Absorption, tissue distribution and bioactivity of vitamin K and related compounds in the rat

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ABSORPTION, TISSUE DISTRIBUTION AND BIOACTIVITY OF VITAMIN K AND RELATED COMPOUNDS IN THE RAT

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof. Dr. A.C. Nieuwenhuijzen Kruseman
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen op
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De enige plaats waar succes eerder komt dan werk is in het woordenboek

Vidal Sassoon

Voor Ed

Voor mijn ouders en Claudia

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ABBREVIATIONS

BGP	bone Gla protein, osteocalcin
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
DTT	dithiothreitol
FII	prothrombin
FLEEL	the pentapeptide phenylalanine-leucine-glutamate-glutamate-leucine
Gla	γ -carboxyglutamic acid
Glu	glutamic acid
HCO-60	polyoxyethylene hydrogenated castor oil derivatives
HPLC	high performance liquid chromatography
MK-n	menaquinone-n
MGP	matrix Gla protein
K ₁	vitamin K ₁ , phylloquinone
K ₂	vitamin K ₂ , menaquinones
KH ₂	vitamin K hydroquinone
KO	vitamin K 2,3-epoxide
NAD(P)H	nicotinamide adenine dinucleotide (phosphate) reduced form
PQ-9	plastoquinone-9
UQ-n	ubiquinone-n

CHAPTER 1

Introduction

1.1 GENERAL INTRODUCTION

1.1.1 Historical background

In 1929 the Danish scientist Henrik Dam described a bleeding disorder in chickens fed a sterol-free diet. The symptoms were not corrected by administration of purified cholesterol.¹ Two years later, McFarlane et al. reported hemorrhages and delayed clotting in chicks fed a diet containing ether-extracted fish or meat meal, which did not occur when ordinary fish or meat meal was offered.² In 1935 the unknown fat-soluble antihemorrhagic factor was defined by Dam as a new vitamin: vitamin K.³ The letter K was the first letter of the word *Koagulation* according to the German and Scandinavian spelling, and a letter in the alphabet not yet occupied by an existing or hypothetical vitamin. In 1939, two forms of vitamin K were isolated from respectively alfalfa (vitamin K1) and putrefied fish meal (vitamin K2) followed by chemical identification.^{4,5} At about the same time, a naturally occurring antagonist of the vitamin was discovered. This antagonist was present in improperly cured sweet clover hay which caused the 'sweet clover disease' in the 1920s among cattle in the American Midwest and Western Canada.⁶ The disease was characterized by comparable haemorrhagic symptoms as described by Henrik Dam. The vitamin K antagonist was isolated and characterized as 3,3'-methylene-bis-(4-hydroxycoumarin) in 1941 and called dicumarol.^{7,8} After the discovery of vitamin K, increasing details of the clotting cascade were elucidated and four clotting factors, II (prothrombin), VII, IX and X appeared to be vitamin K-dependent proteins.⁹ Until 1974, these factors were considered as the only known vitamin K-dependent proteins.

1.1.2 Functions of vitamin K

In 1974 several groups independently established the biochemical role of vitamin K in the promotion of a post-translational modification of glutamic acid (Glu) residues into γ -carboxyglutamic acid (Gla) residues of prothrombin.¹⁰⁻¹² Later on, also the blood coagulation factors VII, IX and X were demonstrated to contain Gla residues, showing the universal function of vitamin K in the post-translational γ -carboxylation. Examples of other known post-translational modifications are phosphorylation, glycosylation, disulfide bond formations and proteolytic cleavages. These modifications take place in the last phase of protein synthesis, shortly before the proteins are secreted by the cell, and occur at the luminal side of the rough endoplasmic reticulum.¹³ Thus far, all Gla-proteins are secretory proteins. The recently discovered proline-rich Gla-proteins which might be membranous receptors, could be exceptions.¹⁴ Secretory Gla-proteins are synthesized in a precursor form that contains a signal sequence necessary for translocation through the endoplasmic membrane, a propeptide and the mature protein. The signal sequence is cleaved off directly after translocation. The propeptide is located at the NH₂ terminal side of the precursor protein and plays a key role in the recognition and binding of the protein to the carboxylase.¹³ The propeptide is proteolytically removed before the proteins are secreted in the plasma via the Golgi apparatus.¹⁵ Gla residues are located near the

amino-terminal region of the vitamin K-dependent proteins. All Gla-proteins known thus far, contain a similar sequence at position +17 to +23: Gla-X-X-X-Gla-X-Cys. At neutral pH the negatively charged Gla residue strongly increases the Ca^{2+} binding capacity of the protein. The Ca^{2+} binding leads to a change in conformation of the vitamin K-dependent blood coagulation proteins thereby facilitating their binding to negatively charged phospholipids.^{13, 16}

Under specific conditions of vitamin K-deficiency, undercarboxylated and biological inactive forms of the blood coagulation factors II, VII, IX and X circulate in plasma which are indicated with the term PIVKA's (Protein Induced by Vitamin K Absence or Antagonists). The occurrence of PIVKA's in plasma from dicoumarol treated patients was already postulated in 1963 by Hemker et al.¹⁷

1.1.3 Chemical structures of vitamin K

Two naturally occurring compounds with vitamin K activity (i.e. antihaemorrhagic activity) are known: phyloquinone (vitamin K1) and the group of menaquinones (vitamin K2). Both compounds have a 2-methyl-1,4-naphthoquinone ring structure in common but differ in the length and degree of saturation of their aliphatic side chain which is attached at the 3-position (figure 1). Phyloquinone is the only form of vitamin K produced by green algae and plants where it plays a role as an electron carrier in the photosynthetic system in the thylakoid membranes of the chloroplasts.¹⁸ The side chain of phyloquinone, named phytyl, consists of 4 isoprenoid units of which only the first one contains an unsaturated bond. Menaquinones are synthesized by intestinal bacteria such as *Escherichia coli*; *Bacteroides fragilis* and *Staphylococcus Aureus*, and possess side chains composed

Figure 1. Chemical structures of phyloquinone (vitamin K1), menaquinone (vitamin K2) and menadione (vitamin K3). n represents the number of isoprenyl units in the side chain.

of repeating (unsaturated) isoprenyl units.¹⁹⁻²¹ Some bacteria may also produce menaquinones in which one or more of the prenyl units is saturated. Menaquinones are generally abbreviated as MK-n, where n stands for the number of isoprenyl units varying from 4-13 in natural menaquinones. In general it can be said that the hydrophobicity of the molecule increases with the length of the side chain.

The 2-methyl-1,4-naphthoquinone structure (menadione or vitamin K3) does not occur in nature as such and has no vitamin K activity by itself. Its K-activity is obtained after conversion into MK-4 in animal tissues.^{22,23} Menadione is used as a supplement in animal food. In humans, menadione is not applied anymore because of its alleged toxic side effects.^{24,25}

1.1.4 Dietary sources of vitamin K

The early vitamin K food analyses were performed with rather insensitive methods, such as the curative chick bioassay, which is quite susceptible for external variations.²⁶ Several food tables containing vitamin K values were published, but these are of questionable value because of methodological pitfalls. In the last two decades, specific and sensitive detection methods using HPLC have been developed for the determination of vitamin K and an increasing number of food composition tables for vitamin K contents became available.^{26,27} Most investigators only considered phyloquinone in their food analyses, but in a limited number of cases also menaquinones were included. The reported values for vitamin K contents in food vary widely, because of differences in detection methods, way of sampling and limited number of determinations per food item. The development of reliable vitamin K food tables will be helpful in dietary counselling of patients on anticoagulant therapy²⁸ and other persons with a risk for subclinical vitamin K-deficiency, e.g. elder people.²⁹⁻³¹

Table 1 lists several nutrients containing high amounts of phyloquinone or menaquinones. Rich sources of phyloquinone are green leafy vegetables as spinach, kale, cabbage and broccoli in a range of 2-8 µg phyloquinone/g, and vegetable oils such as rapeseed, soybean and olive oil containing amounts of about 0.5-2 µg phyloquinone/g.³²⁻³⁴ Booth *et al.* found that vegetables like spinach, collards, lettuce and broccoli contribute for about 40% of the total dietary intake of phyloquinone in a large American population.³⁵ Menaquinones have been demonstrated in dairy products as butter, cheese and eggs; fish and meat.^{36,37} Especially the Japanese food natto (fermented soy beans) contains high amounts of menaquinones, mainly in the form of MK-7. Another dietary form of vitamin K is dihydro-phyloquinone, which is produced during the hydrogenation of vegetable oils and occurs in several (fast-food) products like french fries, fried chicken and apple pie. Its mean daily intake has been estimated to be as much as 30% of the total vitamin K intake in certain age groups of a large American population, but its biological activity is reported to be only about 10% of that of phyloquinone.^{38,39}

Season, degree of maturation and geographical origin of edible plants, food processing

Table 1. Major categories of vitamin K containing nutrients.

Nutrients	Number of references	Phylloquinone (µg/100g)
Vegetables		
Spinach	4	380-1440
Brussels sprouts	2	147-475
Broccoli	4	178-205
Kale	3	618-1657
Cabbage	2	339-719
Swiss chard	1	743-917
Oils & fats		
Rapeseed oil	2	114-188
Soybean oil	2	139-290
Olive oil	2	37-82
Margarin	1	51
Various		
Green tea	1	1430
Parsley	1	550

The data are obtained from various sources³²⁻³⁷ and are expressed as a range of concentrations in pmol vitamin K/g food. The menaquinone content presented in the table is the sum of all menaquinones. The vitamin K concentrations of the nutrients were determined by HPLC-techniques. n.d. stands for not determined.

(e.g. peeling of fruits), heating to temperatures of 185-190°C and exposure to daylight or fluorescent light may all influence vitamin K contents in nutrients.^{34,40} On the other hand, processing techniques like cooking; deep-freezing; heat-sterilization or γ -irradiation (micro-wave oven) did not reduce vitamin K contents of vegetables.^{33, 41}

1.1.5 The vitamin K-cycle

Dietary vitamin K is solely available in the quinone form. The post-translational carboxylation reaction, however, requires the reduced form of vitamin K (vitamin K hydroquinone or KH_2) as a cofactor. Therefore, the vitamin K quinone (K) first has to be converted into KH_2 by NAD(P)H-dependent reductases and/or the dithiol-dependent KO-reductase (see below), which occur both in the cytosol and microsomal membranes.⁴²⁻⁴⁴ During the carboxylation the hydroquinone of vitamin K is oxidized and rearranged into vitamin K epoxide (KO).⁴⁵ This epoxidation provides the energy for the γ -hydrogen abstraction of the Glu residue.⁴⁶ Most probable the reaction mechanism is a diketane ion based proton abstraction leaving a γ -carbanion which subsequently covalently binds CO_2 .⁴⁷ Alternatively, the γ -hydrogen abstraction may be based on a radical mechanism.⁴⁸ In both cases, the final result of the carboxylation reaction is the formation of a second carboxylgroup in

Glu and so the formation of Gla.⁴⁹⁻⁵¹ Although several authors demonstrated that one enzyme may be responsible for the activity of both carboxylase and vitamin K epoxidase *in vitro*, the epoxidation of vitamin KH_2 needs not necessarily to be coupled to the carboxylation.^{45, 52-57} These enzymatical reactions occur at the membranes of the rough endoplasmic reticulum in the liver.

Calculations showed a discrepancy between the low daily vitamin K intake and the relatively high urinary excretion of Gla on a molar base.⁵⁸ This was later explained by the existence of the vitamin K cycle (figure 3). The vitamin K hydroquinone is regenerated from vitamin K epoxide via two subsequent reduction steps both of which may be mediated by the dithiol-dependent KO-reductase.⁵⁹ The physiological cofactor for these reductions is presently unknown. Possible candidates are thioredoxine⁶⁰⁻⁶² and reduced lipoamide.⁶³ Several observations suggest that both reduction steps are performed by the same enzyme: the closely similar variables as the K_i for warfarin⁶⁴, the K_m of dithiol⁴² and, most of all, the observation that the mutation which causes warfarin resistance in rats affects both activities in a similar way⁴⁴. The vitamin K-cycle provides an efficient use of the vitamin which may be recycled 1000 times.⁵⁸ Anticoagulation with coumarins is based on blockade of the KO-reductase.^{59, 65, 66} Consequently, KO accumulates in liver and plasma and the store of KH_2 is exhausted. The blockade can be counteracted by the administration of extra vitamin K which can be converted into KH_2 by coumarin insensitive NAD(P)H-dependent reductases.

Figure 2. The vitamin K-cycle. Vitamin K is reduced to KH_2 via NAD(P)H-dependent reductases. During the oxidation step of KH_2 , glutamic acid residues (Glu) of proteins are converted into γ -carboxyglutamic acid residues (Gla). Vitamin K epoxide (KO) is subsequently reduced in two steps by dithiol-dependent K-reductases into its hydroquinone form.

1.1.6 Human Gla-containing proteins

In 1975, an *in vitro* assay method was developed to measure vitamin K-dependent carboxylase activity in a liver microsomal system.⁶⁷ After 1980, sensitive HPLC detection methods for Gla and vitamin K became available. These developments facilitated the discovery of new Gla-proteins in various tissues. Vitamin K-dependent carboxylase has been found in many tissues like liver, testis, skin, lung and kidney and in cultured celltypes such as hepatocytes; osteoblasts; endothelial cells; renal tubul cells and fibroblasts.⁶⁸⁻⁷⁴ Also vitamin K itself is found in various mammalian tissues, especially in liver, heart and pancreas.⁷⁵⁻⁷⁷ The widespread tissue distribution of γ -glutamylcarboxylase and vitamin K suggests the existence of locally active Gla-proteins. However, only a small number of Gla-proteins has been fully characterized. Various human Gla-proteins will be discussed below and are classified according to their physiological functions in which they are thought to participate.

a. Blood coagulation

Gla-proteins involved in blood coagulation are mainly synthesized in the liver and subsequently released to the blood stream. In 1974, Gla was first discovered in prothrombin¹⁰⁻¹², soon followed by the identification in three other blood coagulation factors VII, IX and X^{78,79}. Other Gla-proteins related to the clotting cascade are: protein C⁸⁰, protein S⁸¹ and protein Z⁸². Activated protein C and S have a function in the inactivation of activated factor V and VIII by limited proteolysis, leading to inhibition of blood coagulation.⁸³⁻⁸⁶ For protein Z, Hogg⁸⁷ postulated an intermediate role in the binding of thrombin with phospholipid vesicles in a Ca^{2+} independent manner, but further experimental evidence for its exact function is lacking at this moment. The vitamin K-dependent clotting factors II, VII, IX and X and the proteins C, S and Z are all typical plasma glycoproteins with molecular weights ranging from 45,000-72,000 Da. The number of Gla residues per molecule in these factors varies from 10 to 12.^{78, 79}

b. Bone metabolism

Extra-hepatic Gla proteins were detected in a wide variety of calcified tissues like: bone; dentin⁸⁸ and atherosclerotic plaques^{89,90}. In bone, several Gla-proteins were demonstrated: bone Gla protein (BGP or osteocalcin)^{91, 92}, matrix Gla protein (MGP)⁹³ and protein S⁹⁴. The precise function of osteocalcin, MGP and protein S in bone metabolism are still not well understood.

Several authors have observed serious bone deformations in newborns whose mothers have taken warfarin during pregnancy.^{95,96} This condition is also known as the Fetal Warfarin Syndrome (FWS) and is characterized by maxillonasal hypoplasia, abnormal calcification of the growth-plate cartilage and cessation of the longitudinal bone growth. In adults on the other hand, the reported effects of long-term oral anticoagulant therapy on bone mass are conflicting, showing both a reduced bone mass^{97, 98} or no significant differences^{99, 100}. Experimental animal studies on the effect of warfarin treatment on bone development gave inconsistent results due to differences in the use of animal species, duration of warfarin

treatment and age of the animals (stage of calcification processes in bone). In two studies, chronically warfarin-treated rats on a strict diet showed bone deformations after the age of 2 months.^{101, 102}

Osteocalcin is the most abundant Gla-protein in man and is produced by osteoblasts and odontoblasts. After its secretion it is bound to the hydroxyapatite matrix of bone.¹⁰³ Osteocalcin may function as a matrix signal in the recruitment of bone-resorbing cells and their differentiation.¹⁰⁴ Remarkably, the absence of osteocalcin genes in adult mice resulted in an increased bone mass and improved mechanical properties.¹⁰⁵ This finding challenges our thoughts about the function of osteocalcin.

Like osteocalcin, MGP is thought to act as a regulator of mineralization in bone and cartilage. In soft tissues MGP may clear extracellular calcium and prevent their calcification.¹⁰⁶ Luo et al. observed inappropriate calcification of various cartilages in MGP-deficient (knock-out) mice older than 2-3 weeks, resulting in short stature, osteopenia and fractures.¹⁰⁷

A relation between protein S and bone metabolism was suggested by Pan et al., who reported the occurrence of severe osteopenia in two patients with inherited protein-S deficiency.¹⁰⁸ Maillard et al. showed that protein S is synthesized and secreted by osteoblasts and suggested that the osteopenia in the two patients as observed by Pan et al. might be related to a deficiency of protein S secretion by the osteoblasts.⁹⁴

c. Vascular biology

In addition to its function in bone metabolism, MGP is thought to play a role in the process of atherosclerosis. In a study in Watanabe heritable hyperlipidemic rabbits, MGP mRNA was markedly increased in the aorta when compared to normal control rabbits. It was suggested that the expression of MGP mRNA is correlated with the progression of atherosclerosis.¹⁰⁹ Shanahan et al. showed that both mRNA and protein for MGP are highly expressed in human atheromatous plaques. The sources of MGP may be both macrophages and vascular smooth muscle cells.¹¹⁰ Furthermore, Luo et al. showed that MGP-deficient mice developed to term, but died within 2 months after birth due to excessive vascular mineralization and rupture of the main blood vessels.¹⁰⁷ The importance of Gla-residues for the mineralization-inhibiting activity of MGP was demonstrated by maintaining standard rats under conditions which specifically inhibit the vitamin K cycle in non-hepatic tissues. After about three weeks these animals developed vascular calcifications, with prominent mineralization of the aorta after 5 weeks (P. Price, personal communication). These observations suggest that MGP is needed to prevent tissues from calcification. If this is true, a serious impact on the vitamin requirement is to be expected.

Recently, another Gla-protein was identified, named Gas6.¹¹¹ Its mRNA was demonstrated in several human and mouse tissues, including heart, kidney and lung.¹¹¹ Gas6 is encoded by the growth-arrest Gas6 gene and can be induced in vitro by Ca^{2+} mobilizing growth factors such as thrombin and angiotensin-II.^{112, 113} Gas6 acts as a ligand for the Axl/Sky receptor tyrosine-kinase subfamily and is reported to stimulate the proliferation of vascular

smooth muscle cells.^{112,114,115} The Gla-residues of Gas6 were demonstrated to be essential for the receptor-binding and its growth-potentiating activity.¹¹³ It is, however, a matter of debate whether vascular smooth muscle cell proliferation causes atherosclerosis or stabilizes the atherosclerotic plaque.¹¹⁶ Therefore, the role of Gas6 on the vascular smooth muscle cell proliferation and process of atherosclerosis *in vivo* needs to be investigated.

d. Other functions of Gla-proteins

Gas6 is also reported to stimulate the proliferation of Schwann cells in the peripheral nervous system *in vitro*. Gas6 acted synergistically with other human Schwann cell mitogens such as heregulin/glia growth factor and forskolin.¹¹⁷ Interestingly, vitamin K was shown to influence the brain sulfatide metabolism in young mice and rats. The brain sulfatide metabolism plays a role in the formation of myeline.¹¹⁸

1.2 VITAMIN K STATUS

1.2.1 Animal vitamin K requirement

The vitamin K requirement varies widely between animal species. Ruminants, for instance, are able to synthesize sufficient vitamin K by rumen micro-organisms to support their own needs.¹¹⁹ On the other hand, chickens have a relatively high need for vitamin K and were reported to be quite susceptible to develop vitamin K-deficiency symptoms.^{120, 121} A possible cause for the latter observation is the relatively poor capacity of chickens to recycle the vitamin K epoxide: the activity of KO-reductase was about 10% of that in rats.¹²² In addition to differences in the recycling, also the practice of coprophagy determines the animal requirement of vitamin K. Laboratory rats consuming a normal purified diet may largely supply their needs by performing coprophagy.¹²³ By this practice, the menaquinones which are synthesized by the colon bacteria become available for absorption in the small intestines. Several studies were conducted to establish the daily requirement of vitamin K in laboratory animals. These data are difficult to compare, however, because of the differences in experimental conditions such as kind of vitamin K used, route of vitamin K administration, criteria used for vitamin K-deficiency, prevention of coprophagy and pre-treatment (germ-free, bile-fistulated, conventional).^{121, 124} For conventional male rats, a minimal daily requirement for phylloquinone between 0.10 and 0.5 µg/g diet was reported.^{125, 126} The dietary vitamin K requirement for female rats is lower than for male rats.¹²⁷ This can possibly be explained by an oestrogen-mediated stimulating effect on vitamin K absorption.¹²⁸

1.2.2 Hemorrhagic disease of the newborn

Due to their low vitamin K-status, newborns are at risk for developing vitamin K-dependent hemorrhagic disease of the newborn (HDN).¹²⁹ The disease has three clinical patterns: early HDN (within 24 hours after birth, occurs very rare), classical HDN (days 2 to 7, incidence: 4 to 17 per 1000) and late HDN (week 2 to 6 months, incidence: 4 to 7 per

100.000).¹³⁰⁻¹³³ Late HDN is often manifested by acute intracranial bleeding.¹³⁴ Several reasons for the low vitamin K-status can be put forward. Firstly, the placental barrier for maternal vitamin K. The ratio of vitamin K levels in cord blood over maternal blood is about 1:30.¹³⁵ Secondly, the low content of vitamin K in human milk.¹³⁶ Therefore, breast-fed babies have an increased risk to develop classical or late HDN.^{133, 137} A third reason may be the intake of medications during pregnancy such as anticonvulsants and tuberculostatics which interfere with vitamin K metabolism.^{138, 139} The third reason is considered to be a main cause for the early form of HDN.

Today, vitamin K (orally or intramuscularly) is routinely given prophylactically to newborns in developed countries.¹⁴⁰ In the early 1990s, Golding et al.^{141, 142} reported the association between intramuscular (i.m.) administration of phyloquinone to newborns and childhood cancer in two British hospitals in a retrospective study. However, a number of large prospective epidemiological studies did not confirm such an association.¹⁴³⁻¹⁴⁵ The finding of Golding et al. subsequently resulted in a change of recommendations in many countries in favor of oral administration of vitamin K instead of i.m. administration. Single oral administration, however, appeared to be less effective in the prevention of the late form of HDN than i.m. administration.^{131, 146-149} Cornelissen et al. showed that one single dose of 1 mg vitamin K orally at birth followed by repeated maintenance doses of 25 µg/day or 1 mg/week of phyloquinone during 3 months would be a better alternative.^{150, 151}

1.2.3 Adult vitamin K requirement

Humans have a low requirement of vitamin K, and clinical vitamin K deficiency is rarely seen in healthy adults. Dietary vitamin K deficiency is difficult to induce in humans.¹⁵² In the past, the human requirement of vitamin K has often been studied in subjects who received a diet deprived of vitamin K combined with antibiotics therapy. From the studies from Frick et al., O'Reilly and Doisy, the minimal daily requirement in adult man can be estimated and ranges between 0.03 and 1.5 µg/ kg body weight/ day.¹⁵³⁻¹⁵⁵ These authors used the prothrombin time (PT) or prothrombin activity as a measure for vitamin K status. In later studies, more sensitive assays were used to establish vitamin K status during dietary phyloquinone restriction and repletion in humans, like PIVKA's, urinary Gla excretion and undercarboxylated prothrombin.^{156, 157} These studies also showed that the human daily requirement of phyloquinone was about 1 µg/ kg body weight. Olson established recommended dietary intakes (RDI) for different age groups ranging from daily 10 µg for infants to 45 µg for adult males.¹⁵⁸ The current Recommended Dietary Allowance (RDA) for vitamin K is partly based on these studies and has been established at approximately 1 µg/kg body weight per day.¹⁵⁹ The daily vitamin K intake is estimated at 50-500 µg per day, so that the diet in general will amply cover the human need.^{35, 156, 160, 161} Whether the current RDA value is also sufficient for extrahepatic Gla-proteins remains to be established. Sokoll et al. and Bach et al. showed that after administration of a "minidose" (1 mg/day) of warfarin to subjects, serum concentrations of undercarboxylated osteocalcin

increased markedly, whereas blood coagulation factor activities (PT, Factor VII concentrations) were hardly influenced except for the plasma prothrombin concentrations in elderly subjects.^{162,163} Ferland and Sadowski showed that a subclinical vitamin K deficiency can be obtained during dietary phyloquinone restriction, without affecting blood coagulation.¹⁵⁷ In a group of postmenopausal women with normal blood clotting activities, vitamin K supplementation of 1 mg/day led to an increase of the hydroxylapatite binding of circulating osteocalcin, a measure for the carboxylation of osteocalcin.³⁰ These studies show that extrahepatic Gla-proteins seem to be more sensitive during vitamin K deficiency than the hepatic vitamin K-dependent clotting proteins.

1.2.4 Absorption and bioavailability of vitamin K

The intestinal absorption processes of vitamin K are highly comparable to that of the other fat-soluble vitamins A, D and E.¹⁶⁴⁻¹⁶⁶ Vitamin K is absorbed by the intestinal mucosa in a mixed micelle complex consisting of bile salts, fatty acids and monoglycerides. The main region of absorption is believed to be the ileum and jejunum. The uptake of bile salts is almost complete at the terminal ileum. The presence of bile salts and pancreatic lipase is a prerequisite for the absorption of vitamin K. This is consistent with the low plasma vitamin K levels as observed in patients suffering from cholestatic disease (e.g. obstructive jaundice) or pancreatic insufficiency. These patients have an impaired absorption of lipids and lipid-soluble compounds leading to an increased risk for vitamin K-deficiency.^{167,168}

The degree of vitamin K absorption was shown to depend on the number of isoprene units in the aliphatic side chain of menaquinones.¹⁶⁹ It also strongly depends on the efficiency with which vitamin K is liberated from the food matrix. The oral availability of phyloquinone from a pharmaceutical formulation may amount up to 80%.¹⁶⁷ The absorption of phyloquinone from food is expected to be lower. In plants, phyloquinone is tightly bound to the thylakoid membranes of the chloroplast.¹⁸ On the other hand, fat-solubilized vitamin K (milk, cheese) may be readily available for absorption. Gijsbers et al. compared the absorption of phyloquinone in a pharmaceutical preparation (Konakion®) with that of spinach.¹⁷⁰ Moreover, the effect of fat on the bioavailability from spinach was estimated. Ingestion of the phyloquinone formulation resulted in approximately 15 times higher peak serum phyloquinone levels than after consumption of the spinach. The availability from phyloquinone from spinach alone was only 4% that from the pharmaceutical preparation. The addition of butter to the spinach meal increased the availability of vitamin K with a factor 4. They concluded that the degree of absorption of vitamin K strongly depends on the source from which it is obtained. Also Uematsu et al. found a stimulating effect of fat on vitamin K absorption from a meal.¹⁷¹

1.2.5 Transport and metabolism of vitamin K

After gut absorption, dietary vitamin K is incorporated into chylomicrons and transported

via lymph and plasma to the liver where they are taken up as chylomicron remnant particles.^{167, 172, 173} After oral administration, both phylloquinone and MK-4 reach a peak in plasma after 2-6 hours.^{170, 174, 175} No specific plasma carrier protein in serum is known for vitamin K as is the case for vitamin A and D. Instead, plasma vitamin K is mainly located in VLDL particles and chylomicrons with a relatively small proportion in the LDL and HDL fractions.¹⁷⁴⁻¹⁷⁶ Four hours after oral administration of 5 mg phylloquinone, 81%, 11% and 7% of the vitamin were found in VLDL, LDL and HDL fractions respectively.¹⁷⁵ On the other hand, intramuscular administration of 5 mg phylloquinone resulted after 10 hours in a different distribution pattern in lipoproteins with relatively high levels of vitamin K in LDL (49%) and HDL (37%) fractions.¹⁷⁵ The difference in plasma lipoprotein distribution of vitamin K after oral or intramuscular administration might result in a different tissue distribution. Because LDL is taken up by extrahepatic tissues, higher vitamin K levels may be expected in these tissues after intramuscular administration of K levels than after oral administration. Since vitamin K is present in the triglyceride rich lipoproteins, it is not surprising that plasma vitamin K levels were shown to be strongly correlated with fasting plasma triglyceride levels.^{177, 178} This is consistent with the high circulating vitamin K levels in patients suffering from hyperlipaemia.¹⁷⁹ Also apolipoprotein E (apoE) was shown to be associated with plasma vitamin K values. The apo E genotype determines the uptake of chylomicron remnants by the liver receptor. The hepatic clearance is highest in the E4 subtype, followed by E3 and E2.¹⁸⁰ A high chylomicron clearance corresponds with low plasma vitamin K levels.^{178, 181} In rats, using radiolabelled compounds, Konishi et al. reported a faster clearance of MK-4 compared to phylloquinone.¹⁸² The half-life time for phylloquinone in human plasma is approximately 4 hours.^{167, 175} Vitamin K is mainly secreted by the bile as metabolites and leaves the body with the faeces. A part of the vitamin K, however, re-enters the plasma via the enterohepatic cycle. Some excretion additionally takes place via the urine as glucuronide conjugates.^{174, 183}

1.2.6 Plasma levels of phylloquinone

The introduction of sensitive HPLC analytical techniques for the assay of vitamin K, resulted in growing insight in the physiological vitamin K distribution. Phylloquinone is the predominant K-vitamin in plasma. Plasma phylloquinone levels in normal healthy adults usually range from 0.1 to 2.0 ng/ml, with a median fasting value around 0.5 ng/ml.^{161, 177, 179, 184, 185} However, large intra- and interindividual differences in plasma phylloquinone levels are present.¹⁶¹ Human fasting plasma phylloquinone concentrations were positively correlated with dietary intake.^{161, 186} High plasma phylloquinone levels were found in several diseases like primary and secondary hyperlipidaemia and hyperlipoproteinaemia.^{161, 176, 179} On the other hand, osteoporosis, coeliac disease, parenteral feeding, and severe pancreatic insufficiency or biliary obstruction were associated with low levels of phylloquinone.^{167, 179, 187} Furthermore, fasting phylloquinone plasma levels were demonstrated to be positively associated with plasma triglycerides and vitamin E and were slightly

influenced by season, alcohol consumption, age and gender.^{177, 178} The use of the ratio of plasma phyloquinone to plasma triglycerides leads to a decrease in intra and inter-individual variations. Therefore, several authors prefer the use of this ratio as an index for vitamin K status rather than the plasma vitamin K levels alone.^{172, 177} Plasma concentrations of phyloquinone may be indicative for tissue reserves. A decrease in plasma phyloquinone concentrations in surgical patients as a result of dietary restriction in vitamin K intake, was paralleled by lower hepatic levels.¹⁸⁸ In normal subjects, dietary restriction of vitamin K also led to decreased plasma phyloquinone levels and, moreover, mild signs of hypoprothrombinaemia what suggests decreased hepatic phyloquinone contents.¹⁵⁶ Also vitamin K concentrations in bone may be associated with plasma vitamin K levels. Low circulating concentrations of vitamin K were found in patients with an increased risk for hip fractures or spinal crush fractures and in subjects with incompletely carboxylated bone protein osteocalcin.^{178, 189, 190}

1.2.7 Tissue distribution of vitamin K

Until recently, only a few data on the tissue distribution of K-vitamins in the organism were published. One of the reasons was the difficulty in the analysis of vitamin K. The development of the current sensitive and accurate HPLC assays and the improvement of the preceding multi-stage extraction assay to remove interfering lipids, enabled the detection of low levels of vitamin K in tissues. Vitamin K is found in a wide variety of rat and human tissues including liver, heart, brain, kidney, bone, lung, pancreas and brain.^{76, 179, 191, 192} Storage of vitamin K mainly occurs in the liver, although bone and heart are also reported to be abundant sources.^{179, 188, 191-193} Vitamin K is found in both cortical and trabecular bone in adipocytes or associated with the matrix.¹⁹² In human liver about 50-90% of the vitamin K store is present as long-chain menaquinones, as calculated on a molar base. Among the large range of menaquinones found in liver (MK-4 to MK-13); MK-7, MK-8 and MK-10 to MK-12 are the predominant homologues, although there exist large inter-individual differences in their relative concentrations.^{77, 188, 193-195} In contrast to the abundance of menaquinones found in the liver, thus far only MK-7 and MK-8 could be detected in human plasma in reasonable amounts.^{179, 188, 196} This suggests that liver has a higher affinity for the more lipophilic forms of vitamin K than plasma. Extrahepatic tissues hardly contain menaquinones except MK-4. Tissue-specific accumulation of MK-4 occurs in the pancreas, salivary glands and brain.^{76, 191} After feeding rats a vitamin K-deficient diet during 9 days, hepatic stores were almost depleted, whereas other tissues like pancreas, brain and sternum still contained relatively high amounts of vitamin K, mainly in the form of MK-4.⁷⁶ This suggests large differences in uptake and metabolism of vitamin K for the tissues. Subcellular fractionation of the rat liver shows an equal distribution of phyloquinone and MK-4 over microsomes and mitochondria, whereas cytosol hardly contains vitamin K.^{76, 197} Higher menaquinones preferentially accumulate in the mitochondria.^{76, 197}

Already in 1960, Billeter and Martius demonstrated that orally administered phyloquinone

can serve as a source for MK-4 in skeletal muscle, heart and kidney but hardly in liver of chickens and pigeons.¹⁹⁸ This observation was confirmed by Thijssen et al. and Yamamoto et al., who found that many rat tissues, including pancreas, sternum, brain and heart, accumulated MK-4 after oral phyloquinone intake.^{76, 191, 199} The latter authors suggested that the MK-4 accumulation in extrahepatic tissues is due to synthesis in these tissues rather than uptake from the circulation. Billeter and Martius showed that intravenous or intraperitoneal administration of phyloquinone did not result in MK-4 tissue accumulation.²⁰⁰ Moreover, they found that intestinal bacteria were able to remove the side chain of phyloquinone resulting in menadione which subsequently can be used by bacteria for further synthesis into other menaquinones. Based on these observations, they proposed an intermediate role for the intestinal bacteria in the conversion of phyloquinone into MK-4. So, two possible locations for the conversion are given: the tissues or the gut bacteria. A third alternative may be a bacterial degradation of phyloquinone into menadione which after absorption is converted into MK-4 by the tissues. Thus far, the exact location of the converting activity remains uncertain, whereas neither the mechanism for this conversion nor a tissue-specific function of MK-4 are known.

