Comparative Distribution, Metabolism, and Utilization of Phylloquinone and Menaquinone-9 in Rat Liver (43915)

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Abstract. **The liver of most species contains a spectrum of bacterially produced menaquinone homologs as well as the major dietary form of vitamin K, phylloquinone. The relative utilization of phylloquinone and menaquinone-9 (MK-9) as substrates for the microsomal vitamin K-dependent γ-glutamyl carboxylase was determined in a rat model. Vitamin K 2,3-epoxide, the co-product of the carboxylation reaction, is recycled to the quinone form of the vitamin by a microsomal vitamin K epoxide reductase. This enzyme activity was blocked by warfarin administration, and the appearance of the hepatic epoxides of phylloquinone and MK-9 was determined as a measure of their utilization by the carboxylase. When the liver contained equimolar amounts of phylloquinone and MK-9, four times as much phylloquinone epoxide as MK-9 epoxide was present in the liver 1 hr after warfarin administration. These data suggest that hepatic MK-9 is not as efficiently utilized as phylloquinone. The data obtained have also demonstrated a previously unrecognized difference in phylloquinone and menaquinone metabolism. MK-9 epoxide, and to a lesser extent MK-9, was preferentially localized in the mitochondria, while higher concentrations of phylloquinone were found in the microsomes.** [P.S.E.B.M. 1995, Vol 209]

Compounds possessing vitamin K activity are 2-methyl-1,4-naphthoquinones with various hydrophobic substitutions at position 3. They are substrates for a microsomal enzyme required for the synthesis of the active form of a number of plasma proteins including coagulation factors II (prothrombin), VII, IX, and X, and proteins C, S, and Z (1). The enzyme catalyzes the carboxylation of specific Glu residues in precursors of these proteins to γ-carboxyglutamyl (Gla) residues in the mature proteins. Phylloquinone, 2-methyl-3-phytyl-1,4-naphthoquinone, produced by plants, is the major dietary form of vitamin K. The menaquinones, synthesized by intestinal and other bacteria, are a series of 2-methyl-3-polyisoprenyl-1,4-naphthoquinones which also possess vitamin K activity.

Although menaquinones synthesized in the lower bowel are often credited with furnishing a substantial part of the daily vitamin K requirement for the human (2), there is little direct evidence for this claim. The major evidence in support of this view is the large number of reported cases of antibiotic-induced hypoprothrombinemia (3, 4). These coagulopathies are usually vitamin K responsive, and inhibition of the synthesis of menaquinones has usually been the assumed cause of this acquired deficiency. However, evidence to support a substantial utilization of intestinal menaquinones in any species is weak. A vitamin K deficiency was readily produced in the rat by Barnes and Fiala (5) when coprophagy was prevented, and a mild or "subclinical" vitamin K deficiency has been produced in healthy human subjects by dietary vitamin K restriction (6–8).

Menaquinones do, however, have biological activ-

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ity *in vivo* (9, 10) and are *in vitro* substrates for the vitamin K-dependent carboxylase (11, 12). Menaquinones have also been shown to represent a substantial portion of the vitamin K present in both human (13–15) and rat liver (16, 17). The relative utilization of the hepatic phylloquinone and menaquinone pools has, however, not been determined. The action of the hepatic vitamin K-dependent carboxylase produces vitamin K-2,3-epoxide as a co-product, and this metabolite is effectively recycled to the quinone and hydronaphthoquinone forms of the vitamin through the action of a vitamin K epoxide reductase (18). The 4-hydroxycoumarin-based oral anticoagulants function by inhibiting this enzyme (18) resulting in a decrease in the enzymatically active, hydronaphthoquinone, form of the vitamin, and the accumulation of the epoxide in liver (19) and plasma (20) during anticoagulant therapy.

The predominant bacterially produced menaquinones in rat liver are MK-8 through MK-11, and MK-9 has previously been used in comparative studies (21) as a model long-chain menaquinone. This report compares the distribution of phylloquinone and menaquinone-9 (MK-9) in rat liver and measures the appearance of their epoxides in the liver of warfarin-treated rats to determine the utilization of the two different vitamers as a substrate for the carboxylase.

