anticonvulsant therapy. Such babies are known to run a higher risk of developing early vitamin K deficiency\textsuperscript{65} and there is some unconfirmed evidence that antenatal vitamin K can substantially reduce this risk.\textsuperscript{64}

**Infant Plasma Concentrations.** Plasma vitamin K concentrations which are very low at birth start to be detectable at around 12–24 h after delivery\textsuperscript{43} and by 3–4 days breast-fed infants have plasma concentrations in the same range as adults.\textsuperscript{43,60} In contrast babies fed on vitamin K supplemented milk formulas have significantly higher concentrations of phylloquinone at 3–4 days.\textsuperscript{43,60} These high plasma levels result from the differential concentrations of phylloquinone in human milk (about 2 μg/l\textsuperscript{15}) compared to formulas which in the studies cited above\textsuperscript{43,60} contained about 50 μg/l. Despite the marked differences in serum phylloquinone concentrations there is no detectable difference in coagulation between breast-fed and formula-fed infants in the first month of life.\textsuperscript{43} On the
other hand sensitive immunoassays for des-gamma-carboxyprothrombin revealed a higher frequency of detection of this marker for vitamin K deficiency in breast-fed infants than in formula-fed infants, none of whom had received vitamin K at birth.60,66

Fetal and Neonatal Liver Reserves. Phylloquinone has been detected in the liver of the human fetus as early as 10 weeks gestation at concentrations of 1-2 ng/g (2.2-4.4 pmol/g).35 Similar concentrations were detected throughout fetal development, the median hepatic concentrations at pre-term (1.4 ng/g) and term (1.0 ng/g) being about one fifth the value in adults.35,53 Although significantly reduced these fetal reserves are not as low as perhaps expected from previous findings of exceedingly low concentrations in fetal and cord plasma.

The major difference between fetal/neonatal reserves and those of adults is that the normal menaquinone complement, which makes up the majority of the total adult stores, are very low in the perinatal period.35,67,68 In fact the concentrations of all menaquinones throughout gestation, at birth and in the first week of life are so low that they are normally undetectable by current assay methods.35,67,68 From limited analyses of post-mortem samples it would seem that there is a gradual build-up of hepatic stores of menaquinones in the neonate but that adult concentrations have still not been reached by 1 month of age.35,68

One implication of these findings is that the needs of the human fetus and neonate are met largely by phylloquinone. It is not known whether this relative deficit of menaquinones is a major factor in the susceptibility of the newborn to vitamin K deficiency or whether these bacterial forms of vitamin K derive from the diet, gut flora or a combination of both sources. A gradual increase in the hepatic stores of menaquinones would of course be consistent with the known colonization of the neonatal gut by enteric microflora but in view of the evidence suggesting their low hepatic turnover, the slow accumulation of menaquinones could also be explained by quite low dietary intakes taken over a prolonged period.

Low fetal reserves of menaquinones are not unexpected. Not only are the concentrations in maternal blood available to the placenta lower than phylloquinone but the efficiency of placental transport itself is likely to be very low for these high molecular weight, lipophilic forms.

Haemorrhagic Disease of the Newborn

Haemorrhagic disease of the newborn (HDN) is a bleeding syndrome caused by a deficiency of vitamin K in early life.69,70 Until about 20 years ago HDN was always thought to present in the first week of life; this syndrome is now known as classical HDN to distinguish it from a late onset form, which was first reported in 1967, and which has a peak incidence at 3-6 weeks.71 A worldwide resurgence of HDN in developed countries, mainly of the late onset form, and its frequent association with intracranial haemorrhagic still makes the disease a serious cause of infant morbidity and mortality.69 Two major risk factors for HDN are breast feeding and not giving vitamin K at birth. As already discussed the protection afforded by formula feeds derives from their higher vitamin K content than breast milk.