1.2.8 Biological activity of vitamin K

In vitro, the cofactor activity of phyloquinone and menaquinones can be measured directly in specific assays for carboxylase and KO-reductase.^{201, 202} The cofactor activity of menaquinones varied with the side-chain length. Based on their V_{sat}/K_m ratios, the higher menaquinones ($n > 5$) were most active.²⁰¹ In vivo, the biological activity of the various K-vitamins is usually tested as their potency to reverse hypoprothrombinaemia. Matschiner and Taggart investigated the biological activity of various K-vitamins at 18 hours after intracardial injection to rats.²⁰³ They found that phyloquinone was about 8 times more active than MK-4, but that menaquinones with longer side chains (MK-7 to MK-10) were up to 25 times more active than phyloquinone. Also, Groenen et al. found a 2-5 fold higher efficacy of phyloquinone compared to MK-4 in reversing hypoprothrombinaemia after either oral or subcutaneous administration in rats.²⁰⁴ In a study feeding rats 1-3 weeks with either phyloquinone or MK-9, it was found that MK-9 was biologically less active.²⁰⁵ So, the results from the study of Matschiner and Taggart differ from that of Will and Suttie, but are difficult to compare because of differences in experimental setup. Firstly, different administration routes were used in their studies. Secondly, Matschiner and Taggart measured the vitamin K activity at one particular time point after injection, whereas Will and Suttie compared the activities of phyloquinone and MK-9 after long-term dietary administration followed by dietary restriction of vitamin K.

Akiyama et al. studied the effect of orally and intravenously administered menadione and MK-1 to MK-14 on hypoprothrombinaemia induced by diet and warfarin in rats.¹⁶⁹ A rapid response (within 3-6 hours after administration) was only observed for MK-4 to MK-8. MK-9 showed an effect on blood coagulation activity after 24 hours, but this effect

was still lower than that of MK-4 at the same time point. The delayed response suggests a slow absorption of MK-9. On the other hand, elevated hepatic concentrations of MK-9 to MK-11 were found six hours after administration. A possible explanation for the discrepancy between the elevated hepatic concentrations and the absence of an effect on hypoprothrombinaemia, may firstly be found in the subcellular localization. As has already been mentioned in paragraph 1.2.7, long-chain menaquinones are localized in mitochondria rather than in microsomes, whereas phyloquinone and MK-4 are equally divided over these two fractions.^{76, 193, 197} According to this subcellular distribution, phyloquinone and MK-4 would be more readily available for vitamin K-dependent enzymes than MK-9, what might result in a higher bioactivity. A second explanation may be a low efficiency of MK-9 in the vitamin K cycle. Reedstrom and Suttie recently showed that phyloquinone was more effectively utilized by γ -glutamyl carboxylase than MK-9 after blocking the K-cycle with warfarin.¹⁹⁷

Thus, the relative bioactivity of the various K-vitamins in vivo seems to vary with the experimental setup and may depend on factors such as intestinal absorption, (subcellular) tissue distribution and their use in the vitamin K cycle.

1.2.9 The contribution of intestinal menaquinones to the human vitamin K status

Historically it was thought that bacterially synthesized menaquinones in the large intestines of mammals were an important source of vitamin K. This view was supported by the lack of appearance of a severe hypoprothrombinaemia in adult humans after dietary restriction of vitamin K, which suggests the availability of vitamin K sources not related to the diet. Moreover, several reports showed an association of antibiotics therapy with the development of serious hypoprothrombinemia, which was assumed to result from a decrease in menaquinone synthesis by the intestinal flora.^{153, 154, 206} Recent findings, however, indicate that the contribution of bacterial menaquinones to mammalian vitamin K utilization may have been overestimated.

Normal human intestines accommodate an enormous bacterial population. Major menaquinone synthesizing bacteria, with their main homologues given between parentheses, are: *Escherichia coli* (MK-8), *Bacteroides* (MK-9, MK-10 and MK-11), and *Eubacteria* (MK-6). The total amount of menaquinones produced in the distal colon contents is about 20 $\mu\text{g/g}$ dry weight.^{19, 20, 207} Human liver also contains substantial amounts of menaquinones with the highest levels for MK-7, MK-8 and MK-10 to MK-12.^{77, 188, 193-195} So, bacterial homologues of menaquinones formed in the human intestines are present in the liver as well, what suggests the absorption of intestinally produced menaquinones.^{20, 207} If absorbed, this large pool of menaquinones would provide an important source of vitamin K. However, the uptake of the highly lipophilic bacterial menaquinones from the colon may be hampered by both the absence of bile salts in the colon and by their tight binding to the cytoplasmic membranes.^{208, 209} Several authors have studied the absorption of menaquinones from

the intestines. From these studies it emerged that both phyloquinone and menaquinones are absorbed in the small intestines if solubilized in bile but that menaquinones are hardly absorbed from the colon in the absence of bile.^{194, 210-212} Several other authors reported no effect on hypoprothrombinaemia after administration of either phyloquinone or MK-4 in the colon, suggesting no absorption.^{194, 204} From these findings it can be argued that the most probable place for absorption of bacterially produced menaquinones would be the terminal ileum and the caecum where both bile salts and MK-producing bacteria are present.^{207, 208, 213} Conly and Stein demonstrated the presence of high amounts of MK-9 and MK-10 (about 8 µg/g dry weight) in the terminal ileum contents of two healthy subjects.²⁰⁷ Shearer and von Kries observed small amounts of MK-6 to MK-10 in ileal juice from six volunteers, although the interindividual variations were large.²¹³ In rats, a positive relation between menaquinone levels in the caecum and hepatic menaquinone contents after various dietary interventions was shown by Ramotar et al.²¹⁴ Furthermore, two studies have shown the uptake and bioactivity of bacterially produced menaquinones after oral administration to anticoagulated human subjects²¹ or after oral inoculation in germ-free rats¹⁹. In these studies, the absorption of menaquinones in the gut most likely occurred in the small intestines. Even when menaquinone absorption would occur at a limited rate, life-time hepatic accumulation of these menaquinones may contribute to vitamin K status. In rats, it was shown that bacterially produced menaquinones become available for absorption by means of coprophagy.¹²³ The prevention of coprophagy with the use of tail cups in combination with a vitamin K-deficient diet, resulted in a severe hypoprothrombinaemia in these rats.^{123, 215} This means that bacterially synthesized menaquinones were not sufficiently absorbed to prevent this condition. If bacterial vitamin K would be utilized by the host, germ-free rats are expected to be more susceptible to develop vitamin K-deficiency and to have a higher need for dietary vitamin K than conventional rats. However, experimental data on germ-free rats are conflicting. After consumption of a vitamin K-deficient diet or fasting, germ-free rats were reported to be more^{216, 217} or equally susceptible²¹⁸ when compared to conventional rats.

The effect of antibiotics on the development of hypoprothrombinaemia was originally ascribed to the suppression of the intestinal menaquinone synthesis. Now it is known that antibiotics that contain the N-methylthioletrazole (NMTT) side chain, can also cause the inhibition of the KO-reductase activity.^{194, 219, 220} As reviewed by Shevchuk and Conly, also antibiotics which did not contain the NMTT side chain could cause hypoprothrombinaemia in debilitated patients.²²¹ This possibly can be explained by the relative deficiency of vitamin K in these patients caused by a low food intake or severe illness, which may predispose persons to hypoprothrombinaemia. Conly and Stein found that post-mortem livers of antibiotics-treated subjects contained lower concentrations of menaquinones than livers from untreated subjects, whereas their phyloquinone levels were comparable.²²² This suggests a relation between a supposed antibiotic-induced reduction of intestinal menaquinone contents and reduced hepatic menaquinone contents. Unfortunately, the

is very limited, we will focuss in these paragraphs on ubiquinone. Ubiquinones, also known as coenzymes Q, have a function in membrane stabilization, as an antioxidant and as electron carrier in the oxidative phosphorylation at the mitochondrial membranes.²²³⁻²²⁶ For its antioxidant function, ubiquinone needs to be in the reduced state. The potential beneficial therapeutical effects of ubiquinone-10 have been tested in cardiovascular and breast cancer patients.²²⁷⁻²³¹ The outcomes of the studies vary widely, however. Several decades ago, Kruse and Dam reported that ubiquinones do not possess vitamin K activity when tested in the chick bioassay.²³² Lowenthaler and MacFarlane found that 3-phytyl substituted analogues of plastoquinone and ubiquinone were also devoid of vitamin K activity when tested in a warfarin anticoagulated rabbit.²³³

Ubiquinone is provided both by *de novo* biosynthesis and by dietary intake.²²⁵ Biosynthesis of ubiquinones occurs in the mitochondria and, to a lesser extent, at the endoplasmic reticulum.^{225,234,235} All tissues investigated thus far contain ubiquinone and mRNA encoding the enzyme 3,4-dihydroxy-5-polyprenylbenzoate methyltransferase that is essentially involved in the ubiquinone biosynthesis.²³⁶⁻²³⁹ Although in nature several homologues are known for ubiquinone with varying numbers of isoprenyl units (UQ-6 through UQ-10), the predominating homologue found in animals is UQ-9, whereas in humans this is UQ-10.^{236,237} Blood levels of UQ-10 of normal humans are on average 0.8 µg/ml, but these can be influenced by external conditions as age, disease, fasting and sport.²⁴⁰⁻²⁴²

Ubiquinones are present in various nutrients like cereals, oils, vegetables, fish, meat and pulses.^{243,244} The concentrations of ubiquinones in nutrients given below are expressed per gram of dry weight. Good sources of UQ-9 are corn oil (150 µg/g); wheat germ (104 µg/g) and lettuce (36 µg/g). UQ-10 has a broader dietary distribution and is found in sardine, mackarel and cattlefish (100-230 µg/g); pork, chicken and beef (80-120 µg/g); spinach, broccoli, rapes and chinese cabbage (70-140 µg/g) and both rapeseed oil and soybean oil (70-100 µg/g). Interestingly, the distribution pattern for ubiquinones in food is comparable to that of vitamin K, although the concentrations of ubiquinones generally are higher.

1.3.2 Absorption, transport, tissue distribution and metabolism of ubiquinones

The pharmacokinetic properties of ubiquinones have mainly been studied using the homologues UQ-7, UQ-9 and UQ-10 in rats. Ubiquinones are poorly absorbed by the rat intestines.^{244,245} After absorption, ubiquinone is recovered in the lymph.²⁴⁶ A peak concentration is reached in blood after about 4 hours but depends on the use of the solvent.^{245,247} In plasma, ubiquinones are mainly transported in LDL, VLDL and chylomicrons and to a lesser extent in HDL.²⁴⁸ In LDL, reduced ubiquinone may serve as a major factor in preventing the oxidation of cholesterol within LDL.²⁴⁸ The character of the solvent used for the ubiquinones, e.g. castor oil (HCO-50) or vegetable oil, determines the rate and extent of absorption as well as the tissue distribution.^{246,249-251} Ubiquinones are excreted via bile and about 60% leaves the body via the faeces within 1-3 days.^{246,252} Only a very small amount of ubiquinones is excreted in the urine.^{245,246} Like vitamin K, ubiquinones

are metabolized to γ -lactones and the metabolites are excreted as conjugates, probably with glucuronide.²⁴⁵ The half-life time of UQ-9 is reported to be about 79 hours for the liver.²⁵³

Ubiquinones have a broad distribution in tissues and cell organells,^{236-238, 244} which underlines their importance. The highest levels of ubiquinone are present in heart, liver and kidney. However, after dietary administration of ubiquinones, accumulation remains restricted to plasma, liver and spleen.^{246, 247, 254, 255} The accumulation of ubiquinones in the latter tissue might be explained by its relatively high blood contents, leaving the liver as the major storage site. In rat liver, saturation is reached after 4 days intake of about 5 mg UQ-10 per day per 100 g body weight.²⁴⁴ Within the hepatic rat cell, the highest amounts of ubiquinones are found in mitochondria, lysosomes, plasma membranes and golgi vesicles.^{238, 255} When absorption, transport and tissue distribution of ubiquinones is compared with that of vitamin K, again a high similarity can be noticed.

1.4 CONTENT OF THIS THESIS

Vitamin K, ubiquinone and plastoquinone show a high degree of structural similarity and all have a function in the electron transport in the photosynthesis in green plants. Both vitamin K and ubiquinones are widely distributed over animal tissues and are present in various food items as vegetables and oils. Because of the similarities in structure, function in green plants and pharmacokinetic properties, we hypothesized a functional similarity or antagonism between these compounds. This hypothesis was tested *in vitro* and *in vivo* (chapter 2). Another prenylquinone, vitamin E quinone, was already shown to be an inhibitor of the vitamin K-dependent *caboxylase*.^{256, 257}

In chapter 3 and 4 of this thesis we have investigated pharmacological aspects and the bioactivity of various forms of vitamin K in rats. Although both phyloquinone and menaquinones possess vitamin K activity *in vitro*, their activity *in vivo* may be different depending on processes like absorption, transport, tissue distribution and metabolism. Thus far, nothing is known about the effect and tissue distribution of high doses of vitamin K in mammals. Nevertheless, doses as high as 45 mg/ day of are recommended in Japan for the treatment of osteoporosis. In Europe, at least three randomized clinical trials are in progress to examine the potential beneficial effect of increased vitamin K intake on bone mass and vessel wall characteristics. Therefore, we have investigated the effect of very high doses of vitamin K on tissue distribution, haemostatic parameters and arterial thrombosis tendency in rats. The results of one of our studies and that of others, led to renewed interest in an old discussion about the biochemical conversion of dietary phyloquinone into . In a germ-free rat model we have investigated whether this conversion could solely be performed by the tissues, or that the presence of a gut bacterial flora is a necessity.

The vitamin K content of human food items, vitamin K consumption and vitamin K status in humans was studied in chapter 5. Most food composition tables on vitamin K that have been published thus far, only included phylloquinone. We have compiled a table with both phylloquinone and menaquinone contents of various nutrients selected from an European diet. In a small human volunteer study we studied the bioavailability of phylloquinone or menaquinone from different meals and their effect on blood coagulation factors during stable oral anticoagulation.

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CHAPTER 2

THE EFFECT OF PRENYLQUINONES ON THE VITAMIN K ACTIVITY IN VITRO AND IN VIVO

- 2.1 Vitamin K-antagonistic effect of plastoquinone and ubiquinone derivatives in vitro
- 2.2 Natural prenylquinones inhibit the enzymes of the vitamin K cycle in vitro
- 2.3 Investigations on the potential effect of natural ubiquinones on the vitamin K status in the rat

CHAPTER 2.1

VITAMIN K-ANTAGONISTIC EFFECT OF PLASTOQUINONE AND UBIQUINONE DERIVATIVES IN VITRO

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Summary

Decyl-ubiquinone and decyl-plastoquinone were used as model compounds to test the potential effect of quinone derivatives on two enzymes of the vitamin K cycle *in vitro*. Substantial inhibition of γ -glutamate carboxylase was found, whereas vitamin K-epoxide reductase was inhibited to a much lesser extent. The inhibitory effect of both decylquinones was eliminated in a time-dependent way by solubilized microsomes, but not by purified carboxylase. Since a wide variety of prenylquinones occur as micronutrients, these results are of potential relevance for the effects of natural quinones in the human diet.

Introduction

Prenylquinones and prenylquinols like phyloquinone (Vitamin K₁), plastoquinone-9 (PQ-9), ubiquinone-10 (UQ-10), and α -tocopherol (vitamin E) are produced by green plants where they are localized in cellular organs (Figure 1). UQ-10 is a genuine constituent of the mitochondria whereas all the others occur in the thylakoid membrane, the photochemically active biomembrane of chloroplasts. Phyloquinone is a component of the photosynthetic electron transport within photosystem I^{1,2}, whereas PQ-9 and its corresponding quinol form a lipophilic redox system.³ α -Tocopherol quinone and α -tocopherol represent another lipophilic redox system of the photochemically active thylakoids. The localization and function of these various compounds in the plant cell have been reviewed recently.⁴

In man prenylquinones are part of the daily nutrition, and several members of this group are regarded as micronutrients. For instance vitamin K is an indispensable cofactor for the mammalian enzyme γ -glutamate carboxylase⁵, a posttranslational enzyme which converts glutamate residues into γ -carboxyglutamate (Gla). Besides phyloquinone, also a number of bacterial menaquinones (generally known by their group-name vitamin K₂) have 'vitamin K activity'. The active cofactor for the carboxylase enzyme is vitamin K quinol (KH₂), which is oxidized into an epoxide (KO) during the carboxylation reaction. In two successive steps KO may subsequently be reduced via the quinone into KH₂, so that it may be re-used several thousand fold. The physiological cofactor for the reductase(s) involved in the recycling of vitamin K is still unknown, for *in vitro* systems dithiols (dithiothreitol, thioredoxin) have proven to be efficient.⁶

Except for vitamin E, which is a weak inhibitor of carboxylase⁷, no information is available concerning the *in vivo* or *in vitro* interaction of prenylquinones with the vitamin K-dependent carboxylase is available at this time. Here we demonstrate that the synthetic derivatives decyl-plastoquinone (d-PQ) and decyl-ubiquinone (d-UQ) may significantly affect the bovine liver vitamin K-dependent carboxylase. It is suggested that also natural (dietary) prenylquinones should be evaluated for their possible effects on human vitamin K status.

Figure 1. Structure formulas of common prenylquinones. For natural plastoquinones and ubiquinones, n represents the number of isoprene residues, which may vary depending on their origin. In the synthetic model compounds used in this paper, the 3-position of the quinones is occupied by an unbranched saturated decyl chain.

Materials and methods

Materials

Phylloquinone (vitamin K₁), menaquinone-4 (MK-4), Triton X-100, dithiothreitol (DTT), 3-((3-cholamidopropyl)-dimethylammonio)-1-propane sulphonate (CHAPS), d-PQ, d-UQ, and L- α -phosphatidyl choline from egg yolk were from Sigma (St. Louis, MO). Before use d-PQ and d-UQ were purified further by high performance liquid chromatography using a reversed phase column (Chrompack, Lichrosorb RP-18) and isocratic elution with 70% (v/v) methanol in H₂O as an eluent. The various quinones were converted to the corresponding quinols by incubating 6 mM of quinone in the presence of 150 mM DTT at pH 8.5 and 37°C overnight in a light-protected tube. KO was prepared according to the method of Tishler et al.⁸ The pentapeptide Phe-Leu-Glu-Glu-Leu (FLEEL) was purchased from Vega Biochemical Co. (Tucson, AZ), NaH¹⁴CO₃ (56 Ci/mol) and Formula 989 from New England Nuclear (Dreieich, Germany). Salt washed microsomes were prepared from normal bovine liver according to earlier described methods⁹, and were used as starting material for the purification of carboxylase according to the method of Wu et al.¹⁰ All chemicals were of analytical grade or better.

Carboxylase assay

Standard reaction mixtures (0.125 ml) contained: 1 mg of microsomal proteins, 0.4% (w/v) CHAPS, 0.5 M NaCl, 25 mM Tris-HCl (pH 7.5), 4 mM FLEEL, 1 M (NH₄)₂SO₄, 1.5 μ Ci NaH¹⁴CO₃, 4 mM DTT, and 200 μ M of either vitamin K, KO or KH₂. Inhibitors were added as indicated. For tests with purified carboxylase, 2 μ g of enzyme was incubated in 0.125 ml reaction mixtures containing 0.1% (w/v) phosphatidyl choline, 0.1% (w/v) CHAPS, 0.15 M NaCl, 50 mM Tris-HCl (pH 7.5), 4 mM FLEEL, 1.5 μ Ci NaH¹⁴CO₃ and 200 μ M

of vitamin KH_2 . Standard incubations were performed for 15 min at 20°C for washed microsomes and 30 min at 20°C for purified carboxylase. The reactions were stopped as detailed earlier.¹¹ All data are presented as the means of three independent experiments.

KO-reductase assay

Standard reaction mixtures (0.25 ml) contained 2 mg of microsomal proteins, other components were as described for the carboxylase assay except for FLEEL and $\text{NaH}^{14}\text{CO}_3$, which were omitted. Extraction and analysis of the samples were performed according to Thijssen.¹²

Analytical Methods

Protein concentrations were determined according to the procedure described by Sedmak and Grossberg.¹³

Results

In this paper vitamin K activity is defined as the ability of a compound to function as a cofactor in the CO_2 -fixation in an in vitro model system containing bovine liver microsomes, the pentapeptide FLEEL, and $\text{NaH}^{14}\text{CO}_3$. In this system we have tested the vitamin K activity of d-UQ and d-PQ both in their quinone and their quinol form. The data are given in Table 1 and demonstrate that neither of these compounds had vitamin K activity.

Table 1. Effects of prenylquinones on the enzymes of the vitamin K cycle

Compound added	Concentration (mM)	Cofactor activity for: carboxylase (pmol $\text{CO}_2 \cdot \text{min}^{-1}$)	Vitamin K-antagonistic activity for:	
			carboxylase (% inhibition)	KO-reductase (% inhibition)
K_1H_2	0.2	100	n.d.	n.d.
K_1	0.2	68	0	n.d.
KO	0.2	52	n.d.	0
d-PQ	1	0	76	11
d-PQH ₂	1	0	78	n.d.
d-UQ	1	0	80	10
d-UQH ₂	1	0	81	n.d.

The cofactor activity for γ -glutamylcarboxylase was measured in the crude microsomal system and is expressed as pmol CO_2 incorporated per minute. Vitamin K-antagonistic activity was tested: a) in a carboxylating system containing a fixed amount of vitamin K_1 quinone (0.2 mM), and b) in the KO-reductase system; the data are expressed as the percentage to which both systems are inhibited. All data are the means of triplicate experiments. n.d. = not determined.

Vitamin K inhibitory activity was tested in a similar system, to which 0.2 mM K_1 quinone had been added. In this way we found substantial vitamin K_1 antagonistic activity of both d-UQ and d-PQ in their quinol as well as in their quinone form. The inhibition as measured in the carboxylase assay (Table 1, 2nd lane) was more pronounced than that in the KO-reductase assay (Table 1, 3rd lane). Since carboxylase and KO-reductase are two distinct enzymes with different functions in the vitamin K cycle, these data indicate that carboxylase is the prime target enzyme for both inhibitors.

In a second set of experiments we have tried to estimate the inhibitor concentration required for half-maximal inhibition. Incubations were performed under standard conditions and varying inhibitor concentrations using 200 μ M of either phyloquinone or menaquinone-4. A typical example of the inhibition curves thus obtained is given in Figure 2, where we have represented the inhibition of various forms of phyloquinone by d-UQ. It is noteworthy that the extent of inhibition was independent of the reduction state of the vitamin, which indicates that among the enzymes of the vitamin K cycle, γ -glutamylcarboxylase is the one most strongly inhibited by d-UQ and d-PQ. This is consistent with the data obtained in Table 1, where - in a direct test - at similar concentrations of d-UQ and d-PQ the inhibition of KO-reductase (lane 3) was 7-8 times less than that of carboxylase (lane 2); 10-50 times

Figure 2. Inhibition of the vitamin K-dependent carboxylation reaction by d-UQ. The reaction was performed with salt-washed microsomes, and was started with 0.2 mM of either vitamin K_1H_2 (●), vitamin K quinone (○) or vitamin KO (x). 100% residual vitamin K activity stands for 15 (for KH_2), 10.5 (for K), and 7.5 (for KO) pmol CO_2 fixed per min, respectively.

Figure 3. d-UQ inhibition in salt-washed microsomes and purified carboxylase. Vitamin K_1H_2 (0.2 mM) was used as a coenzyme. Results are presented as percentage of the non-inhibited reaction. Symbols: (●) salt-washed microsomes; (○) purified carboxylase. 100% residual vitamin K activity stands for 15 pmol CO_2 fixed per min in the microsomal system and for 1.9 pmol CO_2 fixed per min by purified carboxylase.

higher inhibitor concentrations were required for blocking KO-reductase to the same extent as carboxylase (data not shown). In Figure 2 half-maximal inhibition was found at about 400 μM of inhibitor; at 4-fold higher inhibitor concentration the carboxylase reaction was almost completely blocked. From these curves inhibitor concentrations required for 50% inhibition were calculated for all combinations, and all were closely similar: 470 μM and 390 μM for d-PQ and d-UQ in the phyloquinone system, and 460 μM and 450 μM for d-PQ and d-UQ in the menaquinone system, respectively. So it seems that both menaquinone and phyloquinone are antagonized by d-UQ and d-PQ, and that the antagonistic effects of both compounds are of the same order of magnitude.

Recently a purification procedure for carboxylase has been described.¹⁰ Preparations thus obtained are devoid of KO-reductase activity. We have also measured the sensitivity of purified carboxylase for d-UQ, and the results are shown in Figure 3. It turned out that in the purified system 50% inhibition was obtained at 5-fold (91 vs. 430 μM) lower inhibitor concentrations. For d-PQ these figures were 85 and 426 μM , respectively (data not shown).

Finally we have prepared a time course of the inhibited and the non-inhibited reactions. As is shown in Figure 4A the non-purified system is inhibited by d-UQ (and by d-PQ, data not shown) for 10 min, after which the carboxylation reaction proceeds at a normal rate. Preincubation of the inhibitor with the microsomal proteins for 15 min prior to starting

Figure 4. Time-dependent inhibition of the carboxylation reaction by d-UQ. Vitamin K_1H_2 (0.2 mM) was used as a coenzyme, and all reactions were performed at a fixed inhibitor concentration (1 mM). (A) salt-washed microsomes. (B) purified carboxylase. Symbols: (○) non-inhibited reaction; (◐) carboxylation in the presence of inhibitor; (x) carboxylation after preincubation of inhibitor and microsomal proteins for 15 min at 20 °C.

the carboxylation reaction with vitamin K even completely abolished the inhibitory effect. Apparently the inhibitor was degraded or removed from solution during the preincubation period. As is shown in Figure 4B purified carboxylase was unable to neutralize the inhibitory effect of d-UQ even after incubation periods of 40 min and longer.

Discussion

A wide variety of quinone derivatives form part of the human diet, whereas our knowledge about the intestinal absorption and pharmacology of these compounds is still far from complete. Also it is unknown if, and to what extent, prenyl- and other quinones interfere with vitamin K-dependent reactions in liver, bone, and other tissues known to contain γ -glutamate carboxylase. To get an impression about the potential vitamin K or vitamin K-antagonistic activity of these quinones, we have used bovine liver *in vitro* systems, in which two synthetic derivatives of plastoquinone and ubiquinone were tested. It was found that both model compounds (decyl-plastoquinone and decyl-ubiquinone) behaved very similarly. Salt-washed, solubilized microsomes were used to compare the effects on γ -glutamate carboxylase and vitamin KO-reductase. Both quinones turned out to be inhibitors of the enzymes of the vitamin K cycle, but the effect on carboxylase was much stronger than that on KO-reductase. A problem here is that KO-reductase has not yet been purified, so that the experiments had to be performed in the rather crude microsomal system. It cannot be excluded, therefore, that part of the inhibitory activity is masked by non-specific adsorption of the decylquinones to contaminating proteins or phospholipids. This became clear from an experiment in which we compared the inhibitory activity of d-UQ in solubilized microsomes and purified carboxylase. It turned out that the purified enzyme was inhibited at least 5-fold stronger than the non-purified one.

An interesting phenomenon was that in time-course studies in the microsomal system the inhibitory effect of decylquinones decreased after 10-15 min, whereas in the purified system the inhibitory activity persisted, also after very long incubation periods. Pre-incubation of d-UQ with the solubilized microsomes even completely eliminated its inhibitory effect. Whether this elimination forms part of a biochemical degradation pathway is not clear at this time, but if so this pathway must be able to select plastoquinone and ubiquinone derivatives from phyloquinone and menaquinones, because the latter compounds are not affected under the conditions employed.

