

Vitamin K distribution in rat tissues: dietary phyloquinone is a source of tissue menaquinone-4

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The present study was undertaken to determine whether there is selective tissue distribution of vitamin K in the rat and whether this distribution mirrors the distribution of tissue vitamin K metabolism. The effects of feeding a vitamin K-free diet followed by resupplementation with phyloquinone (K_1) were studied. K_1 was recovered in all tissues. In K_1 -supplemented rats, most tissues accumulated K_1 relative to plasma K_1 with the highest levels in liver, heart, bone, and cartilaginous tissue (sternum). Low K_1 levels were found in the brain. In the K_1 -free rats, relatively high K_1 levels were still found in heart, pancreas, bone and sternum. Surprisingly, menaquinone-4 (MK-4) was detected in all tissues, with low levels in plasma and liver, and much higher levels in pancreas, salivary gland and sternum. MK-4 levels exceeded K_1 levels in brain, pancreas, salivary gland and sternum. Supplementation with K_1 , orally and by intravenous infusion, caused MK-4 levels to rise. Some accumulation of K_1 and MK-4 in the mitochondrial fraction was found for kidney, pancreas and salivary gland. In the liver the higher menaquinones (MK-6–9) accumulated in the mitochondria. The results indicate that: (1) there is selective tissue distribution of K_1 and MK-4, (2) dietary K_1 is a source of MK-4. The results also suggest there may be an as yet unrecognized physiological function for vitamin K (MK-4).

Vitamin K: Menaquinone: Phyloquinone: Rat

It is accepted currently that vitamin K undergoes a cyclic interconversion, the vitamin K cycle, comprising the reduction of the vitamin K quinone form into the hydroquinone, the oxidation to the 2,3-epoxide (vitamin K epoxide), and the reduction to the quinone (vitamin K). The epoxidation of the hydroquinone is coupled to a carboxylase which converts glutamyl residues in substrate proteins to essential γ -carboxyglutamates (Gla). In the liver the products of this post-translational vitamin K-dependent carboxylation are the coagulation factors II, VII, IX, and X, and the anticoagulation proteins, protein C and protein S. The reductase(s) of the vitamin K cycle are the target of coumarin antagonists. The vitamin K cycle is embedded in the rough endoplasmic reticulum with the carboxylase facing the luminal site (for reviews, see Suttie, 1987; Vermeer 1990). Enzymes of the vitamin K cycle are also found in non-hepatic tissues but vitamin K-dependent products have been found only in bone (bone Gla protein and matrix Gla protein; Hauschka *et al.* 1989) and endothelial cells (protein S; Fair *et al.* 1986). At the present time no physiological function or dysfunction associated with vitamin K other than haemostasis has been demonstrated, except skeletal malformations due to fetal exposure to warfarin (Hall *et al.* 1980). Thus, the physiological function of the non-hepatic vitamin K cycle is an unresolved phenomenon.

We reasoned that if there is a function for the extrahepatic vitamin K cycle the selective tissue distribution of vitamin K should mirror the biochemical activity of the cycle. Thus,

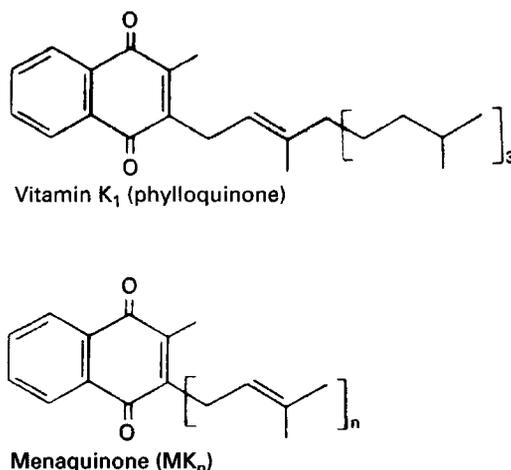


Fig. 1. Structures of phylloquinone (K₁) and menaquinones. The menaquinone-4 (MK-4) contains four isoprenoid units ($n = 4$).

'high' vitamin K levels are anticipated in tissues such as liver, pancreas, bone etc. which have high vitamin K-related activities, with low levels in tissues such as heart and brain which are devoid of any vitamin K cycle-related activity.

