Biochemical Pharmacology, Vol. 46, No. 3, pp. 433–437, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

THE RELATIVE EFFECTS OF PHYLLOQUINONE AND MENAQUINONE-4 ON THE BLOOD COAGULATION FACTOR SYNTHESIS IN VITAMIN K-DEFICIENT RATS

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(*Received* 9 *December* 1992; *accepted* 19 *April* 1993)

Abstract—Rats were made vitamin K-deficient by feeding them a 1:1 (w/w) mixture of a commercial vitamin K-depleted diet and boiled white rice. After one week of treatment the rats had developed severe vitamin K deficiency, resulting in Thrombotest® values of 5–10% of the initial values. In this experimental system the efficacy of phylloquinone (K_1) was compared with that of menaquinone-4 (MK-4) by measuring the extent to which the Thrombotest was normalized after the administration of varying doses of the respective vitamins. Oral administration of the vitamins showed that the efficacy of K_1 was at least two-fold higher than that of MK-4. As comparable results were obtained after subcutaneous administration of the vitamins, we conclude that after oral administration the intestinal absorption had been quick and nearly complete. A less pronounced effect of K_1 and MK-4 was found after colorectal administration. For both forms of vitamin K relatively high amounts (well above the physiological concentration) were required before significant effects on the Thrombotest could be observed. Therefore these data demonstrate the importance of sufficient dietary vitamin K consumption in rats. The efficacy of other menaquinones may be investigated in the same experimental animal model system.

Vitamin K is an essential cofactor in the post-translational processing of several proteins in blood coagulation [1–3]. The term 'vitamin K' refers to at least two different types of quinone: phylloquinone (K_1‡) and menaquinone (MK). K_1 is a single chemical entity, which is exclusively synthesized by plants. Hence vegetables form the main dietary source of K_1 for humans [4]. MK, or vitamin K_2, is a composite name for a family of 2-methylnaphtoquinones differing from each other in the length of their side chains, which may vary from 1 to 12 unsaturated isoprenoid residues. They are generally identified as menaquinone-*n*, where *n* gives the number of isoprenoid residues. MKs are of microbial origin; they are mainly found in fermented foods, as well as in the colon where they are produced by the intestinal flora [5]. The relative importance of K_1 and MK in the total of the vitamin K-dependent processes is not yet clear. Both forms of vitamin K were found to have cofactor activity in an *in vitro* cell-free system, although the activity of the higher MKs decreased at increasing side chain length [6].

The vitamin K status of an organism depends on the K_1 and MK content of its diet, on the intestinal absorption of these vitamins as well as on the extent to which the MKs produced by the intestinal flora are absorbed in the colon. Both K_1 and MK-4 are available as pharmaceutical preparations for oral and parenteral administration. The fact that the former is mainly used in the western part of the world, whereas MK-4 is predominantly used in Japan, is probably based on tradition rather than on comparative data.

To investigate further the absorption of vitamin K in the digestive tract, we have chosen vitamin K-deficient rats as an animal model. Several methods to generate vitamin K-deficiency have been reported in the literature [7, 8], but in our studies the inter-individual variations were high, and did not permit reliable experimentation. Therefore, we have slightly modified the procedure of Mathers *et al.* [9] and it appears that the animals thus obtained form a reproducible model system for studies concerning the *in vivo* effect of vitamin K administration.

MATERIALS AND METHODS

Chemicals. K_1 and MK-4 were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.), and Boseral® (a 30% bovine albumin solution) from Organon Teknika (Boxtel, The Netherlands). Shortly before the start of each experiment the vitamins were dissolved in Boseral by sonication for 30 min with alternating pulses of 5 sec and an amplitude of 4 μ. This treatment resulted in clear and homogeneous solutions of the various vitamins.