From the data presented in this paper we conclude that derivatives of plastoquinone and ubiquinone may form a new class of vitamin K-antagonists. It seems plausible that also natural quinones (e.g. those prominently found in the human diet) may possess vitamin K antagonistic activity *in vitro*. If similar effects will be found *in vivo*, dietary quinones may interfere with vitamin K-mediated processes like blood coagulation and bone metabolism.

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CHAPTER 2.2

NATURAL PRENYLQUINONES INHIBIT THE ENZYMES OF THE VITAMIN K CYCLE IN VITRO

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Summary

Vitamin K belongs to a class of compounds commonly known as prenylquinones. Three other prenylquinones which are abundantly found in food are plastoquinone-9, ubiquinone-9 and ubiquinone-10. Using in vitro assay systems, it was recently found that synthetic derivatives of prenylquinones inhibit the vitamin K-dependent enzyme γ -glutamylcarboxylase and, to a lesser extent, the vitamin K-epoxide reductase. In this paper we describe how natural prenylquinones affect the vitamin K-dependent enzymes in vitro. All three prenylquinones were found to inhibit both the vitamin K-dependent carboxylase and the K-epoxide reductase in a rat as well as in a cow liver system; 50% inhibition was obtained at concentrations in the micromolar range. On the basis of their respective standard redox potentials, a possible mechanism for the inhibitory effect of prenylquinones on the carboxylase enzyme is put forward. It is concluded that natural prenylquinones are potential antagonists of vitamin K and may interfere with vitamin K-dependent reactions in vivo.

Introduction

Prenylquinones like phyloquinone (vitamin K₁), plastoquinone-9 (PQ-9), and ubiquinones-9 and -10 (UQ-9 and UQ-10) are synthesized by green leafy plants. Phyloquinone and PQ-9 serve as electron carriers in the photosynthetically active thylakoid membranes of the chloroplasts, whereas ubiquinones have an electron and proton transport function in the mitochondrial respiratory system.¹ As micronutrients the various prenylquinones form part of the human diet from which they are absorbed via the action of bile salts. Their physiological roles in mammals are widely different, however. Phyloquinone functions as a co-enzyme during the posttranslational conversion of glutamate into γ -carboxyglutamate (Gla), which is an essential step in the formation of the blood coagulation factors II, VII, IX, and X and several other proteins.² The enzyme involved in this reaction is called γ -glutamylcarboxylase. Ubiquinones play a role in the oxidative phosphorylation in the mitochondria³, and have been reported to possess antioxidant and membrane stabilizing properties.^{4,5} The beneficial effect of UQ-10 concentrates in the treatment of cardiovascular diseases has been claimed by various authors.⁶⁻⁸ Therapeutical dosages of 100-300 mg per day are not unusual in this respect.

Ubiquinones are also synthesized endogenously in a number of mammalian tissues: UQ-10 is predominantly formed in humans, whereas UQ-9 is the most common form in rats.⁴ Also a number of enterobacteria are capable of producing ubiquinones: examples are *Escherichia coli*, *Rhodospirillum rubrum* and *Pseudomonas beijerinckii* which synthesize the ubiquinones 1-8; 1-10 and 7-9, respectively.^{9,10} The colonic absorption and the physiological relevance of the bacterially produced ubiquinones remains questionable,

however, because (a) they are tightly bound to the bacterial cell membranes, and (b) the bile salts required for their solubilization and absorption hardly occur in the colon. A mammalian function of plastoquinone has not been reported at this time.

The active form of vitamin K is the quinol (KH_2), the oxidation of which provides the energy required for Gla formation. In this reaction KH_2 is converted into vitamin K epoxide (KO), which is recycled in two reductive steps. *In vitro*, dithiols may serve as the sole reducing cofactor for the reductase(s) involved, but the physiological reductant has not yet been identified unequivocally.^{11,12} Cell-free systems have been developed to test γ -glutamylcarboxylase¹³ and KO-reductase¹⁴ enzymatic activity either separately or in a combined reductase/ carboxylase assay.

In the previous chapter (2.1) we showed that synthetic derivatives of prenylquinones (decyl-plastoquinone and decyl-ubiquinone) inhibit γ -glutamylcarboxylase, and to a lesser extent KO-reductase. In this chapter we report on investigations concerning the vitamin K-antagonistic effect *in vitro* of the three most common prenylquinones: UQ-9, UQ-10 and PQ-9. We have tried to find out at which vitamin K/ ubiquinone ratio a significant inhibition of the carboxylase and reductase systems occurs, and we have put forward a possible mechanism for the observed inhibition.

Materials and methods

Materials.

PQ-9 was isolated from green beech leaves according to Lichtenthaler.¹ Phylloquinone (vitamin K₁, pure compound), dithiothreitol (DTT), Triton X-114, UQ-10, and 3-((chola-midopropyl)-dimethylammonio)-1-propane sulfonate (CHAPS) were from Sigma (St. Louis, MO). KO was prepared as described by Tishler et al.,¹⁵ water-solubilized phylloquinone (Konakion®, Roche, Basel, Switzerland) was converted into KH_2 according to Soute et al.¹⁶ The pentapeptide Phe-Leu-Glu-Glu-Leu (FLEEL) was purchased from Vega Biochemical Co. (Tucson, AZ), and $\text{NaH}^{14}\text{CO}_3$ (56 Ci/mol) from New England Nuclear (Dreieich, Germany). Silica gel-60 plates (20 x 20 x 0.5 cm), 2',7'-dichlorofluorescein spray, trichloroacetic acid, menadione and sodium dithionite were obtained from Merck (Darmstadt, Germany), and the following quinones were supplied by Acros Chimica (Geel, Belgium): 1,2-naphthoquinone, 1,4-naphthoquinone, anthraquinone, and anthraquinone-1,5-disulfonate. Anthraquinone and the various prenylquinones were solubilized by sonication in 10% (w/v) Triton X-114. All other chemicals were of analytical grade or better. UQ-9 was a kind gift from CPC-Europe (Heilbronn, BRD).

In vitro techniques.

Salt-washed microsomes were prepared from the livers of normal cows and from normal and vitamin K-deficient rats of the Lewis strain.¹⁶ For the carboxylase assay, reaction

mixtures (0.125 ml) contained 1 mg of microsomal proteins, 0.4% (w/v) CHAPS, 0.5 M NaCl, 25 mM Tris-HCl (pH 7.5), 4 mM FLEEL, 1 M $(\text{NH}_4)_2\text{SO}_4$, 1.5 μCi $\text{NaH}^{14}\text{CO}_3$, 6 mM DTT, 90 μM of either KH_2 , K, or KO. Unless specified otherwise the reaction mixtures were incubated for 10 min at 20°C, and it was checked that the reaction rate was linear during this period of time. The reactions were stopped with 800 μl trichloroacetic acid (5% (w/v)) and subsequent boiling, to remove free $^{14}\text{CO}_2$. Samples were counted in Formula 989 using a Beckman LS 1803 liquid scintillation counter.¹⁶

KO-reductase was tested in 0.25 ml reaction mixtures; the concentrations of the various constituents were as described for carboxylase, except for FLEEL and $\text{NaH}^{14}\text{CO}_3$, which were omitted. At 20°C the reductase reaction proceeds at a linear rate during at least 10 min. For our experiments the rate of reduction was deduced from samples taken after 0, 4, and 8 min of incubation. Extraction and analysis of the samples were performed as described earlier.¹⁷ Inhibitors and KO were added to the various reaction mixtures in Triton X-114 (as indicated), leading to a final concentration of 0.4 % (v/v) of Triton X-114 in the reaction mixtures. Protein concentrations were determined as described by Sedmak and Grossberg.¹⁸

Redox reactions between various quinones and KH_2 .

Vitamin KH_2 was prepared by dissolving 9 μmol of vitamin K quinone (pure compound) in 2.5 ml diethylether and adding a 30% solution (w/v) of sodium dithionite in 5 ml water. The mixture was shaken overnight at room temperature in a light protected tube, leading to approximately 90% reduction. This preparation was used in experiments in which the chemical oxidation of KH_2 by several quinones in isopropanol was investigated. The etherial KH_2 was diluted in isopropanol with and without the following quinones: UQ-10; menadione; 1,2-naphtoquinone, anthraquinone and anthraquinone-1,5-disulfonate. Final concentrations of KH_2 and the various quinones were 14 and 24 μM , respectively. The vitamin K hydroquinone-quinone oxidation was followed by HPLC.¹⁷

Data-analysis.

The inhibitor concentration necessary for 50% inhibition of the vitamin K activity (I_{50}) was estimated from curve fitting using the computer program Inplot (GraphPad Software, San Diego, CA).

Results

Conditions for maximum inhibition in vitro.

Vitamin K quinol (KH_2) serves as the co-enzyme in the carboxylase assay, which may be performed using a wide variety of glutamate-containing peptides and proteins as substrates. KO is the substrate in the KO-reductase assay. The combined action of reductase

Figure 1. Time course of the carboxylation reaction in the presence (●) or absence (O) of ubiquinone-10 (200 μ M). A rat microsomal system was used as a source for γ -glutamylcarboxylase, vitamin K quinol (90 μ M) was used as a cofactor and FLEEL as the carboxylatable substrate. Carboxylase activity is expressed as pmol CO₂ fixed per mg of microsomal protein. Each point is the mean of duplicate experiments.

Figure 2. Inhibition of vitamin K-dependent carboxylase: effect of different ubiquinone concentrations. The effects of various concentrations of UQ-9 (O) and UQ-10 (●) were tested in a rat microsomal system, with vitamin K quinol (90 μ M) as a cofactor and FLEEL as a substrate. Each point represents the mean of duplicate measurements.

and carboxylase can be measured by using either K quinone or KO as co-enzymes in the carboxylase assay¹⁹ In this paper, vitamin K activity will be defined as the ability to function as a cofactor in the in vitro carboxylase reaction containing either rat or bovine liver microsomes and the pentapeptide Phe-Leu-Glu-Glu Leu (FLEEL) as a substrate. While using different prenylquinones it turned out to be difficult to find solvents which did not inhibit carboxylase or reductase as such. Being highly lipophilic the substances had a strong tendency to precipitate from the aqueous environment in the reaction mixtures. In our hands Triton X-114 was the optimal detergent to solubilize the long-chain prenylquinones up to a concentration of 2-5 mM; after sonication the solutions were miscible with water in all proportions.

The structures of plastoquinone and ubiquinone are very similar to that of phyloquinone (see Figure 1, chapter 2.1). When tested in the absence of KH₂, however, neither PQ-9 nor UQ-9 and UQ-10 showed vitamin K-activity (data not shown). On the other hand, all three compounds showed inhibitory activity towards the enzymes of the vitamin K cycle. An example is given in Figure 1, showing the time course of inhibition by PQ-9 in the combined bovine carboxylase/reductase assay. At the vitamin K₁ concentration chosen for our experiments (90 μ M) the most prominent inhibition was found during the first 10 min. Therefore, an incubation period of 10 min was used for all experiments presented in this paper.

The target enzyme for prenylquinone inhibitors.

Carboxylase inhibition curves were prepared for all three prenylquinones, using the cofactor vitamin K₁ either in the hydroquinone, the quinone or the epoxide form. An example of such an inhibition curve is shown in Figure 2 where we have plotted the inhibition of rat carboxylase by UQ-9 and UQ-10. I_{50} values were estimated for the carboxylating enzyme systems from the cow as well as from the rat (Table 1). I_{50} values for all prenylquinones ranged between 30 and 150 μ M. The inhibitory effect of the corresponding prenylquinols was much weaker and could not be quantified because such experiments would require relatively high prenylquinol concentrations, which could not be reached in the water/detergent test system for carboxylase. The rapid inactivation of rat reductase by Triton X-114 made it impossible to measure the inhibition of the KO- and K-stimulated carboxylase reaction in the rat system. We have also measured the inhibitory effect of PQ-9, UQ-9 and UQ-10 for bovine KO-reductase in the direct reductase assay. The observed I_{50} values were: 112 ± 10 , 156 ± 23 and 162 ± 18 (mean $\pm$ SEM), respectively. These data are of the same order of magnitude as those obtained in the carboxylase assay.

Inhibition of peptide and protein carboxylation.

In the data presented thus far, FLEEL was used as a model substrate for carboxylase. In a subsequent experiment we have tested whether comparable inhibitory activity would be found for natural substrate carboxylation. Microsomes prepared from vitamin K-sufficient animals contain a low but distinct amount of non-carboxylated protein precursors, which may function as endogenous (natural) substrates for carboxylase. If tested under standard conditions (i.e., in the presence of 4 mM of FLEEL), less than 0.5% of the incorporated label is bound to the endogenous substrate both in preparations from cows and rats (data

Figure 3. Protein and peptide carboxylation in the presence of varying concentrations of plastoquinone-9. Vitamin K quinone (90 μ M) was used as a cofactor. Endogenous substrate carboxylation (●) was measured in the absence of FLEEL, using liver microsomes from vitamin K-deficient rats. FLEEL carboxylation (○) was measured in the supernatant of reaction mixtures after precipitation with trichloroacetic acid. Each point represents the mean of duplicate measurements.

Table 1. Effect of various prenylquinones on γ -glutamylcarboxylase in bovine and rat liver microsomal systems

Cofactor:	I_{50} (μ M) in bovine system			I_{50} (μ M) in rat system		
	KH ₂	K	KO	KH ₂	K	KO
Inhibitor						
UQ-10	110 $\pm$ 17	76 $\pm$ 5	91 $\pm$ 15	36 $\pm$ 7	n.d.	n.d.
UQ-9	105 $\pm$ 14	86 $\pm$ 12	72 $\pm$ 8	25 $\pm$ 6	n.d.	n.d.
PQ-9	85 $\pm$ 11	59 $\pm$ 4	41 $\pm$ 7	39 $\pm$ 10	n.d.	n.d.

Either vitamin K quinone, quinol or epoxide (90 μ M) were used as a cofactor (as indicated), and the pentapeptide FLEEL as a substrate. Carboxylase was prepared from the livers of vitamin K-sufficient animals. All data are expressed as the means of triplicate experiments ($\pm$ SEM). n.d., not determined because of the instability of the enzyme. The calculation of the inhibitor concentration necessary for 50% inhibition (I_{50}) is outlined in Section 2.

not shown). Ten to 20-fold higher levels of clotting factor precursors are found in microsomes prepared from livers of vitamin K-deficient animals; therefore we used carboxylase from vitamin K-deficient rats. The experiment was performed using the combined carboxylase/reductase reaction with vitamin K as a cofactor, and the inhibition by PQ-9 was assayed both in the absence and presence of FLEEL. After completion of the carboxylase reaction, FLEEL was separated from the endogenous substrate by trichloroacetic acid precipitation. The results are shown in Figure 3. It turned out that both peptide and protein carboxylation were inhibited to the same extent, with I_{50} values (46 and 54 μ M, respectively) which were comparable with those obtained in microsomes from vitamin K-sufficient animals (cf. Table 1).

Redox reactions between various quinones and KH₂.

In an attempt to elucidate the mechanism of inhibition in the vitamin K-dependent reactions, we have checked whether a number of quinones having redox potentials higher and lower than of vitamin K, will induce the chemical oxidation of K quinol. Such a reaction is expected to decrease the available cofactor for γ -glutamylcarboxylase, and the substrate for KO-reductase, respectively. Figure 4 shows the rapid oxidation of KH₂ into K in the presence of three quinones with a higher redox potential than phyloquinone: UQ-10; 1,2-naphthoquinone and menadione. Epoxidation of K quinone did not occur. No oxidation was observed in the presence of both anthraquinones, the redox potentials of which are lower than that of phyloquinone. Redox potentials (E_0 -values) for the several quinones and their inhibitory effect on the carboxylase reaction are given in Table 2. Quinones with a redox potential exceeding that of vitamin K₁, showed strong inhibition of the KH₂ mediated carboxylation reaction, whereas the anthraquinone derivative, with a relatively low redox potential, had no effect. Because of the hydrophobic properties of anthraquinone

Figure 4. Time course of the oxidation of vitamin K hydroquinone by various quinones in isopropanol. The oxidation of 14 μM KH_2 was measured in the absence of other quinones ($\bullet$), and in the presence of 24 μM of either UQ-10 (Δ); menadione ($\circ$); 1,2-napthoquinone ($\blacksquare$), anthraquinone-1,5 disulfonate ($\square$), or anthraquinone ($\blacktriangle$). The mixtures were analysed by HPLC at 250 nm.¹⁸ The conversion of KH_2 into K was deduced from the respective areas under the curve. Points are means of duplicate measurements.

Table 2. Normal oxidation-reduction potentials and I_{50} values for various quinones

Quinones tested	E_0 (mV)	I_{50} (μM)
Ubiquinon-10	122	110
1,2-Napthoquinone	156	57
1,4-Napthoquinone	65	60
2-Methyl-1,4-napthoquinone (menadione)	2	121
Phylloquinone (vitamine K_1)	-57	-
2,3,5,-Trimethylbenzoquinone	110	n.d.
Plastoquinone-9	Not known	85
Anthraquinone	-266	n.d.
Anthraquinone-1,5-disulphonate	-170	No effect

The standard two electron oxidation-reduction potentials refer to values obtained with the normal hydrogen electrode at pH 7.0. The E_0 values are from Refs. 36 and 37; for plastoquinone-9 the E_0 is not known, but may be expected to be closely similar to that of 2,3,5-trimethylbenzoquinone. The I_{50} values were determined in a bovine carboxylase system with KH_2 as a cofactor. n.d., not determined. For further details, see Table 1.

it could not be dissolved in Triton X-114, hence this compound was not tested as a carboxylase inhibitor. The non-enzymatic oxidation of reduced vitamin K by quinones possessing a higher E_0 than vitamin K may provide an explanation for the inhibition of vitamin K-dependent carboxylase.

Discussion

After we had established in the previous chapter that vitamin K-dependent reactions are inhibited by a number of synthetic quinones, the data presented in this chapter clearly show that natural prenylquinones may act as potent inhibitors of the vitamin K cycle in vitro. The inhibition was observed both in rat and cow liver microsomal systems. As was reported by Uotila²⁰ another prenyl derivative, d- α -tocopherylquinone (vitamin E), also acts as an inhibitor of vitamin K-dependent carboxylase with I_{50} values in the micromolar range. The extent of inhibition was found to depend on the form in which vitamin E was applied as well as on the concentration of vitamin K in the reaction mixtures. A possible explanation for the inhibition of γ -glutamylcarboxylase was given by Dowd and Zheng²¹, who found that vitamin E quinone acts as an inhibitor. The authors postulated a mechanism involving a covalent binding of vitamin E with active site thiols of carboxylase, leading to inactivation of the enzyme. On the other hand, our results show that inhibition of the carboxylation reaction was not only obtained with benzoquinones but also with several naphthoquinone ring structures. They all have in common that their standard redox potentials are higher than that for phyloquinone. An alternative explanation, therefore, is that these inhibitors may chemically interact with the substrate KH_2 , thus exhausting the pool of KH_2 available for the carboxylation reaction. This hypothesis was supported by the fact that compounds with higher redox potentials than the vitamin K/ KH_2 couple appeared to be inhibitors, whereas compounds with lower redox potentials were not (Table 2). Obviously, the high redox potentials of prenylquinones do not provide an explanation for their inhibition of vitamin K-epoxide reduction. In this case competition for the substrate binding place forms the most plausible mechanism.

In our studies it was a considerable problem to solubilize the various prenylquinones in a water environment without affecting the activities of the various enzymes. It cannot be excluded that the solubilization was less complete than expected, and that the data presented here underestimate the true inhibitory potential of the various compounds. As was reported earlier, the binding of the pro-peptide may alter the affinity of carboxylase for its cofactor KH_2 ²², the fact that no substantial differences were found between I_{50} values measured for FLEEL carboxylation and endogenous precursor carboxylation demonstrates that the extent of inhibition is not affected by the binding of the propeptide.

The prenylquinones tested in our experiments (UQ-9, UQ-10, and PQ-9) belong to the most abundant natural quinones and occur in many human food items, such as green leafy vegetables, oils and cereals.²³ The concentrations in which they occur as micronutrients in the daily diet exceed that of phyloquinone by 1-2 orders of magnitude.²⁴ Additionally, ubiquinones are produced endogenously in mammals.²⁵⁻²⁷ The highest concentrations are found in liver, heart and kidney but they occur in nearly all other tissues.²⁸ It is important to know the extent to which these compounds are absorbed in the gastrointestinal tract, and whether they accumulate in the liver. Concentrations of 915 pmol/ml of UQ-10²⁹

and 1.2 pmol/ ml of phyloquinone³⁰ were reported for normal human plasma, which is a difference of three orders of magnitude. Takahashi et al.³¹ and Kalén et al.²⁷ analysed the UQ-9 content of rat liver microsomes, and found concentrations ranging from 0.15 - 0.19 nmol/ mg of protein. This is at least 100-fold higher than the cumulative amounts of phyloquinone and menaquinones as reported by Thijssen and Drittj-Reijnders³², and supports the assumption that the various prenylquinones are absorbed - at least in part - from the intestinal contents.

Nothing is known about the *in vivo* effect of the various prenylquinones on the synthesis of Gla-proteins. On one hand they might act as mild, competitive antagonists of vitamin K action, leading to the appearance of undercarboxylated Gla-proteins like descarboxy prothrombin (effect in liver) or descarboxy osteocalcin (effect in bone). In this way a high intake of ubiquinones or plastoquinone might have an anticoagulant effect. On the other hand it is possible that due to intracellular trafficking or rapid metabolism the active inhibitors either do not reach the endoplasmic enzymes of the vitamin K cycle, or are converted into inactive metabolites. The latter possibility is consistent with our observation that the inhibitory effect decreases after prolonged incubation of the prenylquinones with microsomes.

Our findings may have two clinical implications. First, the as yet putative mild anticoagulant effect of a number of prenylquinones provides additional theoretical support for the frequently reported beneficial effects of UQ-10 concentrates in the treatment of cardiovascular disease.³³ Second, at least 10% of the patients on coumarin anticoagulant therapy show substantial instability of the level of anticoagulation.³⁴ This may lead to either bleeding or thrombosis. It is at least feasible that even weak inhibitors might disturb the rather delicate balance which is aimed at to reach the therapeutic window required for effective oral anticoagulation. It would be interesting, therefore, to investigate whether fluctuations in the dietary intake of prenylquinones are correlated with the observed instability of anticoagulation.

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CHAPTER 2.3

INVESTIGATIONS ON THE POTENTIAL EFFECT OF NATURAL UBIQUINONES ON THE VITAMIN K STATUS IN THE RAT

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Summary

Phylloquinone, ubiquinones and plastoquinones all belong to the group of prenylquinones and show a high degree of similarity in structure and occurrence in food items as green leafy plants and oils. In previous studies we found a vitamin K antagonistic effect of both synthetic derivatives and natural forms of ubiquinones and plastoquinone-9 (see chapter 2.1 and 2.2). In this chapter we investigated the potential inhibitory effect of ubiquinone-9 (UQ-9) and ubiquinone-10 (UQ-10) on vitamin K activity in a rat model. Various doses ranging from 1 to 10 mg/ day of UQ-9 or UQ-10 were orally or subcutaneously administered during two weeks, while feeding the rats a marginal phylloquinone containing diet. Tissue analysis of ubiquinone contents showed an increase in both serum and liver as a result of both orally or subcutaneously administered UQ-9 or UQ-10. In the liver, however, the exogenously administered ubiquinones only exceeded the endogenously produced UQ-9 levels 3 fold at the highest dose of UQ-10. Subcellular fractionation of the liver showed a similar distribution pattern for ubiquinones and vitamin K, with the highest accumulation in microsomes, mitochondria and nuclei. Endogenous levels of UQ-9 and UQ-10 appeared to be many thousand times higher than for vitamin K. Despite the observed high hepatic ratio between UQ and vitamin K concentrations, neither UQ-9 nor UQ-10 showed significant effects on plasma prothrombin concentrations. We therefore conclude, that natural ubiquinones as UQ-9 and UQ-10 do not interfere with vitamin K activity in the rat.

Introduction

Phylloquinone (vitamin K₁) and ubiquinones belong to the group of prenylquinones and are synthesized by green leafy plants. Their structures show a high degree of similarity. Phylloquinone exists of a 2-methyl-1,4-naphthoquinone ring structure with a phytyl as side chain at position 3. Ubiquinones have a 2-methyl- 5,6-dimethoxy-1,4-benzoquinone ring structure, with polyisoprene side chains of different lengths. The chemical structures of phylloquinone and ubiquinone are shown in chapter 1, paragraph 2.2. The most abundant ubiquinones homologues are ubiquinone-9 (UQ-9) and ubiquinone-10 (UQ-10) which, like phylloquinone, occur in many food items such as green leafy vegetables, oils and cereals.¹⁻³ The concentrations at which UQ-9 and UQ-10 occur as micronutrients in various food items exceed that of phylloquinone by 1-2 orders of magnitude. Ubiquinones can also be synthesized endogenously by mammals. In humans, the predominant homologue is UQ-10⁴ whereas for the rat this is UQ-9.⁷ UQ-10 is increasingly used as a food supplement. Several clinical trials with UQ-10 have been undertaken in cardiovascular and cancer patients to test its potential favourable effects on healing.⁸⁻¹⁰ Doses of 100-300 mg per day are not unusual in this respect. The underlying mechanism is thought to be

the antioxidant activity of UQ-10, but strong clinical or biochemical evidence is lacking. The potential beneficial effect of UQ-10 was recently reviewed by Greenberg and Frishman.¹¹ In chapter 2.1 and 2.2 experimental evidence was given that synthetic derivatives of prenylquinones (decyl-PQ and decyl-UQ) as well as natural forms, e.g. plastoquinone-9 (PQ-9), UQ-9 and UQ-10 may inhibit the vitamin K-dependent enzymes γ -glutamyl-carboxylase and KO-reductase in vitro. The objective of the study reported here was to test the hypothesis that natural prenylquinones with structural analogy to vitamin K may act as vitamin K-antagonists in vivo.

Materials and methods

Materials

Phylloquinone and menaquinone-9 were kind gifts of Hoffmann-LaRoche (Basel, Switzerland). UQ-9 was kindly granted by CPC-Europe (Heilbronn, BRD). UQ-10, sodium dodecylsulphate and butylated hydroxytoluene were obtained from Sigma (St. Louis, MO). HCO-60 (polyoxyethylene hydrogenated castor oil derivatives) was purchased from Nikko Chemicals (Tokyo, Japan). All other chemicals were HPLC grade or better.

Animals and diets

All studies were performed in male rats of the Lewis strain, which were 12 weeks old and had a body weight of 309 ± 5.6 g (mean $\pm$ SEM) at the start of the experiment. The animals were housed in normal cages in an environment with a 12-h light-dark cycle, controlled temperature ($20 \pm 2^\circ\text{C}$) and humidity ($50 \pm 10\%$). All animals underwent a dietary pre-treatment of 2 weeks followed by 2 weeks of treatment with UQ. In the first week of pre-treatment, the animals were housed in individual metabolism cages and were fed an irradiated (0.9 Mrad) vitamin K-deficient diet (Hope Farms, Woerden, The Netherlands) mixed with cooked and dried rice in a 1:1 ratio, to deplete serum and hepatic vitamin K levels. In the second week of the pre-treatment period, they received a controlled, low vitamin K diet ($0.9 \mu\text{g K}_1/\text{g food}$). After this dietary pre-treatment period the treatment with UQ-9 and UQ-10 was started while continuing the vitamin K supplemented diet. The rats had free access to the various diets. Blood (0.15 ml) was taken from the tail vein and collected in 0.14 M trisodium citrate (9:1) at the start of the experiment, after 1 week of vitamin K-deficiency, after 1 week of controlled vitamin K-intake and during the ubiquinone-treatments at the indicated days. Rat livers were collected at the end of each experiment, on average 4.5 hrs after the last administration of ubiquinones. Under light diethyl anaesthesia blood was withdrawn from the abdominal aorta. Immediately after death the liver was perfused with ice-cold 0.15 M NaCl via the portal vein, excised and rinsed in 0.15 M NaCl. Serum was obtained by incubating the blood at room temperature for 3 hrs and subsequent centrifugation for 15 min at 1700 g. Sera, plasmas and livers

were stored at -80°C until use. The experimental protocols were approved by the Experimental Animal Ethics Committee of the University of Maastricht.

Protocol for UQ-9 treatment (Experiment A)

UQ-9 was mixed at a concentration of 2 mg/ml with buffer A (0.15 M NaCl, 0.05 M Tris-HCL, pH 7.5) containing 7% (w/v) of the non-ionic surfactant HCO-60. A clear solution was obtained after sonication at an amplitude of 6 micron. Rats ($n=5$) received 2 mg of UQ-9 per day during 6 days via an oral cannula. Subsequently they received 2 mg of UQ-9 per day during 7 days via subcutaneous injections. The control rats ($n=5$) received the solvent only.

Protocol for UQ-10 treatment (Experiments B and C)

Two concentrations of UQ-10 were prepared: 1 and 10 mg/ml, by mixing the UQ-10 with buffer A containing 7% (w/v) HCO-60 and sonication at an amplitude of 6 micron until a clear solution was obtained. One group of rats ($n=5$) received UQ-10 via an oral cannula (experiment B): 1 mg/day during the first 6 days and 10 mg/day during the second week. A second group of rats (experiment C, $n=5$) received 1 and 10 mg respectively of UQ-10 per day via subcutaneous injections during respectively 6 and 7 days. Control rats were treated either orally ($n=5$) or subcutaneously ($n=5$) with the solvent only.

Plasma prothrombin concentration

Plasma prothrombin concentrations were determined as described in the materials and methods section of chapter 3.1. A calibration curve was made with pooled plasmas from normal male Lewis rats. The data obtained during the pretreatment period are expressed as means $\pm$ S.D. as a percentage of the individual starting values (day 0 of the pretreatment period). The data from experiments A-C are expressed as means $\pm$ S.D. for 5 rats as a percentage of the individual values obtained at day 14 of the pre-treatment period.

Analysis of UQ-9 and UQ-10 in serum and in liver homogenates

Liver homogenates were prepared by diluting 1 g of liver in ice-cold 0.15 M saline (1:3 w/v; wet-weight basis) and homogenization in a blender (Ultra Turrax; Janke & Kunkel, Staufen, Germany). The extraction of ubiquinones was performed according to the procedure described by Lang et al.¹² with slight modifications. The following solutions were added to the serum and liver samples: butylated hydroxytoluene (BHT, 50 mg/ml ethanol), which was used as an antioxidant; sodium dodecylsulphate (SDS, 0.3 g/ml water) and an internal standard, menaquinone-9 (MK-9, 100 $\mu\text{g/ml}$ ethanol). Serum samples (0.2 ml) were supplemented with 40 μl of MK-9, 20 μl of SDS, 10 μl of BHT and 780 μl water. The samples were mixed with 2 ml ethanol and extracted with 3.5 ml hexane in a glass tube fitted with a Teflon-lined screw cap. Because ubiquinones are light sensitive, all procedures were performed under yellow light. Liver homogenates (0.8 ml) were supplemented with 50

μ l MK-9, 200 μ l SDS and 20 μ l BHT. Extracts thus obtained were evaporated under a constant stream of nitrogen at 30°C, the residues were dissolved in 0.2 ml propane-2-ol and analysed for their UQ-9 and UQ-10 content by HPLC using a C-18 reversed phase column (150 x 4.6 mm, Altech, Deerfield, USA) and UV detection at 274 nm. Isocratic elution was performed with a mixture of 2.5% dichloromethane and 97.5% methanol at a flow rate of 1 ml/min. Authentic UQ-9 and UQ-10 were used to determine the elution times, and to prepare calibration curves. The detection limit for both UQ-9 and UQ-10 was established to be 5 ng.