In the present study we report on the tissue distribution of phylloquinone (K₁) in the rat and the effect of dietary K₁ restriction. The results show that the selective distribution of K₁ in tissues is not strictly related to biochemical activity. Surprisingly, the completely prenylated variant of K₁, i.e. menaquinone-4 (MK-4; Fig. 1), was found in all extrahepatic tissues in high amounts. Furthermore, the results of the dietary experiment suggested that the presence of MK-4 is related to the supply of K₁.

MATERIALS AND METHODS

Male Wistar rats (200–220 g) were divided in two groups of five animals and fed for 9 d on either a K₁-deficient diet or a diet which was supplemented with K₁. The rats were housed individually in metabolism cages with raised wired bottoms to prevent coprophagy. Rats were maintained in a 12 h light–dark cycle at a temperature of $21 \pm 1^\circ$ and 50% humidity. The K₁-free diet was supplied by Hope Farms (Woerden, The Netherlands) and consisted of (g/kg): vitamin K-free casein 20, methionine 2, choline 4, maize starch 100, sunflower oil 50, cellulose 50, glucose 480, and standard amounts of minerals, trace elements and vitamins (except menadiene). The diet was subjected to γ -irradiation (2 MRad) to destroy any natural K₁. The K₁ content was less than 5 ng/g (our own analyses). The diet was powdered and mixed 1:1 (w/w) with commercially available ordinary baby rice flour. It has been shown previously by Mathers *et al.* (1990) that a rice diet suppresses menaquinone-producing intestinal bacteria in the rat. The K₁-supplemented diet was prepared by thoroughly mixing a powdered K₁ formulation (Konaktion formulation (50 g K₁/kg); Hoffman La Roche, The Netherlands) with the diet. It was intended to prepare a diet containing 2 μ g K₁/g diet, i.e. about the level of normal lab chow. However, the analyses at the end of the experiment revealed a K₁ content of 4.2 μ g/g. The diet was presented *ad lib*. The rats exhibited a normal feeding pattern, with a weight gain of 35–45 g/week.

In a second experiment, which arose from the results of the first experiment, eight rats were fed on the vitamin K-deficient diet for 6 d. From day 7 on, three rats were given K₁

(Konaktion in mixed micelles) by continuous intravenous infusion ($2 \mu\text{g/h}$) via an implanted cannula in the *vena jugularis*. The vitamin was administered using a subcutaneously implanted osmotic minipump (Alza Corporation, Palo Alto, CA, USA). Three rats were given the diet supplemented with K_1 ($2 \mu\text{g/g}$). The remaining two rats served as controls. The experiment ended at day 11. The protocols were approved by the University Ethical Committee for experimental animals.

At the end of the experiments the rats were killed; under light diethyl ether anaesthesia blood was withdrawn from the abdominal aorta and the blood was mixed 9:1 (v/v) with 0.1 M-trisodium citrate. Immediately after death the rats were perfused with cold (4°) saline (9 g NaCl/l) via the heart and the following organs (tissues) were excised: pancreas, liver, kidney, heart, lung, submaxillary gland, brain, muscle (thigh), femur bone, and sternum. The organs (except bone) were immersed immediately in ice-cold saline. Tissue homogenates in phosphate-buffered saline (1:3 w/v; wet-weight basis) with (for pancreas and salivary gland) or without a cocktail of protease inhibitors (soya-bean trypsin (EC 3.4.21.4) inhibitor $50 \mu\text{g/ml}$, aprotonin $1 \mu\text{g/ml}$, benzamidine 0.1 mg/ml ; PMFS 0.1 mg/ml ; EDTA 1 mM), were prepared with a blender (Ultra Turrax; Janke & Kunkel, Staufen, Germany) followed by further treatment in a Potter homogenizes (three strokes at 1100 rpm ; Braun, Melsungen, Germany). The homogenates were stored at -70° until assayed. The femur bone and the sternum was scraped free of adhering tissue as much as possible. The femur was slivered carefully and the marrow was dispersed in 0.5 ml cold phosphate-buffered saline by vortex mixing on a whirl mixer and the bone slivers were separated.

Tissue mitochondrial, microsomal and cytosolic fractions (100000 g supernatant fraction) were prepared by standard procedures from pooled organs of two rats fed on standard lab chow. The mitochondria as well as the microsomes were repeatedly washed by resuspension and resedimentation respectively.