Animals. All experiments were performed with 12 week old male rats of the Lewis strain, which were housed in coprophagy reducing metabolic cages. The rats were fed *ad lib.* with a diet prepared fresh each day consisting of a radiated vitamin K-deficient diet (Hope Farms, Woerden, The Netherlands), mixed with cooked and dried white rice in a 1/1 (w/w) ratio [9]. The commercial vitamin K-deficient food contained (g/kg): vitamin-free milk proteins, 300; synthetic methionine, 2; cellulose, 50; sugars, 450; starch, 70; sunflower oil, 50, and

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‡ Abbreviations: K_1, phylloquinone; MK-4, menaquinone-4.

standard amounts of minerals, vitamins (except vitamin K) and trace elements. Blood was taken by venipuncture from the tail vein in 0.1 vol. of 0.14 M tri-sodium citrate at the following time points: before starting a 7 day vitamin K-deficient regimen as well as shortly before ($t = 0$) and 6, 24 and 48 hr after vitamin K administration. The experimental protocol was approved by the Animal Investigation Committee of the University of Limburg.

Administration of vitamin K. For all experiments the various forms of vitamin K were freshly solubilized before administration. The volume of the sample was 0.5 mL. Oral administration was performed via a syringe equipped with a plastic cannula, which was protruded into the esophagus. For colorectal administrations the cannula was introduced via the rectum, and moved up to the colon loop, where the vitamin was applied. Subcutaneous administration was performed in the neck of the animals.

Tests. The blood coagulation characteristics were measured with a coagulation test known to be sensitive for deficiencies of the vitamin K-dependent coagulation factors (Thrombotest®, Nycomed AS, Oslo, Norway). Before starting an experiment blood was taken from each rat to determine the initial values. All further data are expressed as a percentage of the initial values for each individual rat. Each point in Figs 1–3 represents the mean ± SEM of five different rats.

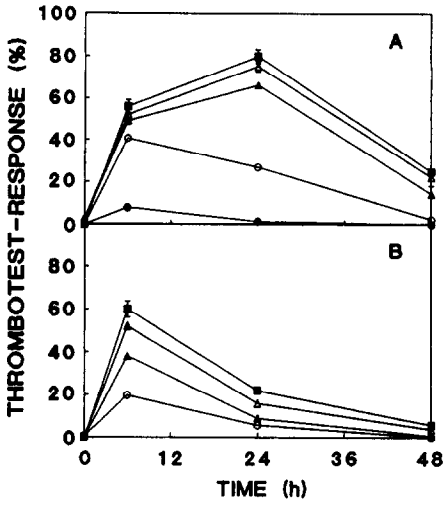

Fig. 1. Thrombotest time course after oral administration of vitamin K to vitamin K-deficient rats. At $t = 0$ the following amounts of vitamin K were given: 4 (●), 20 (○), 40 (▲), 100 (△) and 200 (■) μg/kg body weight. The values of the placebo group (Boseral only) were subtracted from corresponding values in the vitamin K-treated groups. (A) K_1; (B) MK-4. Each point represents the mean (± SEM) of duplicate tests in five different animals. In those cases in which no error bar is indicated, it falls within the size of the symbol.

RESULTS

In this paper we describe experiments with partly vitamin K-deficient rats. The vitamin K status of the animals was checked with an overall clotting test sensitive for the vitamin K-dependent coagulation factors (Thrombotest), and vitamin K deficiency is defined as a state in which the Thrombotest values are between 5 and 10% of the initial values. To obtain a quick and reproducible test system it was essential that these levels were reached within 1 week after starting the vitamin K-deficient regimen, and that on continuation of this regimen the Thrombotest remained constant for a longer period. Probably because of variable MK ingestion via coprophagy, these requirements were not met during feeding the animals various vitamin K-deficient diets. Mathers *et al.* [9] described a procedure by which the intestinal MK production is strongly reduced by feeding the animals a low fibre diet (boiled white rice), but obviously this will lead to a wide range of deficiencies. Therefore we have modified this procedure by supplementing the rice with a mixture of all essential nutrients except vitamin K. Optimal results were obtained if (a) the boiled and dried rice was freshly prepared each day, and (b) the animals were housed in coprophagy reducing metabolic cages. Under these conditions vitamin K levels in serum and in liver tissue had decreased within 1 week to less than 1% of those in untreated animals (H. H. W. Thijssen, unpublished data), whereas in the same period the Thrombotest values had dropped to 5–10% of the starting values. On continuation of this regimen the blood Thrombotest values did not change further, and no animals were lost because of bleeding.