Detection and Incidence of Subclinical Deficiency

Several attempts have been made to document the incidence of subclinical vitamin K deficiency amongst normal babies using the detection of the precursor molecule, des-gamma-carboxyprothrombin as a biochemical marker. These abnormal molecules (also known as PIVKA or proteins induced by vitamin K absence or antagonism) are released into plasma in situations of vitamin K deficiency or antagonism. Although functional assays have been used to detect des-gamma-carboxyprothrombin the most useful assays are immunoassays either using crossed immunoelectrophoresis or even more sensitive enzyme-linked immunoabsorbent methods. The use of several assay methods with different sensitivities15 often makes the interpretation of these studies difficult.22 Some trends however can be seen. Whether measured by immunoelectrophoresis23 or enzyme-linked immunoabsorbent assay24 the incidence of detection of des-gamma-carboxyprothrombin is lower in cord plasma than 3-5 days after birth. In many respects more relevant results may be obtained using a less sensitive method such as crossed immunoelectrophoresis since the detection of very small amounts of des-gamma-carboxyprothrombin by some immunoassays may reflect some other facet of liver metabolism rather than vitamin K deficiency. For instance abnormal prothrombin molecules are rarely detected in cord plasma by immunoelectrophoresis but at 4-5 days the incidence of detection rises to about 50% in all babies and about 70% in solely breast fed babies.66 These results suggest that while the fetus derives sufficient transplacental transfer of vitamin K to ensure that prothrombin is fully or almost fully carboxylated at birth the existing reserves are not normally sufficient to meet the demands on this post-translational function through the first few days of life. As would be expected an important determinant of vitamin K status immediately after birth is the volume of milk ingested. It has been shown that the average daily intake in babies with des-gamma-carboxyprothrombin is significantly lower than in babies without this marker19 and that babies with des-gamma-carboxyprothrombin who also had low factor II and VII activities had failed to increase their milk intake beyond 100 ml/day by the third and fourth day of life.66 The delay which is often seen before lactation becomes established would thus account for the temporal dip in vitamin K status. A
cultural change in developed countries which may have contributed to the resurgence of HDN has been the widespread abandonment of early cow’s milk supplements because of the risk of cow’s milk protein intolerance.

Aetiology of Late HDN

By 1–2 months of age the incidence of detection of des-gamma-carboxyprothrombin in healthy breastfed infants (not given vitamin K at birth) falls to about 10% using the monoclonal immunoabsorbent assay and to less than 1% by immunoelectrophoresis. Once again the protective effect of formula feeding is seen by the inability of the very sensitive monoclonal assay to detect abnormal prothrombin molecules in some 50 formula-fed infants at 1-month and beyond.

The aetiology of late HDN seems to be different from that of the classical syndrome. For example although breast-feeding is a strong risk factor the volume of milk intake is unlikely to be so important since the peak incidence occurs well beyond the time taken for breast feeding to become established. Also mothers of affected infants do not necessarily have particularly low breast milk concentrations of vitamin K. Some studies have reported mild abnormalities of liver function which may be transient, mild and self correcting but sufficient to create a temporary cholestasis and impairment of vitamin K absorption. Affected infants have no accompanying sign of deficiencies of other fat-soluble vitamins except for evidence of lower than normal plasma levels. Both infectious and environmental agents have been listed as possible triggering factors of liver dysfunction leading to late IIDN but the evidence is still largely speculative.

Prophylaxis

Because of the high risk and potentially disastrous consequences of intracranial haemorrhage, and the inability to detect individual infants at risk, many countries are now favouring a policy of vitamin K prophylaxis for all babies. Predictions made from the first survey in the British Isles suggest that without prophylaxis some 40 cases of intracranial haemorrhage due to vitamin K deficiency would occur annually. Both epidemiological and biochemical evidence of the incidence of des-gamma-carboxyprothrombin suggest that parenteral prophylaxis gives almost total protection against late HDN. On the other hand there is equally clear epidemiological and biochemical evidence that a single oral dose at birth, though protecting against classical HDN, gives less protection against the late onset disease than a single parenteral dose. Comparative blood measurements in infants have shown that the bioavailability of phyloquinone after oral administration is much lower than after intramuscular administration. Of more concern is the great variability in the efficiency of intestinal absorption of phyloquinone from traditional oil-based preparations.

Despite its efficacy the practice of intramuscular prophylaxis is not ideal, there are proper concerns about the medical risks and social acceptability of injecting newborn babies and even that the very high tissue levels achieved by intramuscular regimens may be excessive. Future policies should aim at finding more efficient regimens of oral prophylaxis probably involving newer, better absorbed vitamin K preparations and possibly repeated dosages.