Prothrombin time was assayed using the thrombotest (R) reagent (Nycomed AS, Oslo, Norway) according to the manufacturer's instructions.

Vitamin K analyses

As vitamin K is light sensitive, all procedures were performed under orange-yellow light. Homogenates (0.5 ml and 0.25 ml for the vitamin K-free and -supplemented samples respectively) in a glass tube with a Teflon-lined screw cap were made up to 1 ml with water and mixed thoroughly with 2 ml ethanol containing 400 pg 2',3'-dihydrophyloquinone as internal standard for 30 min on a horizontal shaker. The mixture was extracted with $3\text{--}5 \text{ ml}$ hexane. After evaporation to dryness under N_2 at 30° , the residue was dissolved in 5 ml hexane and loaded onto a silica column (500 mg silica $60, 40\text{--}63 \mu\text{m}$, Merck, Darmstadt, Germany). The column was eluted with 5 ml hexane containing 5 ml ethyl acetate/ 1 (fraction discarded) whereafter the vitamin K-containing fraction was eluted with 5 ml ethyl acetate in hexane (20 ml/l). Loading, washing and elution were performed using a solid-phase extraction manifold (Supelco, Leusden, The Netherlands). The bone and sternum were extracted as follows: the chopped sternum and the bone slivers in a glass tube were placed under vacuum (solid-phase extraction manifold) whereafter 0.5 ml ethanol containing the internal standard was injected. Following an overnight period, 0.5 ml water was added and the samples were homogenized using the Ultra Turrax.

The vitamin K fraction was analysed by fluorescence detection following HPLC separation and post-column reduction (Thijssen & Drittij-Reijnders, 1993). A Jasco 821-FP fluorescence detector (excitation 244 , emission 430 nm) was used. Calibration curves for vitamin K measurements were prepared using solutions of K_1 and MK-4 in albumin in

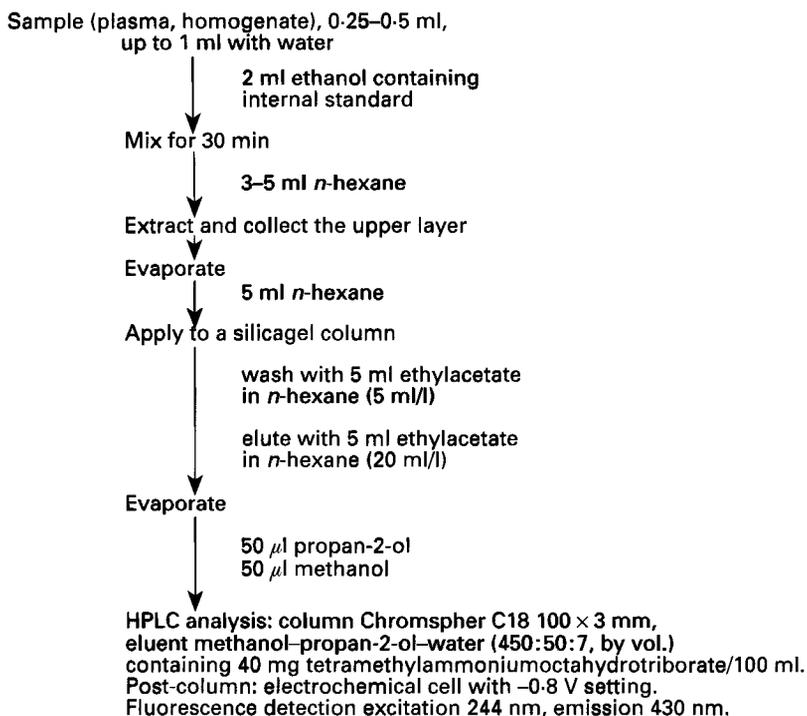


Fig. 2. Flow diagram of vitamin K analysis.

phosphate-buffered saline (40 g/l) and in plasma of K_1 -deficient rats. The calibration curves of K_1 and menaquinones-4–9 (MK-4–9) were linear up to at least 4 ng/ml. Fig. 2 shows a flow diagram of the vitamin K analysis.