Before generating vitamin K deficiency blood was taken from each rat. This value was used to assess the animal's initial Thrombotest value. All further data are expressed as a percentage of the initial values. During the next 7 days the animals were subjected to the vitamin K-deficient regimen, and after this pretreatment period the Thrombotest values were checked in a second blood sample (this point is referred to as $t = 0$). In a first experiment six groups of five vitamin K-deficient rats received a single oral dose of K_1, varying from 0 (the placebo group, which received only Boseral) to 200 μg/kg body weight. Both K_1 and MK-4 are strongly hydrophobic compounds, which are generally dissolved with the aid of detergents. As detergents may change the intestinal absorption and pharmacokinetic parameters of hydrophobic drugs, however, the vitamins were dissolved in a 30% albumin solution. It was found that the effect of 4 μg K_1 per kg body weight was almost negligible, but that for all other dosages the vitamin induced a rapid increase of the Thrombotest values during the first few hours after oral administration (Fig. 1A), with a good dose–response relation between 0 and 20 μg/kg. The data in this figure are in agreement with several other experiments (data not shown), in which we consistently found a maximal effect after 6 hr when using dosages below 40 μg/kg. After that time there was a gradual decrease of the Thrombotest values in the low dose groups, which is seen in the samples taken after 24 and 48 hr. For the higher

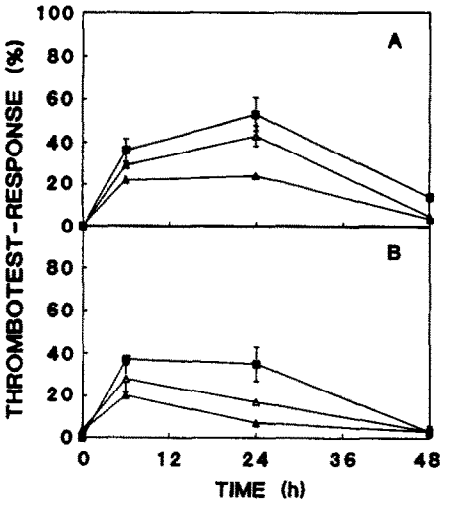

Fig. 2. Thrombotest time course after colorectal administration of vitamin K to vitamin K-deficient rats. Further details are as described in the legend to Fig. 1.

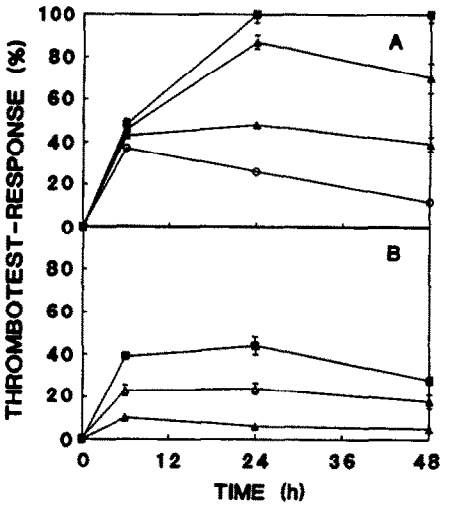

Fig. 3. Thrombotest time course after subcutaneous administration of vitamin K to vitamin K-deficient rats. Further details are as described in the legend to Fig. 1.

dosages (up to 200 μg/kg) the increase of the Thrombotest continued until a maximum was reached at 24 hr. After that time there was a decline of the Thrombotest values in all cases, which is seen in the samples taken shortly before the animals were killed ($t = 48$ hr). In this paper the surface area under the Thrombotest–response curve is taken as a marker for the efficacy of the treatment.