Fractionation of hepatic cell organelles

Liver homogenates were used as a starting material for further fractionation. Livers of three rats from experiment C were pooled. Plasma membranes and nuclei were isolated by centrifugation for 10 min at 600 x g, mitochondria for 20 min at 6,500 x g, and microsomes for 1 h at 105,000 x g. Lysosomes were removed from the microsomes by 15 min centrifugation at 10,000 x g and discarded. After each centrifugation step the pellets were washed three times by resuspending in buffer B (0.1 M NaCl, 50 mM Tris-HCl, pH7.4) and repeated centrifugation. The supernatants were cleaned by repeating the centrifugation step three fold. UQ-9 and UQ-10 were analyzed as described above, and the data are expressed per mg of protein. Protein concentrations were determined according to the Sedmak method.¹³

Detection of vitamin K in serum and liver

The extraction and analysis of vitamin K in serum, liver homogenate and hepatic subcellular fractions was performed as described in the material and methods section of chapter 3.2.

Statistical analysis

Plasma prothrombin concentrations and ubiquinone values in serum and liver were statistically compared with their control groups with the non-parametric Mann-Whitney test with $p = 0.05$. All data are expressed as means $\pm$ S.D.

Results

Before starting the administration with ubiquinones in experiments A-C, all rats were treated with a vitamin K-deficient diet followed by a diet containing phylloquinone in a concentration which was only slightly above the minimal daily requirement. This was done to bring the rats in a marginal vitamin K status with hepatic vitamin K levels just sufficient to maintain prothrombin synthesis at a normal level. At the start of the pre-treatment period, plasma prothrombin values were $86\% \pm 11$ of reference pooled plasma (mean $\pm$ S.D.) which declined

Table 1. Plasma prothrombin concentrations after UQ-9 treatment (2 mg daily): experiment A

Day	Oral treatment			Subcutaneous treatment		
	0	2	5	7	8	12
Treatment						
UQ-9	100 ± 0	110 ± 9.7	116 ± 11	94 ± 8.4	83 ± 14	105 ± 26
Control	100 ± 0	105 ± 11	108 ± 11	122 ± 21	116 ± 34	115 ± 10

Effect of UQ-9 treatment on plasma prothrombin concentrations in rats. Rats were pre-treated as indicated in the materials and methods section. At $t=0$ days, the actual treatment was started with daily oral administrations of 2 mg UQ-9, followed by subcutaneous injections of 2 mg UQ-9 from $t=6$ days. Plasma prothrombin concentrations were determined at the indicated days. The data are expressed as the means of 5 rats $\pm$ S.D.

Table 2. Plasma prothrombin concentrations after oral or subcutaneous UQ-10 treatment: experiments B and C

Day	Dose: 1 mg daily			Dose: 10 mg daily	
	0	2	5	9	12
Treatment					
UQ-10 orally	100 ± 0	99 ± 17	90 ± 24	91 ± 7.4	84 ± 5.8
Control orally	100 ± 0	101 ± 8.7	101 ± 18	91 ± 5.7	81 ± 8.9
UQ-10 subcut.	100 ± 0	103 ± 13	94 ± 12	92 ± 7.9	81 ± 7.9
Control subcut.	100 ± 0	118 ± 23	93 ± 9	99 ± 4.2	85 ± 1.7

Effect of UQ-10 treatment on plasma prothrombin concentrations in rats. Rats were pre-treated as indicated in the materials and methods section. UQ-10 was daily administered orally or subcutaneously (subcut.) in two concentrations: a low dose (1 mg/day) during the first period of 6 days and a high dose (10 mg/day) during the second period of 7 days. Plasma prothrombin concentrations were determined at the indicated days. The data are expressed as the means of 5 rats $\pm$ S.D.

to $6.5\% \pm 7$ after 1 week feeding a vitamin K-deficient diet. The controlled, low vitamin K diet (daily $0.9 \mu\text{g K}_1/\text{g food}$) restored these values to a level of $91\% \pm 12\%$ of reference pooled plasma (mean $\pm$ S.D.).

Effects on plasma prothrombin values

The effects of UQ-9 and UQ-10 on plasma prothrombin concentrations are summarized in Table 1 (experiment A) and 2 (experiments B and C). After the pre-treatment period,

the rats in experiment A received either UQ-9 (2 mg/day, $n=5$) or solvent ($n=5$). During the first week the solutions were given via an oral cannula, during the second week via subcutaneous injections. The plasma prothrombin concentrations at the start of the treatment period were arbitrarily taken as 100%. As is shown in Table 1, no effect on plasma prothrombin concentrations was observed as a result of oral administration of UQ-9. Following the start of the subcutaneous injections the plasma prothrombin concentration in the UQ-9 treated group tended to decline, and a maximal effect was seen at 3 days after the start of subcutaneous administration. However, this tendency was not statistically significant. After 7 days of UQ-9 treatment, prothrombin concentrations were almost similar as those observed in the control group.

In experiments B and C the effect of UQ-10 administration during 2 weeks was studied. In the first week the UQ-10 dose was 1 mg/day, in the second week it was increased to 10 mg/day. In experiment B rats received either UQ-10 ($n=5$) or solvent ($n=5$) orally, whereas in experiment C the rats received either UQ-10 ($n=5$) or solvent ($n=5$) subcutaneously. No significant difference between the UQ-10 treated and control group was found, irrespective of the dose given or route of administration (see Table 2).

Effects on serum and liver UQ levels

At the endpoints of experiments A-C, the rats were killed and blood and livers were collected from 3 rats per group for analysis of their UQ-9 and UQ-10 content. The livers from three rats of experiment C were also used as a starting material for further fractionation to investigate the subcellular distribution of UQ-contents. Because of the limited amount of blood that can be obtained during tail puncture, we only measured endpoint serum UQ-contents. The serum concentrations of UQ-9 and UQ-10 are shown in Figure 1. Treatment with the solvent only, resulted in endogenous levels of UQ-9 and UQ-10 which were 0.29 ± 0.10 and 0.13 ± 0.12 $\mu\text{g/ml}$ respectively. After UQ-9 treatment in experiment A, the UQ-9 level was 20-fold higher (6.00 ± 0.36 $\mu\text{g/ml}$). Since oral administration preceded subcutaneous injections in the same animals, no data are available from rats after oral UQ-9 intake. UQ-10 levels in serum after UQ-9 treatment were not detectable. Oral UQ-10 treatment (experiment B) raised the serum UQ-10 concentration to a level of 0.9 ± 0.14 $\mu\text{g/ml}$, which is 7-fold the endogenous level of UQ-10. Subcutaneous UQ-10 treatment (experiment C) led to circulating UQ-10 levels of 54 ± 5.1 $\mu\text{g/ml}$, which is about 400 times the endogenous UQ-10 level. Circulating UQ-9 levels were not influenced by oral or subcutaneous UQ-10 treatment, which were 0.30 ± 0.04 and 0.21 ± 0.03 $\mu\text{g/ml}$ respectively.

The hepatic concentrations of UQ-9 and UQ-10 are shown in Figure 2. The UQ-9 levels in the rats subcutaneously treated with UQ-9 (experiment A) were more than 2-fold higher compared to the control rats, 93 ± 22.4 versus 42 ± 9.9 $\mu\text{g/g}$. After oral or subcutaneous UQ-10 treatment the endogenous hepatic UQ-9 levels were statistically not different, and were 54 ± 14 and 38 ± 5.8 $\mu\text{g/g}$ respectively. Endogenous UQ-10 levels in control rats were fairly low (3.7 ± 1.1 $\mu\text{g/g}$) and remained the same after UQ-9 treatment: 4.1

Figure 1. Serum concentrations of UQ-9 and UQ-10 after oral and subcutaneous administration. The two bars on the left part of this figure represent the serum UQ-9 levels of rats treated 6 days orally and 7 days subcutaneously with either the solvent or UQ-9 (2 mg daily). The three bars on the right part represent serum UQ-10 levels in the control rats or rats treated orally or subcutaneously with UQ-10 (1 mg/day during 6 days, followed by 10 mg/day during 7 days). The data are expressed as means of 3 rats $\pm$ S.D. For further details see Material and Methods section. * : $p = 0.05$ for ubiquinone treated group versus control group.

Figure 2. Liver concentrations of UQ-9 and UQ-10 after oral and subcutaneous administration. The two bars on the left part of this figure represent the liver UQ-9 levels of rats treated 6 days orally and 7 days subcutaneously with either the solvent or UQ-9 (2 mg daily). The three bars on the right part represent liver UQ-10 levels in the control rats or rats treated orally or subcutaneously with UQ-10 (1 mg/day during 6 days, followed by 10 mg/day during 7 days). Data are expressed as means of 3 rats $\pm$ S.D. For further details see Material and Methods section. * : $p = 0.05$ for ubiquinone treated group versus control group.

± 1.3 µg/g. Both oral and the subcutaneous UQ-10 administration (experiments B and C) induced an increase in the UQ-10 levels to 18 ± 4.3 µg/g and 120 ± 13 µg/g, respectively.

Subcellular hepatic distribution of UQ and vitamin K

A possible explanation for the absence of inhibition of plasma prothrombin synthesis in the rat, might be a difference in subcellular liver distribution of vitamin K and ubiquinones. Therefore, we compared the subcellular distribution of vitamin K with that of ubiquinones in rats subcutaneously treated with UQ-10 (experiment C). As is shown in Table 3, endogenous UQ-9 was equally distributed over the microsomes, the mitochondria and the nuclei, whereas it was hardly present in the cytosol. This is consistent with its hydrophobic nature, by which nearly all UQ-9 will be bound to phospholipids and the membrane fractions. After subcutaneous administration with UQ-10, the supplemented

Table 3. Vitamin K, UQ-9 and UQ-10 concentrations in subcellular liver fractions of UQ-10 treated rats (pmol/mg protein)

	UQ-10 treated		
	K	UQ-9	UQ-10
Microsomes	0.42	566	1112
Mitochondria	0.18	792	1506
Nuclei	0.43	855	1066
Cytosol	0.03	38	46

n.d. = not detectable. Vitamin K concentrations are the sum of epoxides and quinones. For both the control and the treated groups from experiment C, the livers from 3 rats were pooled.

UQ-10 shows the same subcellular distribution as endogenous UQ-9, with similar levels in the microsomes, mitochondria and nuclei. The vitamin K distribution over the subcellular fractions shows the same pattern as for the ubiquinones. In general, the vitamin K levels are 3 orders of magnitude lower than those of UQ's.

Discussion

In chapter 2.1 and 2.2 it was shown that both synthetic and natural prenylquinones inhibit the vitamin K-dependent enzymes carboxylase and KO-reductase in vitro. An inhibition of 50% was obtained with PQ-9, UQ-9 and UQ-10 in micromolar concentrations. The next step was to investigate whether this inhibition would occur in vivo (rat) as well. Because the degree of the in vitro inhibition of vitamin K by UQ-9 and UQ-10 was comparable, we hypothesized that an eventual inhibitory effect in vivo would be similar for both compounds as well. Before starting the ubiquinone administrations, the rats were pre-treated with diets aimed to lower their serum and hepatic vitamin K values. This was done to make the rats more sensitive for the potential effects of vitamin K antagonists. The minimal dose of phylloquinone required for normal prothrombin synthesis was established earlier at 0.6 µg K₁/g food (see chapter 3.2). We offered our rats a somewhat higher concentration, i.e. 0.9 µg K₁/g food. Neither UQ-9 nor UQ-10 significantly affected the plasma prothrombin concentrations in the dosages tested. This suggests that ubiquinones do not inhibit vitamin K action in vivo. To find an explanation for the discrepancy between the observed in vitro and in vivo effects, we measured UQ levels in serum and liver. Since we measured only the oxidized form of ubiquinones we underestimated the total amount of ubiquinones (reduced and oxidized). The percentage of reduced ubiquinones was shown to be roughly

50% of total ubiquinones for serum and liver respectively.^{5,7} In rats fed a commercial diet, UQ-9 and 10 are mainly found in heart, kidney and liver.^{5,7,14} On the other hand, oral administrations of UQ-10 are exclusively recovered in plasma and liver.^{5,15,16} If the liver is the main target of exogenous administered ubiquinones, a possible interaction with the vitamin K-dependent enzymes cannot be ruled out. The amounts of ubiquinones administered during the experimental phase in our experiments exceed the amount found in the regular diet many times. Daily subcutaneous injections with UQ-9 or UQ-10 highly increased the levels of UQ in serum and liver. Yet, the levels at the highest intakes were only 2-3 times higher than the endogenous levels of UQ-9. The increase in serum and hepatic levels of after oral administration of UQ-10 was relatively low, suggesting a poor intestinal absorption. This is in agreement with results from Zhang et al.⁵ who estimated that not more 2-3% of total dietary UQ-10 is taken up in rats. The endogenous UQ-10 levels in both serum and liver were not influenced by UQ-9 treatment and vice versa, showing that processes as interconversion or downregulation are not involved. Because vitamin K-dependent enzymes are known to be located at the microsomal membranes, we were interested whether the subcellular distribution pattern in liver of both vitamin K and ubiquinones would be comparable. UQ-9 and 10 (endogenous compounds and exogenously administered ones) and vitamin K showed a similar subcellular distribution pattern with the highest levels in microsomes, mitochondria and nuclei and a neglectable amount in the cytosolic fraction. Other authors^{5,7,16} reported the highest concentrations of UQ-9 and 10 in mitochondria, plasma membranes and lysosomes, whereas vitamin K was observed to accumulate mainly in mitochondria and microsomes of rat liver.¹⁷ By comparing the amount of ubiquinones in rat liver microsomes with that of vitamin K, it appears that the difference is three orders of magnitude. For the *in vitro* inhibition of vitamin K-dependent carboxylase a molar ratio of about 1 (UQ/ vitamin K) turned out to be sufficient for 50% inhibition. It is remarkable therefore, that no inhibition occurs even with this high molar ratio of UQ/ vitamin K *in vivo*. Possibly, intracellular transport systems are capable of preventing the 1000-fold excess of endogenous and nutritional ubiquinones from interacting with the enzymes of the vitamin K-cycle. In chapter 2.2 we discussed several mechanisms for the *in vitro* inhibition of γ -glutamylcarboxylase by prenylquinones. One of these mechanisms implies the inhibition of enzymes, the other, the oxidation of vitamin K-hydroquinone. From our data, however, a mechanism or clear explanation cannot be given for the absence of an inhibitory effect of ubiquinones *in vivo*. One difference between the experiments *in vitro* and *in vivo* may be the degree of water solubility of the prenylquinones. The *in vitro* inhibition was obtained in a solubilized microsomal system in which the interaction between vitamin K and ubiquinones might be easier than *in vivo*.

In conclusion, we have found that the *in vitro* vitamin K-antagonistic effect of prenylquinones could not be demonstrated in an *in vivo* rat system. If the same holds true for humans, the beneficial effect in cardiovascular diseases claimed for UQ-10 cannot be explained via inhibition of the vitamin K-dependent prothrombin synthesis.

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CHAPTER 3

BIOAVAILABILITY, TISSUE DISTRIBUTION AND CONVERSION OF VITAMIN K IN THE RAT

- 3.1 Bioavailability of phylloquinone and menaquinones after oral and colorectal administration in vitamin k-deficient rats
- 3.2 Tissue distribution of K-vitamins under different nutritional regimens in the rat
- 3.3 Intestinal flora is not an intermediate in the phylloquinone-menaquinone-4 conversion in the rat

CHAPTER 3.1

BIOAVAILABILITY OF PHYLLOQUINONE AND MENAQUINONES AFTER ORAL AND COLORECTAL ADMINISTRATION IN VITAMIN K-DEFICIENT RATS

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Summary

Rats were made vitamin K-deficient by feeding them a diet devoid of vitamin K and by rigorously preventing coprophagy. After one week, circulating prothrombin concentrations were between 5 and 10% of initial values, and various amounts of phylloquinone, menaquinone-4, and menaquinone-9 were given in a single dose either subcutaneously, orally, or colorectally. The relative 'vitamin K-activities' of these compounds were assessed by comparing their ability to support prothrombin synthesis after subcutaneous injection. Intestinal and colonic absorption were deduced from the difference between subcutaneous and either oral or colorectal administration of the vitamers. It is concluded that the colonic absorption of all three forms of vitamin K is extremely poor, suggesting that physiological menaquinones in the colon do not contribute substantially to vitamin K status in rats. Furthermore, the stimulation of prothrombin synthesis by menaquinone-9 lasted much longer than that by the two other K-vitamers, resulting in a substantially higher 'vitamin K activity' of menaquinone-9.

Introduction

Vitamin K is a group name for a series of related compounds that share the ability to serve as a cofactor for the microsomal enzyme γ -glutamylcarboxylase. This enzyme is involved in the posttranslational conversion of peptide-bound glutamate residues into γ -carboxyglutamate (Gla), which occurs in a number of blood coagulation factors and bone proteins.¹⁻³ Natural forms of vitamin K are phylloquinone (K_1) and menaquinones (K_2). They share a naphthoquinone ring structure, but differ in their aliphatic side chain. In phylloquinone this side chain contains 4 isoprenoid residues, one of which is unsaturated; in menaquinone the side chain consists of a variable number of isoprenoid residues, all of which are unsaturated. The common nomenclature for menaquinones is MK- n , where n represents the number of isoprenoids. The most abundant menaquinones in human food are MK-4, MK-7, MK-8, and MK-9.^{4,6} Phylloquinone becomes available exclusively via the diet (green vegetables and dairy produce), whereas menaquinones occur in food (dairy produce, meat, and fermented products) as well as in the colon, where they are produced by the intestinal flora. The extent to which the various nutritional and colonic menaquinones contribute to the biosynthesis of the various Gla-proteins is a matter of debate at this time.⁷⁻¹¹

On the basis of a diet including 50% (w/w) white rice to reduce intestinal menaquinone production¹² and using anal cups to prevent coprophagy¹³, we have developed a protocol according to which rats can be made vitamin K-deficient in one week. Previously we used a similar technique to compare the absorption of phylloquinone and MK-4 after oral and

colorectal administration.¹⁴ In that study the bioavailability of both vitamins was assessed from the shortening of the clotting time in a standard overall blood coagulation test. A problem in this type of study is the high lipophilicity of vitamin K, which increases even with increasing side chain length. Detergents used to solubilize the K-vitamins may substantially influence their intestinal absorption. For that reason we previously used detergent-free 30% albumin in saline to bring phyloquinone and MK-4 into a water environment.¹⁴ Higher menaquinones cannot be solubilized in this way, however.

In the present study we have used plasma prothrombin concentration as a sensitive blood coagulation marker, and have solubilized three forms of vitamin K (K_1 , MK-4, and MK-9) using the detergent HCO-60, which has been shown not to interfere with the colonic absorption of compounds of widely differing lipophilicity.¹⁵ If not specified further, the term 'vitamin K' will be used hereafter to designate all three K-vitamins. 'Vitamin K-activity' is defined as the ability of a compound to stimulate prothrombin synthesis after its subcutaneous injection in vitamin K-deficient rats. Intestinal and colonic absorption were deduced from the difference between subcutaneous and either oral or colorectal administration of the vitamins. In this model system we have measured (a) whether the data obtained for the bioavailability of K_1 and MK-4 were comparable to those observed when the albumin-solubilized vitamins were used, and (b) the importance of the number of isoprenoid residues in menaquinones for their vitamin K-activity and intestinal absorption.

Materials and methods

Chemicals

K_1 and MK-4 were obtained from Sigma (St. Louis, MO), MK-9 was a kind gift from Hoffmann-La Roche (Basel, Switzerland). The non-ionic surfactant HCO-60 (polyoxyethylene hydrogenated castor oil derivatives) was purchased from Nikko Chemicals (Tokyo, Japan).

Animals and diets

All studies were performed in male rats of the Lewis strain, which were 12 weeks old when entering the experiment. The animals were housed in individual, metabolic cages with a 12-hr light-dark cycle and controlled temperature ($20 \pm 2^\circ\text{C}$) and humidity ($50 \pm 10\%$). They had free access to a radiated (0.9 Mrad) vitamin K-deficient diet (Hope Farms, Woerden, The Netherlands), mixed with cooked and dried white rice in a 1:1 (w/w) ratio.¹² The contents of the commercial vitamin K-deficient food has been detailed earlier¹⁴, and it was verified not to contain detectable amounts of phyloquinone or menaquinone. All food was powdered with a blender and mixed in a professional food processor before use. Starting at day 5 of the vitamin K-deficient regimen until the end of the experiment, the rats were equipped with anal cups to exclude coprophagy.¹³ At day 7 prothrombin

concentrations were observed to be between 5 and 10% of initial values, and vitamin K was administered as described below. Blood (0.5 mL) was taken by venipuncture of the tail vein in 0.05 mL of 0.14 M trisodium citrate before the experiment (= initial value), after 7 days of vitamin K-deficiency, and at 6, 10, 24, and 48 hr after vitamin K administration. These time points were chosen on the basis of pilot-experiments that showed that the increase of plasma prothrombin continued from 6 hr (low dose of phyloquinone) up to 24 hr (high dose of phyloquinone) following vitamin K administration. The protocol for this experiment was approved by the Experimental Animal Ethics Committee of the University of Limburg.

Vitamin K administration

Stock solutions were prepared containing 3.5 g/L HCO-60 and 1 g/L of either K₁, MK-4, or MK-9 in buffer A (0.15 M NaCl, 0.05 M Tris-HCl, pH 7.5). The K-vitamins were dissolved by sonication during five pulses of 5 sec with an amplitude of 6 μ m. Solutions thus obtained were clear, homogeneous, and stable. Shortly before vitamin K administration the stock solutions were diluted five times with buffer A, leading to a final HCO-60 concentration of 0.7 g/L. Further dilutions (as required) were made with 0.7 g/L HCO-60 in buffer A. Each dilution step was followed by sonication as described above. In all cases vitamin K was administered to the rats in 0.5 mL samples, with amounts ranging between 0 and 50 nmol (as indicated). Subcutaneous administration was performed in the neck of the animals. Oral doses were given via a syringe equipped with a plastic cannula, which was protruded into the esophagus. For colorectal administration the animals were anaesthetized with carbon dioxide and a canula introduced via the rectum and moved up to the colon loop, where the vitamin was applied.

Blood coagulation assays

One-stage prothrombin concentration was determined with a coagulometer (KC-4, Amelung, Germany), using a commercial thromboplastin preparation (Thromborel S[®]) and clotting factor II-deficient plasma (both from Behringwerke AG, Marburg, Germany). Prothrombin concentrations were calculated with the aid of a reference curve from pooled normal rat plasma. The initial prothrombin level was calculated for each rat, and all later data are expressed as a percentage of the respective initial values. Each point in Figures 1-3 represents the means $\pm$ SEM for five rats.

Results

Circulating prothrombin concentrations were measured after the administration of 2-5 different dosages of either K₁, MK-4, or MK-9, and in all cases three different routes of administration were compared: subcutaneous, oral, and colorectal. The dose-response

Figure 1. Effect of subcutaneous administration of vitamin K on circulating prothrombin concentrations in vitamin K-deficient rats. Vitamin K was administered as a detergent-solubilized preparation in a single dose. Each point represents the means of five different rats $\pm$ SEM. If no error bar is shown, the SEM falls within the symbol. Blank values (solvent without vitamin K) are subtracted (A) phyloquinone (K_1); (B) menaquinone-4 (MK-4); (C) menaquinone-9 (MK-9). The following amounts were given: 0.2 nmol ($\bullet$), 1 nmol ($\circ$), 5 nmol ($\blacksquare$), 10 nmol ($\square$), 25 nmol ($\blacktriangle$), and 50 nmol ($\triangle$).

curves are given in Figures 1-3, and it is clear that after colorectal administration the response of all three vitimers was extremely poor. Both after oral and subcutaneous administration, on the other hand, the response to K_1 and MK-4 was rapid, reaching a maximum after 6-10 h. The response to MK-9 was slightly slower, but the curves for oral and subcutaneous administration were of a similar shape, suggesting that intestinal absorption is not the rate-limiting step in the transport of the K-vitimers to the liver. Remarkably, the duration of the response to MK-9 was much longer than expected on the basis of previous experiments with K_1 and MK-4. Even after 48 hrs, prothrombin concentrations were still high in most cases; since the animals were killed at this time point, all calculations given below will result in an underestimation of the true vitamin K activity of MK-9.

The vitamin K-activity of the various K-vitimers were calculated from the dose-response curves as the Area Under Curve (AUC), and is expressed per nmol vitamin K given to the rats. In Table 1 the results are given for 5 and 10 nmol of the three K-vitimers after subcutaneous and oral administration, and for 50 and 100 nmol of vitamin K after colorectal administration. If expressed per nmol of vitamin K, the highest biological effect was obtained at the lowest doses. For all vitimers the efficacy of subcutaneous injection was

Figure 2. Effect of oral administration of vitamin K on circulating prothrombin concentrations in vitamin K-deficient rats. Symbols are as in Figure 1.

approximately two-fold higher than the values obtained after a similar oral dose, indicating that roughly half of the vitamin had adsorbed in the intestines. This value is an upper estimate, based on the assumption that 100% of the subcutaneously administered vitamin K will reach the circulation. From Table 1 it is also clear that K_1 was 2-3 times as effective as MK-4, but that its efficacy was 40-60% that of MK-9. The bioavailability of colorectally applied MK-9, a typical product of the intestinal flora, was less than 0.2% of that following oral administration, suggesting that the direct colonic absorption of this product is negligible.

Discussion

In this paper we have demonstrated that the colonic absorption of three abundant forms of vitamin K is extremely poor. If compared with earlier reports¹⁴, the technique for application of the solubilized vitamers in the colon has been improved by executing all manipulations while the animals were under carbon dioxide anaesthesia. This resulted in a complete release of muscle tension, so that injury of the colonic epithelium (with occasional bleeding)

Figure 3. Effect of colorectal administration of vitamin K on circulating prothrombin concentrations in vitamin K-deficient rats. Symbols are as in Figure 1. The greatest amount of vitamin K applied was 100 nmol (+).

Table 1. Biological effect of various forms of vitamin K as tested in vitamin K-deficient rats

Route of administration	Amount (nmol)	Apparent vitamin K-activity (AUC, mm ² /nmol)		
		K ₁	MK-4	MK-9
Subcutaneous	5	334 ± 39	80 ± 9	534 ± 59
Oral	5	134 ± 15	64 ± 6	352 ± 41
Colorectal	50	5 ± 1	10 ± 2	7 ± 2
Subcutaneous	10	212 ± 26	104 ± 14	329 ± 31
Oral	10	163 ± 17	56 ± 8	298 ± 26
Colorectal	100	6 ± 1.5	10 ± 3	4 ± 1

The total effect of each vitamer was calculated from the appearance and disappearance of plasma prothrombin for 48 hr following a single dose of purified detergent-solubilized vitamin K. AUC is the area under the dose-response curve, the data are given ± SEM. For further details see text.

was prevented. A second improvement was the rigorous prevention of coprophagy by equipping the rats with anal cups throughout the experiment. The fact that the bioavailability of colonic vitamin K decreased from the previously reported 30-50%¹⁴ to less than 0.2-2% of the values obtained after oral ingestion of the vitamin shows that epithelial damage and unexpected coprophagy are factors which may lead to substantial overestimation of the physiological importance of this pathway. If adequate precautions are taken, the absorption of the various K-vitamins is negligible. In nontreated rats the total amount of colonic menaquinones was reported to be 3-6 μg , most of which are MK-7, MK-8, and MK-9.^{11,16} It should be realized, however, that most of these menaquinones are not available for absorption because they are tightly bound to insoluble material (bacteria, membrane remnants, etc.⁶). In our colonic absorption experiment up to 100 nmol (80 μg) was used, but even at this extremely high dose of well-solubilized menaquinone, the biosynthesis of prothrombin was hardly affected. It must be expected, then, that the direct absorption of menaquinones produced by bacteria in the gut hardly contributes to the vitamin K status in the rat.

The three routes via which vitamin K may enter the human circulation are: (a) absorption in the small intestine (from the diet), (b) absorption in the colon (from the intestinal flora), or (c) by subcutaneous injection. In our experimental design we have tried to mimic these three routes as closely as possible. The response to intravenous injection may be quicker than that to any of the three routes mentioned above¹⁷, but in humans intravenous injection is not recommended because of the risk of anaphylactic shock. Hence, we have excluded this route from our experiments. Furthermore, it was decided to follow the circulating prothrombin concentrations prospectively in the individual rats, so that the total effect of a single dose of vitamin K could be measured. Obviously this choice implies that hepatic vitamin K levels cannot be determined simultaneously. In a recent paper, however, Akiyama et al. showed that at 1 and 3 hr after oral administration of various menaquinones their hepatic concentration was increased.¹⁸ This is consistent with our data showing increasing plasma prothrombin concentrations during the first hrs following vitamin K administration.

We did not observe differences in response time after either oral or subcutaneous administration of the K-vitamins. This suggests that the intestinal absorption after oral ingestion is rapid. In all cases the effect of MK-9 was slower than that of the other K-vitamins, but its biological half-life was substantially longer. From the curves shown in Figure 1 it seems that, if measured over longer periods (e.g., 1 or 2 weeks) the total activity of MK-9 will be at least two-fold higher than that suggested by the data given in Table 1. From our data it appears that the relative molar activity of the three vitamins is: MK-9 > phyloquinone > MK-4. Similar data were reported by Matschner and Taggart, who administered the vitamins by intracardial injection in partly vitamin K-deficient rats.¹⁹ On the basis of these data it is to be expected that the relative importance of long-chain menaquinones in food is larger than generally assumed. Our observation that both long- and short-chain menaquinones are readily absorbed after oral ingestion is consistent

with data recently published by Conly and Stein, who showed that, if taken orally, long-chain menaquinones are absorbed and counteract the effect of vitamin K antagonists.^{7,20} Our data do not support their conclusion that menaquinones synthesized by the intestinal flora are directly absorbed in the bowel. On the other hand, it is well known that - at least in the human - bacteria also inhabit to some extent the terminal ileum, and the possibility exists that some bacterially synthesized menaquinones may be absorbed from this region by a bile-salt mediated pathway.⁶

The data obtained after subcutaneous and oral administration of detergent-solubilized K₁ and MK-4 were comparable to those obtained with the albumin-solubilized vitamins.¹⁴ Since solubilization in HCO-60 was quick, practical, and even applicable for very lipophilic compounds, this detergent is the solvent of choice for experiments such as described in this paper. From Table 1 it seems that the gastrointestinal absorption of vitamin K is 50-75% that after subcutaneous administration, irrespective of the side chain length. This is remarkable, because there is a substantial difference in lipophilicity between long- and short-chain menaquinones. A clear difference between the short-chain and the long-chain vitamins was that the response to a single dose of MK-9 lasted significantly longer than that to a dose of K₁ or MK-4. This is consistent with the observation that the hepatic turnover rate of phyloquinone is three times higher than that of MK-9.²¹ As a consequence, the apparent vitamin K activity of MK-9 - notably after oral administration - was much higher than that of MK-4. It is to be expected that this effect will be even more pronounced if the response-curve is measured over a longer time period.