K_1 and MK-4 were obtained from Sigma Chemicals (St Louis, MO, USA). MK-6–9 and 2',3'-dihydrophyloquinone were provided by Hoffman La Roche (Basle, Switzerland).

RESULTS

Rats fed on the K_1 -deficient diet showed a prolonged prothrombin time, although the response of the individual rats differed greatly (Table 1).

In addition to K_1 MK-4 was present in extracts from all tissues (Fig. 3). Traces of MK-6 were found also in some liver extracts, but higher menaquinones were not found (Fig. 3). As a control for the homogeneity of the vitamin K peaks, samples of all tissues were initially rerun without post-column reduction. This showed that liver extracts contained endogenous fluorescing compounds, one of which interfered with MK-6 (Fig. 3). The other tissue extracts appeared to be free of endogenous fluorescing peaks.

Fig. 4 shows tissue K_1 and MK-4 distribution in the rat. In K_1 -supplemented rats, all tissues examined showed levels in the nanogram range and, except for brain tissue, the tissue levels were higher than those for plasma. High K_1 levels were found in liver and heart (101 (SD 28) and 77 (SD 21) ng/g wet weight tissue respectively). Relatively high K_1 levels were also found in bone tissue, particularly in bone marrow (13 (SD 2) ng/g bone).

MK-4 distribution revealed remarkable results. Selective tissue distribution is apparent, with very high levels (> 100 ng/g) in pancreas and salivary gland, low levels in plasma and

Table 1. The effect of the phylloquinone (K_1)-free diet on prothrombin time
(Mean values and standard deviations for five rats)

Diet	Thrombotest (s)					
	Day 4			Day 9		
	Mean	SD	Range	Mean	SD	Range
K_1 supplement	28.7	2.5		27.6	3.5	
K_1 -free	39.8	9	30.2-53.6	74.3	39.7	38.7-117.5

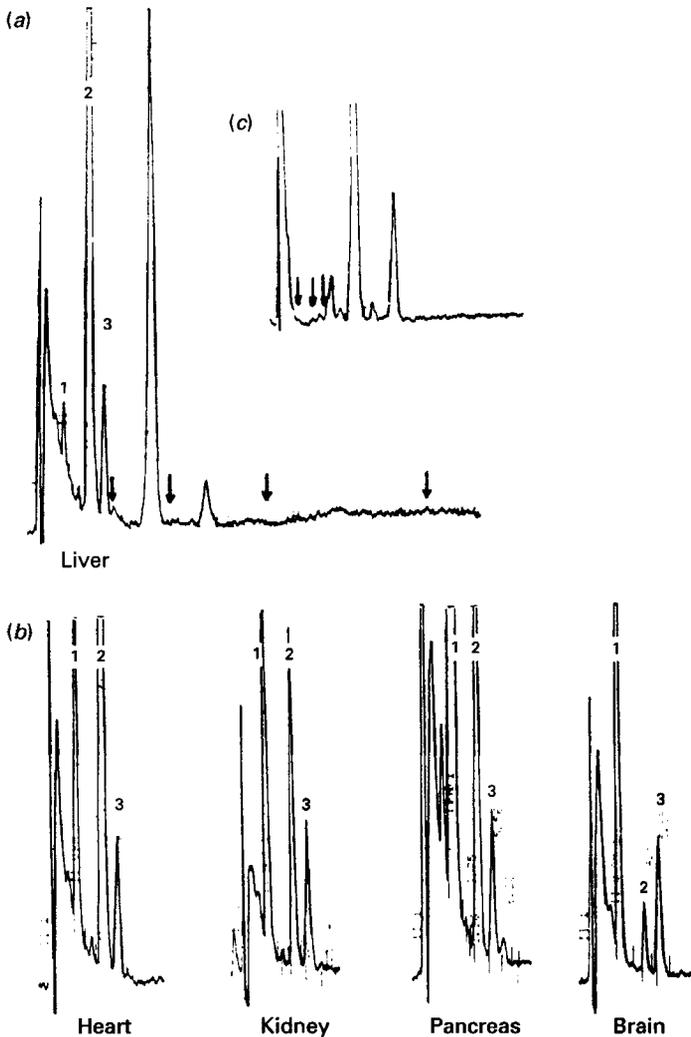


Fig. 3. (a, b), Chromatograms of extracts of tissues of rats fed on a phylloquinone (K_1)-supplemented diet. \downarrow , Menaquinone (MK)-6, MK-7, MK-8 and MK-9 respectively. Peaks 1, 2 and 3 represent MK-4, K_1 and internal standard respectively. (c), Chromatogram of a liver extract without post-column reduction. \downarrow , MK-4, K_1 and internal standard respectively. For details of diets and procedures, see pp. 416-418.

