# Intestinal, hepatic, and circulating vitamin K levels at low and high intakes of vitamin K in rats

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The aim of the present study was to assess how high doses of dietary vitamin K influence the intestinal profile of K-vitamins in vitamin K-deficient rats, and whether the induced changes are reflected in the hepatic vitamin K store. Vitamin K-deficient rats were fed for 10 d on diets containing different forms of vitamin K, and it was determined how these diets affected the vitamin K concentration at various sites of the instestine, serum, and the liver. It was found that the absorption of phylloquinone from standard food is not more than 10 %, while the absorption of pharmacological doses of oil-solubilized phylloquinone and menaquinone-4 was also far from complete (18 and 55 % respectively). High intakes of phylloquinone suppress the colonic production of all higher menaquinones. High menaquinone-4 intake induces very high menaquinone-8 concentrations, both in the colonic contents as well as in the liver. These data suggest that menaquinone-4 may be converted into menaquinone-8 (but not into other menaquinones) via a metabolic pathway which has not been reported previously.

**Phylloquinone: Menaquinone: Vitamin K**

Vitamin K serves as a cofactor during the post-translational synthesis of γ-carboxyglutamic acid (Gla) in several proteins. Since its discovery in the early 1930s, vitamin K was believed to play an exclusive role in the process of blood coagulation; only recently has it become clear that Gla-containing proteins are synthesized in most mammalian tissues, and that the functions of these proteins include regulation of bone growth (osteocalcin; Ducy *et al*. 1996), prevention of vascular mineralization (matrix Gla-protein; Luo *et al*. 1997), and stimulation of cell growth (growth-arrest-specific gene protein 6, Nakano *et al*. 1997). Natural forms of vitamin K are phylloquinone (also known as vitamin $K_1$) and the group of the menaquinones ($K_2$ vitamins; Shearer, 1995). Phylloquinone is synthesized by plants and the highest levels are found in plant oils and green vegetables (Booth *et al*. 1995; Koivu *et al*. 1997; Pironen *et al*. 1997). Menaquinones are of microbial origin: they are found mainly in cheese and fermented foods (Hirauchi *et al*. 1989; Schurgers *et al*. 1999). In addition, the intestinal microflora produces substantial amounts of menaquinones, but the extent to which these products are absorbed has remained a matter of debate (Suttie, 1995). In the present paper the various menaquinones will be designated as MK-*n*, where *n* stands for the number of isoprenoid residues in the aliphatic side-chain. Menadione (vitamin $K_3$) is a synthetic form of vitamin K which is generally used in commercial animal feed (for rodents, poultry, cows). Its vitamin K activity results from its *in vivo* transformation into MK-4 (Dialameh *et al*. 1971).

The vitamin K status of an organism depends on the phylloquinone and menaquinone contents of its diet, on the extent to which these vitamins are absorbed in the intestines, as well as on the colonic absorption of menaquinones produced by intestinal microflora. It is generally assumed that absorption of vitamin K mainly takes place in the jejunum and ileum in the form of mixed micelle complexes with bile salts (Hollander, 1981). Since the absorption of bile salts is virtually completed in the distal ileum, uptake of K-vitamins from the colon is less likely (Uchida *et al*. 1978; Ichihashi *et al*. 1992; Suttie, 1995).

In the present study we fed vitamin K-deficient rats for 10 d on either standard diets or diets containing pharmacological doses of oil-solubilized, purified vitamin K, and we investigated how the vitamin K content of the diet affects the vitamin K concentration at various sites in the intestine and in the faeces. Moreover, we investigated the uptake and metabolism of K-vitamers by recording their respective concentrations in plasma and in the liver.

**Abbreviations:** Gla, γ-carboxyglutamic acid residue; MK, menaquinone.

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## Materials and methods

### *Chemicals and animal food*

MK-4 and phylloquinone were purchased from Sigma (St Louis, MO, USA); a set of higher menaquinones (MK-5 to MK-10) as well as 2′,3′-dihydrophylloquinone were kind gifts from Hoffmann-La Roche (Basel, Switzerland). A powdered vitamin K-deficient, γ-irradiated (0·9 Mrad) diet and a standard animal diet were purchased from Hope Farms (Woerden, The Netherlands); the detailed food contents have been described previously (Groenen-van Dooren *et al*. 1993). On analysis, the vitamin K-deficient diet did not contain detectable amounts of either phylloquinone or menaquinone (<20 pg/g diet), the standard diet contained phylloquinone (1·95 μg/g diet) and menadione (6·23 μg/g diet). All solvents and chemicals were of analytical quality or HPLC-grade purity.