Materials and Methods

Animals. All animal protocols were approved by the University of Wisconsin—Madison College of Agricultural and Life Sciences Animal Care Committee. Vitamin K epoxide formation was studied in male 230–250 g rats (Sprague-Dawley, Madison, WI) housed in wire-bottom cages and fed either Purina (St. Louis, MO) Rat Chow or a soybean protein, sucrose and cornstarch, vegetable oil-based, vitamin K-deficient diet (Teklad #81053, Madison, WI). This diet contains less than 0.02 nmol phylloquinone/g diet by analysis. It was supplemented with 1.10 nmol phylloquinone/g diet and 0.38 nmol MK-9/g diet and fed for 8 days. Both vitamers were added to the diet in corn oil which contributed less than 0.003 nmol phylloquinone/g diet. To determine the relative utilization of each vitamer, the rats were injected ip with 5 mg Na warfarin/kg in 0.9% NaCl and killed at the indicated times after warfarin injection. To standardize the data relative to periods of diet consumption, the timing of the warfarin injection was altered so that all animals were killed between 2 and 3 hr after the animal room lights came on at 6:00 AM.

In a separate experiment designed to measure hepatic metabolism and distribution of each vitamer and its epoxide, male Sprague-Dawley 250–270 g rats were housed in the coprophagy preventing cages (22) and fed the vitamin K-deficient diet for 5 days to reduce endogenous hepatic vitamin K. The rats were then injected ic with 17 nmol of either the epoxide or quinone forms of phylloquinone and MK-9 in 100 μl of ethanol and sacrificed at 1, 3, or 6 hr. Livers of noninjected animals were analyzed to confirm that concentrations of liver vitamin K were low at the start of the experiment.

Tissue Preparation. After ether anesthesia, animals were killed by decapitation and the livers removed. Whole liver homogenates were prepared using a Polytron tissue homogenizer in one volume of 0.25 *M* sucrose, 0.25 *M* imidazole (pH 7.3) per gram of liver, and stored in 2 ml aliquots at −70°C. Mitochondria and microsomes were prepared from livers homogenized in a Potter-Elvehjem homogenizer in two volumes of 0.25 *M* sucrose, 0.25 *M* imidazole, and 1 m*M* EGTA (pH 7.4) (SIE buffer) per gram of liver, and centrifuged at 10,000*g* for 15 min. The supernatant was decanted and centrifuged at 105,000*g* for 1 hr to pellet the microsomal fraction. The 10,000*g* pellet was rehomogenized in SIE buffer, diluted to a 10% homogenate, and centrifuged at 700*g* for 10 min. The supernatant was centrifuged at 7000*g* for 10 min to pellet the mitochondria. The mitochondrial pellet was resuspended once in 20 ml SIE buffer and centrifuged at 7000*g*. Samples were stored at −70°C. Marker enzyme assays for microsomes (glucose-6-phosphatase) and mitochondria (succinate dehydrogenase with tetrazolium as an electron acceptor) were performed on fresh preparations (23) to determine the purity of the preparations. Protein content of liver homogenates and subcellular fractions was determined by the method of Lowry *et al.* (24) using bovine serum albumin as the standard. The vitamin K and vitamin K epoxide content of mitochondria and microsomal subcellular fractions are expressed as vitamin concentration in these fractions per gram of total liver protein assuming (25) that microsomes and mitochondria account for 24% and 16% respectively of total liver protein.

Vitamin K and Vitamin K Epoxide Determinations. All procedures were conducted under subdued or yellow light on samples thawed at 37°C. Liver homogenate, microsomal, and mitochondrial samples were resuspended in 1.5 ml of water. To each sample, 0.2 ng of trans-^{3}H-phylloquinone (2400 Bq/ng) and 6.0 ng of 6-methyl-menaquinone-9 were added as internal standards and incubated at room temperature for 1 hr with frequent mixing. Two milliliters of ethanol and 4.0 ml of diethyl ether were added, and the solution was mixed on a vortex mixer for 1 min. After 10 min, 5.0 ml of hexane was added, the suspension was vortexed for 1 min, centrifuged for 10 min at 4°C, and the hexane layer removed and dried under filtered air as previously described (26).