Similar curves were prepared for MK-4 (Fig. 1B), and it was found that the efficacy of this vitamin was about 50% lower, and that the persistence of its effect was shorter than that of K_1. The differences with K_1 after 6 hr were relatively small and may be the result of a slower or less efficient intestinal absorption, or of a decreased bioavailability or biological activity of MK-4. The fact that the differences were more pronounced after 24 hr seems to indicate that MK-4 was eliminated much faster than K_1.

The second route of vitamin K administration investigated was the colorectal one, and the results are summarized in Fig. 2. By comparing Fig. 2A and B it can be seen that the efficacy of MK-4 was about 60% that of K_1, but that the effects of both vitamins after 6 hr were comparable. By comparing Figs 1 and 2 it is clear that the efficacy of oral administration of the vitamins is 1.5 (MK-4) to 3 (K_1)-fold higher than that of rectal administration.

Finally, we also investigated the effect of subcutaneous administration of both vitamins. Under these conditions the efficacy of K_1 was 3 to 5-fold that of MK-4 (cf. Fig 3A and B). This figure shows the striking slow decline after 24 hr, which is probably related to depot formation. By comparing Figs 1, 2 and 3 it can be seen that the efficacy of MK-4 is more or less similar for all three routes of administration, whereas for K_1 the effect of oral and subcutaneous administration is more prominent than that of rectal administration.

DISCUSSION

The question to what extent the MKs produced by the intestinal flora contribute to a subject's vitamin K status has been a matter of debate for many years. Initial experiments in rats, the intestinal flora of which was destroyed with antibiotics, showed the rapid development of hypoprothrombinaemia [10]. This was regarded as strong evidence for a direct absorption in the colon of bacterial MKs. An alternative explanation, however, is that because rats eat their faeces, the vitamin K originating from the intestinal flora forms an important dietary source of vitamin K for the rat. The destruction of this flora would therefore result in a strong decrease of their dietary vitamin K intake [11]. Later experiments, in which rats were fed a vitamin K-deficient diet, and in which coprophagy was effectively prevented, demonstrated that the animals immediately developed hypoprothrombinaemia despite the presence of bacteria in their colons [12]. More recently Suttie *et al.* performed experiments with human volunteers receiving a vitamin K-restricted diet [13]. Also these authors concluded that the intestinal MKs are of minor importance for maintaining a subject's vitamin K status. An alternative mechanism for the antibiotic-induced hypoprothrombinaemia was provided by the demonstration that many antibiotics, especially those containing thiol-leaving groups, are direct inhibitors of the vitamin K-dependent step in the blood coagulation factor synthesis [11, 14]. These drugs must be classified, therefore, as vitamin K-antagonists, which explains their effect on blood coagulation.

Direct studies concerning the absorption of MK were performed by Hollander *et al.* [15], who used preparations of the rat colon. A drawback of these studies is that the authors used bile salts to solubilize their vitamin K. The expected effects of bile salts

and other detergents are (a) that they extract and dissolve the MKs which under physiological conditions are bound to the bacterial membranes and not available for intestinal absorption, and (b) that they form micelles via which exogenous and endogenous vitamin K may penetrate through the intestinal mucosa. Since *in vivo* the absorption of cholates in the small intestine is nearly complete, the strongly hydrophobic MKs produced by the colonic bacteria are not expected to be in a milieu of bile salts, which makes these results difficult to interpret. Based on our present knowledge we must assume that the MKs present in the colon are bound to lipophilic proteins and/or phospholipids originating from bacterial membranes after cell lysis.