Recently Akiyama et al. used the warfarin-treated rat as a model to study the effectiveness of various menaquinones.¹⁸ A major discrepancy between their result and ours is that they found MK-9 to be substantially less active than MK-4. An explanation for this discrepancy may be found by comparing the different model systems used. Our results were obtained in vitamin K-deficient animals, which means that the enzymes of the vitamin K cycle were operational. Under these conditions vitamin K is substantially recycled via the dithiol-dependent enzyme vitamin K-epoxide reductase.²² Akiyama et al., however, used warfarin-treated animals for their experiments, which means that in their system the epoxide reductase was blocked, and vitamin K could only be converted into the active metabolite via an NADH-dependent enzyme, which is unable to complete the recycling of the vitamin.²³ The author's conclusion, namely, that MK-4 is more active than MK-9, is only valid for the reduction of vitamin K via the NADH-dependent pathway, but not for the efficiency with which the vitamin is recycled via the natural dithiol-dependent system. We want to put forward the hypothesis that the NADH-dependent and the dithiol-dependent reductases differ substantially with respect to their ability to reduce MK-4 and MK-9. This point is subject to further *in vitro* research.

Since nutritional menaquinones are generally dissolved in the lipid fraction of products such as dairy produce and meat^{4,5}, these K-vitamins are likely to be incorporated into well-absorbable micelles and chylomicrons through the action of bile salts. The efficacy

with which phyloquinone is extracted from the thylakoid membranes of green vegetables in the gastrointestinal tract, on the other hand, may be variable and far more incomplete. It is our opinion, therefore, that the physiological importance of nutritional menaquinones is presently underestimated. To be able to assess the relative contribution of both phyloquinone and menaquinones to human vitamin K status, it is important that concentrations of the various forms of vitamin K in food as well as their respective bioavailability, are determined in human volunteers.

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CHAPTER 3.2

TISSUE DISTRIBUTION OF K-VITAMERS UNDER DIFFERENT NUTRITIONAL REGIMENS IN THE RAT

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Summary

Two forms of vitamin K (phylloquinone (K_1) and menaquinone-4 (MK-4)) were added to vitamin K-deficient rat food in varying amounts. These diets were given as the sole source of nutrition to rats for one week. The minimal dietary requirements (MDR) to attain maximal prothrombin synthesis were determined to be 0.6 and 6–10 $\mu\text{g/g}$ of food for K_1 and MK-4, respectively. The difference between both vitamers could be explained by the limited hepatic accumulation of MK-4. Next, vitamin K was offered to rats at concentrations ranging between 0.6 and 3000 $\mu\text{g/g}$ of food, and the tissue distribution of vitamin K was investigated after one week of administration. Accumulation of K_1 and MK-4 was found in all tissues investigated, but both the absolute tissue concentration and the ratio between K_1 and MK-4 were tissue-dependent. Highest values were found in liver and in heart, but since the heart contains no γ -glutamylcarboxylase, the function of vitamin K in this tissue remains obscure. High tissue concentrations of MK-4 were also found in pancreas and testis after a diet containing K_1 exclusively. The data indicate that this conversion is tissue-specific, but neither the reason nor its mechanism are known.

Introduction

Vitamin K is a group name for a number of related compounds that are all capable of serving as a cofactor for the mammalian enzyme γ -glutamylcarboxylase. In liver, this enzyme is involved in the synthesis of four blood coagulation factors, including prothrombin.¹ γ -glutamylcarboxylase has also been detected in most soft tissues and bone², but, in most cases, the products formed in these tissues have not yet been identified. The recommended dietary allowance (RDA) for vitamin K in humans is 1 $\mu\text{g/day}$ per kg body weight³ and this is based on the dose required for normal coagulation factor synthesis. This amount is amply covered by the normal western diet, but whether it is also sufficient for the extrahepatic carboxylases is presently unknown.

Natural K-vitamers are phylloquinone (vitamin K_1) and the menaquinones (vitamin K_2). Only phylloquinone and menaquinone-4 (MK-4) have therapeutic applications. To prevent haemorrhagic disease of the newborn (HDN), 1 mg of K_1 is administered at birth in most developed countries, followed by moderate oral doses (e.g. 25 $\mu\text{g/day}$ or 1 mg/week) during the first three months of life.⁴ In Japan, MK-4 is used both in the form of a pharmacological drug or as a food supplement in newborns. For instance, doses of 45 mg per day are used for the treatment of postmenopausal osteoporosis^{5,6}, and this treatment may be continued for several years. During moderate intake, vitamin K is mainly absorbed in the ileum and distributed to the various tissues. Studies in experimental animals have shown that after oral ingestion, vitamin K is distributed to a wide variety of tissues, with

substantial accumulation in liver, heart, pancreas and sternum.^{7,8} No data are available about the absorption and tissue distribution of K-vitamins during long-term high-dose administration. In vitro, K₁ and MK-4 are comparably active in their function as cofactor for γ -glutamylcarboxylase⁹, whereas Thijssen et al.⁸ showed that in vivo inhibition of vitamin K recycling with warfarin leads to accumulation of both K₁-epoxide and MK-4-epoxide in liver. This suggests that both vitamers are active. Groenen-van Dooren et al.¹⁰ reported that in vitamin K-deficient rats, K₁ is about twice as active as MK-4 in maintaining prothrombin synthesis. Remarkably, in rats^{7,8} and chickens^{11,12}, it was found that a diet in which K₁ was the only source of vitamin K lead to accumulation of MK-4 in various extrahepatic tissues. Neither the reason nor the mechanism involved in this apparent conversion of K₁ into MK-4 are known.

In the studies reported in this paper, we have used the rat as an experimental model, in which the MDR value was defined as the Minimal Dietary Requirement of vitamin K to attain full prothrombin synthesis. The tissue distribution of K₁ and MK-4 was analyzed at a constant intake of low-moderate doses (1-10 times the respective MDR values) over one week, and compared with the distribution at high intakes (up to 5000 times the MDR value).

Materials and methods

Chemicals and animal food

Menaquinone-4 and K₁ were purchased from Sigma (St. Louis, MO, USA), 2',3'-dihydrophyloquinone was a kind gift from Hoffmann-La Roche (Basel, Switzerland). Powdered vitamin K-deficient γ -irradiated (0.9 Mrad) food and high vitamin K₁ food (3 g K₁/kg) were obtained from Hope Farms (Woerden, Netherlands). Diets containing various menaquinone-4 concentrations were prepared by mixing the vitamin K-deficient food with menaquinone powder in a food processor. Diets containing various phyloquinone concentrations were prepared by mixing the high K₁ diet with vitamin K-deficient food in the desired ratio. In all cases, the vitamin K concentration in the food was checked after hexane extraction and HPLC analysis.

Animals and diets

Male Lewis rats (12 weeks old, mean weight 307 $\pm$ 24 g) were housed in metabolism cages and maintained in a 12-hr light-dark cycle at a temperature of 20 $\pm$ 2 °C and 50 $\pm$ 10% humidity. Upon starting the experiments, all rats received the vitamin K-deficient diet, mixed with cooked and dried white rice in a 1:1 (w/w) ratio during one week, followed by one week of K-deficient food supplemented with either phyloquinone or menaquinone-4 in concentrations ranging from 0-3 mg/g food (as specified below). The diets were given ad libitum, the average food intake was 20 g per day. Blood (0.5 ml) was taken from the

tail by venipuncture and collected in 0.05 ml of 0.14 M trisodium citrate. Plasma prothrombin concentrations were determined at start, after 1 week of vitamin K deficiency and at the end of the experiment. Tissue concentrations of vitamin K were determined in pancreas, heart, kidney, testis, femoral bone, serum and liver. For this purpose, the animals were brought under ether anesthesia before the various organs were perfused with 0.15 M NaCl via the abdominal aorta, and subsequently excised from the body. All tissues were immersed in a cold saline solution and stored at -80 °C until analysis.

Vitamin K detection

After thawing, tissue homogenates were diluted in 0.15 M NaCl (1:3 w/v, wet-weight basis) and homogenized using a blender (Ultra Turrax; Janke and Kunkel, Staufen, Germany). Tissue homogenates were diluted 10- to 1000-fold with 0.15 M NaCl, depending on the amount of vitamin K that was expected. Samples of tissue homogenate (0.5 ml) and bone fragments were supplemented with 2 ng of 2',3'-dihydrophyloquinone as an internal standard, extracted with hexane, and pre-purified on silica columns as described previously.⁷ Quantification of vitamin K was performed by HPLC (column: Chromspher C₁₈, 100 x 3 mm) with fluorescence detection (excitation, 244 nm; emission, 430 nm) after post-column reduction on a 10 x 0.2 cm column filled with zinc powder (ϕ < 200 mesh, Johnson Matthey, Karlsruhe, Germany) at 40 °C.¹³ The mobile phase consisted of methanol/ propan-2-ol/ buffer A at a ratio (v/v) of 360:40:16 (buffer A: 1M ZnCl₂, 1M NaOAc, 1M AcOH), and was degassed continuously with helium.

Prothrombin

Plasma prothrombin concentrations were measured in a one-stage coagulation test using human thromboplastin (Thromborel S®) and clotting factor II-deficient plasma (Behringwerke AG, Marburg, Germany) as described in chapter 3.1. The individual prothrombin levels after the experimental diet period were expressed as a percentage of their respective initial values.

Data analysis. Plasma prothrombin values are presented as means $\pm$ S.D. for 5 rats. Tissue vitamin K contents are expressed as the means of three rats $\pm$ S.D.

Results

Assessment of the MDR values for K₁ and MK-4

For each K-vitamin, the MDR value for maximal prothrombin synthesis was established in 45 rats that were fed with a vitamin K-deficient diet for one week before they were subdivided into 9 groups of five rats each, receiving diets containing vitamin K at concentrations varying from 0 -10 μ g per g of food. Blood was collected at 0, 5, and 7

Figure 1. Rat plasma prothrombin concentrations after dietary intake (7 days) of varying amounts of phyloquinone (○), or menaquinone-4 (●). Values are the means $\pm$ SEM of 5 rats.

days after the start of the experiment. It appeared that circulating prothrombin concentrations had reached a steady state at day 5. At day 7, dose-response curves for both K_1 and MK-4 were made in which all data are expressed as a percentage of the individual starting values. The MDR value is defined as the lowest concentration of vitamin K necessary to reach the initial plasma prothrombin concentration. As can be seen from Figure 1, the MDR values for K_1 and MK-4 were 0.6 and 6-10 $\mu\text{g/g}$ food, respectively. To explain this difference between both vitamers, we decided to measure vitamin K levels in serum, liver, and in various other tissues, using the same steady-state model and a wide range of dietary vitamin K concentrations.

Tissue distribution of K-vitamers at low dietary intake

A low dietary vitamin K intake was defined as the range between 1- and 10- times the MDR value. Therefore, we compared serum and tissue vitamin K in the steady-state model at 0.6 and 6 μg K_1/g food and at 6 and 60 μg MK-4/g food. As before, the diets were administered for seven days following a one-week period of vitamin K-deficiency. All data are compared with a control group that received a vitamin K-deficient diet for a total period of 14 days. Each group consisted of three rats. As is shown in Table 1, neither K_1 nor MK-4 were detectable in the livers of the K-deficient rats. Also long-chain menaquinones were undetectable (data not shown). This is consistent with the very low prothrombin concentrations in the corresponding plasmas. In all other tissues analyzed, low vitamin K levels remained detectable; in pancreas and testis, the residual levels of MK-4 were

Table 1. Tissue distribution of K-vitamins at low levels of dietary intake of K₁ or MK-4

Tissue	Vitamin K-active compound	Dietary vitamin K intake (µg/g food)				
		0	K ₁ (0.6)	K ₁ (6)	MK-4 (6)	MK-4 (60)
Serum	K ₁	0.4 ± 0.1	0.3 ± 0.1	2.0 ± 0.2	0.9 ± 0.0	n.d.
	MK-4	0.1 ± 0.0	0.37 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	65 ± 63
Pancreas	K ₁	5.7 ± 2.6	5.3 ± 0.7	9.8 ± 2.8	12 ± 1.9	8.3 ± 1.2
	MK-4	239 ± 134	339 ± 119	570 ± 84	810 ± 133	3490 ± 1153
Heart	K ₁	4.3 ± 0.3	6.0 ± 0.7	13 ± 2.6	9.3 ± 0.2	7.8 ± 0.6
	MK-4	0.9 ± 0.2	1.9 ± 0.6	1.3 ± 0.9	5.1 ± 0.5	1447 ± 203
Testis	K ₁	2.7 ± 0.3	1.1 ± 0.1	n.d.	5.5 ± 0.9	5.6 ± 0.2
	MK-4	107 ± 27	91 ± 16	70 ± 12	230 ± 42	409 ± 146
Liver	K ₁	n.d.	5.4 ± 2.3	38 ± 0.8	n.d.	n.d.
	MK-4	n.d.	n.d.	n.d.	1.8 ± 0.2	313 ± 41
Kidney	K ₁	0.9 ± 1.2	1.3 ± 0.8	2.0 ± 0.1	4.4 ± 0.9	4.8 ± 1.4
	MK-4	1.3 ± 0.7	2.7 ± 0.7	2.5 ± 0.6	3.9 ± 1.0	504 ± 180
Bone	K ₁	0.5 ± 0.2	5.5 ± 3.5	4.5 ± 4.5	0.7 ± 0.2	1.1 ± 0.4
	MK-4	2.2 ± 0.3	3.7 ± 0.8	8.2 ± 2.1	2.9 ± 1.6	15 ± 3.1

Values are expressed as means ± S.D. (n=3) in ng/g tissue or ng/ml serum. n.d. = not detectable
 Total of vitamin K in the table is an enumeration of vitamin K-quinone and vitamin K-epoxide.

even remarkably high. From Table 1, it can also be seen that using a diet containing 6 µg of vitamin K per g of food, the serum concentration of K₁ after K₁ intake was only slightly higher, but the hepatic K₁ concentration was about 20-fold higher than that of MK-4 after MK-4 intake. Even at 0.6 µg K₁/g food, the hepatic K₁ stores were 3- times higher than those of MK-4 at 6 µg/kg food. The differences in hepatic accumulation fully explain the different MDR values.

By comparing the vitamin K accumulation rates in extrahepatic tissues, it was found that, in pancreas, the intake of K₁ resulted in the accumulation of MK-4. Since the animals had no access to MK-4, this phenomenon can only be explained by assuming that nutritional K₁ had been converted into MK-4. As a logical consequence, the sum of tissue K₁ + MK-4 will have to be regarded as the accumulated vitamin K after a certain dietary regimen. It can also be seen from Table 1 that the amounts of accumulated vitamin K differ widely from one tissue to another, and that the liver is unique in its selective accumulation of K₁. At equal dietary dosages, other tissues (like pancreas and testis) seem to have a

Table 2. Tissue distribution of K-vitimers following a high dietary intake of K₁ or MK-4

Tissue	Vitamin K-active compound	Dietary vitamin K intake (µg/g food)					
		K ₁ (6)	K ₁ (600)	K ₁ (3000)	MK-4 (6)	MK-4 (600)	MK-4 (3000)
Liver	K ₁	2.0 ± 0.2	2536 ± 1234	6194 ± 2674	0.9 ± 0.0	n.d.	n.d.
	MK-4	0.4 ± 0.1	n.d.	n.d.	0.5 ± 0.1	1985 ± 970	7267 ± 2822
Pancreas	K ₁	9.8 ± 2.8	7470 ± 4371	6359 ± 1040	12 ± 1.9	9.7 ± 0.8	11 ± 2.3
	MK-4	570 ± 84	1471 ± 735	938 ± 35	810 ± 133	5212 ± 670	10648 ± 1978
Heart	K ₁	13 ± 2.6	18066 ± 6719	19484 ± 447	9.3 ± 0.2	6.6 ± 0.8	10 ± 2.6
	MK-4	1.3 ± 0.9	n.d.	n.d.	5.1 ± 0.5	4217 ± 1462	12931 ± 4728
Testis	K ₁	n.d.	285 ± 154	896 ± 242	5.5 ± 0.9	4.8 ± 0.3	3.1 ± 0.7
	MK-4	70 ± 12	85 ± 76	343 ± 91	230 ± 42	467 ± 277	734 ± 145
Spleen	K ₁	38 ± 0.8	16463 ± 7700	53161 ± 35160	n.d.	n.d.	n.d.
	MK-4	n.d.	n.d.	n.d.	1.8 ± 0.2	2990 ± 361	18884 ± 3114
Kidney	K ₁	2.0 ± 0.1	1998 ± 747	2940 ± 628	4.4 ± 0.9	2.5 ± 0.6	1.9 ± 0.3
	MK-4	2.5 ± 0.6	n.d.	n.d.	3.9 ± 1.0	731 ± 133	1407 ± 525
Bone	K ₁	4.5 ± 4.5	352 ± 218	404 ± 13	0.7 ± 0.2	0.5 ± 0.2	0.9 ± 0.3
	MK-4	8.2 ± 2.1	12 ± 1.2	9.6 ± 2.9	2.9 ± 1.6	121 ± 93	355 ± 65

Values are expressed as means ± S.D. (n=3) in ng/g tissue or ng/ml serum. n.d. = not detectable

Total of vitamin K in the table is an enumeration of vitamin K-quinone and vitamin K-epoxide.

substantially higher preference for MK-4 than for K₁. The diets used in the study below will therefore not be defined on the basis of the respective MDR values, but on a weight per weight basis.

Tissue distribution of K-vitimers at high dietary intakes

To achieve high vitamin K intakes, diets were prepared containing 600 and 3000 µg of either K₁ or MK-4 per g of food. Although these dosages were up to 500- (for MK-4) and 5,000 (for K₁)-times the MDR values, no adverse health effects were observed during the daily inspection of the rats. Plasma prothrombin concentrations remained stable, at around 100%, in the highest intake groups (data not shown). The tissue levels of vitamin K levels at these high intakes are summarized in Table 2, and it may be concluded that liver and heart are capable of accumulating very high levels of vitamin K. Serum levels increased in a dose-dependent way, and circulating vitamin K levels after K₁ or MK-4 intake were comparable. In some tissues, accumulation increased proportionally with increasing intake (e.g., in liver and testis), in other tissues (heart, pancreas, and bone), plateau levels were reached at 600 µg vitamin K₁ per g of food. Even at the highest K₁ dosages, the conversion of K₁ into MK-4 remained restricted to pancreas and, to a lesser

extent, the testis. In no case was the reverse reaction, i.e. conversion of MK-4 into K_1 , observed. Vitamin K epoxides were detected in all tissues (notably at the higher intake levels) in a range of 1.5-20% of the quinone levels (data not shown), suggesting the actual participation of both vitamers in the vitamin K-cycle.

Discussion

The minimal dietary requirement of vitamin K_1 for normal prothrombin synthesis in our experimental rat model was found to be 0.6 $\mu\text{g/g}$ of food. This value may be subject to slight variations, dependent on rat strain, age and experimental conditions, but it is in good agreement with data from other laboratories (0.1-0.5 $\mu\text{g/g}$ food^{14,15}). Taking into account the mean weight of the rats (307 g) and their daily food intake (about 20 g), their minimal daily requirement was about 40 μg of K_1 per kg body weight. Since the recommended dietary allowance (RDA) for vitamin K in humans is not more than 1 $\mu\text{g/kg}$ body weight³, it seems that the vitamin K requirement in the rat is much higher than in man. This may be related to a lower absorption or to a higher turnover of vitamin K in the rat. In our experiments, we also demonstrate that the efficacy of K_1 in restoring normal prothrombin synthesis is about 10-15 times higher than that of MK-4. Previously, however, we had found that both vitamers had comparable activity *in vitro*.⁹ In our attempts to explain this apparent discrepancy, we found that, at a comparable vitamin K intake level, the serum concentrations of both vitamers are similar, but that the hepatic accumulation of K_1 is at least 10-fold higher than that of MK-4. For both vitamers, the minimal required daily intake corresponded to hepatic vitamin K concentrations of between 2 and 5 ng/g tissue. It is not clear from our data whether the difference in accumulation results from a more efficient uptake of K_1 or from a more rapid metabolism of MK-4. The latter possibility is supported by data from Konishi et al.¹⁶, who showed a faster hepatic clearance of MK-4 compared to K_1 . Independent of the underlying mechanism, the difference in hepatic accumulation fully explains the differences between the MDR values for both vitamers.

From our data, it is clear that the extent to which K-vitamers accumulate in extrahepatic tissues may differ substantially from that of the liver, both with respect to the steady-state level at a certain vitamin K intake and to the maximal level at very high intakes. Also, we found tissue-specific differences between the relative accumulation of K_1 and MK-4. So, MDR values can only be defined for the synthesis of hepatic clotting factor, but they are meaningless for extrahepatic vitamin K accumulation. It appeared that heart and liver had very high storage capacities for both forms of vitamin K. Notably, the accumulation in heart tissue is intriguing because, on the basis of *in vitro* enzyme assays, no vitamin K-dependent carboxylase activity was found in this tissue.² Recently, however, substantial amounts of mRNA coding for carboxylase were reported in heart.¹⁷ It might be possible,

therefore, that for *in vitro* testing of heart carboxylase, unusual conditions, or as yet unrecognized peptide substrates, are required. Alternatively, the accumulation of vitamin K in heart might serve other, as yet unknown, physiological functions. All other tissues investigated in this paper are known to contain the vitamin K-dependent carboxylase, although, in most cases, the γ -carboxyglutamate containing proteins produced have been characterized insufficiently. At moderate levels of vitamin K intake (up to 60 μg MK-4/g food), the accumulation in bone tissue was poor, with vitamin K concentrations hardly exceeding the values found in vitamin K-deficient rats. Only at 600 and 3000 $\mu\text{g/g}$ food was substantial accumulation of K-vitamins in bone observed. This is consistent with the claim that only at very high intake (45 mg/day) is vitamin K effective in the treatment of postmenopausal osteoporosis.⁵

As early as 1958, it was reported that menadione, which was supplemented in animal food, was converted into MK-4.¹⁸ Later it was found that feeding animals with K_1 was also followed by an apparent conversion of this vitamin into MK-4.¹¹ Experimental evidence was provided of the degradation of K-vitamins to menadione by intestinal bacteria.¹⁹ It was recently reported that the MK-4 content of rat tissues following dietary vitamin K_1 supplementation is due to local MK-4 synthesis rather than to MK-4 uptake from a central source.⁸ The latter suggestion is supported by our data: if the conversion of K_1 into MK-4 would be accomplished by gut bacteria, the absorption and tissue distribution of the thus formed MK-4 would be comparable to that after direct oral ingestion of MK-4. This was definitely not the case. The liver, for instance, was capable of accumulating large amounts of MK-4 from the diet, but no accumulation of MK-4 was found when a diet containing K_1 was given. In the studies presented in this paper, we used rats of the Lewis strain, and it was found that the apparent conversion of K_1 into MK-4 remained restricted mainly to two tissues, the pancreas and testis. The fact that, using Wistar rats, Thijssen et al.^{7,8} observed conversion in several other tissues as well, suggests that the tissue distribution of the K_1 -converting activity may depend on the particular strain of rat used. We postulate that the tissues in question contain an, as yet hypothetical, enzyme, involved in the conversion of either K_1 or a K_1 -metabolite into MK-4. The K_1 -metabolite might be menadione formed by gut bacteria¹⁹ or elsewhere. Apparently, the K_1 converting activity is saturable, because at low intake, the majority of the available K_1 was transformed into MK-4, whereas on increasing intake, this percentage declined to 10-20% conversion at very high levels of K_1 intake.

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CHAPTER 3.3

INTESTINAL FLORA IS NOT AN INTERMEDIATE IN THE PHYLLOQUINONE - MENAQUINONE-4 CONVERSION IN THE RAT

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Summary

To elucidate the role of intestinal bacteria in the conversion of phyloquinone into menaquinone-4 (MK-4) we investigated the tissue distribution of vitamin K in germ-free rats. The rats were made vitamin K deficient by feeding a vitamin K-free diet for 13 days. In a subsequent period of 6 days, phyloquinone and menadione were supplied via the drinking water in concentrations of 10 and 50 $\mu\text{mol/L}$. Menadione supplementation led to high levels of tissue MK-4, particularly in extrahepatic tissues like pancreas, aorta, fat and brain. Liver and serum were low in MK-4. Phyloquinone supplementation resulted in higher phyloquinone levels in all tissues when compared with vitamin K-deficient values. The main target organs were liver, heart and fat. Remarkably, tissue MK-4 levels were also higher after the phyloquinone supplementation. The MK-4 tissue distribution pattern after phyloquinone intake was comparable with that found after menadione intake. Our results demonstrate that the conversion of phyloquinone into MK-4 in extrahepatic tissues may occur in the absence of an intestinal bacterial population and is tissue specific. A specific function for extrahepatic MK-4 or a reason for this biochemical conversion of phyloquinone into MK-4 remains unclear thus far.

Introduction

Natural vitamin K occurs in two forms: phyloquinone (vitamin K₁) and the group of menaquinones (vitamin K₂). The former, mainly present in vegetables, contains a phytyl group at the 3-position of the 2-methyl-1,4-naphtoquinone nucleus. Menaquinones are from bacterial origin and contain a polyisoprenoid side chain instead. The provitamin menadione (vitamin K₃) may be prenylated in the active menaquinone-4 (MK-4) in birds and mammals.^{1,2} According to present knowledge, the biological function of vitamin K in eukaryotes is to serve as a cofactor for the post-translational carboxylation of glutamate residues. More than 3 decades ago, Billeter and Martius demonstrated that orally administered phyloquinone can serve as a source for MK-4. Birds showed a higher conversion capacity than rats, i.e., more MK-4 from administered phyloquinone was recovered in tissues of chicks and pigeons.³ Later on, the same authors reported on the dealkylation of the side chain of both phyloquinone and MK-4 after dietary intake and replacement by a newly introduced geranyl-geranyl side chain to give MK-4. The exchange of the side chain, however, was not observed after a single intravenous or intraperitoneal administration of phyloquinone. Furthermore, experimental evidence was given suggesting that intestinal bacteria degrade K-vitamins to release menadione, which can be used by other bacteria for menaquinone synthesis and also by the host for MK-4 synthesis.⁴

Recent findings have renewed the interest of the apparent conversion of phyloquinone

to MK-4. Will et al.⁵ and Guillaumont et al.⁶ found higher MK-4 levels in the liver of rats and chicks after intravenous administration. Thijssen and Drittij-Reijnders⁷ found that rats accumulate MK-4 mainly in extrahepatic tissues. The levels increased after phyloquinone supplementation, either via the diet or via chronic intravenous infusion.⁷ The same MK-4 distribution pattern was observed when rats were fed solely menadione, showing high MK-4 levels in, e.g. pancreas, heart and brain, but low levels in liver and plasma.⁸ Furthermore, evidence was given that the extrahepatic MK-4 accumulation was due to local MK-4 synthesis rather than MK-4 uptake. Also, human tissues were found to contain MK-4.⁹ Because of the low concentration of MK-4 in nutrients, human MK-4 may also originate from dietary phyloquinone. The question that has to be answered is whether tissues mediate the conversion or whether they use menadione released by gut flora activity. The fact that also iv administration of phyloquinone resulted in higher MK-4 levels⁵⁻⁷ may point to tissue conversion without the need of the intestinal flora. Enterohepatic recirculation of phyloquinone, however, cannot be ruled out. Here, we present data on the MK-4 disposition in germ-free rats after different dietary vitamin K regimens. The results show unequivocally that intestinal bacteria are not obligatory in the phyloquinone-MK-4 conversion.

Materials and methods

Chemicals

Phylloquinone in a Konakion® formulation, packed in sterile, sealed ampules, and 2',3'-dihydrophyloquinone as a kind gift were obtained from Hoffmann-LaRoche (Basel, Switzerland). Menadione sodium bisulphite was purchased from Sigma (St. Louis, MO, USA). Thromborel S (a commercial human thromboplastin supplemented with calcium) and human clotting factor II-deficient plasma were from Behringwerke AG (Marburg, Germany). All other chemicals were of analytical grade and were from commercial suppliers.

Animals and diets

The experiment had the approval of the local ethical committee for animal experiments. Conventional and germ-free male WKY Wistar rats, aged 14-18 weeks, were used for the various experiments. The germ-free rats were kept and handled in standard cages within an isolator, under conditions as required for germ-free animals.¹⁰ Air was filtered with bacterial filters (Miller Filters, Millipore, U.S.A.). Diets, drinking water and other materials were sterilized by autoclaving at 120 °C during 20 min. Faeces samples of germ-free rats were weekly controlled for aerobic and anaerobic bacterial contamination. Both germ-free and conventional rats were housed under conditions of a temperature of $20 \pm 2^\circ\text{C}$, a humidity of $50 \pm 10\%$ and a 12 h light-dark cycle. The conventional rats were housed in standard cages. During the vitamin K-deficient diet period, the conventional rats were

kept in individual metabolism cages to minimize coprophagy. All rats were in good condition during the study, as was established by daily visual inspection. Rats were made vitamin K-deficient in 13 days by feeding them a powdered, γ -radiated (0.9 Mrad) vitamin K-deficient diet supplied by Hope Farms (Woerden, The Netherlands). In a subsequent period of 6 days, phyloquinone and menadione bisulphite were offered to the rats via the drinking water. For phyloquinone, the Konakion preparation was used which was administered aseptically to autoclaved water. The stock solution of menadione bisulphite was sterilized by filtration through a sterile 0.22 μm Millex-GS filter (Millipore SA, Molsheim, France) and further diluted in the isolator with autoclaved water. Germ-free rats were offered 10 and 50 $\mu\text{mol/L}$ of both vitamins whereas conventional rats received 10 $\mu\text{mol/L}$ of phyloquinone and 50 $\mu\text{mol/L}$ of menadione bisulphite. Diets and drinking water were consumed ad libitum. Volumes of drinking water were recorded every other day for conventional rats and by measuring the volumes before and after the experiment for germ-free rats. To prevent degradation of vitamin K by daylight, drinking water bottles were wrapped in foil. In total, 14 germ-free rats were used in the experiment, 4 rats served as vitamin K-deficient control, 5 rats were set on menadione bisulphite (10 $\mu\text{mol/L}$, $n=2$, and 50 $\mu\text{mol/L}$, $n=3$, in the drinking water), 5 rats obtained phyloquinone (10 $\mu\text{mol/L}$, $n=2$, and 50 $\mu\text{mol/L}$, $n=3$, in the drinking water). 6 conventional rats, 2 deficient controls, 2 on menadione bisulphite (50 $\mu\text{mol/L}$) and 2 on phyloquinone (10 $\mu\text{mol/L}$) were used.