Hepatic Metabolism and Vitamin K Antagonism

Vitamin K antagonists based on 4-hydroxycoumarin or indanedione structures were introduced into clinical practice long before their biochemical basis was understood. Leading from the finding in 1970 that oral anticoagulants caused the hepatic accumulation of the metabolite, vitamin K 2,3-epoxide, it is now known that all oral anticoagulants interfere with a metabolic cycle in the liver known as the vitamin K-epoxide cycle (Fig. 3).

Although the dietary form of vitamin K has the stable quinone structure the vitamin K-dependent carboxylase (denoted E1 in Fig. 3) which catalyses the conversion of glutamate residues to gamma-carboxyglutamate residues can only utilize the quinol form. During gamma-glutamyl carboxylation vitamin K quinol is simultaneously converted to vitamin K 2,3-epoxide by vitamin K epoxidase (E2). The similarities in location and requirements of the carboxylase and epoxidase suggest that the same enzyme (E1) may be responsible for both activities. The epoxide produced is recycled by two enzyme activities; in the first step the epoxide is reduced to vitamin K quinone by a dithiol-dependent vitamin K epoxide reductase (E2) while in the second step vitamin K quinone is reduced back to the active quinol form. Although this vitamin K reductase activity is denoted in Figure 3 by a separate enzyme (E3) there is evidence that this conversion may be effected by the same enzyme that reduces the epoxide to the quinone (E2).

Warfarin and other oral anticoagulants are strong inhibitors of the dithiol-dependent vitamin K epoxide reductase (E2) and vitamin K reductase (E3) activities. This accounts for both their pharmacological action and the accumulation of vitamin K epoxide. The dithiol-dependent activity, however, is not the only pathway by which vitamin K can be reduced to the quinol. A second NAD(P)H-dependent pathway (E2) is relatively insensitive to warfarin. During warfarin anticoagulation this enzyme is able to bypass the inhibited dithiol-dependent enzyme. When the epoxide cycle is completely blocked, as in anticoagulant overdose, the NAD(P)H-dependent enzyme
provides the only route by which the antidotal effect of vitamin K may be mediated.93

In humans the inhibition of the vitamin K epoxide reductase by coumarins results in the dose-dependent plasma accumulation of the epoxide metabolite when a pharmacological dose of the vitamin is administered.94,95 This effect provides a ready screening test for possible vitamin K antagonists even when other coagulation indices are normal. In the early 1980's a group of compounds which were suspected of having antivitamin K activity and which caused the release of vitamin K epoxide into plasma49,96 were antibiotics containing an N-methyl-thiotetrazole (NMTT) side chain (e.g. moxalactam, cefamandole etc). Experimental studies in animals have since confirmed that the target of this inhibition is vitamin K epoxide reductase though the active inhibitor is not known.97 These drugs were initially investigated because they had been shown to be associated with an increased risk of bleeding amongst hospitalized patients. The fact that patients at greatest risk were those with low serum phylloquinone concentrations and/or other indications of malnourishment48,49 and that antibiotic-induced hypoprothrombinaemia could not be induced in healthy volunteers92 shows the importance of vitamin K status to the pharmacological expression of these unusual vitamin K antagonists. As shown in Figure 3, in the presence of an inhibitor of vitamin K epoxide reductase, the supply of the quinol to the carboxylase can only be augmented by the input of dietary vitamin K quinone into the cycle and its conversion to the quinol by an alternative NAD(P)H-dependent reductase; therefore in states of dietary depletion even weak inhibitors can sufficiently interrupt the supply of quinol co-factor to cause hypoprothrombinaemia and bleeding.

The enzyme vitamin K epoxide reductase seems to be particularly susceptible to various antagonists with widely different structures. Other compounds with weak antagonistic activity include salicylate,99–101 lapachol102 and sulphaquinoxaline.103 A group of more powerful and potentially dangerous inhibitors of vitamin K epoxide reductase are the so called superwarfarins developed to combat warfarin resistance. Such compounds (e.g. difenacoum, brodifacoum) are characterized by the introduction of tetralin and aromatic moieties at the 3-position providing a more bulky and lipophilic character with apparently much greater binding characteristics than conventional anticoagulant drugs (Fig. 4). This results in very long biological half-lives. Several cases of accidental poisoning with superwarfarins have been reported104–108 including multiple fatalities when poisoned rice bait was eaten by peasant farmers in Indonesia.109 Poisoning with these rodenticides requires very careful clinical management because the requirement for vitamin K to maintain carboxylation is enormously increased and can only be met by frequent transfusion and/or administration of massive doses of vitamin K over a prolonged period of time.86