Tissue extracts were partially purified on Waters

(Milford, MA) silica SepPak cartridges. The SepPak cartridge was washed with 8 ml hexane; the sample dissolved in 1.0 ml hexane was loaded; the column was washed with 8 ml hexane and vitamin K eluted with 8 ml 5% ether in hexane. To achieve further purification of the crude extract, samples were dried under filtered air dissolved in 250 μl hexane, injected onto a Waters μPorasil HPLC column (30 cm × 3.9 mm i.d.), and eluted with 25% dichloromethane in hexane with a flow rate of 1.5 ml/min. The eluant was monitored at 254 nm, and the fractions containing phylloquinone, phylloquinone epoxide, MK-9, and MK-9 epoxide as determined by a previous standard injection, were collected and dried under nitrogen. If the retention time of phylloquinone on this straight phase column did not exceed 8 min, the sample was not sufficiently purified to quantitate phylloquinone in the next step. The presence of lipid in the tissue extracts was found to influence chromatography of the menaquinone fraction. The retention times of 6-Me-MK-9, MK-9, and MKO-9 standards on this column were 12.6, 12.8, and 13.2 min, respectively. When standards were added to a lipid extract of rat liver homogenate, the peak elution time of MKO-9 was shortened by 30 sec, MK-9 by 60 sec, and 6-Me-MK-9 by 90 sec. Because of this influence of other lipids on retention time, collection of the menaquinone-9 fractions from the μPorasil column was started 2.5 min before the 12.9 midpoint of this group of compounds and was continued for 4 min. The dried samples from the μPorasil column were dissolved in 250 μl methanol. Recovery of ^{3}H-phylloquinone, added as an internal standard from the extraction and preparative column steps, was assessed by adding 30 μl of the sample in methanol to 4 ml of BioSafe (Research Products International Corp., Mount Prospect, IL) and determining the radioactivity of the internal standard in a liquid scintillation spectrometer.

To quantitate phylloquinone and its epoxide, 200 μl of the remaining methanol solution was injected onto a Dupont NEN (Wilmington, DE) reversed-phase C-18 Zorbax ODS analytical HPLC column (25 cm × 4.6 mm i.d., 5 μ*M* particle size). The sample was eluted with 15% dichloromethane in methanol containing 5 ml of a 2.0 *M* $ZnCl_2$, 1.0 *M* sodium acetate, 1.0 *M* acetic acid aqueous solution per liter of mobile phase with a flow rate of 1.0 ml/min. To quantitate MK-9, MK-9 epoxide, and 6-methyl-MK-9 (the internal standard for MK-9), the same Zorbax ODS column was used with a mobile phase of 30% dichloromethane in methanol containing 5 ml of the same $ZnCl_2$/Na acetate/acetic acid solution per liter of mobile phase. Vitamin K was detected fluorometrically (Ex = 330 nm, Em = 430 nm) after post column metallic zinc reduction of the quinones and epoxides to vitamin K hydronaphthoquinone (27). The detection limits for the vitamers in this analytical step were phylloquinone, 25 pg; phylloquinone epoxide, 40 pg; MK-9, 150 pg; MK-9 epoxide, 250 pg.

Chemicals. Phylloquinone was purchased from Sigma Chemical Co. (St. Louis, MO) and MK-9 was a gift from Hoffman-LaRoche. Phylloquinone and MK-9 were converted to their epoxides (28) and purified by preparative HPLC using a Waters C18 μBondapak 10 μ*M* semipreparative column. HPLC grade solvents were purchased from American Burdick and Jackson (Muskegon, MI). The 6-methyl-MK-9 was synthesized by the method of Fieser *et al.* (29). Sodium warfarin was obtained from the Wisconsin Alumni Research Foundation (Madison, WI).