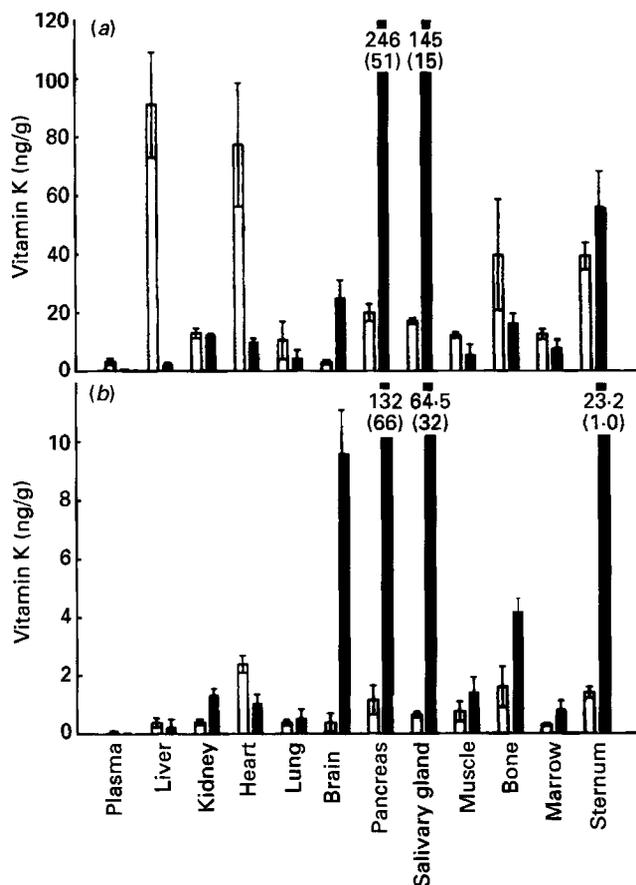


Fig. 4. The distribution of (□) phylloquinone (K₁) and (■), menaquinone-4 (MK-4) in the rat. (a), Rats fed on, a vitamin K-free diet supplemented with K₁ (4 μg/g); (b), Rats fed on a vitamin K-free diet. Values are the means and standard deviations represented by vertical bars for five rats. The femur bone was used to represent bone tissue. For details of diets and procedures, see pp. 416–418.

liver (0.4 and 2.3 ng/g), and intermediate levels in brain (25.5 (SD 6) ng/g) and sternum (57 (SD 12) ng/g).

The tissue K₁ levels in rats maintained on the K₁-deficient diet for 9 d were low (< 1 ng/g), except for heart and bone which contained about 2 ng/g. The K₁-deficient rats had lower MK-4 levels compared with the K₁-supplemented rats. In most tissues the MK-4 levels were less than 10% of those found in the K₁-supplemented rats except for the pancreas, salivary gland, brain and sternum where the MK-4 levels were about half those of the K₁-supplemented rats (Fig. 4).

The comparatively high MK-4 levels found in the K₁-supplemented rats raised the question of whether MK-4 originates from the K₁ supplement or whether it reflects the conservation of MK-4 stores present at the start of the feeding. A second experiment was designed to study the effect of K₁ supplementation of vitamin K-deficient rats. The results are shown in Fig. 5; distribution patterns were similar to those found in the first experiment (Fig. 4). Tissue MK-4 levels increased in response to K₁ administration either orally or by intravenous infusion. The relative rise in MK-4 differed between tissues; liver levels increased 10–30-fold, although the absolute contents remained low (< 10 ng/g). Pancreas

