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Metabolism of Menadione-6,7-^{3}H in the Rat*

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ABSTRACT: Menadione-6,7-^{3}H was prepared from tetrasodium 2-methyl-1,4-naphthoquinol-6,7-^{3}H diphosphate with specific activities to 44 Ci/mmole. Distribution of radioactivity in rats 18 hr after administration of a physiological dose (10 μg) of the tritiated vitamin to deficient rats showed low levels in liver, heart, kidney, carcass, and viscera while 78–83% of the administered tritium was recovered in the urine during this period. The lipophilic metabolite of menadione, menaquinone-4, was found in all tissues examined. The identity of this compound was supported by chromatographic comparison with authentic material on adsorption and partition columns and on thin-layer plates in the presence and absence of silver nitrate. The concentration of menaquinone-4 ranged from 2.4 to 48.4 pmoles per g depending upon the tissue studied. The administration of Dicumarol® in amounts sufficient to inhibit coagulation response lowered these values to 0.8–8.2 pmoles/g.

A study of vitamin K under conditions which relate physiological response to the vitamin with its metabolic fate has been undertaken as an approach to elucidating the function of this fat-soluble vitamin. A metabolic study under these conditions requires labeled vitamin with a specific activity higher than previously attained. The work of Andrews *et al.* (1962) resulted in highly radioactive vitamin K in the form of tetrasodium 2-methyl-1,4-naphthoquinol-6,7-^{3}H diphosphate.[1] Although originally used as a cancer chemotherapeutic, this compound is easily converted to menadione-6,7-^{3}H with specific activities much higher than the labeled preparations presently available (Lee *et al.*, 1953; Martius and Esser, 1958; Billeter and Martius, 1960).

The preparation of menadione-6,7-^{3}H, its purification and the results of some metabolic studies are the subject of this report.

Materials and Methods[2]

Preparation of Menadione-6,7-^{3}H. Tetrasodium 2-methyl-1,4-naphthoquinol-6,7-^{3}H diphosphate was obtained in saline solution and used immediately or stored at 4°. New samples were sufficiently pure for direct conversion into menadione; however radiodecomposition of the diphosphate during storage is a major problem (Evans, 1966). Older samples were purified by the paper chromatographic system of Andrews *et al.* (1962): saturated ammonium sulfate–1 M sodium acetate–2-propanol (75:25:2, v/v) (R_F = 0.60).

Menadione-6,7-^{3}H was prepared from the diphosphate by acid hydrolysis in the presence of ceric sulfate according to Andrews *et al.* (1962). Up to 10 ml of solution of the radioactive diphosphate was mixed with 10 ml of hot 10% ceric sulfate in 20% H_2SO_4, allowed to stand for 2 min and washed into a separatory funnel with cold water. The resulting mixture was extracted with ether, washed with water, dried with anhydrous sodium sulfate, and evaporated without heat under a stream of nitrogen or at partially reduced pressure in a

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[1] Different forms of vitamin K are designated according to the recommendation of the IUPAC–IUB Commission on Biochemical Nomenclature (*J. Biol. Chem. 241*, 2989, 1966). Menadione is 2-methyl-1,4-naphthoquinone; phylloquinone is 2-methyl-3-phytyl-1,4-naphthoquinone; and menaquinone-4 (abbreviated MK-4) is 2-methyl-3-geranylgeranyl-1,4-naphthoquinone.

[2] Nuclear-Chicago Corp., Des Plaines, Ill.