Blood (0.15 ml) from conventional rats was taken from the tail vein and collected in 15 μl of 0.14 M trisodium citrate at the start, at days 8 and 13 of vitamin K-deficiency to determine prothrombin values. At day 13 of vitamin K-deficiency and at the end of the vitamin K supplementation period, both conventional and germ-free rats were killed by aortic exsanguination under light ether anaesthesia. Blood was collected, diluted 9:1 (v/v) with 0.1 M trisodium citrate and centrifuged during 15 min by 1700 $\times g$ for the preparation of plasma. The body was perfused via the abdominal aorta with saline and the following tissues were removed: abdominal fat; aorta; brains; heart; kidneys; liver; pancreas and sternum. All organs were immersed and rinsed immediately in the saline solution, and subsequently stored by -80°C until assayed.

Vitamin K analysis

Phyloquinone, MK-4 and epoxides were extracted from tissue homogenates as described previously.⁵ The extraction of fat homogenates differed slightly from that of other tissues. Fat homogenates were heated to 40°C , diluted 1:1 (v/v) with acetone and sonicated in an ultrasonic sound bath before extraction with hexane. Analysis was performed by fluorescent detection following HPLC and post-column reduction. For the reduction, a Zinc-column was used as firstly described by Haroon et al.¹¹ The mobile phase consisted of a mixture of acetonitrile/ propane-1-ol/ H_2O (v/v/v; 80/10/3). The water part contained 1 M/L of each ZnCl_2 , NaAc and HAc and was degassed continuously with helium.

Plasma prothrombin assay

Plasma prothrombin concentrations were measured in a one-stage coagulation test with a coagulometer (KC-4, Amelung, Germany) as described by Groenen-van Dooren et al.¹² The individual prothrombin levels of the rats were expressed as a percentage of a normal plasma pool of 18 weeks old WKY germ-free or conventional rats fed a standard diet.

Statistical analysis

The data are given as mean $\pm$ SEM if not indicated otherwise. To compare plasma prothrombin values in vitamin K-deficient germ-free and conventional rats, the non-parametric Mann-Whitney test was used, with $\alpha=0.05$.

Results

For the vitamin K supplementation we originally aimed to mix the vitamin with the powdered diet. It turned out, however, that autoclaving led to a loss of at least 70% of phyloquinone. Therefore, the K-vitamins were supplied via the drinking water. Conventional rats appeared not to prosper upon autoclaved diet. They suffered from severe diarrhoea, probably due to incomplete intestinal degradation of sugars, which had caramelized during the autoclaving process. Therefore, these rats received the usual vitamin K-deficient diet without sterilising pretreatments. Daily consumption of drinking water in the conventional rats was 44 ± 12 and 61 ± 14 ml (mean $\pm$ S.D.) in the phyloquinone supplemented and menadione supplemented group, respectively. For the germ-free rats, daily consumed drinking water volumes were about 39 and 44 ml, respectively.

We first established the time period which was necessary to obtain nutritional vitamin K-deficiency. At day 8 of vitamin K-deficiency, plasma prothrombin values were about 50% of normal reference pooled plasma in both germ-free and conventional rats. After 13 days, plasma prothrombin concentrations were $8.0\% \pm 2.3$ and $36\% \pm 6.2$ in germ-free ($n=4$) and conventional rats ($n=6$), respectively, $p < 0.05$. From day 13, the rats were provided for 6 days with K-vitamins via the drinking water. The effect of vitamin K supplementation on plasma prothrombin was as follows, all vitamin K regimens restored plasma prothrombin to normal except for the $10 \mu\text{mol/L}$ menadione bisulphite regimen in germ-free rats, here plasma prothrombin reached only to about 35% of normal. With $10 \mu\text{mol/L}$ of menadione bisulphite, prothrombin had increased to about 35% of normal pool plasma.

Tissue distribution of vitamin K in conventional rats after intake of phyloquinone and menadione

For comparative purposes and to confirm previous findings in tissue MK-4 accumulation, conventional vitamin K-deficient rats were offered phyloquinone ($10 \mu\text{mol/L}$, $n=2$) and

Figure 1. Tissue distribution of MK-4 in conventional rats after administration of phyloquinone and menadione via the drinking water. The MK-4 shown in the figure is an enumeration of both the quinone and epoxide forms. Conventional rats were made vitamin K-deficient in 13 days and subsequently received vitamin K during 6 days. Open bars represent the vitamin K-deficient rats (mean, $n=2$), closed bars the rats supplemented with phyloquinone in a concentration of $10 \mu\text{mol/L}$ (mean, $n=2$) and the hatched bars the rats supplemented with menadione bisulphite in a concentration of $50 \mu\text{mol/L}$ (mean $\pm$ SEM, $n=3$).

Figure 2. Tissue distribution of MK-4 in germ-free rats after menadione supplementation via the drinking water. The MK-4 shown in the figure is an enumeration of both the quinone and epoxide forms. Germ-free rats were made vitamin K-deficient in 13 days and subsequently received vitamin K during 6 days. Open bars represent the vitamin K-deficient rats (mean $\pm$ SEM, $n=4$) and the hatched bars the rats supplemented with menadione in a concentration of $50 \mu\text{mol/L}$ (mean $\pm$ SEM, $n=3$).

menadione bisulphite (50 $\mu\text{mol/L}$, $n=2$) via the drinking water. In agreement with reported findings, phyloquinone supplementation led to accumulation of phyloquinone particularly in heart (52 and 83 ng/g) and liver (139 and 174 ng/g). Also fat tissue accumulated high amounts of phyloquinone (113 and 145 ng/g), which highly exceeded circulating phyloquinone levels (12 and 18 ng/ml serum). Menadione supplementation had no influence on tissue phyloquinone concentrations. Figure 1 shows that both after phyloquinone and menadione supplementation MK-4 levels had increased in most tissues compared to the vitamin K-deficient starting values. Relatively, MK-4 was increased most pronounced in heart, fat and kidney. Steady-state levels of MK-4 remained low in serum and liver.

Tissue distribution of vitamin K in germ-free rats; the effect of menadione

The vitamin K-deficient controls ($n=4$) contained no detectable phyloquinone or MK-4 levels in the liver. The levels in other tissues ranged from 1 ± 0.4 (brain) to 55 ± 14 (fat) ng phyloquinone and 2 ± 0.3 (heart) to 265 ± 39 (pancreas) ng MK-4 per g tissue. These starting values are also presented in Figures 2 and 3.

At the lowest level of menadione intake (10 $\mu\text{mol/L}$ drinking water), hepatic MK-4 levels remained below the level of detection which explains the low blood coagulation activity. Extrahepatic MK-4 levels, however, were higher than the vitamin K-deficient starting values: for example, brain was 2 fold, fat 4 fold, and heart 5 fold higher. The effect of 50 $\mu\text{mol/L}$ menadione bisulphite is shown in Figure 2. Accumulation of MK-4 was found in all tissues. Liver and serum contained the lowest concentrations (5.7 ± 0.3 ng/g and 4.9 ± 1.0 ng/ml, respectively) and pancreas the highest (650 ± 20 ng/g). The relative increase of MK-4 was most pronounced in kidney (40 fold); heart (30 fold); brain (10 fold) and aorta (5 fold) (Figure 2). Phyloquinone tissue levels remained unchanged after menadione supplementation. The 2,3-epoxide of MK-4 was detected in pancreas, kidney, aorta, sternum, brain and liver and ranged from 6% (aorta) to 20% (sternum) of total of epoxide and quinone of MK-4.

Tissue distribution of vitamin K in germ-free rats; the effect of phyloquinone

A dose-dependent increase in phyloquinone concentrations was observed in all tissues (Figure 3A). The main target organs were heart (117 and 1143 ± 90 ng/g), liver (85 and 1420 ± 178 ng/g) and fat (251 and 2404 ± 496 ng/g) at intake levels of 10 (mean of $n=2$) and 50 (mean $\pm$ SEM, $n=3$) $\mu\text{mol/L}$ of phyloquinone in the drinking water, respectively. Brain remained low in phyloquinone, 8 and 46 ± 3.9 ng/g. Also tissue MK-4 levels had increased after phyloquinone intake, its accumulation occurred in all tissues investigated, except for liver and serum (Figure 3B). Moreover, the MK-4 accumulation was dependent on the level of phyloquinone intake. For example, MK-4 levels in the heart were 2 ± 0.3 , 16.6, and 108.6 ± 7.6 ng/g for the vitamin K-deficient ($n=4$), and the 10 ($n=2$) and 50 ($n=3$) $\mu\text{mol/L}$ phyloquinone supplemented rats, respectively. Absolutely, the pancreas had the highest increase. Liver and serum remained low in MK-4 (7.8 ± 1.1 ng/g and $4.7 \pm$

Figure 3. (A) Tissue distribution of phyloquinone in germ-free rats after administration of phyloquinone via the drinking water. The vitamin K shown in the figures is an enumeration of both the quinone and epoxide forms. Germ-free rats were made vitamin K-deficient in 13 days and subsequently received vitamin K during 6 days. Open bars represent the vitamin K-deficient rats (mean $\pm$ SEM, $n=4$), closed bars the rats supplemented with phyloquinone in a concentration of 10 $\mu\text{mol/L}$ (mean, $n=2$) and the hatched bars the rats supplemented with phyloquinone in a concentration of 50 $\mu\text{mol/L}$ (mean $\pm$ SEM, $n=3$). (B) Tissue distribution of MK-4 in germ-free rats after administration of phyloquinone via the drinking water. The vitamin K shown in the figures is an enumeration of both the quinone and epoxide forms. For explanation of the experimental set up and the symbols see A.

1.0 ng/ml, respectively), and were comparable with the MK-4 levels after menadione intake. Most organs accumulated MK-4 to concentrations well above serum levels; tissue / serum ratios of MK-4 ranged from about 10 in the heart to 400 in pancreas. The vitamin K distribution in fat tissue revealed unexpected differences for phyloquinone and MK-4. Although absolute phyloquinone levels in fat after phyloquinone intake were higher than

the MK-4 levels, the fat/ serum ratios were 8-16 fold higher for MK-4 than for phyloquinone, indicating that the accumulation of MK-4 in fat was relatively better.

The epoxides of both MK-4 and phyloquinone were generally observed in all tissues in a range of 1-21% of the total of epoxides + quinones. No MK-4 epoxides were found in heart, serum and fat.

Discussion

The objective of this study was to clarify if intestinal bacteria play an essential role in the conversion of phyloquinone into MK-4. Such a role was previously suggested by Billeter et al.⁴ The data in the present study, however, clearly show that conversion of phyloquinone into MK-4 in the rat may occur independent of the presence of a bacterial population. In the germ-free rat we observed a dose-dependent increase in phyloquinone as well as MK-4 contents in a variety of tissues after dietary phyloquinone intake. Generally, the tissue specificity of MK-4 accumulation in the germ-free rat was the same as found for conventional rats and resembles the distribution found after menadione supplementation (this study and Refs.^{7,8}).

The synthesis of MK-4 from menadione has been well established^{1,14} and is probably performed by geranylgeranylation of the reduced menadione.¹⁵ The route of the phyloquinone-MK-4 conversion is completely unclear at the moment. Possibilities are a stepwise degradation of the phytyl side chain of phyloquinone, for instance via β -oxidation, or by removal (dealkylation) of the complete phytyl group. Billeter et al.^{3,4} recovered from dove tissue a compound resembling the methylester of phytanic acid after oral administration of phyloquinone, labeled with ¹⁴C in the phytyl side chain. This points to the complete dealkylation route.

The question arises whether the complete conversion is performed by the tissues themselves, or, alternatively, that the degradation of phyloquinone and the release of menadione occurs at a central place, e.g. the liver. The menadione thus formed, may be absorbed by the target tissues and used as starting material for MK-4 synthesis. Arguments for a central source of menadione are given by the finding that the MK-4 distribution is similar after menadione and phyloquinone administration (this study and Ref.⁸). Furthermore, it was observed that menadione could serve as a source for MK-4 synthesis in cell cultures, whereas phyloquinone could not (Thijssen, personal communication). In both possible routes of MK-4 synthesis, the tissues play an important role in the conversion of at least the last step of the MK-4 synthesis. Liver and serum hardly contained MK-4 after phyloquinone and menadione intake, probably because of the high hepatic clearance of MK-4.^{8,13} Alternatively, liver may have a limited capacity of MK-4 synthesis.

In agreement with previous observations,^{7,8} liver and heart accumulate high amounts

of phyloquinone, brain offers a barrier. The amounts of phyloquinone and menadione that were offered to the rats in the study were relatively high, 0.4 to 2.5 μmol daily. Rats on normal lab chow consume about 0.1 and 1 μmol , respectively. Even with the high dosage the phyloquinone distribution remained different among the tissues (Figure 3A). Because vitamin K is a fat soluble compound, high amounts of vitamin K were expected and could be detected in fat tissue, which makes fat an important storage tissue. A remarkable difference between phyloquinone and MK-4 accumulation in fat was observed, when fat concentrations were related to serum levels. The higher ratio of the less lipophilic MK-4 suggests that its accumulation is not a mere distribution phenomenon. Apparently, MK-4 synthesis is also present in fat tissue. The epoxide metabolites of MK-4 and phyloquinone were observed in most tissues suggesting an operating vitamin K cycle. It has been suggested that MK-4 might be the preferred vitamin K substrate in extra-hepatic tissues.⁸

In this study a 13-day period of feeding with vitamin K-deficient food was necessary to minimise plasma prothrombin values in both conventional and germ-free rats. Conventional rats seemed to be more resistant to vitamin K-deficiency than were germ-free rats. After this period, phyloquinone and MK-4 levels in livers of both germ-free and conventional young rats were depleted to values below the level of detection. Our observation is in agreement with that of Uchida et al.,¹⁶ who found significant lower plasma prothrombin concentrations and prolonged prothrombin times in germ-free rats compared to conventional rats, when these rats were fed a vitamin K-deficient diet for 3 days. This suggests that long-chain menaquinones, produced by the gut flora and probably provided via coprophagy, may partly prevent the manifestation of vitamin K deficiency.

In summary, our results clearly show that the conversion of phyloquinone into MK-4 in extrahepatic tissues of the rat is a process which is independent of the presence of intestinal bacteria. Whether the conversion is an intrinsic capacity of the tissues or whether there is a central source releasing menadione remains to be resolved. The function of tissue MK-4 synthesis and its accumulation is a subject of future studies.

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CHAPTER 4

MODULATION OF ARTERIAL THROMBOSIS TENDENCY IN RATS BY VITAMIN K AND ITS SIDE CHAINS

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Summary

Vitamin K is involved in the biosynthesis of a number of blood coagulation factors and bone proteins. It has been suggested that the vitamin K requirement of bone tissue is higher than that of the liver. Here we report that in rats very high doses of vitamin K affected neither the blood coagulation characteristics nor the blood platelet aggregation rate. This was observed for both phyloquinone and menaquinone-4. Both vitamers were also tested for their effects on the arterial thrombosis tendency in the rat aorta loop model. The mean obstruction times were prolonged at a high intake of menaquinone-4 (250 mg/kg body weight/day), and shortened after a similarly high phyloquinone regimen. Since (a) both vitamers only differ in their aliphatic side chains; and (b) a similar trend was observed after administration of phytol and geranylgeraniol, we conclude that the modulation of the arterial thrombosis tendency is accomplished by the side chain of vitamin K.

Introduction

In mammals, vitamin K functions as a cofactor for the enzyme γ -glutamylcarboxylase, which is involved in the post-translational conversion of glutamate into γ -carboxyglutamate (Gla) residues in a limited number of proteins.¹⁻³ Its cofactor function is accomplished via the naphthoquinone group, which is a common feature in a number of structurally related compounds possessing 'vitamin K activity'. Well known Gla-containing proteins are found in blood (prothrombin and related coagulation factors) and in bone tissue (osteocalcin, matrix Gla-protein).³⁻⁵ Vitamin K-deficiency results in undercarboxylated or descarboxy proteins in which the number of Gla-residues is reduced. Hence, the occurrence of undercarboxylated proteins is a strong indication for a biochemical vitamin K-deficiency in the tissues from where these proteins originate.

Undercarboxylation of coagulation factors due to dietary vitamin K-deficiency is rarely seen in healthy subjects. Recently it has become clear that undercarboxylated osteocalcin is found in a substantial part of the population, notably in elderly women.⁶⁻⁸ This suggests that under conditions of hepatic vitamin K-sufficiency, the vitamin K status of bone tissue may be poor. Independently, a number of authors have associated low bone and plasma vitamin K concentrations with low bone mass and osteoporotic bone fractures.⁹⁻¹² Pharmacologic doses of vitamin K₁ (phyloquinone, 1-10 mg/day) induced an increase of serum markers for bone formation and in some studies also a decrease of urine markers for bone resorption and urinary calcium loss.^{6,7,13} In a Japanese trial the administration of 45 mg/day of menaquinone-4 (vitamin K₂₍₂₀₎) induced a significant reduction of bone loss in postmenopausal osteoporotic women.¹⁴ On the basis of the latter study the therapeutic use in osteoporosis of menaquinone-4 is now encouraged in Japan.

Side-effects of high doses of vitamin K₁ or menaquinone-4 have not been reported in the literature. The risk of increased procoagulant activity by a high vitamin K intake may also be excluded on the basis of theoretical considerations. First, the upregulation of coagulation factor synthesis by high doses of vitamin K may be excluded, because vitamin K acts neither on the level of transcription nor on that of translation. Second, overcarboxylation of Gla-proteins has never been reported, but even if it could occur this would lead to additional negative charges in the protein molecules and hence to inactivation and denaturation rather than to supranormal procoagulant activity. So the introduction of a hypercoagulability state by vitamin K administration seems to be highly unlikely. In an experimental animal model we have confirmed these theoretical considerations, and also demonstrated that the different forms of vitamin K (phylloquinone, menaquinone-4) may affect the arterial thrombosis tendency in an unexpected way.

Materials and methods

Chemicals

Phylloquinone, phytol, bovine fibrinogen (type IV) and adenosine diphosphate (ADP) were purchased from Sigma (St. Louis, MO, USA), menaquinone-4 and geranylgeraniol were kind gifts from Eisai (Tokyo, Japan). Thromborel S (a commercial thromboplastin preparation) and human clotting factor II-deficient plasma were from Behringwerke (Marburg, Germany); Innovin (recombinant tissue factor) was from Baxter-Dade (Düdingen, Switzerland), and the chromogenic substrate S-2238 from Kabi (Stockholm, Sweden). Thrombin and collagen were from Kordia (Leiden, Netherlands) and Hormonchemie (München, Germany), respectively. All other chemicals were of analytical grade or better.

Animals and diets

Male Wistar rats were used in all studies; they entered the experiment at the age of 12 weeks, and were housed in individual wired-bottom cages with a 12-h light-dark cycle and controlled temperature ($20 \pm 2^\circ\text{C}$) and humidity ($50 \pm 10\%$). All foods were supplied in a powdered form. Vitamin K-deficient food was obtained from Hope Farms (Woerden, Netherlands) and its composition has been described previously.¹⁵ A diet containing a high vitamin K₁ content was also produced at Hope Farms, by mixing of phylloquinone (which is an oil) in the oil fraction of the food to a final concentration of 3 mg/g of food. Diets containing intermediate concentrations of vitamin K were prepared by mixing vitamin K-deficient food with vitamin K-containing food in a professional food processor. In the same way menaquinone-4 (as a powder) was mixed with vitamin K-deficient food to produce the high-dose menaquinone-4 diet. Phytol and geranylgeraniol were dissolved in hexane (20 g in 100 ml), which was mixed with 30 g of rice flower and dried under a stream of nitrogen. The remaining residues were mixed with 6.25 kg of normal (i.e. normal vitamin

K concentration) food. All animals were allowed to eat and drink ad libitum during the entire experimental period. The protocol for these experiments was approved by the Experimental Animal Ethics Committee of the Maastricht University.

Biochemical tests

For blood coagulation studies blood (0.5 ml) was taken from each rat by puncturing the tail vein and collected in 0.05 ml of 0.1 M trisodium citrate. Plasma prothrombin concentrations were determined with the one-stage coagulation assay as described in chapter 3.1. The plasma thrombin potential was recorded according to Hemker et al.¹⁶ with manual removal of the clot formed after activation (C. Nieuwenhuys et al., unpublished data). Shortly, the total amount of thrombin formed after activation of the blood coagulation system with innovin was recorded by sub-sampling at 30 different time points using the chromogenic substrate S-2238 to test the actual thrombin concentration. For blood platelet aggregation studies, 6 ml of blood was taken per rat by aortic puncture under ether anaesthesia and collected in 0.6 ml 0.1 M trisodium citrate. The platelets were washed and tested for aggregation tendency as described by Heemskerk et al.¹⁷ Serum vitamin K concentrations were assessed after extraction with hexane and prepurification on silica columns. The analytic step was performed by HPLC with fluorescence detection.¹⁸

Arterial thrombosis tendency

After a 10 day period during which the rats were fed with one of the various diets (as specified below), aorta loops consisting of a polyethylene cannula were inserted into the abdominal aorta, according to Hornstra and Lussenburg.¹⁹ In this technique the wound is closed in such a way that the loop protrudes from the abdomen and projects into the external environment. At places where the aorta loop is in permanent contact with the vessel wall, endothelial damage and flow disturbances result in the production and growth of a thrombus, which reaches an occlusive state after about five days. This moment of total obstruction is indicated by a change in color of the blood in the translucent loop, and is established by visual inspection. The period elapsed between insertion of the loop and complete obstruction of the blood flow is called the obstruction time, and is inversely correlated with the arterial thrombosis tendency.

Statistical evaluation

Since obstruction times show a log-normal distribution pattern,¹⁹ logarithmic transformation of these values (designated as 'log-OT') was performed before the differences between the groups were tested by one-way ANOVA using the Bonferroni post-hoc procedure. Mean obstruction times were calculated from the mean log-OT values. Effects of treatment on blood coagulation and platelet aggregation were tested non-parametrically for each group using the Mann-Whitney *U* test. All statistics were performed with the statistical program Statview 4.1, Abdacus Concepts 1992-94.

Results

Definition of normal and high dose vitamin K diets

The minimal dose required for normal prothrombin synthesis was established in an experiment in which rats were fed for three weeks with a vitamin K-deficient diet supplemented with known amounts of phyloquinone. It was found that plasma prothrombin concentrations decreased in a dose-dependent way if the food contained less than 0.6 μg of phyloquinone/g of food. At higher phyloquinone intakes the plasma prothrombin concentrations remained at their initial values (data not shown). It should be pointed out that rats have a high vitamin K metabolism: based on their mean food consumption (20 g/day) and body weight (250 g) we have calculated that the minimal vitamin K requirement in the rat is about 50 $\mu\text{g/day/kg}$ body weight, which is 50-100 times the human requirement. To prevent minor changes in the plasma prothrombin concentrations due to daily fluctuations of food intake, we decided that the 'standard diets' used in all experiments described below should contain three times the minimal dose of phyloquinone required, i.e. 1.8 $\mu\text{g/g}$. A high-dose phyloquinone diet was defined as containing 5000 times the minimal dose required, i.e. 3 mg (6.7 μmol)/g of food. In experiments in which we used either menaquinone-4, phytol, or geranylgeraniol these compounds were added to the standard diet in the same molar concentration.

The efficiency of absorption of these very high doses of vitamin K was studied in an experiment in which 4 groups of three rats each were fed ad libitum with diets of known vitamin K concentration for one week. The various diets contained 0, 60, 600 and 3000 μg of either phyloquinone or menaquinone-4 per g. At the end of the week blood was taken and analysed for vitamin K. It appeared that even at the high vitamin K intakes used in our studies there was a clear dose-response, with very high circulating phyloquinone and menaquinone levels (Figure 1).

Figure 1. Serum vitamin K concentrations at increasing vitamin K intake. Each point represents the average of 3 animals $\pm$ S.D. Symbols: $\bullet$, phyloquinone; $\circ$, menaquinone-4. For further details see the text.

Effects of high vitamin K intakes on blood coagulation and platelet aggregation

Three groups of 12 rats each received either the standard diet (group A), the same food mixed with 3 mg/g (6.7 μ mol/g) phyloquinone (group B), or the standard food mixed with 3 mg/g (6.7 μ mol/g) menaquinone-4. The treatment was continued for 10 days, and blood was taken before and after treatment. In the citrated plasma we determined the prothrombin concentration and the thrombin potential. Washed blood platelets were used to measure the platelet aggregation rate after induction with either collagen, with thrombin, or with ADP + fibrinogen. The large blood volume required for isolation of the platelets did not allow us to measure the platelet aggregation at the start of the study. As is shown in Table 1 the blood coagulation characteristics before and after treatment are similar, and there is a difference neither among the three groups at the start nor at the end of the experiment. This demonstrates that even at very high vitamin K intakes neither phyloquinone nor menaquinone-4 are able to increase the procoagulant activity to values above the normal level. Also, the blood platelet aggregation was not affected by high doses of vitamin K: independent of the trigger, the rate of platelet aggregation was comparable for all three groups.

Effects of high vitamin K intakes on arterial thrombosis tendency

Three groups of 72 rats each were subjected to one of the nutritional regimen mentioned above (group A: standard food; group B: high phyloquinone; group C: high menaquinone-4) for 10 days. After this pre-treatment period, all animals underwent surgery in which an aorta loop was inserted in the abdominal aorta, and the obstruction times were recorded.

Table 1. Effects of high vitamin K intake on blood coagulation and blood platelet aggregation

Marker tested	Group A (normal K treatment)		Group B (high K ₁ treatment)		Group C (high MK-4 treatment)	
	Before	After	Before	After	Before	After
Plasma prothrombin (%)	100 $\pm$ 1.1	99 $\pm$ 1.3	99 $\pm$ 1.1	95 $\pm$ 1.2	101 $\pm$ 1.6	96 $\pm$ 1.7
Thrombin potential (nmol/min)	615 $\pm$ 18	608 $\pm$ 21	618 $\pm$ 20	612 $\pm$ 15	607 $\pm$ 14	571 $\pm$ 13
Platelet aggregation (%T/min)						
Collagen-induced	n.d.	26.3 $\pm$ 1.6	n.d.	25.8 $\pm$ 1.4	n.d.	28.6 $\pm$ 1.3
Thrombin-induced	n.d.	67.6 $\pm$ 5.4	n.d.	68.1 $\pm$ 3.7	n.d.	65.8 $\pm$ 1.2
ADP-induced	n.d.	5.9 $\pm$ 0.9	n.d.	6.1 $\pm$ 1.2	n.d.	6.6 $\pm$ 4.1

Plasma prothrombin concentrations are expressed as the percentage of a reference pool of normal rat plasma. The thrombin potential is given in nmol of S-2238 converted/min. Platelet aggregation was quantitated as the percentage transmission of visible light; activation was accomplished by the addition of either collagen (10 μ g/ml), thrombin (5 nM), or ADP (80 μ M) + fibrinogen (20 μ M). All data are mean values for 12 different rats and are given $\pm$ SEM.

The data are summarized in Figure 2, (individual data in Figure 2A, and mean values after log-transformation in Figure 2B). It was found that menaquinone-4 prolonged the mean obstruction times substantially from 117.4 h in the controls to 139.8 h in the menaquinone-treated group and that the difference of the log-OT values was statistically significant ($P<0.05$). Remarkably, the effect of phyloquinone was the opposite and showed a significant shortening of the mean obstruction time to 88.7 h ($P<0.001$). Since both vitamers differ only in their aliphatic side chain, we have tested whether comparable data could be obtained by feeding the animals a diet containing either phytol (the side chain of phyloquinone) or geranylgeraniol (the side chain of menaquinone-4).

Effects of phytol and geranylgeraniol on arterial thrombosis tendency

Three groups of 24 rats each were fed with either the standard food as such (group A), standard food supplemented with 6.7 $\mu\text{mol/g}$ (2 mg/g) of phytol (group B), or standard food supplemented with 6.7 $\mu\text{mol/g}$ (2 mg/g) of geranylgeraniol (group C). In each group one rat died during surgery, so that the calculations are made for 23 animals per group. In Figure 3 the same trend was observed for the aliphatic side chains as observed earlier for the complete vitamins: geranylgeraniol (side chain of menaquinone-4) caused a longer

Figure 2. Effects of phyloquinone and menaquinone on the arterial thrombosis tendency in rats. A, the number of rats with an open loop as a function of time since surgery. B, mean values for the three groups after log transformation; the difference between the menaquinone-4 and the control group was significant at $P<0.05$, the difference between the phyloquinone and control groups was significant at $P<0.0001$.

Figure 3. Effects of phytol and geranylgeraniol on the arterial thrombosis tendency in rats. A, the number of rats with an open loop as a function of time since surgery. B, mean values for the three groups after log transformation; the difference between the phytol and geranylgeraniol groups became only apparent at longer obstruction times (see text).

mean obstruction time (167 h), and phytol caused a slightly shorter mean obstruction time (135 h) than the controls (140 h). The difference between the phytol group and the control group was very small, and may represent the normal fluctuation. The mathematical difference between phytol and geranylgeraniol was substantial (32 h), with a 95% confidence interval for the difference (Δ) between the geranylgeraniol group and the phytol group of $-0.023 < \Delta < +0.217$. The rather small group size ($n=23$), combined with the unexpected large SEM in the geranylgeraniol group meant that statistical significance was not reached in this case (see also discussion). By comparing Figure 2 and Figure 3 it can be observed that the mean obstruction times in the control groups differ substantially (117.4 versus 140 h). This is a well known phenomenon and demonstrates that obstruction times (as absolute values) can only be compared within one experiment, but not in experiments separated in time. In this case the time lag between the experiments in Figures 2 and 3 was 6 months.

Discussion

In a number of recent clinical trials vitamin K supplementation has been evaluated for the prevention^{6,7} or treatment¹⁴ of postmenopausal osteoporosis. The dose of vitamin K (either in the form of phylloquinone or menaquinone-4) given in these studies ranges

from 20 µg/day/kg body weight⁶ to 1 mg/day/kg body weight.¹⁴ The latter figure is about 1000 times the recommended dose for the maintenance of normal human hemostasis. To investigate the safety of long term administration of these high doses of vitamin K (both phyloquinone and menaquinone-4), we have used a rat model system in which the animals received 250 mg/day/kg body weight, which was 5000 times the minimal dose required for their normal prothrombin production. It turned out that even at these very high doses, neither the plasma prothrombin concentration nor the blood platelet aggregation tendency were affected. Also the plasma thrombin potential, which is a sensitive overall coagulation test, remained unchanged by the high vitamin K intake.