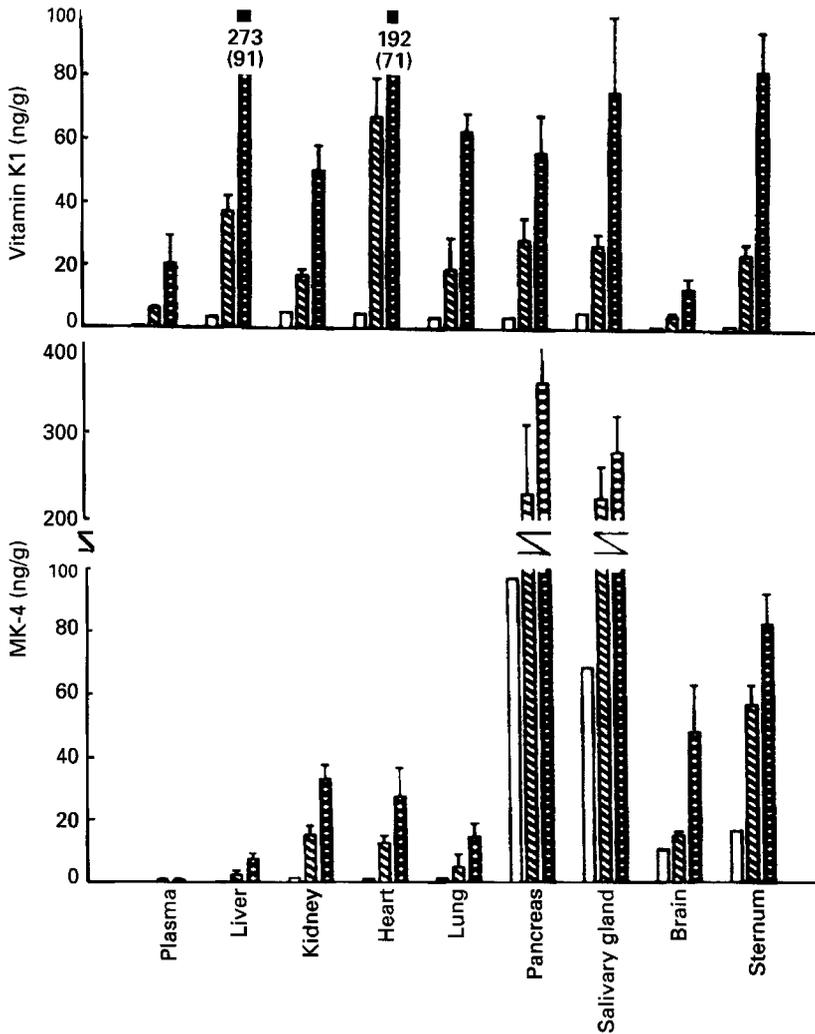


Fig. 5. Tissue distribution of phylloquinone (K_1) and menaquinone-4 (MK-4) following K_1 supplementation (4 d) to vitamin K-deficient rats. (□), Vitamin K-deficient rats (n 2); (▨), K_1 -supplemented via the diet ($2 \mu\text{g/g}$; n 3); (▤), K_1 -supplemented via intravenous infusion ($2 \mu\text{g/h}$; n 3). Values are means and standard deviations represented by vertical bars. For details of diets and procedures, see pp. 416–418.

and salivary gland showed a 2–4-fold increase; their levels were high: (97, 229 and 360 ng/g for the pancreas and 68, 225 and 280 ng/g for salivary gland in the K_1 -deficient and orally and intravenously K_1 -supplemented rats respectively). High K_1 levels were found in liver and heart of the K_1 -supplemented rats.

The tissue distribution as well as the cellular distribution of K_1 and MK-4 was estimated in rats (n 2) on normal lab chow (K_1 content about $2 \mu\text{g/g}$). The data are presented in Table 2. As for rats on the K_1 -supplemented diets (Figs. 4 and 5), there was selective tissue distribution of K_1 and MK-4; elevated K_1 levels occurred in liver, heart, and pancreas, and low levels ($< 1 \text{ ng/g}$) in brain tissue. Very high levels ($> 100 \text{ ng/g}$) of MK-4 were found in pancreas and salivary gland and high levels ($> 10 \text{ ng/g}$) in kidney, brain, and heart. In liver also MK-6–9 were recovered (menaquinones higher than MK-9 were not investigated).