### *Animals and preparation of diets*

This study was performed in fifteen male rats of Lewis strain, which were 12 weeks old and had a body weight of 303 (SD 49·7) g at the start of the experiment. The animals were housed individually in flat bottomed cages under conditions of a 12 h light–dark cycle, controlled temperature (20 ± 2 °) and humidity (50 ± 10 %). One group of three animals was kept on the standard diet throughout the experiment and served as the normal reference group. The remaining twelve animals were divided into four groups (three rats/group), all of which received the vitamin K-deficient diet for 7 d before the start of the experiment. After that period three groups of three rats received vitamin K-deficient diet supplemented with 20 μg/g of either phylloquinone, MK-4, or menadione. The last group served as the zero-intake reference and received vitamin K-deficient diet throughout the experiment. The various diets were prepared by dissolving the respective vitamins in 10 ml maize oil, which was added to 750 g vitamin K-deficient diet; the same amount of maize oil (without vitamin K added) was added to the diet of animals receiving either the standard diet or the vitamin K-deficient diet. Faeces of the rats were collected on each day of the experiment and lyophilized before analysis. After the animals had received these diets for 10 d, they were killed by aortic exsanguination under light diethyl ether anaesthesia. Blood was collected, diluted 9 : 1 (v/v) with 0·1 M-trisodium citrate and centrifuged for 15 min (2000 ***g***) for the preparation of plasma. The body was perfused with saline via the abdominal aorta, whereafter the liver and intestine were removed for further analysis.

### *Vitamin K analysis*

The spectra of K-vitamers (phylloquinone and MK-4–10) were analysed in nine parts of the intestinal tract, faeces, livers and plasma. In all cases the intestines were similarly divided as follows: duodenum in total (segment 1), first 100 mm of jejunum (segment 2), last 100 mm of ileum (segment 6), middle part of jejunum and ileum in three equal parts (segments 3, 4 and 5), caecum in total (segment 7), and colon in two equal parts (segments 8 and 9). The content of each part was collected with careful rinsing of the lumen and freeze-dried; dry intestinal contents were weighed and 0·1 g was used for analysis. Faecal vitamin K contents were analysed in 0·2 g (dry weight) of samples collected at days 5, 7 and 9 of the experiment. In plasma, only the data for phylloquinone and MK-4 are provided. Hepatic and circulating vitamin K concentrations were measured in 1 g liver (wet weight) and in 0·5 ml plasma. Extraction and detection of vitamin K were performed as described earlier (Thijssen & Drittij-Reijnders, 1993; Gijsbers *et al*. 1996), 2′,3′-dihydrophylloquinone served as an internal standard. In short, samples were mixed with 2·0 ml distilled water (for plasma 1·5 ml), 4 ml ethanol and internal standard (1–40 ng, as required) and extracted with 8 ml hexane. Liver and faeces were homogenized using an Ultra Turrax homogenizer (Janke and Kunkel, Staufen, Germany) before extraction. Extracts were centrifuged (2000 ***g*** for 5 min), evaporated under a stream of $N_2$ gas, and redissolved in 2 ml hexane. Pre-purification of the samples was performed with silica Sep-pack cartridges (Millipore, Milford, MA, USA) as described previously (Gijsbers *et al*. 1996). Quantitative analysis of the samples was performed by reversed-phase HPLC using a C-18 reversed phase column and fluorescence detection (Thijssen & Drittij-Reijnders, 1993). Because of the long retention times for the long-chain menaquinones (MK-7 to MK-10) the flow was increased from 0·5 to 1·0 ml/min at 11 min after injection. K-vitamers in the eluate were reduced electrochemically using a Coulochem 5010 analytical cell (ESA, Bedford, MA, USA) maintained at potential of −1·5 V. Phylloquinone and menaquinones were recorded in the same run. Reference curves were prepared from purified phylloquinone and each of the menaquinones, and a linear dose-response was obtained for the entire range in which the sample concentrations were measured. The vitamin K contents of the samples were quantified with the internal standard method based on the ratio of peak heights. Detection limits were 0·015 ng/ml for phylloquinone, MK-4 and MK-5, 0·04 ng/ml for MK-6 and MK-7, 0·10 ng/ml for MK-8, and 0·12 ng/ml for MK-9 and MK-10. Intestinal absorption was calculated by expressing the difference between dietary intake and faecal excretion as a percentage of the intake, for each animal separately.