In this paper we describe a simple experimental animal model to develop vitamin K deficiency in rats. The model is based on a protocol given by Mathers *et al.* [9] who demonstrated that exclusively feeding rats with boiled, white rice induced severe hypoprothrombinaemia within 1 week. In these rats both the phylloquinone intake as well as the menaquinone production by enteric bacteria were strongly reduced. A disadvantage of the white rice protocol is that the animals rapidly become deficient of more essential nutrients. Therefore we have supplemented the low-fibre diet with a mixture of essential nutrients (except vitamin K). Additionally the animals were housed under coprophagy-reducing conditions. In this way hypoprothrombinaemia (Thrombotest values 5–10% of the initial values) was provoked within 1 week. After this period the Thrombotest values did not decrease further, but remained at these low levels. We assume that this residual coagulation activity reflects a low intake of vitamin K, which may originate either from the rice (2 μg K_1/kg), from direct MK absorption in the colon, or from intestinal MK resorption after coprophagy. Nevertheless this experiment demonstrates the importance of a dietary source of vitamin K.

Subcutaneous injection of either K_1 or MK-4 generally led to a good response (Fig. 3), the extent of which was comparable with that obtained after oral administration of the same amount (cf. Fig. 1). Notably at the higher dosages, however, the effect of subcutaneous administration lasted at least 2-fold longer than that after an oral dose. This phenomenon is probably due to subcutaneous depot formation. The maximal effect of vitamin K at 6 hr after both oral and subcutaneous administration was an increase of the Thrombotest values to 60% of the normal values (see Figs 1 and 3), and we think that this number represents the maximum synthesis rate of the vitamin K-dependent coagulation factors during this time period. It was obtained at dosages ± 20 μg K_1 per kg, and ± 100 μg MK-4 per kg body weight. At least for K_1 this amount is of the same order of magnitude as the daily K_1 requirements of rats (10 μg/kg, [16]). On the other hand, the colorectal administration of the vitamins had a poor effect on the Thrombotest values. The physiological amounts of enteric MKs in rats range from 15 to 20 μg/kg body weight [17], and at these concentrations the effect of exogenous MK-4 was negligible. The results obtained after colorectal K_1 administration were slightly better, but it may be calculated from the dose–response curves that also in this case the efficacy was less than 10% from that after oral or subcutaneous administration. The fact that colorectal dosages of MK-4 required for a significant effect on the blood coagulation characteristics were substantially higher than the physiological amounts present in the rat colon suggests that the absorption of enteric MKs is of minor importance for the vitamin K status of the rat. Another conclusion is that, whereas in the large intestine the efficacy of K_1 and MK-4 administration is comparable (see Fig. 2), K_1 seems to be more effective than MK-4 after oral and subcutaneous administration (cf. Fig. 1). These data are consistent with recent data from Will and Suttie [18]. Using a rat model system slightly differing from ours these authors compared the efficacy of K_1 and MK-9 exclusively given via the oral route, and they concluded that if MK-9 is the only dietary source of vitamin K, its requirement is substantially higher than that of K_1.

In our studies we restricted ourselves to the comparison of K_1 and MK-4 because both compounds are very similar with respect to mass and hydrophobicity. *In vivo* K_1 turned out to be about 2 to 5-fold (depending on the route of administration) more active than MK-4. It should be realized, however, that MK-4 forms a very small fraction (less than 0.5%) of the total pool of intestinal MKs. It is to be expected that the absorption of the far more hydrophobic higher MKs ($N > 7$), which form more than 95% of the total, is less efficient because they are firmly bound to the bacterial membranes and no detergents (bile salts) are present to mediate their solubilization in the colon. This was confirmed recently for MK-9 by Ichihashi *et al.* [19], who demonstrated that the absorption rates of the MKs in the colon markedly decrease with increasing numbers of isoprenoid units. Therefore our data do not suggest a dominant physiological role of the intestinal MKs in the synthesis of blood coagulation factors in the rat.