Results

The increase in hepatic vitamin K epoxide following administration of warfarin to block the reduction of vitamin K epoxide to vitamin K quinone and vitamin K hydronaphthoquinone was quantitated as a measure of the relative utilization of phylloquinone and MK-9 as a substrate for the carboxylase. Utilization of the two different vitamers was assessed in rats which had roughly equal (40–50 pmol/g liver protein) hepatic concentrations of phylloquinone and MK-9. Based on the results of preliminary studies in which molar ratios of phylloquinone:MK-9 of 2:1, 3:1, and 5:1 were fed, this was achieved by feeding rats a diet containing 1.1 nmol phylloquinone and 0.38 nmol MK-9 per gram of diet for 8 days. After warfarin was administered to these rats, the total hepatic concentration of both phylloquinone and MK-9 decreased while the concentration of phylloquinone and MK-9 epoxides increased (Fig. 1). In the first 3 hr following warfarin administration the appearance of MK-9 epoxide lagged that of phylloquinone epoxide in both whole liver homogenate and microsomes. Phylloquinone epoxide also increased in the mitochondria but at a slower rate than that observed in the microsomes and the magnitude of the increase was not as great. This delay in the appearance of phylloquinone epoxide in the mitochondria was not seen in the case of MK-9 epoxide, and the increase of this metabolite in mitochondria was much greater than in microsomes.

The hepatic concentration of both vitamin K epoxides had leveled off or dropped by the end of the 12-hr study. Further metabolism and biliary excretion of the vitamin would account for this result, which was particularly evident in the case of phylloquinone epoxide. The large amounts of both phylloquinone and MK-9 epoxide that accumulated in the mitochondria were of particular interest since very little vitamin K-dependent carboxylase/epoxidase activity has been observed in the mitochondria (30). Assays of glucose-6-phosphatase activity as a microsomal marker (data not shown) demonstrated that the mitochondrial

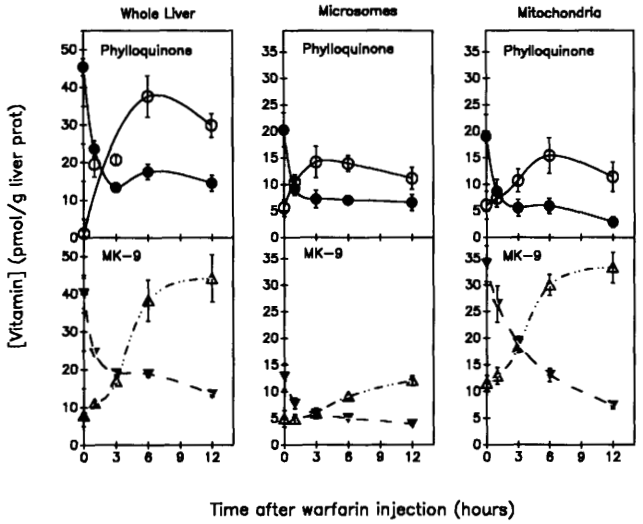

Figure 1. Effect of warfarin injection on hepatic vitamin K and vitamin K epoxide. Male rats were fed a vitamin K-deficient diet supplemented with (1.1 nmoles phylloquinone + 0.38 nmoles MK-9)/g diet for 8 days, injected with 5 mg warfarin/kg body wt ip, and sacrificed at the times indicated after injection. Vitamin and epoxide concentrations were determined in total liver homogenate, liver microsomes, and liver mitochondria. Each point represents the mean ± SEM with four rats per group. When SEM was smaller than the data point symbols, it is not indicated. (●) Phylloquinone; (○) phylloquinone epoxide; (▼) MK-9; (▽) MK-9 epoxide.