Table 2. *The intracellular distribution of vitamin K in rat tissue**

Tissue	Vitamin K-active compound	ng/mg protein			Total	
		Mitochondria	Cytosol	Microsome	ng/mg protein in homogenate	ng/g tissue wet wt
Heart	K ₁	0.08	0.04	0.12	0.27	11.2
	MK-4	0.06	0.00	0.09	0.27	11.4
Kidney	K ₁	0.10	0.01	0.04	0.07	2.90
	MK-4	0.63	0.01	0.28	0.99	38.6
Lung	K ₁	0.15	0.02	0.09	0.07	1.44
	MK-4	0.25	0.03	0.21	0.18	3.64
Pancreas	K ₁	0.13	0.01	0.06	0.21	8.07
	MK-4	4.54	0.05	2.55	7.49	293.0
Salivary gland	K ₁	0.28	0.03	0.09	0.12	3.30
	MK-4	1.12	0.04	0.51	5.03	141.5
Brain	K ₁	0.01	0.01	0.15	0.02	0.64
	MK-4	0.49	0.00	0.56	1.08	30.4
Liver	K ₁	0.50	0.03	0.40	0.44	23.7
	KO	0.20	0.03	0.24	0.12	6.78
	MK-4	0.04	0.00	0.05	0.04	2.33
	MK-6	0.10	0.00	0.01	0.05	2.57
	MK-7	0.16	0.00	0.03	0.05	2.81
	MK-8	0.22	0.00	0.02	0.06	3.00
	MK-9	0.12	0.00	0.02	0.01	0.81

K₁, phyloquinone; MK-4-9, menaquinones-4-9; KO, 2,3-epoxide of phyloquinone.

* For details of procedures, see pp. 416-418.

There appeared to be some mitochondrial accumulation of K₁ and MK-4 in kidney, pancreas, and salivary gland. In the liver there was selective distribution in the mitochondria for the higher menaquinones (MK-6-9).

DISCUSSION

The present study was undertaken to determine whether tissue K₁ distribution is related to tissue vitamin K metabolism, i.e. do tissues known to be rich in, for example, carboxylase activity also accumulate K₁? The results did not follow the pattern expected: K₁ was recovered not only in carboxylase-rich tissues (e.g. liver, kidney, lung, bone etc.) but also in tissues low in carboxylase (heart, brain and muscle). In particular the large accumulation of K₁ in heart tissue from K₁-supplemented rats was interesting. Hirauchi *et al.* (1989) reported on the distribution of vitamin K-active compounds in a limited number of tissues of the rat; they found that heart tissue contained K₁ in amounts comparable with those of liver.

Another unexpected finding was the recovery of MK-4 in all tissues examined. However, the identification of MK-4 and other vitamin K-active compounds in the tissue extracts was based on chromatographic data and on the reduction-dependent fluorimetric response (Fig. 3). The distribution pattern of MK-4 appears to be tissue-selective, showing that for most tissues the levels of MK-4 were higher than those of K₁, with very high levels in the pancreas and salivary gland; cartilaginous tissue (sternum) also appeared to be rich in MK-4. In fact, only liver and plasma appeared to contain low levels of MK-4. The findings also showed, contrary to expectation, that in the rat there was a relatively high body store of the vitamin (as MK-4), the majority being extrahepatic. Furthermore, the results suggested

a direct relationship between tissue K_1 and MK-4; reduced MK-4 stores are replenished by feeding K_1 .

While the present study was in progress, two independent groups reported high MK-4 levels in chick livers and found a relationship between MK-4 levels and dietary K_1 supply (Guillaumont *et al.* 1992; Will *et al.* 1992).

The intriguing question is: what is the source of MK-4? In the early days of vitamin K research, *in vivo* conversion of K_1 to MK-4 was reported by Billeter & Martius (1960). The conversion of K_1 to MK-4 is believed to proceed via menadione as an intermediate followed by the introduction of the geranylgeranyl side chain. The first step, the dealkylation of K_1 , has been suggested to be mediated by bacterial (intestinal flora) metabolism (Billeter *et al.* 1964). The hypothesis that MK-4 is the true physiologically active form and that it is derived from dietary K_1 through bacterial metabolism can still be found in text books (Mutschler, 1991).