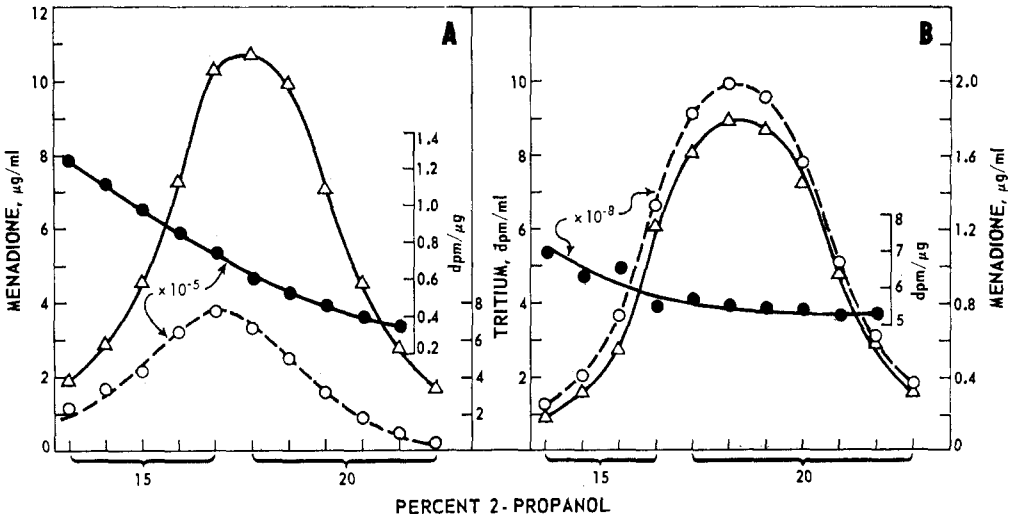

FIGURE 1: Chromatography of menadione-6, 7-^{3}H with specific activities of 5 (A) and 44 Ci/mmole (B) on reversed phase partition columns (stationary phase, hexane; movable phase, mixtures of 2-propanol in water containing 5% acetic acid). Each fraction was analyzed spectrophotometrically for menadione (△—△) and for tritium (○- -○) by scintillation spectrometry. The specific activity of each fraction (disintegrations per minute per microgram) (●—●) is also shown.

rotary evaporator.[3] The residue was chromatographed on the reversed-phase partition column described by Matschiner and Taggart (1967). The fractions containing menadione were determined by ultraviolet absorption and by radioassay[4] and were extracted with ether. The ether solution was washed with 1 M sodium bicarbonate, finally with water and evaporated to dryness. The residue of purified menadione-6,7-^{3}H was dissolved in saline for metabolic experiments.[5] The yield of radioactive menadione from 2 mg of pure diphosphate was approximately 70%.

Radiopurity of Menadione-6,7-^{3}H. Figure 1A shows the results of a reversed-phase chromatogram of a freshly prepared sample of menadione-6,7-^{3}H at 5 mCi/mmole. Less than 5% of the chromatographed radioactivity was eluted in fractions other than those containing menadione. The separation of isotope through the zone of elution of menadione shown in this figure was most prominent in samples of low specific activity. Separate chromatograms of the combined leading fractions, combined middle fractions, and combined tailing fractions from Figure 1A gave a pattern of elution similar to the original chromatogram. At 75% isotope enrichment (44 Ci/mmole), separation of ^{3}H from menadione detected by ultraviolet absorption was nearly absent (Figure 1B). The specific activity dropped slightly through the zone of elution of menadione but not as markedly as shown in Figure 1A.[6] These results resemble the characteristics of isotope separation studied extensively by Cejka *et al.* (1966) and others. Thus the prospect of isotopic separation must be considered during the identification of metabolites and derivatives of this form of tritiated vitamin K. Characterization of the radioactive menadione by crystallization of the quinone and its diacetate is shown in Table I.

TABLE I: Radiopurity of Menadione-6, 7-^{3}H.

Crystallizations	Sp Act. (μCi/mmole)
Menadione[a]	
Aqueous methanol (2)	0.99
Aqueous methanol (2)	0.89
Menadiol diacetate	
Aqueous methanol (4)	0.90

[a] Menadione-6, 7-^{3}H (4.3×10^6 dpm) (sp act. 354 mCi/mmole) was diluted with 340 mg of unlabeled menadione to give a diluted specific activity of 0.98 μCi/mmole. Number of crystallizations given in parentheses.

Metabolic Studies. Adult male rats of the Saint Louis University strain were made deficient in vitamin K according to previously described procedure (Matschiner and Doisy, 1965). Rats were selected at 13 ± 1 weeks of age with body weights 350 ± 50 g. They were fed for 2 weeks a deficient diet containing 21% soy

[3] Care must be taken during evaporation of solvent since small amounts of menadione may sublime under ordinary laboratory conditions.

[4] Radioassays were carried out in a Packard Model 314 liquid scintillation spectrometer with internal standardization.

[5] The following extinction coefficients were used for the determination of menadione in saline solution: λ_{337} (ε 2400), λ_{262} (ε 14,800), λ_{252} (ε 17,500), and λ_{248} (ε 15,800). Absorption spectra were determined with a Cary Model 14 spectrophotometer.

[6] Bioassay of this sample of menadione-6,7-^{3}H (44 Ci/mmole) by intracardial injection in deficient adult male rats (Matschiner and Taggart, 1968) gave a biological response similar to that of authentic unlabeled menadione.