Surprisingly, however, we found a substantial, and statistically significant, decrease of the arterial thrombosis tendency by menaquinone-4 and an opposite effect caused by phyloquinone. The difference between the effects induced by both vitamers was highly significant ($P < 0.0001$). Since phyloquinone and menaquinone-4 have comparable vitamin K activity both *in vitro*²⁰ and *in vivo*,^{15,21} it seems unlikely that their opposite effects have anything to do with post-translational Gla formation. The only structural difference between both vitamers is found in the degree of saturation of their aliphatic side chain, and in this respect it is interesting that Hara et al.²² observed different activities of phyloquinone and menaquinone-4 on the proliferation and differentiation of osteoclasts in a co-culture system. In the same paper, these authors demonstrated that the effect persisted if the complete vitamers were replaced by the respective side chains phytol and geranylgeraniol, which suggests a new, formerly unrecognized function for the side chain of one or more K-vitamers. Therefore, we have repeated the aorta loop experiment using either phytol or geranylgeraniol and the same trend was observed as in the experiment in which the complete vitamers were used. It is striking that the three curves shown in Figure 3A coincide during the first 4 days following surgery, with 18 open loops in each group at 96 h. After this time the curves diverge, with a maximal difference between geranylgeraniol and phytol treatment. This could mean that the pretreatment period during which the animals were fed with geranylgeraniol and phytol was too short and that more pronounced differences may be observed after a longer intake of these compounds. Alternatively, there may be differences in the absorption and metabolic handling of the alcohol side chains compared to the intact vitamers. Because of this late effect the SEM in the latter experiment was larger than expected, so that the differences between the phytol and the geranylgeraniol groups were only of borderline significance. If we exclude from each group the 5 animals with obstruction times shorter than 96 h, the difference between the phytol and the geranylgeraniol group is statistically significant ($P < 0.05$), however. These data are consistent with the conclusion drawn from the experiment with the complete vitamers, and strongly suggest that - given in high doses - the side chains of vitamin K may affect the arterial thrombosis tendency. The mechanism underlying the observed effects is still unclear, but it seems unlikely that it takes place via γ -glutamylcarboxylase and the formation of Gla residues. Since the aorta loop model is based on the thrombus formation after

endothelial damage due to the insertion of the loop, an effect of vitamin K on either the secretion of endothelial procoagulants (tissue factor), or on the rate of vascular repair might influence the obstruction time in our rat model system. Alternatively, increased vitamin K intake might affect the invasion of monocytes and macrophages in the vascular lesions. The latter possibility should be considered in the light of the observation by Kameda et al.²³ who reported that menaquinone-4 (but not phyloquinone) inhibits osteoblastic bone resorption by specifically inducing osteoclast apoptosis. Osteoclasts originate, like macrophages, from the pluripotent hemopoietic stem cells in the bone marrow. Precursor cells are released into the blood stream as monocytes and collect either at the bone surface to form multinucleated osteoclasts, or they collect at the degenerating vessel wall to form macrophages which stimulate the development of small vascular lesions into atheromatous surfaces with procoagulant activity. Because of the close resemblance between osteoclasts and macrophages, it is at least conceivable that macrophages also are affected by one or more K vitamers and that stimulation of apoptosis is a common mechanism, which underlies both the menaquinone-induced reduction of bone loss as well as the modulation of arterial thrombosis tendency.

It should be pointed out that the doses of vitamin K used in the experiments presented in this paper were extremely high. Dose-response studies are needed to reveal whether the same effects will be found at lower vitamin K intakes. At this time it remains unclear whether these data may be extrapolated to man and, if so, what their physiological and clinical relevance may be.

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CHAPTER 5

ASSESSMENT OF K-VITAMINS IN HUMAN FOOD: ESTIMATION OF POTENTIAL DIETARY EFFECTS ON ORAL ANTICOAGULANT THERAPY

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Summary

A sensitive HPLC technique was used to determine various forms of vitamin K in foods. It was confirmed that spinach, sauerkraut and some margarines are rich in vitamin K₁. Higher menaquinones (MK-8 and MK-9) were abundant in curd cheese and cheese, whereas relatively high concentrations of MK-4 were found in meat, goose liver, butter, and egg yolk. Based on these data, meals were composed which were rich in either K₁ (1.8 mg) or K₂ (sum of menaquinones: 0.3 mg). Both meals were ingested by two volunteers who had been mildly anticoagulated with phenprocoumon. Following the consumption of the K₁-rich meal the serum K₁ levels increased; serum menaquinones were hardly affected by the K₂-rich meals. No post-prandial changes of either INR-values, plasma prothrombin or factor VII were observed. Our data suggest that the consumption of a single vitamin K-rich meal is not likely to substantially affect INR (International Normalized Ratio) values during phenprocoumon treatment.

Introduction

Vitamin K serves as a cofactor in the post-translational carboxylation of glutamate (Glu) residues into γ -carboxyglutamate (Gla). Well-known Gla-containing proteins are prothrombin and the blood coagulation factors II (prothrombin), VII, IX and X. Deficiency of vitamin K leads to non-functional coagulation factors, prolonged coagulation times, and bleeding.¹ Vitamin K-antagonists (coumarin derivatives) act by inhibiting the recycling of vitamin K and are used for the treatment and prevention of thrombosis. Pharmacological doses of vitamin K can overcome this blockade via the coumarin insensitive NAD(P)H-dependent K-reductase. Instability of INR-values is a well-recognized problem occurring in 20-25% of all patients on oral anticoagulant therapy.² Besides interfering drugs, age, poor compliance and concurrent diseases,³⁻⁷ variations in the dietary vitamin K intake has repeatedly been mentioned as one of the causes for this instability.⁸⁻¹² Reliable data on the vitamin K content of a wide range of food items may therefore be helpful in the dietary counselling of anticoagulated patients.¹³

Nutritional vitamin K occurs in two different forms: phyloquinone (K₁) and menaquinone (K₂), which is a group name for 2-methyl,3-prenylquinones with variable side chain length. The various menaquinones are usually designated as MK-n, where n indicates the number of repeating isoprenoid units in the side chain. Both vitamin K₁ and menaquinones were reported to possess biological activity *in vivo*.^{14,15} Several food composition tables have been published for K₁,¹⁶⁻¹⁹ but only limited data is available for the menaquinones. Thus far menaquinones of variable chain length were identified in dairy produce, fish, meat, and fermented soy beans (natto).²⁰⁻²²

In the present study we have intended to give a more elaborate list of menaquinone contents of foods commonly used in the European countries. Since in the same analyses also K_1 was quantified, the latter vitamin is included in our data. On the basis of these data we have composed different meals containing the highest possible quantities of either K_1 or menaquinones which can be ingested in a single meal. Subsequently we have measured in two mildly anticoagulated volunteers the disposition of K-vitamins in the circulation, and the potential effect of a single vitamin K-rich meal on INR-values and plasma prothrombin and factor VII concentrations.

Materials and methods

Materials

Phylloquinone (K_1) was obtained from Sigma (St. Louis, MO), and a set of menaquinones (MK-4 through MK-10) and 2',3'-dihydrophyllloquinone (to be used as reference compounds and internal standard, respectively) were kind gifts from Hoffmann-La Roche (Basel, Switzerland). Wild animals were obtained from a hunter, and free-range eggs were purchased directly from a farmer. All common foods were obtained at a local supermarket. Creamed spinach was from Iglo Ola B.V. (Utrecht, Netherlands). Silica Sep-pak cartridges were purchased from Millipore (Milford, MA, USA). All other chemicals were of the highest analytical grade commercially available.

Extraction of food

At least two samples were taken from each food item and analyzed. The extraction, pre-purification and analysis of vitamin K from beverages and dairy produce (except butter and cheese) was performed as described for serum^{23,24} using 2',3'-dihydro-phyllloquinone as an internal standard. Raw vegetables and fruits were processed as described for spinach.²⁴ Unless indicated otherwise the extraction was performed by the addition of 2 ml of distilled water, 4 ml of ethanol, 8 ml of hexane, and 20 μ l of internal standard (2',3'-dihydrophyllloquinone, 50 μ g/l in propanol-2) to 1 g of sample. For some of the nutrients slightly modified procedures were followed. Cheese and butter were cut into small pieces, 1 g of which was supplemented with 4 ml of propanol-2, 100 μ l (5 ng) of internal standard and 2 ml of distilled water. The mixture was homogenized with a blender (Ultra Turrax; Janke & Kunkel, Staufen, Germany) at a temperature of 60 °C, and extracted with 8 ml of hexane. Vitamin K was separated from the excess of fat by chromatography of the hexane phase on Sep-pak silica columns.²⁴ Raw meat and raw fish fillet were cut into small pieces, 1 g of which was supplemented with 2 ml 0.15 M NaCl and 20 μ l (1 ng) of internal standard, homogenized for 2 x 20 s in a blender and sonicated for 30 s at an amplitude of 6 micron. After adding 4 ml of ethanol and 8 ml of hexane the mixtures were shaken for 2 min and centrifuged at 2,000 x g for 5 min. The hexane phases were

purified on Sep-pak silica columns as described above. Bread was dried and ground to powder in a mortar, 1 g aliquots were supplemented with 20 μ l (1 ng) of internal standard and extracted according to the standard procedure.

Vitamin K detection

Vitamin K was analysed by HPLC using a C-18 reversed phase column and fluorometric detection after post-column electrochemical reduction as described previously.^{23,24} K₁ and menaquinones were recorded in the same run. Because of the long elution times of the long-chain menaquinones (MK-7 through MK-10) the flow was increased from 0.5 to 1 ml/min at 11 min after injection. Detection limits were 0.015 ng/ml for K₁ and MK-4, 0.04 ng/ml for MK-7, 0.10 ng/ml for MK-8, and 0.12 ng/ml for MK-9. The interday variation in serum vitamin K determinations was 6-7%.

Biochemical tests

All blood coagulation tests were performed with blind assessment in the routine of patient control of the Laboratory for Haematology of the University Hospital Maastricht. Prothrombin times (PT) were determined with an automated analyser (STA, Diagnostica Stago, Asnières, France) using Hepatoquick (Boehringer-Mannheim, Germany) as a reagent. The data were expressed as International Normalized Ratio (INR) values. The INR is calculated by using the formula: $INR = (\text{Subject's PT} / \text{laboratory control PT})^{ISI}$. In this formula ISI stands for International Sensitivity Index that compares each thromboplastin's sensitivity to an international reference thromboplastin with an ISI of 1.00.²⁵ Plasma prothrombin concentrations were measured in a one-stage coagulation test with a KC-4 coagulometer (Amelung, Germany) using human thromboplastin (Thromborel S) and clotting factor II-deficient plasma (Behringwerke AG, Marburg, Germany) as described previously.²⁶ Factor VII was measured in a coagulometer, ACL 300 Research (Instrumentation Laboratory, Milan, Italy) using factor VII-deficient plasma (Organon, Oss, The Netherlands) and human thromboplastin. All prothrombin and factor VII concentrations were expressed as a percentage of a normal human adult plasma pool. Triglycerides were determined by standard enzymatic techniques (Boehringer Mannheim, Germany), automated on a Beckman Synchron CX 7-2 apparatus (Fullerton, CA, USA).

Human volunteer studies

Two healthy male volunteers (ages: 27 and 28 years; weights: 82 and 95 kg; heights: 1.90 and 1.95 m, for subject 1 and 2 respectively) participated in the study. Both were non-smokers, regular sporters and moderate alcohol consumers. The volunteers were anticoagulated with phenprocoumon (Marcoumar[®]) during a 15-day period at an intended INR-value of 1.5. Anticoagulant control was performed in the setting of the routine of the Haematologic Laboratory of the University Hospital. INR-values were checked on days 4, 7, 10, 11, 12, 13 and 15. During the experimental period the subjects had no dietary

restrictions except some vitamin K-rich nutrients (broccoli, spinach, kale, cheese and curd cheese), which were only allowed in the experimental meals. On days 11 through 15 blood samples were taken by venipuncture at regular intervals (as indicated). Base-line data were assessed on day 11, the next day 450 g of creamed spinach (a deep-frozen product containing 1.8 mg of K_1) was prepared and consumed by the participants at 9 a.m. after an overnight fast. Day 13 served as a wash-out day, whereas on day 14 the same participants consumed a menaquinone-rich breakfast containing 225 g of baked chicken, 10 g of butter, 240 g of cheese (Gouda) and 450 g of curd cheese, with a total menaquinone content of about 0.3 mg. The subjects consumed identical meals. The design of this experiment was approved by the local Medical Ethics Committee.

Data analysis

The areas under the curve (AUC) were calculated according to the trapezoid rule, using the computer program Inplot (GraphPad Software, San Diego, CA). In all cases the data were corrected for the corresponding fasting base-line level.

Results

Food composition table for phyloquinone and menaquinones

Specimens from 6 categories of food were analysed for vitamin K: meat, fish, dairy produce, beverages, vegetables & fruit, and bread. The data are summarized in Table 1. High amounts of K_1 were found in spinach, sauerkraut and margarine. Meat, fish and dairy produce contained both K_1 and MK-4, with relatively high MK-4 concentrations in goose meat and liver, butter and egg yolk. Long chain mena-quinones were mainly found in curd cheese and in both hard and soft cheeses. No substantial differences were found between free-range eggs and those from factory-farms. Sauerkraut (fermented cabbage) contained a wide range of menaquinones, but no trace of menaquinones was found in fermented beverages like beer and wines. Icetea contained small amounts of K_1 , probably originating from the vitamin K-rich tea leaves.²⁰

Dietary effects on circulating vitamin K

Based on the data in Table 1, food items were selected with the aim to prepare palatable meals with a maximal content of either vitamin K_1 or K_2 . The spinach-based meal contained 1.8 mg of vitamin K_1 , the highest content of vitamin K_2 that could be reached in the chicken- and cheese-based meal was considerably less (sum of menaquinones: 0.3 mg). The two types of meals were consumed on different days by two mildly anticoagulated, healthy men. The protocol for the experiment is detailed in the Materials and Methods section. Serum vitamin K_1 levels in both subjects increased after consumption of the meal rich in K_1 , but not after the K_2 rich meal (see Figure 1). In both subjects a peak was reached

Table 1. Concentrations of vitamin K₁ and menaquinones in food

Nutrients	n	Vitamin K content in foods (in µg/100 g or µg/100 ml)							
		K ₁	MK-4	MK-5	MK-6	MK-7	MK-8	MK-9	MK-10
Meat									
Beef	5	0.6	1.1	-	-	-	-	-	-
Chicken	5	-	8.7	-	-	-	-	-	-
Pork	5	0.3	2.1	-	-	-	-	-	-
Minced meat ^a	5	2.4	6.7	-	-	-	-	-	-
Animal liver ^b	3	0.2	0.3	-	-	0.5	1.1	-	-
Goose liver pate	5	1.1	37	-	-	-	-	-	-
Luncheon meat	3	3.9	7.7	-	-	-	-	-	-
Salami	3	2.3	9.0	-	-	-	-	-	-
Wild hare	4	4.8	0.06	-	-	-	-	-	-
Wild deer	3	2.0	0.7	-	-	-	-	-	-
Wild duck	3	1.9	3.6	-	-	-	-	-	-
Wild goose	5	4.1	31	-	-	-	-	-	-
Fish									
Prawn	3	0.1	0.03	-	-	-	-	-	-
Mackerel	3	1.2	0.4	-	-	-	-	-	-
Herring	3	0.05	0.04	-	-	-	-	-	-
Plaice	3	0.05	0.2	-	0.3	0.05	1.6	-	-
Eel	3	0.3	1.7	-	0.1	0.4	-	-	-
Salmon	3	0.1	0.5	-	-	-	-	-	-
Vegetables & fruits									
Sauerkraut	5	25	0.4	0.8	1.5	0.2	0.8	1.1	1.3
Spinach	2	399	-	-	-	-	-	-	-
Banana	2	0.3	-	-	-	-	-	-	-
Apple	2	3.0	-	-	-	-	-	-	-
Orange	2	0.1	-	-	-	-	-	-	-
Bread									
Rye bread	3	0.7	-	-	-	-	-	-	-
Brown bread	3	1.1	-	-	-	-	-	-	-
Sourdough bread	3	1.0	-	-	-	-	-	-	-
Buckwheat bread	3	3.0	-	-	-	1.1	-	-	-

at 6 h after consumption of the meal, but the difference in peak height was large. After 24 h the circulating K₁ levels had not yet completely returned to the starting values. AUC-values were calculated for the 24 h period following the K₁-rich meal, and were found to be 18 pmol.h.ml⁻¹ for subject 1 and 89 pmol.h.ml⁻¹ for subject 2. In the same samples we have also assessed the triglyceride concentrations. Before ingestion of the K₁ rich meal, the triglyceride levels in subject 2 were about twice as high compared to subject 1. It turned out that, whereas the differences between subject 1 and subject 2 in peak height and AUC₀₋₂₄ for circulating K₁ were about 5 fold, the interindividual variation was reduced to less than 2-fold if the data were expressed as the K₁/triglyceride ratio (Table

Nutrients	n	Vitamin K content in foods (in µg/100 g or µg/100 ml)							
		K ₁	MK-4	MK-5	MK-6	MK-7	MK-8	MK-9	MK-10
Dairy produce & eggs									
Whole milk	3	0.5	0.8	0.1	-	-	-	-	-
Milk ^c	3	-	-	-	-	-	-	-	-
Milk ^e	3	1.1	2.1	-	-	-	-	-	-
Buttermilk	5	0.04	0.3	0.1	0.1	0.1	0.6	1.4	-
Whole yoghurt	3	0.4	0.6	0.1	-	-	0.2	-	-
Yoghurt ^c	3	0.02	-	-	-	-	0.06	-	-
Curd cheese	5	0.3	0.4	0.1	0.2	0.3	5.1	19	0.4
Whipping cream	3	5.1	5.4	-	-	-	-	-	-
Margarin	3	93	-	-	-	-	-	-	-
Butter	3	15	15	-	-	-	-	-	-
Gouda cheese	5	4.4	5.2	0.3	0.6	1.1	16	38	-
Camembert	5	2.6	3.7	0.3	0.5	1.0	11	40	-
Blue castello	5	2.5	7.4	0.1	0.6	1.2	11	26	-
Goat cheese	4	10	4.7	1.5	0.8	1.3	17	51	-
Egg yolk	4	2.1	31	-	0.7	-	-	-	-
Egg albumen	4	-	8.7	-	-	-	-	-	-
Egg yolk ^d	3	1.2	29	-	-	-	-	-	-
Egg albumen ^d	3	0.03	0.5	-	-	-	-	-	-
Beverages									
Beer	10	-	-	-	-	-	-	-	-
Red wine	2	-	-	-	-	-	-	-	-
Lemonades	6	-	-	-	-	-	-	-	-
Icetea	2	0.3	-	-	-	-	-	-	-

All values are given as the means of determinations in different samples, where *n* stands for the number of samples tested, and a hyphen for not detectable. ^a pork/bovine; ^b pork; ^c skimmed; ^d free-range; ^e evaporated.

2). In subject 2 the peak height after ingestion of 1.8 mg of K₁ was about 450 times the detection limit. Since the MK-4 content of the menaquinone-rich meal was only 34 µg, a response might be expected in subject 2 if the absorption and pharmacokinetic behaviour of MK-4 would be comparable to that of K₁. No MK-4 could be detected in any of the samples, however. Long-chain menaquinones were more abundant in the menaquinone-rich meal, with the highest content for MK-9 (175 µg). In subject 1 circulating menaquinones were undetectable throughout the experiment. In subject 2 we observed MK-7 and MK-8 in all samples, for MK-9 the values exceeded the lower detection limit only at 24 and 36 h after consumption of the menaquinone-rich diet. Also MK-8 was slightly increased in these samples (see Table 3). This is consistent with the experimental animal data in chapter 3.1, showing a relatively slow absorption of the higher menaquinones.

Dietary effects on INR-values and individual blood coagulation factors

During the institution of the oral anticoagulant treatment the INR-values increased to values between 1.7-1.8 in subject 1, and between 1.3-1.4 in subject 2 (see Table 4). During

Figure 1. Serum vitamin K₁ levels after consumption of vitamin K-rich meals. During an experimental period of 5 days two male volunteers consumed a vitamin K-restricted diet except on two occasions: at 9 a.m. on day 2 breakfast was high in vitamin K₁, and at 9 a.m. on day 4 breakfast was high in menaquinones. The data are shown as means of duplicate determinations for subject 1 (●) and subject 2 (○).

Table 2. Serum triglyceride levels and vitamin K/triglyceride ratio's after consumption of the vitamin K₁ rich meal

Time since ingestion (hrs)	Serum triglyceride concentrations (mmol/l)		10 ⁶ . Ratio serum vitamin K/ triglycerides	
	Subject 1	Subject 2	Subject 1	Subject 2
0	0.42	1.01	1.79	1.50
3	0.52	1.21	3.27	4.04
6	0.62	1.71	4.82	7.06
9	0.62	1.62	2.68	3.93
24	0.42	1.23	2.93	2.71

the various dietary regimens the INR-values did not change noticeably. Because individual clotting factors might be more sensitive to dietary effects than the overall prothrombin time, we also assessed the individual plasma prothrombin and factor VII concentrations (Figure 2). Indeed, small fluctuations of the plasma factor VII concentrations were observed during the day following ingestion of the vitamin K₁-rich meal, but these fluctuations did not exceed those in the base-line measurements. In both subjects the prothrombin concentrations did not change noticeably during the dietary vitamin K interventions.

Table 3. Serum menaquinone levels after consumption of a vitamin K₂ rich meal

Time since ingestion (hrs)	Serum concentration of K-vitamer (pmol/ml)		
	MK-7	MK-8	MK-9
0	0.22	0.25	< 0.15
3	0.17	0.24	< 0.15
6	0.19	0.29	< 0.15
9	0.21	0.28	< 0.15
24	0.21	0.37	0.20
36	0.23	0.35	0.27

All data are presented as means of duplicate determinations. During the first 9 hrs of the experiment the peak of MK-9 was below the detection limit (0.15 pmol/ml).

Table 4. INR-values during controlled vitamin K intake

Days	Subject 1	Subject 2
4	1.15	0.99
7	1.73	1.28
10	1.79	1.32
11	1.83	1.37
12	1.76	1.36
13	1.72	1.43
15	1.77	1.38

Figure 2. Effect of vitamin K-rich meals on plasma factor VII and prothrombin concentrations in mildly anticoagulated subjects. Clotting factor concentrations are expressed as a percentage of normal reference plasma. A: factor VII; B: prothrombin. Symbols: ●, subject 1; ○, subject 2. All values represent means of duplicate determinations.

Discussion

A wide range of nutrients was analysed for their K_1 and menaquinone content. Food composition tables published by other groups had shown high levels of phylloquinone in green vegetables like kale, spinach, cabbage and broccoli, and in various plant oils such as rapeseed, soybean and olive oil.¹⁶⁻¹⁹ Our data for K_1 are in good agreement with those published previously. In the present study we have focussed ourselves on the menaquinone content of various foods. Long-chain menaquinones were mainly found in curd cheese and cheese, whereas MK-4 was found in nearly all animal products (meat, dairy produce, eggs). For animals kept in factory-farms this may be explained from the high concentration of menadione, which is present in the fortified food given to these animals. It is well known that in vivo, menadione is readily converted into MK-4.^{27,28} To

test whether fortified animal food might be the source of MK-4 in animal products, we have also analysed the meat from a number of wild animals caught in hunting. It turned out, however, that also these animals (notably birds like goose and duck) contained variable amounts of MK-4, the origin of which remains to be established. Also eggs from factory-farms and those produced under free-range conditions had comparable MK-4 contents. In this respect it is interesting that notably in birds (and to a lesser extent in mammals) not only menadione, but also vitamin K₁ was reported to be rapidly metabolized into MK-4.^{29,30}

From Table 1 and from the previous literature data, it can be seen that in most cases the daily intake of K₁ will exceed that of menaquinones by far. Since the efficacy of intestinal extraction and absorption of the K-vitamins from the food may depend on both their structure and the food matrix in which they occur, we have compared the disposition of K-vitamins in the circulation after meals which were rich in either K₁ or menaquinones. Both meals contained sufficient fat to facilitate adequate intestinal absorption of the vitamins. Whereas it was relatively easy to prepare a meal containing about 2 mg of K₁, it turned out to be impossible to compose a meal in which the accumulated menaquinone content was above 0.3 mg. The difference between both meals is even larger if their vitamin K content is expressed on a molar base. In this respect it is important to realize, however, that in animal studies MK-9 was found to stimulate prothrombin synthesis at least 5 times more effectively than did either K₁ or MK-4 (see chapter 3.1). After consumption of the K₁ rich meal, the serum K₁ levels were elevated in both subjects, with much higher values for subject 2. In the latter case the post-prandial triglyceride serum levels were also high, which is consistent with the hypothesis that circulating levels of vitamin K₁ and triglycerides are correlated.³¹ Previous data suggested that the interindividual variation in serum vitamin K levels after ingestion of a vitamin K₁ preparation (Konakion®) is larger than the intraindividual variation.²⁴ No MK-4 was detectable in serum after consumption of the menaquinone-rich meal, and only in subject 2 we found trace amounts of the long-chain menaquinones. This means that either the intestinal absorption of menaquinones is not substantially better than that of K₁, or the hepatic uptake of menaquinones is more efficient than that of K₁. We have tried to check the latter possibility by mildly anticoagulating the subjects before the start of the nutrition experiment, and measuring the effects of both diets on their INR-values and on the individual blood coagulation factor levels. However, no substantial disturbances were observed, and in all cases the values the biochemical variables remained within the base-line range.

In the literature several cases have been reported of acquired warfarin resistance attributed to a high intake of dietary vitamin K.^{8,9,11} Two experimental studies showed that the consumption of vitamin K-rich vegetables during one day had only small effects on the blood coagulation parameters in anticoagulated patients.^{10,12} In our experimental setting no diet-associated fluctuations in either INR-values or individual coagulation factor concentrations were registered after testing the samples in the daily routine for patient control. Our data do not support the hypothesis that variations in the dietary vitamin K-intake

form a major cause for unstable INR-values during oral anticoagulant therapy. This does not exclude the possibility of fluctuating INR-values due to the repeated intake of more than one vitamin K-rich meal within a relatively short period of time, or due to the use of vitamin concentrates and food supplements. In this respect it should also be mentioned that among the three anticoagulants commonly used, phenprocoumon (used in our experiment) has the longest half-life time (150 h). The half-life times of warfarin (55 h) and acenocoumarol (8 h) are substantially shorter. It has been shown that the most stable level of anticoagulation is obtained by using phenprocoumon.³² Patients taking oral anticoagulants with shorter half-life times may be more susceptible to fluctuations of dietary vitamin K intake, therefore. Moreover, it should be noticed that our *in vivo* data were obtained in only two (healthy) volunteers. Obviously these results need to be confirmed in larger numbers of patients to assess whether the sensitivity for dietary vitamin K shows substantial interindividual variations, and whether it is dependent on other factors, like the level of anticoagulation, and the type of anticoagulant used. Food composition tables for K₁ and menaquinones as presented in this paper are a basic requirement for these studies.

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CHAPTER 6

Summary and general discussion/
Samenvating en algemene discussie

Summary and general discussion

Vitamin K has a well-established function as a cofactor for the mammalian enzyme γ -glutamylcarboxylase which converts glutamate (Glu) residues into γ -carboxyglutamate (Gla) residues of several proteins. Vitamin K-dependent proteins include the blood coagulation factors II (prothrombin), VII, IX and X, protein C, S and Z; the bone proteins BGP (osteocalcin) and MGP as well as a recently discovered protein, Gas-6. **Chapter 1** reviews literature on vitamin K in general (discovery, functions, chemical structures and sources); the mammalian requirement of vitamin K as well as the pharmacokinetic properties of vitamin K and two other structurally strongly related compounds: ubiquinone and plastoquinone.

Vitamin K and prenylated benzoquinones (ubiquinone and plastoquinone) are highly comparable regarding their structure, their function in plants and with respect to the food items in which they occur. Because of these similarities, we have investigated whether ubiquinone and plastoquinone have either vitamin K activity or vitamin K-antagonistic activity *in vitro* (**chapter 2.1 and 2.2**) and *in vivo* (**chapter 2.3**). Neither decyl-derivatives nor authentic compounds of ubiquinone and plastoquinone could act as cofactors for γ -glutamylcarboxylase. In contrast, they showed vitamin K-antagonistic activity in the *in vitro* γ -glutamylcarboxylase and KO-reductase assays. The inhibition may be due to a mechanism on an enzymatic level, e.g. the compounds may interact with the substrate binding site. On the other hand, a more chemical based mechanism of interaction cannot be excluded. We found that redox-active compounds having higher redox potentials than that of the K-KH₂ equilibrium all inhibited the KH₂-dependent carboxylase. *In vivo*, we did not observe any effects on plasma prothrombin values in rats after oral or subcutaneous administrations of ubiquinone-9 and 10 (1 to 10 mg per day) during 2 weeks. Tissue analysis revealed that both serum and liver ubiquinone levels increased after oral or subcutaneous administration of ubiquinone-9 or 10. Subcellular fractionation of the liver showed an equal distribution for vitamin K and ubiquinones with a relatively high degree of accumulation in microsomes, mitochondria and nuclei. Remarkably, the concentration of vitamin K in microsomes was about 3 orders of magnitude lower than that for ubiquinones, whereas no inhibition of the γ -glutamylcarboxylase was measured. So, the discrepancy between *in vitro* and *in vivo* data cannot be explained on the basis of differences in (subcellular) tissue distribution of vitamin K and ubiquinones. Possibly, intracellular transport systems are capable of preventing the 1000-fold excess of endogenous and nutritional ubiquinones from interacting with the enzymes of the vitamin K-cycle. Our results suggest that the reported beneficial effect of administration of high doses of ubiquinones to patients suffering from cardiovascular diseases, is not associated with an anticoagulant effect of ubiquinones.¹ Alternatively, the antioxidant activity of ubiquinones may contribute to the prevention of lipid peroxidation of membranes and LDL.

In **chapter 3** we have focussed on the absorption, tissue distribution and bioactivity of compounds known to possess vitamin K-activity, using the rat as an experimental animal model. We have investigated the dose-response of phyloquinone (vitamin K₁), menaquinone-4 (MK-4) or menaquinone-9 (MK-9) on plasma prothrombin synthesis in time after three administration routes: orally, subcutaneously and colorectally (**chapter 3.1**). The rats were rigorously prevented from coprophagy by the use of tail cups. Our results showed that the K-vitamins were hardly absorbed from the colon. After both oral and subcutaneous administration, a maximal effect of phyloquinone on prothrombin synthesis was noticed after 6-10 hours, whereas for MK-9 this was much later. We found that menaquinone-9 had a sustained effect on prothrombin synthesis. On a molar base, the vitamin K bioactivity in rats was in the order: MK-9 > phyloquinone > MK-4. In **chapter 3.2** we studied the effect of chronic administration of a large range of accurately defined doses of vitamin K on both plasma prothrombin concentrations and on the distribution of vitamin K in various tissues. The minimal dietary requirement (MDR) for both phyloquinone and MK-4 to attain maximal prothrombin synthesis was established. The MDR-values were 0.6 and 6-10 µg/g food for phyloquinone and MK-4 respectively and the difference was explained by the limited hepatic accumulation of MK-4. The MDR-value can only be defined for the synthesis of vitamin K-dependent clotting factors and is not necessarily similar for other Gla-proteins. Vitamin K was found in a wide variety of tissues. The distribution at low to moderate intake levels of vitamin K is tissue-specific, with relatively high MK-4 levels in pancreas and testis versus relatively high phyloquinone levels in liver and heart. At high to very high intake levels of vitamin K (3 mg/g food), the main vitamin K stores appeared to be liver and heart. In humans, Thijssen and Driessens² observed a comparable tissue-specific distribution pattern for phyloquinone and MK-4. Remarkably, we also found accumulation of substantial amounts of MK-4 after dietary intake of phyloquinone. This effect was most prominent in pancreas and testis and suggests that phyloquinone is converted into MK-4. This conversion has been observed previously in various animal tissues by others.^{3,6} In 1964, Billeter and Martius suggested a role for the intestinal bacteria in this conversion.⁴ To elucidate whether gut bacteria are involved in the conversion of phyloquinone into MK-4, we studied in **chapter 3.3** the tissue distribution of vitamin K in germ-free rats. These rats do not possess an intestinal flora and are kept in sterile isolators. Our results showed MK-4 accumulation after oral intake of phyloquinone in most extrahepatic tissues of the germ-free rat. The tissue-specific distribution of MK-4 was comparable with that observed in conventional rats after phyloquinone intake. Moreover, a similar tissue distribution pattern was also observed after menadione supplementation in germ-free rats. Our data demonstrate that the conversion of phyloquinone into MK-4 is not mediated by intestinal bacteria. Neither the mechanism nor the reason for this conversion are known.