### *Data analysis*

All groups consisted of three animals, and mean values for each group are given throughout this paper. Differences between groups were compared using the Mann–Whitney U-test, and were considered to be significant at $P = 0{\cdot}05$.

## Results

### *Intestinal absorption of dietary vitamin K*

In the experiments described in the present paper the experimental animals had been on a constant diet for 10 d before their intestinal contents were analysed. During the entire experiment the individual daily food intake was recorded, and was found to be 19·0 (SD 1·9) g (average for all groups). The average faeces production was 3·2 (SD 1·3) g (wet weight), and the weight gain was comparable in all

**Table 1.** Dietary intake and faecal excretion of phylloquinone and menaquinone-4 (MK-4) by rats fed on different diets, on days 5, 7 and 9 of a 10 d feeding period‡

(Mean values for three animals§)

| Type of diet . . . | Vitamin K-deficient | | | Standard diet | | | High phylloquinone | | | High MK-4 | | | Menadione | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Day no. . . . | 5 | 7 | 9 | 5 | 7 | 9 | 5 | 7 | 9 | 5 | 7 | 9 | 5 | 7 | 9 |
| Dietary intake (μg/d) | | | | | | | | | | | | | | | |
| phylloquinone | 0 | 0 | 0 | 38 | 42 | 40 | 392 | 401 | 397 | 0 | 0 | 0 | 0 | 0 | 0 |
| MK-4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 405 | 389 | 398 | 0 | 0 | 0 |
| menadione | 0 | 0 | 0 | 109 | 120 | 114 | 0 | 0 | 0 | 0 | 0 | 0 | 377 | 393 | 412 |
| Faecal excretion (μg/d) | | | | | | | | | | | | | | | |
| phylloquinone | 0·5 | 0·7 | 0·5 | 35* | 37* | 36* | 316† | 332† | 317† | 0·2 | 0·7 | 0·8 | 0·4 | 0·6 | 0·5 |
| MK-4 | 0·5 | 0·1 | 0·2 | 2·1* | 2·6* | 1·9* | 6·1† | 5·0† | 3·2 | 178 | 187 | 180 | 4·9† | 7·2† | 5·1† |
| Absorption (%) | | | | | | | | | | | | | | | |
| phylloquinone | – | – | – | 8 | 12 | 10 | 19† | 17† | 20† | – | – | – | – | – | – |
| MK-4 | – | – | – | – | – | – | – | – | – | 57 | 54 | 55 | – | – | – |

Mean values were significantly different from those for the vitamin K-deficient group: *$P < 0.05$.
Mean values were significantly different from those for the standard diet group: †$P < 0.05$.
‡ For details of diets and procedures, see p. 186.
§ Standard deviations were all <15 %.

groups. To demonstrate that the animals were in a steady-state condition at the time of death, we assessed the amounts of K-vitamins in the faeces at days 5, 7 and 9 (see Table 1). No significant differences between these three days were found. From Table 1 it can also be seen that the mean absorption (as calculated from the difference between dietary intake and faecal excretion) of phylloquinone from the standard diet was 4 μg/d (about 10 % of the food content), whereas from the enriched diets the absorption of phylloquinone and menaquinone was substantially higher, both in absolute amounts (75 μg/d and 216 μg/d) and as a percentage of the daily intake (18 % and 55 % respectively).

### *Effect of diet on intestinal vitamin K profile*

The intestinal vitamin K profile in rats receiving the standard diet is shown in Fig. 1. K-vitamers recovered were mainly the higher menaquinones (MK-8, MK-9, and MK-10), which were almost exclusively present in the caecum and colon. Other menaquinones were barely detectable or absent in this group, as well as in the other groups described later. In animals receiving the vitamin K-deficient diet, phylloquinone and MK-4 were low (<0·05 μg/g) in all segments of the intestinal tract (Fig. 2(a)). Long-chain menaquinones were not found in the duodenum, jejunum or ileum, whereas segments 7–9 (caecum and colon) contained considerable amounts of MK-8, MK-9 and MK-10, the concentrations were comparable to or even slightly