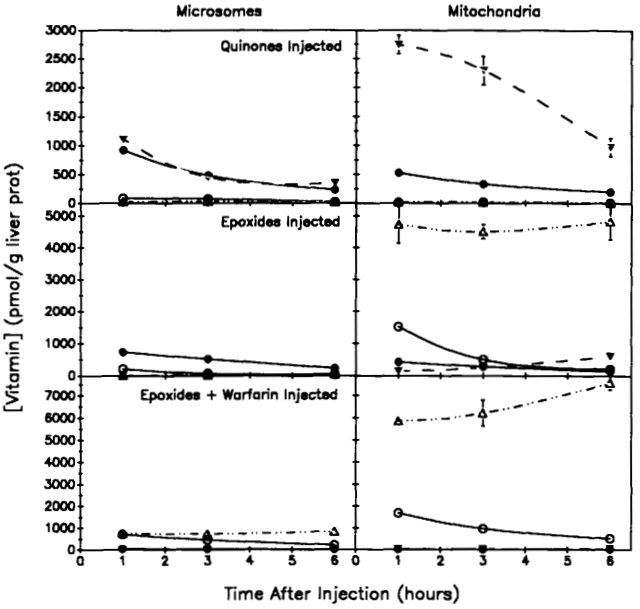

Figure 2. Vitamin K quinone and epoxide concentrations in microsomal and mitochondrial fractions following injection of the quinone or epoxide forms of phylloquinone and menaquinone-9. Male rats were housed in coprophagy-preventing cages for 5 days to deplete hepatic phylloquinone, injected ic with 100 μl ethanol containing 17 nmoles of phylloquinone and MK-9 (top panel), 17 nmoles of phylloquinone epoxide and MK-9 epoxide (middle panel), or 17 nmoles of each epoxide and 5 mg warfarin/kg body wt ip (bottom panel). Rats were sacrificed at the times indicated after injection, and vitamin and epoxide concentrations were determined in liver microsomes and mitochondria. The concentration of phylloquinone, MK-9, and their epoxides in the vitamin K-deficient rats before vitamer administration were less than 10 pmol/g liver protein in both microsomes and mitochondria. Each point represents the mean ± SEM with four rats per group. When SEM was smaller than the data point symbols, it is not indicated. (●) Phylloquinone; (○) phylloquinone epoxide; (▼) MK-9; (▽) MK-9 epoxide.

fraction was less than 10% contaminated with microsomes.

Interpretation of the data in Figure 1 was complicated by a lack of knowledge of the rate of metabolism of phylloquinone and MK-9 and their epoxides, and by a lack of understanding of the manner in which the vitamin and its metabolites redistribute between subcellular pools. The hepatic metabolism and distribution of phylloquinone, phylloquinone epoxide, MK-9, and MK-9 epoxide was therefore compared in vitamin K-deficient rats injected intracardially with equimolar amounts of the quinone or the epoxide form of both vitamers. The concentrations of phylloquinone, phylloquinone epoxide, MK-9, and MK-9 epoxide were followed over time, and the microsomal and mitochondrial concentrations of the vitamers and their epoxides are shown in Figure 2. A number of aspects of vitamin K localization and metabolism are apparent from these data.

Following intracardial administration of the two vitamers or their epoxides, hepatic uptake and metabolism was rapid, as both microsomal and mitochondrial concentrations had peaked or were decreasing at the initial sampling period of 1 hr. As the amount of vitamin injected in a single ic dose was slightly more than the normal daily intake, the liver concentrations of the vitamins and their epoxides were much higher than those seen in the previous study. Following phylloquinone administration, phylloquinone epoxide was detected in microsomes at 82 ± 8, 75 ± 18, and 21 ± 5 pmol/g liver protein at 1, 3, and 6 hr. Administration of MK-9 resulted in 20 ± 2, 18 ± 2, and 10 ± 4 pmol MK-9 epoxide/g liver protein at the same time periods. Concentrations of both phylloquinone epoxide and MK-9 epoxide in the mitochondrial fraction were <20 pmol/g liver when the quinones were administered (Fig. 2, top panels).

Administered phylloquinone epoxide was detected in microsomes at a level of 212 ± 5 pmol/g liver protein at 1 hr and then declined. The predominant form of phylloquinone in the microsomes when phylloquinone epoxide was administered was, however, the quinone (Fig. 2, left middle panel). Much higher concentrations of injected phylloquinone epoxide were found in mitochondria at 1 hr, but rapidly declined (Fig. 2, right middle panel). When MK-9 epoxide was administered, only 13 ± 7 pmol/g liver protein was found in microsomes at 1 hr, and this increased to only 42 ± 3 pmol/g liver protein at 6 hr. Very little reduction of MK-9 epoxide occurred, and MK-9 concentrations in the microsomes did not exceed 20 pmol/g liver protein over the 6-hr period stud-

ied. In contrast to the small amount of MK-9 epoxide in microsomes, its concentration in mitochondria was very high (>4000 pmol/g liver protein) at 1 hr and did not decrease over the 6-hr period of the experiment.