Acknowledgements—The authors would like to thank Dr M. M. W. Ulrich for assistance with animal handling. This research was supported in part by grant 88.004 from the Dutch Thrombosis Foundation.

REFERENCES

1. Furie B and Furie BC, The molecular basis of blood coagulation. *Cell* **53**: 505–518, 1988.
2. Suttie JW, Vitamin K-dependent carboxylase. *Annu Rev Biochem* **54**: 459–477, 1985.
3. Vermeer C, γ-Carboxyglutamate-containing proteins and the vitamin K-dependent carboxylase. *Biochem J* **266**: 625–636, 1990.
4. Shearer MJ, Allan V, Haroon Y and Barkhan P, Nutritional aspects of vitamin K in the human. In: *Vitamin K Metabolism and Vitamin K-Dependent Proteins* (Ed. Suttie JW), pp. 317–327. University Park Press, Baltimore, 1980.
5. Gibbons RJ and Engle LP, Vitamin K compounds in bacteria that are obligate anaerobes. *Science* **146**: 1307–1309, 1964.
6. Buitenhuis HC, Soute BAM and Vermeer C, Comparison of the vitamins K_1, K_2 and K_3 as cofactors

for the hepatic vitamin K-dependent carboxylase. *Biochim Biophys Acta* **1034**: 170–175, 1990.
7. Mameesh MS and Johnson BC, Production of dietary vitamin K deficiency in the rat. *Proc Soc Exp Biol Med* **101**: 467–468, 1959.
8. Kindberg CG and Suttie JW, Effect of various intakes of phylloquinone on signs of vitamin K deficiency and serum and liver phylloquinone concentrations in the rat. *J Nutr* **119**: 175–180, 1989.
9. Mathers JC, Fernandez F, Hill MJ, McCarthy PT, Shearer MJ and Oxley A, Dietary modification of potential vitamin K supply from enteric bacterial menaquinones in rats. *Br J Nutr* **63**: 639–652, 1990.
10. Kornberg A, Daft FS and Sebrell WH, Mechanism of production of vitamin K deficiency in rats by sulfonamides. *J Biol Chem* **155**: 193–200, 1944.
11. Lipsky JJ, Antibiotic-associated hypoprothrombinaemia. *J Antimicrob Chemother* **21**: 281–300, 1988.
12. Barnes RH and Fiala G, Effects of the prevention of coprophagy in the rat. VI. Vitamin K_1. *J Nutr* **68**: 603–614, 1959.
13. Suttie JW, Mummah-Schendel LL, Shah DV, Lyle BJ and Greger JL, Vitamin K deficiency from dietary vitamin K restriction in humans. *Am J Clin Nutr* **47**: 475–480, 1988.
14. Creedon KA and Suttie JW, Effect of *N*-methylthiotetrazole on vitamin K epoxide reductase. *Thromb Res* **44**: 147–153, 1986.
15. Hollander D, Muralidhara KS and Rim S, Colonic absorption of bacterially synthesized vitamin K_2 in the rat. *Am J Physiol* **230**: 251–255, 1976.
16. Lefevere MF, de Leenheer AP, Claeys AE, Claeys IV and Steyaert H, Multidimensional liquid chromatography: a breakthrough in the assessment of physiological vitamin K levels. *J Lipid Res* **23**: 1068–1072, 1982.
17. Uchida K and Komeno T, Relationships between dietary and intestinal vitamin K, clotting factor levels, plasma vitamin K, and urinary Gla. In: *Current Advances in Vitamin K Research* (Ed. Suttie JW), pp. 477–492. Elsevier, New York, 1988.
18. Will BH and Suttie JW, Comparative metabolism of phylloquinone and menaquinone-9 in rat liver. *J Nutr* **122**: 953–958, 1992.
19. Ichihashi T, Takagishi Y, Uchida K and Yamada H, Colonic absorption of menaquinone-4 and menaquinone-9 in rats. *J Nutr* **122**: 506–512, 1992.