The formation of MK-4 from menadione is well established (Taggart & Matschiner, 1969), in particular chick liver shows high (*in vitro*) activities in this respect (Dialameh *et al.* 1971). The standard commercial rat food used in our laboratory contained menadione (6 $\mu\text{g/g}$, value given by the manufacturer: Hope Farms, The Netherlands) as the added vitamin K-active compound. The K_1 content from natural sources, which varied from batch to batch, was found to be 1–2 $\mu\text{g/g}$ (our own analyses). Thus, the tissue MK-4 in rats fed on standard commercial food (Table 2) may be derived from dietary menadione, as may be MK-4 in the K_1 -supplemented rats described in Fig. 4. Results shown in Fig. 5, however, rule out menadione; neither the vitamin K-deficient diet nor the K_1 formulations (Konaktion dry formulation (50 g/kg) and Konaktion mixed micelles formulation) that were used for supplementation of the diet and for intravenous infusion contained menadione. Two possible sources of MK-4 under the experimental conditions used could be: (1) MK-4 derived from K_1 , (2) MK-4 of bacterial (intestinal flora) origin. The latter option assumes high bacterial production of MK-4 considering its poor colonic absorption (Ichihashi *et al.* 1992). However, MK-4 has been reported to be a minor bacterial product (Ramotar *et al.* 1984; Kindberg *et al.* 1987). Moreover, as was shown by Mathers *et al.* (1990), the addition of rice to the diet successfully suppressed bacterial menaquinone synthesis since there was an absence of higher menaquinones in liver (Fig. 3 cf. Table 2). Consequently, the most likely explanation is that MK-4 was derived from dietary K_1 . The present findings do not distinguish between a possible bacterial pathway (Billeter *et al.* 1964) and tissue metabolism of K_1 to MK-4. The fact that the intravenous K_1 administration also was associated with raised tissue MK-4 levels (Fig. 5), as has been found to occur in chicken liver (Will *et al.* 1992), may point to tissue metabolism. However, entero-hepatic circulation of K_1 can not be ruled out. Assuming that MK-4 is derived from K_1 , either by total K_1 conversion or by alkylation of bacterially-derived menadione, the question arises as to whether the tissue MK-4 is formed locally or whether it is delivered from a metabolic source, e.g. the liver. The low circulating MK-4 levels, even with intravenous administration of K_1 , where high tissue MK-4 levels are encountered (Fig. 5), suggest specific tissue formation of MK-4.

Clearly, rather than bridging gaps in our understanding of potential extrahepatic functions of vitamin K, this study has generated new gaps in our knowledge. It is hard to believe that the high levels of the vitamin in tissues such as heart, pancreas, salivary gland etc. are related to a possible vitamin K-dependent post-translational carboxylase reaction. Its presence in the brain may be related to the reported vitamin K-dependent brain sulphotransferase activity (Sundaram & Lev, 1990). Consequently, additional physiological (biochemical) functions have to be attributed to the vitamin, in particular to MK-4. Based on the chemical similarity with ubiquinone, a function in the electron transport of the

mitochondrial respiration chain could be considered. Mitochondrial accumulation of vitamin K-active compounds was apparent in some tissues; however no mitochondrial accumulation was found in the heart (Table 2). Studies of K_1 biochemical vitamin K metabolism, i.e. vitamin K-epoxidation, in the extrahepatic tissues are in progress.

Considering the high density of the warfarin receptor (Thijssen & Baars, 1991) it was anticipated that K_1 would be present in pancreas and salivary gland tissue. Experiments have been planned to determine whether any relationship exists between the warfarin receptor and the high accumulation of MK-4. In this respect it is of interest to note that Taggart & Matschiner (1969) found that dicoumarol inhibits the *in vitro* alkylation of menadione to form MK-4.

The role of the bacterially-derived higher menaquinones in liver vitamin K metabolism is as yet unanswered. The livers of rats on the K_1 -free diet did not contain detectable amounts of these vitamin K-active compounds demonstrating the effectiveness of rice supplementation (see p. 416). However, there was no clear-cut relationship between the prolongation of the prothrombin time and the individual liver K_1 levels (data not shown).

In summary, the present study shows a selective distribution of MK-4 and K_1 in the rat. The body store of MK-4 can be derived from the dietary K_1 supply. The findings suggest there is an as yet unrecognized physiological function of vitamin K. Information on tissues of other species have to be gathered to determine how widespread the observations are.

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