Warfarin administration effectively blocked the microsomal conversion of both vitamer epoxides to their quinones (Fig. 2, left bottom panel), and the microsomal content of both epoxides was much higher when warfarin was injected to block conversion to the quinone. The amount of phylloquinone epoxide in mitochondria when the epoxide was administered was greater than that observed in microsomes and was not greatly influenced by warfarin administration (Fig. 2, right middle and bottom panels). When MK-9 epoxide was administered, very high concentrations were observed in mitochondria in both the presence and absence of warfarin, and these high concentrations were maintained over a 6-hr period.

The interpretation of the data in Figure 1, which were obtained over a 12-hr period, is obviously complicated by the variations in localization and metabolism of the two forms of the vitamin and their epoxides which are illustrated in Figure 2. The increase in the epoxide form of the two vitamers following warfarin administration was therefore analyzed at shorter time points (Fig. 3) in an attempt to obtain a measure of microsomal epoxide production rather than changes in distribution or metabolism. In this experiment, the initial microsomal phylloquinone concentration was about double that of the first experiment, but the microsomal MK-9 concentration was similar. Following warfarin administration, the microsomal pool of phylloquinone epoxide increased 5.4 times that of the MK-9 epoxide pool in 1 hr (13.2 pmol/g liver protein compared with 2.4 ± 0.6 pmol/g liver protein). These data clearly indicate a more rapid utilization of the phylloquinone pool for epoxide formation. The corresponding values for the first hour from the data in Figure 1 were 4.3 times as much phylloquinone epoxide as MK-9 epoxide (7.0 ± 2.0 pmol/g liver protein compared with 1.6 ± 0.9 pmol/g liver protein). The data in Figure 3, where metabolism and cellular distribution of the vitamin are minimized, are therefore supported by the early data.

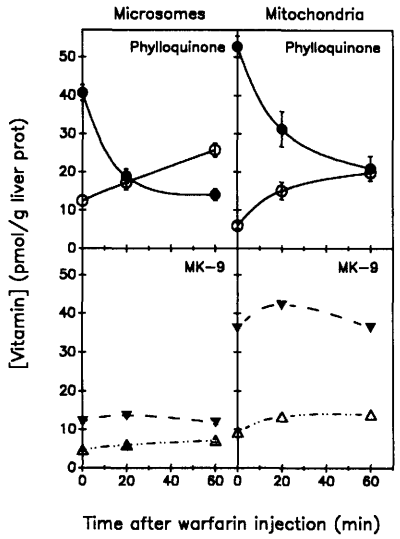

Figure 3. Effect of warfarin injection on hepatic vitamin K and vitamin K epoxide. Male rats were fed a vitamin K-deficient diet supplemented with (1.1 nmoles phylloquinone + 0.38 nmoles MK-9)/g diet for 7 days injected with 5 mg warfarin/kg body wt ip and sacrificed at the times indicated after injection, and vitamin and epoxide concentrations were determined in liver microsomes and mitochondria. Each point represents the mean ± SEM with four rats per group. When SEM was smaller than data point symbols, it is not indicated. (●) Phylloquinone; (○) phylloquinone epoxide; (▼) MK-9; (▽) MK-9 epoxide.

Discussion

Although the extent to which bacterially synthesized long-chain menaquinones can satisfy the human requirement for vitamin K remains an open question, oral and intravenous bioassays in animal models (9, 10, 31) have clearly established that they are active forms of the vitamin. The relative biopotency of various long-chain menaquinones undoubtedly depends on a number of factors such as absorption, distribution, and metabolism. A recent study (21) has demonstrated that an equimolar dietary concentration of MK-9 was less effective than phylloquinone in promoting vitamin K-dependent clotting factor synthesis. In this rat model, hepatic concentrations of the two forms of the vitamin were similar, and the data suggested that the hepatic γ-glutamyl carboxylase does not utilize tissue MK-9 as efficiently as phylloquinone.