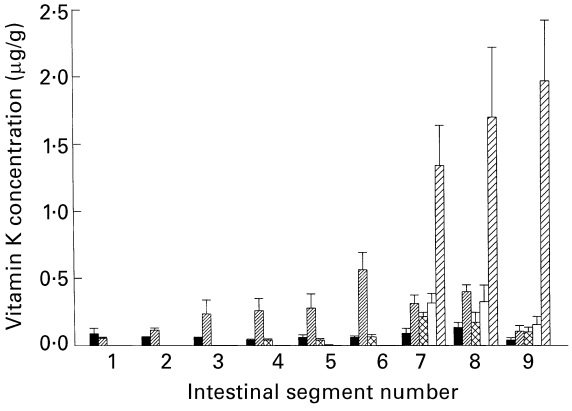


**Fig. 1.** Concentrations of vitamin K in intestinal segments of rats fed on a standard diet. The intestinal segments analysed were: 1, duodenum; 2, proximal jejunum; 3, distal jejunum; 4, proximal ileum; 5, middle ileum; 6, distal ileum; 7, caecum; 8, proximal colon; 9, distal colon. (■), Menaquinone (MK)-4; (▨), phylloquinone; (▩), MK-8; (□), MK-9; (▨), MK-10. Values are means for three rats with standard deviations represented by vertical bars.

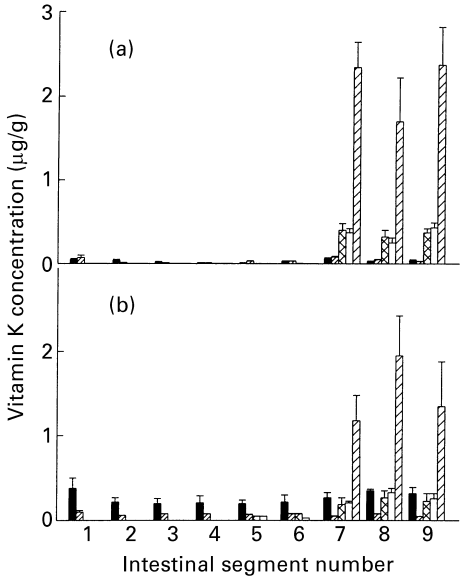


**Fig. 2.** Concentrations of vitamin K in intestinal segments of rats fed on (a) a vitamin K-deficient diet and (b) a high-menadione diet. For details of diets, see p. 186. The intestinal segments analysed were: 1, duodenum; 2, proximal jejunum; 3, distal jejunum; 4, proximal ileum; 5, middle ileum; 6, distal ileum; 7, caecum; 8, proximal colon; 9, distal colon. (■), Menaquinone (MK)-4; (▨), phylloquinone; (▩), MK-8; (□), MK-9; (▨), MK-10. Values are means for three rats with standard deviations represented by vertical bars.

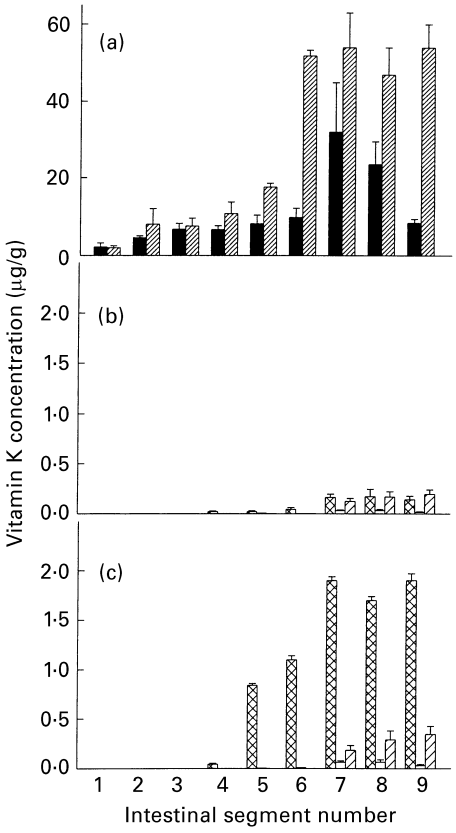


**Fig. 3.** Concentrations of vitamin K in intestinal segments of rats fed on diets rich in phylloquinone or menaquinone (MK)-4. For details of diets, see p. 186. The intestinal segments analysed were: 1, duodenum; 2, proximal jejunum; 3, distal jejunum; 4, proximal ileum; 5, middle ileum; 6, distal ileum; 7, caecum; 8, proximal colon; 9, distal colon. (■), MK-4; (▨), phylloquinone; (▩), MK-8; (□), MK-9; (▨), MK-10. Values are means for three animals with standard deviations represented by vertical bars. (a) Phylloquinone after the phylloquinone-rich diet and MK-4 after the MK-4-rich diet; (b) higher menaquinones after the phylloquinone-rich diet; (c), higher menaquinones after the MK-4-rich diet.

higher than those in rats on standard diet (see Figs. 1 and 2(a)). Because the rats in the vitamin K-deficient group had not received any vitamin K for 17 d the menaquinone profile shown in Fig. 2(a) must be the result of menaquinone synthesis by the intestinal flora. Supplementation with menadione did not increase the bacterial menaquinone production (Fig. 2(b)). As compared with the vitamin K-deficient animals, the mean values of MK-4 were about 10-fold elevated in the upper part of the intestinal tract.