Non-hepatic Metabolism

Gla-containing proteins and/or vitamin K-dependent carboxylase activity have been located in a number

![Chemical structures of two 'superwarfarin' rodenticides (left) difenacoum and (right) brodifacoum.](image)
of extrahepatic tissues including bone, cartilage, dentin, kidney, placenta, pancreas, spleen, lung and testis. Likewise vitamin K reductase, vitamin K epoxide reductase and/or vitamin K epoxidase activities have also been detected in various extrahepatic tissues or cell lines. This evidence suggests that the vitamin K-epoxide cycle is operational in all tissues capable of synthesizing vitamin K-dependent (Gla-containing) proteins.

Little is known however of the function of non-coagulation Gla-proteins or indeed other aspects of vitamin K metabolism in extrahepatic tissues. Vermeer and colleagues reasoned that anticoagulant drugs may also inhibit vitamin K-dependent processes in extrahepatic tissues with possible deleterious consequences in patients on long term anticoagulant therapy. Although their first results indicated very little inhibition of vitamin K epoxide reductases of extrahepatic soft tissues more recent data suggest that coumarin drugs do inhibit hepatic and extrahepatic enzymes to a similar extent. Oral anticoagulants also affect circulating levels of the bone protein osteocalcin leading to both a decrease in total antigenic protein released into the bloodstream and a decreased Gla-content. Despite these demonstrable biochemical effects, there has been little to suggest that even long term treatment with oral anticoagulants has any adverse effect on the structure and function of mature bone. Two recent reports suggest that anticoagulant therapy may increase urinary calcium loss or reduce bone mass in some patients but this requires confirmation. On the other hand there is an increasing realization and clear clinical evidence that warfarin interferes with the normal process of mineralization in rapidly growing bone. Fetuses whose mothers have been exposed to warfarin in early pregnancy may present with congenital defects known collectively as the fetal warfarin syndrome characterized by excessive calcification of the epiphyses and irregular growth of the facial and long bones. Strong circumstantial evidence that the bony defects of warfarin embryopathy are mediated via its antagonism of the vitamin K-epoxide cycle has come from detailed studies in an infant, who in addition to a selective deficiency of the vitamin K-dependent coagulation factors had identical clinical features to the fetal warfarin syndrome and a specific biochemical defect consistent with a congenital deficiency of vitamin K epoxide reductase. A deficiency of this enzyme would be expected to mimic the antagonistic effect of warfarin and prevent the normal recycling of vitamin K.

It should perhaps be emphasized that the inability to ascribe a function to the vitamin K-dependent proteins osteocalcin and matrix Gla-protein is not unusual; these are just two of several non-collagenous proteins present in bone tissue (others are sialoproteins, proteoglycans, phosphoproteins etc) for which no proven metabolic or structural function are yet known but which many investigators believe are essential to the mineralization process. By analogy to the essential function that gamma-carboxyglutamate residues play in calcium binding of the blood coagulation factors it is not unreasonable to assume that the Gla-containing moieties of osteocalcin and matrix Gla-protein may play an important subtle role in bone mineralization. This raises the question of whether the vitamin K status of an individual can influence normal bone metabolism or affect the progression of disease. Some tantalizing observations are worthy of further exploration and explanation. Firstly it has been shown that circulating phylloquinone was low in osteoporotic patients who had sustained either spinal crush-fractures or fractures of the neck of femur. Secondly some findings in post-menopausal women indicate that supplementation with pharmacological doses of phylloquinone increases both the total and the Gla-content of circulating osteocalcin and may decrease urinary calcium loss particularly when this is already high.

In the last decade then, our conception of the role of vitamin K has changed from that of a rather specific regulatory function in the synthesis of six blood coagulation proteins to the possibility that the vitamin has a more diverse role in calcium homoeostasis. A major problem, however, is that whereas the essential functions of the coagulation Gla-proteins have been delineated those of the non-coagulation Gla-proteins remain to be discovered.

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