Mammalian vitamin K status depends on two potential sources: 1) the diet and 2) the intestinal bacteria which produce a wide range of menaquinones. As is discussed in paragraph

1.2.9, the contribution of the intestinal flora to human vitamin K status has remained uncertain, leaving the diet as the major source of vitamin K.

Since the development of sensitive HPLC methods to determine vitamin K, several food composition tables for phyloquinone have been published. We have assessed the concentration of both phyloquinone and menaquinones in various nutrients selected from an European diet (**chapter 4**). High levels of phyloquinone were observed in vegetables such as spinach and sauerkraut. Margarin based on plant oils also contains relatively high amounts of phyloquinone. MK-4 was mainly found in products of animal origin, whereas long-chain menaquinones were predominantly present in dairy produce, notably cheese and curd cheese. The food composition of a Dutch meal makes it likely that phyloquinone contents in the food are higher than the total content of menaquinones. However, differences in absorption, transport and cofactor activity between phyloquinone and menaquinones from food may determine the *in vivo* vitamin K-activity. The uptake of vitamin K from food is highly variable and depends on several factors such as 1) the amount of fat in the diet;⁷ 2) the degree to which it is bound to components in the foodmatrix (for example, the binding to thylakoid membranes in vegetables or the solubilization in fat like in cheese). Our study showed that if meals of generally accepted size and composition are consumed, a single menaquinone-rich meal (cheese, curd cheese, chicken) hardly affected vitamin K serum levels, whereas a phyloquinone-rich meal from green vegetable origin increased serum vitamin K levels. Food composition tables on vitamin K contents may prove to be a valuable tool for the management of patients on oral anticoagulant therapy. Instability of INR-values frequently occurs in these patients and variations in dietary vitamin K intake has been mentioned as one of the possible causes of this instability. In addition to the measurement of food contents of vitamin K, the bioavailability of vitamin K from the diet needs to be determined to know if dietary vitamin K can influence the level of anticoagulation. Therefore, we offered the earlier mentioned meals separately to two mildly anticoagulated male volunteers and measured the effect on blood coagulation. No marked changes were observed in the INR-values or at the plasma factor VII and factor II levels as a consequence of the meals. It should be noticed that our results were obtained in only two volunteers and need to be confirmed in a larger population. A follow-up study should also include experimental conditions like the frequency of consumption of vitamin K-rich meals and use of oral anticoagulants with different half-life times.

Nowadays, high doses of MK-4 (45 mg per day) are prescribed in Japan for the treatment of osteoporosis in elderly women. In Europe, several trials with post-menopausal or osteoporotic women are conducted with high doses of phyloquinone. Thus far, side-effects of high doses of vitamin K have not been reported in the literature. In **chapter 5** we have studied whether such high doses of phyloquinone or MK-4 could affect thrombosis tendency, platelet aggregation and clotting parameters in the rat. We used the rat aorta loop model to study the arterial thrombosis tendency. This model is based on a method by which a loop-shaped polyethylene cannula is inserted into the abdominal aorta of a rat. At places

where the cannula is in permanent contact with the vessel wall, endothelial damage and flow disturbances result in the formation and growth of a thrombus, which reaches an occlusive state after about five days. The period between insertion and complete obstruction of the loop is called the obstruction time (OT) and is a measure for the arterial thrombosis tendency of the rat. The obstruction time is inversely correlated with the arterial thrombosis tendency. High doses of either phyloquinone or MK-4 (3 mg K/g diet) did not significantly affect platelet aggregation, thrombin potential or plasma prothrombin concentrations. On the other hand, MK-4 significantly prolonged the mean obstruction time, whereas phyloquinone shortened the OT at equal intake levels. Since both vitamers only differ in their aliphatic side chain, we also tested the effect of their side chains on the arterial thrombosis tendency: geranylgeraniol in the case of MK-4 and phytol in the case of phyloquinone. A similar trend on arterial thrombosis tendency was observed for the side chains as for the whole vitamin K compounds, suggesting that the modulation of the arterial thrombosis tendency is accomplished by the side chain of vitamin K. Hara et al. observed that both MK-4 and geranylgeraniol by itself showed an activity which differed from that of phyloquinone. Also in the case of these authors, this did not concern the effect on the carboxylation reaction but, instead, the affection of the proliferation and differentiation of osteoclasts in a co-culture system.⁹ These observations suggest a new function for vitamin K which is not related to the cofactor activity for the carboxylation reaction in which the naphthoquinone structure plays an essential role. The mechanism which underlies the effect of vitamin K on arterial thrombosis tendency is not clear at the moment.

The presence of vitamin K in a wide variety of tissues suggests the existence of thusfar unknown physiological functions for vitamin K or vitamin K-dependent proteins. One of these functions may be related to the above described effect on arterial thrombosis tendency. Furthermore, Vervoort et al.¹⁰ suggested an antioxidant function for phyloquinone in preventing lipid peroxidation induced by various prooxidants in vitro.

During the last few years, many new Gla-proteins have been discovered which are not involved in the blood coagulation process. Examples are osteocalcin (regulation of bone growth), matrix Gla-protein (inhibition of arterial mineralization), Gas6 (induction of cell growth) and two new so-called proline-rich Gla-proteins with a thus far unknown function.¹¹ The fact that vitamin K is involved in so many different physiological processes, makes that the scientific interest for this thus far relatively unknown vitamin increases rapidly.

Samenvatting en algemene discussie

In zoogdieren heeft vitamine K een goed gedocumenteerde functie als cofactor voor het γ -glutamylcarboxylase enzym, dat in sommige eiwitten glutaminezuur residuen (Glu) omzet in γ -carboxyglutaminezuur residuen (Gla). Tot de vitamine K-afhankelijke eiwitten behoren de bloedstollingsfactoren II (protrombine), VII, IX en X, de eiwitten C, S en Z; de boteiwitten BGP (osteocalcine) en MGP evenals een recent ontdekt eiwit, Gas-6. **Hoofdstuk 1** geeft een literatuuroverzicht omtrent vitamine K in het algemeen (ontdekking, functies, chemische structuren en bronnen); de vitamine K behoefte van zoogdieren evenals de farmacokinetische eigenschappen van vitamine K en twee structureel nauw verwante stoffen: ubiquinon en plastochinon.

Vitamine K en geprenyleerde benzochinonen (ubichinon en plastochinon) zijn sterk vergelijkbaar zijn wat betreft hun structuur, hun functie in planten en de voedingsmiddelen waarin ze voorkomen. Geïnspireerd door deze overeenkomsten, hebben we onderzocht of ubiquinon en plastochinon vitamine K activiteit dan wel vitamine K-antagonistische activiteit vertonen in vitro (**hoofdstuk 2.1** en **2.2**) en in vivo (**hoofdstuk 2.3**). Noch de deciel-derivaten noch het authentieke ubiquinon en plastochinon konden als cofactor fungeren voor het γ -glutamylcarboxylase. Integendeel, ze vertoonden een vitamine K-antagonistische werking by de γ -glutamylcarboxylase en KO-reductase analyses in vitro. De remming zou mogelijk toe te schrijven zijn aan een mechanisme op enzymatisch niveau, de genoemde stoffen zouden bijvoorbeeld een interactie kunnen aangaan met de bindingsplaats voor het substraat. Anderzijds kan een mechanisme dat gebaseerd is op een meer chemische interactie niet worden uitgesloten. We hebben gevonden dat redox-actieve bestanddelen met een hogere redoxpotentiaal dan die van het K-KH₂ evenwicht, allen het KH₂-afhankelijke carboxylase remmen. In vivo hebben we geen effecten kunnen waarnemen op plasma protrombine concentraties in ratten na orale of subcutane toedieningen van ubiquinon-9 en -10 (1 tot 10 mg per dag) gedurende 2 weken. Weefselanalyse onthulde dat na orale of subcutane toediening van ubiquinon-9 of -10, de ubiquinon niveau's toenamen in zowel serum als lever. Subcellulaire fractionering van de lever toonde een gelijke verdeling aan voor vitamine K en ubiquinonen met een relatief sterke accumulatie in de microsomen, de mitochondriën en de kernen. Het is opvallend dat de vitamine K concentratie in de microsomen ongeveer 3 ordes van grootte lager was dan die van de ubiquinonen, terwijl het γ -glutamylcarboxylase niet meetbaar werd geremd. De discrepantie tussen de in vitro en in vivo data kan dus niet worden verklaard op basis van verschillen in (subcellulaire) weefselverdeling van vitamine K en ubiquinonen. Mogelijk zijn intracellulaire transport-systemen in staat om te voorkomen dat de 1000-voudige overmaat aan endogene- en voedingsubichinonen een interactie aangaan met de enzymen van de vitamine K-cyclus. Onze resultaten suggereren dat het gerapporteerde gunstige effect van toediening van hoge doses ubiquinonen aan cardiovasculaire patiënten, niet geassocieerd is met een

anticoagulant effect van ubiquinonen.¹ Aan de andere kant zou de antioxidant activiteit van ubiquinonen kunnen bijdragen aan het voorkomen van lipide peroxidatie van membranen en LDL.

In **hoofdstuk 3** hebben we ons gericht op de absorptie, weefselverdeling en bioactiviteit van stoffen waarvan bekend is dat ze vitamine K-activiteit bezitten, waarbij we gebruik hebben gemaakt de rat als experimenteel diemodel. We hebben de respons onderzocht van een éénmalige dosis fylochinon (vitamine K₁), menachinon-4 (MK-4) of menachinon-9 (MK-9) op de plasma protrombinesynthese, waarbij gebruik gemaakt werd van drie toedieningsroutes: oraal, subcutaan en colorectaal en waarbij het effect werd vervolgd in de tijd (**hoofdstuk 3.1**). Door gebruikmaking van aan de staart vastgehechte opvangbuizen voor de faeces werd coprofagie door de ratten rigoreus voorkomen. Onze resultaten toonden aan dat de K-vitamines nauwelijks werden geabsorbeerd in het colon. Na zowel orale als subcutane toediening, werd na 6-10 uur een maximaal effect van fylochinon op de protrombinesynthese gezien, voor menachinon-9 was dat veel later. We vonden dat menachinon-9 een relatief langdurig effect had op de protrombine synthese. Op molaire basis was de bioactiviteit van vitamine K in ratten als volgt: MK-9 > fylochinon > MK-4. In **hoofdstuk 3.2** hebben we het effect bestudeerd van het chronisch toedienen van vitamine K in verschillende en nauwkeurig bepaalde doseringen op zowel de protrombineconcentraties in plasma, alsmede de verdeling van vitamine K over verscheidene weefsels. De minimale voedingsbehoefte (Engelse afkorting: MDR) voor zowel fylochinon als MK-4 om maximale protrombinesynthese te bereiken werd vastgesteld. De MDR-waarden waren 0.6 en 6-10 µg/g voedsel voor fylochinon en MK-4 respectievelijk en het verschil werd verklaard door de beperkte hepatische accumulatie van MK-4. De MDR-waarde kan alleen worden gedefinieerd voor de synthese van vitamine K-afhankelijke stollingsfactoren en hoeft niet noodzakelijk hetzelfde te zijn voor andere Gla-eiwitten. Vitamine K werd in een grote variëteit aan weefsels gevonden. De verdeling van vitamine K is niet gelijkmatig over alle weefsels: bij lage tot matige vitamine K-inname vonden we relatief hoge MK-4 niveau's in het pancreas en de testis en relatief hoge fylochinon niveau's in de lever en het hart. Bij hoge tot zeer hoge inname niveau's van vitamine K (3 mg/g voeding), bleken de lever en het hart de voornaamste vitamine K opslagplaatsen te zijn. In de mens werd door Thijssen en Drijt-Reijnders² een vergelijkbaar weefsel-specifiek verdelingspatroon voor fylochinon en MK-4 waargenomen. We vonden verder een opmerkelijke accumulatie van MK-4 na orale toediening van fylochinon. Dit effect was het grootst in het pancreas en de testis en suggereert dat fylochinon wordt omgezet in MK-4. Deze omzetting is ook door anderen beschreven^{3,6} in verscheidene dierlijke weefsels. In 1964 suggereerden Billeter en Martius⁴ een rol voor de darmbacteriën in deze omzetting. Om de mogelijke betrokkenheid van darmbacteriën bij de omzetting van fylochinon in MK-4 op te helderen, hebben we in **hoofdstuk 3.3** de weefselverdeling van vitamine K in kiemvrije ratten bestudeerd. Deze ratten bezitten geen darmflora en worden in steriele isolatoren gehuisvest. Onze resultaten toonden MK-4 accumulatie na orale inname van

fylochinon in de meeste extra-hepatische weefsels van de kiemvrije rat. De weefsel-specifieke verdeling van MK-4 was vergelijkbaar met die in conventionele ratten na fylochinon inname. Bovendien werd een soortgelijk weefselverdelingspatroon ook waargenomen na menadion suppletie in kiemvrije ratten. Onze data tonen aan dat bij de omzetting van fylochinon in MK-4, darmbacteriën niet zijn betrokken. Zowel het mechanisme als de reden voor deze omzetting zijn niet bekend.

De vitamine K status in zoogdieren is afhankelijk van twee potentiële bronnen:

1) de voeding en 2) de darmbacteriën die een reeks menachinonen produceren. Zoals is bediscussieerd in paragraaf 1.2.9, is de bijdrage van de darmbacteriën aan de humane vitamine K status onzeker, waardoor de voeding overblijft als de voornaamste bron van vitamine K. Sinds de ontwikkeling van gevoelige HPLC methoden om vitamine K te bepalen, zijn er meerdere voedingstabellen voor fylochinon gepubliceerd. We hebben de concentratie bepaald van zowel fylochinon als menachinonen in een aantal voedingsmiddelen die zijn geselecteerd uit een Europees dieet (**hoofdstuk 4**). Hoge fylochinon niveau's werden aangetoond in groenten als spinazie en zuurkool. Ook plantaardige margarine bevat relatief hoge hoeveelheden fylochinon. MK-4 werd voornamelijk gevonden in producten van dierlijke oorsprong, terwijl lange-keten menachinonen overwegend aanwezig waren in zuivelprodukten, met name kaas en kwark. De voedingssamenstelling van een Nederlandse maaltijd maakt het waarschijnlijk dat het gehalte aan fylochinon in het voedsel hoger is dan het totaal aan menachinonen. Echter, verschillen in absorptie, transport en cofactor activiteit tussen fylochinon en menachinonen uit de voeding kunnen een belangrijke factor zijn voor de in vivo vitamine K-activiteit. De opname van vitamine K uit voedsel varieert sterk en is afhankelijk van o.a. de hoeveelheid vet in de voeding⁷,⁸ en de mate waarin het gebonden is aan componenten uit de voedselmatrix (bijvoorbeeld de binding aan thylakoïde membranen in groenten of de vetoplosbaarheid in kaas). Onze studie toonde aan dat wanneer maaltijden van algemeen geaccepteerde grootte en samenstelling worden geconsumeerd, een enkele menachinon-rijke maaltijd (kaas, kwark, kip) de vitamine K gehaltes in serum nauwelijks beïnvloedde, terwijl een fylochinon-rijke maaltijd van groene groente-oorsprong de serum vitamine K niveau's liet stijgen. Voedingstabellen over vitamine K gehaltes kunnen een waardevol instrument zijn voor de behandeling van patiënten op orale antistollingstherapie. Instabiliteit van INR-waarden komt frequent voor in deze patiënten en variaties in de voedingsinname van vitamine K werd genoemd als één van de mogelijke oorzaken van deze instabiliteit. Naast de meting van de vitamine K gehaltes in voeding, moet ook de biologische beschikbaarheid van vitamine K uit het dieet worden bepaald om te weten of vitamine K uit voeding het niveau van antistolling kan beïnvloeden. Daarom hebben wij de eerder genoemde maaltijden apart aan twee mild geantistolde mannelijke vrijwilligers aangeboden en het effect op de bloedstolling gemeten. Er werden geen opvallende veranderingen waargenomen als gevolg van de maaltijden op de INR-waarden of op de plasma factor VII en factor II concentraties. Het moet opgemerkt worden dat onze resultaten in slechts twee

proefpersonen zijn verkregen en dat ze bevestigd moeten worden in een grotere populatie. Een vervolgstudie zou ook experimentele condities als de consumptiefrequentie van vitamine K-rijke maaltijden en het gebruik van orale antistollingsmiddelen met verschillende halfwaardetijden moeten bevatten.

Tegenwoordig worden in Japan hoge doses MK-4 (45 mg per dag) voorgeschreven voor de behandeling van osteoporose in oudere vrouwen. In Europa worden verscheidene onderzoeken uitgevoerd waarbij hoge doses fylochinon worden gegeven aan postmenopauzale of osteoporotische vrouwen. Tot nu toe zijn er in de literatuur nog geen bijwerkingen van hoge doses vitamine K gerapporteerd. In **hoofdstuk 5** hebben we bestudeerd of zulke hoge doses fylochinon of MK-4 van invloed zouden kunnen zijn op de tromboseneiging, plaatjesaggregatie en stollingsparameters in de rat. We hebben het aorta-lus model gebruikt om de arteriële tromboseneiging te bestuderen. Dit model is gebaseerd op een methode waarbij een lus-vormige polyethyleen buis wordt geplaatst in de abdominale aorta van de rat. Op de plaatsen waar de buis in permanent contact verkeert met de vaatwand, resulteren endotheliale beschadigingen en wervelingen in de bloeddorstroming in de aanmaak en groei van een trombus, die na ongeveer vijf dagen een afgesloten toestand bereikt. De periode tussen het plaatsen en de complete afsluiting van de lus wordt de obstructietijd (OT) genoemd en is een maat voor de arteriële tromboseneiging van de rat. De obstructietijd is omgekeerd evenredig met de arteriële tromboseneiging. Hoge doses fylochinon of MK-4 (3 mg vitamine K/g voeding) beïnvloedde de plaatjesaggregatie, trombine potentiaal of plasma protrombine concentraties niet significant. Anderzijds verlengde MK-4 de gemiddelde obstructietijd significant, terwijl fylochinon de OT verkortte bij gelijke innameniveaus. Aangezien beide vitamines alleen verschillen in hun alifatische zijketen, hebben we ook het effect getest van hun zijketens op de arteriële tromboseneiging: geranylgeraniol in het geval van MK-4 en fytol in het geval van fylochinon. Een soortgelijke trend op de arteriële tromboseneiging werd waargenomen voor de zijketens als voor de hele vitamine K bestanddelen, wat suggereert dat de modulatie van de arteriële tromboseneiging wordt gemedieerd door de zijketen van vitamine K. Hara et al.⁹ namen waar dat zowel MK-4 als geranylgeraniol zelf een activiteit bezitten die verschilt van fylochinon. Ook in het geval van deze auteurs betrof dit niet een effect op de carboxyleringsreactie maar een beïnvloeding van de proliferatie en differentiatie van osteoclasten in een co-cultuur systeem. Deze observaties suggereren een nieuwe functie voor vitamine K die niet gerelateerd is aan de cofactor activiteit voor de carboxyleringsreactie, waarbij de naftochinon structuur een essentiële rol speelt. Het mechanisme dat ten grondslag ligt aan het effect van vitamine K op de arteriële tromboseneiging is nog niet duidelijk op dit moment.

De aanwezigheid van vitamine K in een grote verscheidenheid aan weefsels suggereert het bestaan van tot nu toe onbekende fysiologische functies voor vitamine K of vitamine K-afhankelijke eiwitten. Eén van deze functies zou gerelateerd kunnen zijn aan het hierboven beschreven effect op de arteriële tromboseneiging. Bovendien suggereerden Vervoort

et al.¹⁰ een antioxidant functie voor fylochinon waarbij lipide peroxidatie, geïnduceerd door verschillende pro-oxydanten in vitro, kan worden voorkomen.

De laatste jaren zijn er vele nieuwe Gla-eiwitten ontdekt, die niet betrokken zijn bij het bloedstollingsproces. Voorbeelden zijn osteocalcine (regulatie botgroei), matrix Gla-proteïne (remming arteriële mineralisatie), Gas6 (inductie celgroei) en twee nieuwe zogenaamde proline-rijke Gla-eiwitten, waarvan nog geen functie bekend is.¹¹ Het feit dat vitamine K betrokken blijkt bij zoveel verschillende fysiologische processen maakt dat de wetenschappelijke belangstelling voor dit tot op heden betrekkelijk onbekende vitamine snel toeneemt.

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2. Groenen-van Dooren, M.M.; Ronden, J.E.; Soute, B.A.M. & Vermeer, C. Bioavailability of phyloquinone and menaquinones after oral and colorectal administration in vitamin K-deficient rats. *Biochem. Pharmacol.* 1995; 50 (6): 797-801.
3. Ronden, J.E.; Soute, B.A.M.; Thijssen, H.H.W.; Saupe, J. & Vermeer, C. Natural prenylquinones inhibit the enzymes of the vitamin K cycle in vitro. *Biochem. Biophys. Acta* 1996; 1298: 87-94.
4. Ronden, J.E., Thijssen, H.H.W. & Vermeer, C. Absorption and transport of various K-vitamins in the rat. *Int. J. Vit. Nutr. Res.* 1996; 66: 276 (abstract).
5. Ronden, J.E.; Groenen-van Dooren, M.M.C.L.; Homstra, G. & Vermeer, C. Modulation of arterial thrombosis tendency in rats by vitamin K and its side chains. *Atherosclerosis*, 1997; 132: 61-67.
6. Vervoort, L.M.T., Ronden, J.E. & Thijssen, H.H.W. The potent antioxidant activity of the vitamin K cycle in microsomal lipid peroxidation. *Biochem. Pharmacol.* 1997; 54: 871-876.
7. Ronden, J.E., Groenen-van Dooren, M.M.C.L., Thijssen, H.H.W. & Vermeer, C. Tissue distribution of K-vitamins at different nutritional regimens in the rat. *Biochem. Biophys. Acta*, *In press*.
8. Ronden, J.E., Vermeer, C. & Thijssen, H.H.W. Intestinal flora is not involved in the phyloquinone - menaquinone-4 conversion in the rat. *Biochem. Biophys. Acta*, *In press*.
9. Ronden, J.E., Schürgers, L.J., Gijsbers, B.L.M.G., Hamulyák, K., Thijssen, H.H.W. & Vermeer, C. Assessment of K-vitamins in human food: estimation of potential dietary effects on oral anticoagulant therapy. *Br. J. Nutr.*, submitted for publication.

Curriculum vitae

De auteur van dit proefschrift werd geboren op 13 juni 1968 te Heerlen. De middelbare schoolperiode werd doorlopen aan de scholengemeenschap 'Stella Maris' te Meerssen en in juni 1987 afgesloten met het VWO-B diploma. Vervolgens startte zij met de studie Gezondheidswetenschappen aan de toen nog zo geheten Rijksuniversiteit Limburg, waar in het tweede jaar werd gekozen voor de afstudeerrichting Biologische Gezondheidkunde. De afstudeerstage werd verricht aan het onderzoeksinstituut TNO-Voeding te Zeist. In het laatste jaar van de studie volgde zij nog een aantal aanvullende stages en modules voeding, inspanningsfysiologie en farmacologie waarna zij in november 1992 afstudeerde. In januari 1993 werd begonnen met een promotieonderzoek als AIO bij de vakgroep Biochemie, divisie vitamine K, aan de inmiddels in naam omgedoopte Universiteit Maastricht. Het promotieonderzoek, waarvan de resultaten staan beschreven in dit proefschrift, werd uitgevoerd op twee laboratoria onder begeleiding van Dr. C. Vermeer (vakgroep Biochemie) en Dr. H.H.W. Thijssen (vakgroep Farmacologie).

Dankwoord

Als startend AIO kun je nog wel eens de illusie hebben dat jij het werk in je uppie gaat doen. Gaandeweg blijkt echter dat zonder samenwerking en hulp van anderen, promoveren bijna onmogelijk wordt. Zowel binnen de vitamine K groep van de afdeling Biochemie als daarbuiten bleek dit het geval.

Allereerst wil ik mijn twee co-promotoren, Cees Vermeer (afdeling Biochemie) en Henk Thijssen (afdeling Farmacologie), bedanken voor de algehele begeleiding bij het doorlopen van het AIO-traject. Beiden hadden een eigen inbreng in deze.

Cees opereert als een heuse wetenschapsmanager. Hij verstaat de kunst om wetenschap, leidinggeven en financiële stromen goed te integreren, iets waarvan velen nog wat kunnen leren, waaronder ikzelf. Door zijn efficiënte en structurele manier van werken heb ik, terwijl ik m'n artikeltjes schreef, nauwelijks hinder ondervonden van zijn vele werkbezoeken over de aardbol afgelopen jaar. Was hij er wel, dan was hij meestal direct beschikbaar om te discussiëren over nieuwe ideeën, artikelen en andere onderwerpen.

Henk is een wetenschapper pur sang, een senior scientist die zelf het lab nog opgaat om onder andere de detectielimieten voor vitamine K verder aan te scherpen. Hij vormde voor mij een levende bibliotheek, voor hot items op literatuurgebied kon ik meestal bij hem terecht. Hij wist vaak nog iets extra's toe te voegen aan de in dit proefschrift beschreven artikelen, en data op een andere manier te tonen. Soms na gebruikmaking van bijna eindeloze omrekeningsfactoren.

Op het lab kon ik altijd rekenen op de man-met-de-gouden-handjes: Berry Soute. Hij heeft mij ingewijd in de wereld van het carboxyleren en KO-reduceren. Van zijn praktisch vermogen heb ik dankbaar gebruik gemaakt bij mijn prenylchinonproefjes waarbij na lang testen, alcoholen geen oplossing bleken te bieden. Hij wist met een heerlijke dosis humor op de juiste momenten te kunnen relativeren.

Met Monique Groenen ben ik de proefdierwereld binnengetreden. In de proefdierstallen hadden we een goede taakverdeling. Tijdens de bloedafnames mocht ik o.a. de ratten uit de kooien halen en onder controle houden (soms met bijtrisico!) terwijl Monique als de expert de staartpuncties uitvoerde (onder verdoving, jawel). Het was fijn samenwerken met haar, ze was altijd opgewekt en vrolijk. Organiseren en coördineren zijn haar op het lijf geschreven.

De laatste twee jaar heb ik in de proefdierstallen goede hulp gekregen van Nicole Bitsch. Haar judokwaliteiten heeft ze gelukkig nooit hoeven te gebruiken tijdens het hanteren, stipt verzorgen en opereren van de ratten.

Leon Schürgers is de diplomatieke lab-man die nooit nee zegt tegen grote aantallen te analyseren monsters op vitamine K-gehaltes. Hij heeft een grote bijdrage geleverd aan mijn laatste artikel waarbij we op een leuke manier hebben samengewerkt. Ons gedeeld enthousiasme over sport vergemakkelijkte dat alleen maar.

Judy Wolf vertegenwoordigde ons lab tot in de hoogste regionen: in het Mir ruimtestation. Hiervoor moest ze veel op pad. Als ze weer in Maastricht was vormde ze een gezellige thuisbasis waar ik altijd terecht kon voor een praatje. Daarnaast is ze ook mijn badminton- en fysiotherapiemaatje geweest.

Marjo Knapen heeft mijn bewondering gewekt door haar capaciteit om massa's data

na de nodige berekeningen in SPSS(X) te reduceren tot een paar tabelletjes. Wat er ook gebeurde, ze wist altijd haar rust te bewaren. De adviezen die zij gaf, kwamen meestal goed van pas en werden door mij in dank afgenomen.

Op computergebied was ik ten opzichte van Henri Spronck maar een kleintje, toch wilde hij nog van zijn niveau af te dalen om mij 't een en ander bij te brengen.

Roger Houben was nooit te beroerd om een behulpzaam handje uit te steken op het lab en daarbuiten. Ook was hij een eersteklas sfeermaker tijdens de CARIM cursussen en de NVTH dagen te Houthalen, samen met de andere Biochemie AIO's.

Alexandra Craciun is our Rumanian guest. The parallels in our research work brought us together. I enjoyed the time you were in Maastricht and I wish you a lot of succes with finishing your PhD study.

Gedurende mijn AIO-schap heb ik niet met iedereen even intensief samengewerkt, toch wil ik ook Paul, Birgit, Asiong, Lavienja, Marieke, Marjon en Marjolijn bedanken voor hun gezelschap en nuttige adviezen.

Veel werkzaamheden heb ik uitgevoerd bij de afdeling Farmacologie onder Henk's leiding. Hier heb ik prettig mogen samenwerken met Marie-José Drittij-Reijnders; Lily Vervoort; Mark Fischer en Rob Hermans. Marie-José en ik hadden beiden hetzelfde doel: het extraheren en meten van een enorme voorraad vitamine K-monsters op dezelfde apparatuur die meer dan eens haperde. Een goede onderlinge werkafstemming, begrip en gezelligheid, maakt dat ik goede herinneringen aan onze tijd samen heb overgehouden.

Van een aantal andere afdelingen wil ik de volgende mensen bedanken: Frans Weekers, Ton van de Boogaard, Eep van Dam en **alle** andere medewerkers van de Centrale Proefdienvoorziening; Jos Kop van het C-lab; Michel en Gerrie van de spoelkeuken voor het op tijd en apart reinigen van al mijn reageerbuisjes; Mariet Moolenaar en Trees Camphuizen voor de administratieve ondersteuning en hun interesse, de adequate medewerkers van het magazijn, technische diensten en de inkoop.

Verder bedank ik Prof. Dr. H.C. Hemker voor het vertrouwen dat hij de afgelopen jaren in mij heeft gesteld en de leden van de beoordelingscommissie: Prof. Dr. W.H.M. Saris; Prof. Dr. H.F.P. Hillen; Prof. Dr. P.J. Brombacher; Dr. E.A.M. Cornelissen en Dr. M.J. Shearer voor de beoordeling van dit proefschrift.

Aangezien het AIO-zijn zich niet alleen afspeelt binnen de universiteitsmuren, maar ook (ver) daarbuiten, ben ik vooral Ed en mijn ouders grote dank verschuldigd. Lieve Ed, jij bent mijn belangrijke achterban en continue stimulans geweest, al ervoer je het laatste jaar de computer als een grote concurrent. We hebben onze lange-afstands-relatie al die tijd goed weten te overbruggen en zijn nu eindelijk samen. Op kleinere afstand zijn het mijn ouders geweest die altijd voor mij klaar stonden.