In the group receiving a diet rich in phylloquinone (Fig. 3(a)), substantial amounts of this vitamin were found in all parts of the intestinal tract, but the concentration increased from segment 1 to segments 6–9. So it seems that the absorption of the dietary phylloquinone was far from complete, and that its concentration increased during the passage through the upper part of the intestinal tract because other components of the diet were absorbed more readily. Remarkably, levels of the long-chain menaquinones were extremely low in all parts of the intestine (Fig. 3(b)). Also, in the group receiving MK-4 the dietary vitamin was recovered from the intestinal contents, but in general the concentrations were lower than in the phylloquinone group (Fig. 3(a)). So it seems that MK-4 is absorbed somewhat better than phylloquinone. As in the phylloquinone group, the intestinal production of MK-9 and MK-10 was decreased in animals receiving the menaquinone-rich diet. Surprisingly, however, relatively high amounts of MK-8 were found in animals on the MK-4 diet (Fig. 3(c)). The faecal MK-8 levels in these animals were substantially higher even than those in vitamin K-deficient rats.

### *Effect of dietary vitamin K on circulating and hepatic vitamin K concentrations*

In the various plasma samples only phylloquinone and MK-4 were detectable (Table 2). Levels of both vitamins were extremely low in the vitamin K-deficient group, and in the menadione group they were found to be only slightly higher. In the groups of controlled phylloquinone and

**Table 2.** Concentrations of K-vitamers in the plasma and liver of rats fed on diets containing different amounts and forms of vitamin K for 10 d
(Mean values for duplicate measurements from three rats, with standard deviations)

| | Type of diet | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | K-deficient | | Menadione | | Phylloquinone | | MK-4 | |
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| K-vitamin in plasma (ng/ml) | | | | | | | | |
| phylloquinone | 0·4 | 0·1 | 1·2* | 0·2 | 51* | 4·5 | 0·2 | 0·1 |
| MK-4 | 0·1 | 0·05 | 0·6* | 0·2 | 0·5* | 0·2 | 87* | 5·0 |
| K-vitamin in liver (ng/g) | | | | | | | | |
| phylloquinone | 2·7 | 1·4 | 6·9 | 5·1 | 200* | 43 | 17* | 2·0 |
| MK-4 | 1·1 | 0·8 | 28* | 10·5 | 14* | 1·6 | 410* | 96 |
| MK-6 | 9·1 | 2·5 | 9·8 | 5·6 | 9 | 3·4 | 8·7 | 0·9 |
| MK-7 | 9·7 | 1·8 | 10 | 7·5 | 14 | 3·3 | 21 | 13 |
| MK-8 | ND | | ND | | ND | | 71* | 17 |
| MK-9 | ND | | ND | | ND | | ND | |
| MK-10 | ND | | ND | | ND | | ND | |

MK, menaquinone; ND, not detectable (<0·05 ng/g tissue).
Mean values were significantly different from those for the vitamin K-deficient group: *$P < 0·05$.

MK-4 diets high concentrations of the respective vitamins were found in plasma. In the livers of both the menadione-supplemented and the phylloquinone-supplemented groups MK-4 was slightly increased, levels of other K-vitamins were comparable to those in the K-deficient group. In the MK-4 supplemented group the most remarkable observation was the prominent increase of MK-8 from less than 0·05 ng/g to 71 ng/g tissue, which is consistent with the high colonic contents of MK-8.