The data presented here provide more direct support for a preferential utilization of hepatic phylloquinone rather than MK-9, and presumably other long-chain menaquinones. These data have also raised a number of new questions regarding the metabolism and subcellular distribution of vitamin K. The data in Figure 1 demonstrate the rapid appearance of vitamin K epoxide, the co-product of the carboxylation reaction when epoxide reduction is blocked by warfarin administration. There were nearly equal amounts of phylloquinone and MK-9 in the liver when warfarin was administered, and the appearance of phylloquinone epoxide in the first hour was over four times that of MK-9 epoxide. However, by 6 hr total liver MK-9 epoxide was similar to that of phylloquinone epoxide. It is likely that this is the result of a much slower hepatic turnover of MK-9 epoxide than phylloquinone epoxide. The hepatic turnover of phylloquinone has been shown (21) to be two to three times that of MK-9. The data in Figure 2, although not sufficient to calculate turnover rates, also indicate that MK-9 epoxide is removed from the liver at a much slower rate than phylloquinone epoxide.

When the subcellular distribution of the vitamins

is examined (Fig. 1), it can be seen that the large amount of hepatic MK-9 epoxide is not in the microsomal fraction where γ-glutamyl carboxylation is occurring, but rather is predominantly in the mitochondrial fraction. Previous studies (35–35) have indicated that mitochondria contained substantial amounts of phylloquinone and suggested that the mitochondrial vitamin pool was increased relative to the microsomal pool as the hepatic concentration of vitamin K was increased. The data in Figure 1 and 2 confirm the presence of this substantial mitochondrial pool of phylloquinone, and also demonstrate a preferential localization of MK-9 in the mitochondria. In addition, these data have shown localization of both epoxides in the mitochondria. This was particularly evident in the case of MK-9 epoxide. A mitochondrial epoxidase, which may or may not be coupled to carboxylase activity, has previously been reported (36). Whether the concentration of MK-9 epoxide in the mitochondria observed in this study is the result of epoxide production within this organelle or if it is transferred there following microsomal production cannot be determined from the available data. The data in Figure 2, which show high concentrations of MK-9 epoxide in the mitochondria after injection of the vitamin K epoxides in the absence of warfarin and very little MK-9 epoxide in the microsomes, suggest that mitochondria are an effective trap of MK-9 epoxide and are in agreement with previous reports (30) that the epoxide reductase activity is at a low level or lacking in this organelle.

Inspection of the data in Figure 1 indicates that the total hepatic concentration of quinone plus epoxide for either form of the vitamin has not been altered substantially over the 12-hr period studied, and suggests that most of the changes in vitamer concentrations seen are the result of cellular redistribution rather than metabolism. However, this is not a closed system, and this interpretation is complicated by the unknown quantity of vitamin absorbed from the intestine or metabolized and excreted in the bile during the 12-hr experiment.

The complications involved in interpreting the 6 or 12 hr data suggest that the short time period (1 hr) data shown in Figure 3 might be the best indication of the utilization of the two forms of the vitamin. The production of five times as much microsomal phylloquinone epoxide relative to that of MK-9 epoxide in 1 hr would indicate a preferential utilization of phylloquinone. More phylloquinone than MK-9 was present in the liver at 0 time in this experiment, but in the experiment shown in Figure 1, where the initial hepatic concentration of phylloquinone and MK-9 were equal, microsomal phylloquinone epoxide production in the first hour was still four times that of MK-9 epoxide. Overall, these data have demonstrated that a long chain menaquinone such as MK-9 is less effectively utilized than phylloquinone as a source of vitamin K, even when it is present in substantial quantities in the liver. This could be the result of less rapid utilization of microsomal MK-9 or the fact that much of the MK-9 pool is sequestered in the mitochondria and not available to the microsomal carboxylase.

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