## Discussion

According to the protocol the animals in our study were not treated with a single dose of vitamin K, but they were fed with diets containing well-defined concentrations of either menadione, phylloquinone, or MK-4 until they were in a steady-state condition. From Table 1 it can be seen that the dietary uptake and faecal excretion of phylloquinone and menaquinone were constant on days 5, 7 and 9 of the experiment. From these data it may also be calculated that the total absorption of phylloquinone from the standard diet was not more than 10 %. An explanation for this poor uptake may come from the fact that the phylloquinone in this diet originates from grass, which forms the fibre component. In human subjects the uptake of vitamin K from green vegetables was 5–15 %, depending on the fat content of the meal (Gijsbers *et al*. 1996). In the supplemented diets, the added K-vitamins had been dissolved in maize oil, from which they are more readily extracted by the action of bile salts. Here a marked difference was observed between phylloquinone and the slightly more hydrophylic MK-4, the latter being absorbed substantially better than phylloquinone. In this respect it is noteworthy that Ichihashi *et al*. (1992) reported some colonic MK-4 absorption even in the absence of bile salts.

After 17 d of vitamin K-deficiency, long-chain menaquinones (notably MK-10) were abundantly present in the caecum and the colon, and certainly not at lower levels than in animals fed on the standard diet (Figs. 1 and 2). Apparently, the vitamers are not absorbed from this part of the tract, because two animals in the vitamin K-deficient group were bleeding at the end of the experiment. Also, the higher menaquinones were undetectable in the liver (see Table 2). A menadione-rich diet induced an increase of MK-4 in the liver, but not in the colon. Probably because of the enterohepatic cycle, MK-4 was slightly increased in the upper part of the digestive tract in this group. These data are consistent with the view that menadione may be converted into MK-4 in the liver (Dialameh *et al*. 1971), but they also demonstrate clearly that this conversion is absent in the colonic bacteria. Whether menadione may be used as a source for the intestinal production of higher menaquinones remains to be seen, although it is clear that menadione does not increase the menaquinone concentrations in the gut.

We observed that high phylloquinone intake suppressed the intestinal menaquinone production. Apparently the flora is capable of using this vitamin, thereby shutting down its own menaquinone production. It is also clear that, despite the high phylloquinone concentration in the diet, the phylloquinone concentration was low in the upper part of the digestive tract. A possible explanation may be that in all animals (also in the other groups) the ileum was virtually empty, so that mucosal material and not digested food were the main components of what was assembled from its contents. This effect would have been negligible in the caecum and colon, where a high degree of intestinal filling was observed. The gradual increase of phylloquinone downstream in the digestive tract as well as its relatively high concentration in colon and faeces suggests that its absorption is far from complete. In the group receiving a diet rich in MK-4 the recovery of the corresponding vitamer from the intestinal contents was substantially lower than in the phylloquinone group, which is consistent with a better absorption of MK-4 than of phylloquinone (Table 1). This seems to be in contrast to previous data from Groenen-van Dooren *et al*. (1993, 1995), who showed that the utilization of nutritional phylloquinone for prothrombin synthesis is better than that of MK-4. Differences between the previous protocols and the present experiment are that Groenen-van Dooren *et al*. (1993, 1995) used a single dose of vitamin K, and that the vitamers were dissolved in detergent (HCO-60). When used in a steady-state situation and dissolved in a natural compound (maize oil) it seems that MK-4 and phylloquinone accumulate in the liver to comparable levels.

As in the group receiving the phylloquinone diet, in animals receiving MK-4 the intestinal concentrations of MK-9 and MK-10 were also low. Surprisingly, however, the diet containing MK-4 induced a remarkable increase of colonic MK-8. These data suggest that either the intestinal flora or the liver is capable of using MK-4 for the exclusive synthesis of MK-8, which is a hitherto unknown metabolic pathway. A second striking observation was that in this group the high MK-8 levels were reflected in the liver vitamin K concentrations (Table 2). Since this seems to be in contrast to the poor absorption of higher menaquinones in the vitamin K-deficient animals (see earlier), we believe that the conversion of MK-4 into MK-8 may take place in the liver, and reach the digestive tract via the enterohepatic pathway. This hypothesis implies that re-absorption of MK-8 from the intestines is poor, so that it accumulates to the high concentrations in the colon as observed in the present study. Investigations in germ-free animals (Ronden *et al*. 1998) may help to verify this hypothesis. The data presented here confirm that the absorption of phylloquinone from its natural food matrix is extremely poor. Previously this has also been reported in a limited number of human volunteers (Gijsbers *et al*. 1996). Absorption from oil-solubilized preparations is substantially better but still far from complete. This supports the very high efficacy of hepatic vitamin K recycling during the synthesis of blood clotting factors, and raises the question of whether a similar efficacy is obtained in other tissues, for instance in bone and vessel wall during the production of osteocalcin and matrix Gla-protein.

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