TABLE II: Distribution of Radioactivity in Whole Tissue and Lipids 18 hr after Injection of Menadione-6, 7-^{3}H.[a]

	Deficient		Deficient + Dicumarol	
Tissue	Whole Tissue (%)[c]	Lipid (%)[b]	Whole Tissue (%)	Lipid (%)[b]
Liver	1.1 ± 0.2	0.4 ± 0.06	1.2 ± 0.2	0.6 ± 0.07
Heart	0.2 ± 0.03	0.1 ± 0.01	0.1 ± 0.03	0.07 ± 0.009
Kidneys	0.5 ± 0.09	0.3 ± 0.02	0.5 ± 0.04	0.08 ± 0.01
Carcass	9.5 ± 3.2	5.1 ± 0.2	8.0 ± 0.1	5.2 ± 0.9
Viscera	17.1 ± 0.9	6.3 ± 0.8	13.7 ± 1.6	7.1 ± 1.0
Blood	0.8 ± 0.2		0.5 ± 0.07	
Feces	3.4		0.02	
Urine	78.3 ± 2.7		83.2 ± 0.6	
Totals	110.9		107.2	

[a] 56 nmoles (4.4×10^7 dpm) per animal. After administration of the radiolabeled menadione to deficient rats, average prothrombin level was 85% of normal; in rats fed Dicumarol it was 5%. [b] Lipid was extracted with ether after dehydration of the tissue with Na_2SO_4 as described in the text. [c] The data are the average of three rats per group expressed as per cent of administered radioactivity plus and minus the standard error.

protein, 43% cornstarch, 22% glucose monohydrate, 5% corn oil, and an adequate supply of minerals and vitamins except for vitamin K. Blood samples taken after 1 week by cardiac puncture were analyzed for prothrombin activity by the method Hjort *et al.* (1955). For metabolic studies, rats were selected so that the concentration of prothrombin was approximately 40% of normal for each experimental group of three rats.

The amount of menadione required for detectable physiological response in these depleted animals is approximately 2 μg (Matschiner and Taggart, 1968). For the present studies near maximal response was attained by injecting each animal intracardially with 9.6 μg (56 nmoles) of menadione-6,7-^{3}H (specific activity 354 mCi/mmole). After 18 hr, blood was drawn for prothrombin assay, the animals were decapitated and the liver, kidney, heart, remaining viscera, and carcass were separated and frozen. In order to determine possible alterations in metabolism due to coumarin drugs, some deficient rats were fed 2 mg of Dicumarol at 8-hr intervals beginning 6 hr prior to injection of radioactive menadione. These studies were undertaken since menadione is a poor antidote for the anticoagulants (Lowenthal and Taylor, 1959; Douglas, 1962) and since it has been suggested that conversion of menadione into its active fat-soluble form may be inhibited by these drugs (Douglas, 1962).

Weighed portions of tissues were prepared for radioanalysis by digestion in Nuclear-Chicago Solubilizer (0.5 ml/50 mg) for 4–12 hr at 50°. The digested samples were dissolved in 10 ml of toluene-based scintillation cocktail containing 1% anhydrous methanol to minimize chemiluminescence.

Lipid extracts were prepared by grinding the remaining tissues with anhydrous sodium sulfate and thoroughly extracting the resulting mixture with ethyl ether. Preliminary experiments showed that more polar solvents such as chloroform–methanol (3:1) did not extract more radioactivity from the tissue.

TABLE III: Chromatographic Elution[a] of Reference Compounds and Lipid-Soluble Radioactivity from Rat Liver after Administration of Menadione-6,7-^{3}H.

	% Chromatographed		
Fraction[b]	Tritium[c]	Phyllo-quinone	Menadione
1			
2			
3		79	
4	32	19	
5	19		
6	1		
7	30		79
8	24		18
Total	106	98	97

[a] Chromatography on hydrated silicic acid according to Matschiner *et al.* (1967). [b] Fraction 1 was eluted with 15% benzene in Skellysolve B; fractions 2–6 were eluted with benzene concentrations increasing at 7.5% increments; fraction 7 was eluted with ether; fraction 8 with methanol. [c] Ether-soluble radioactivity extracted from liver as described in text.

Chromatographic separations were carried out on silicic acid (Matschiner *et al.*, 1967) on partition columns (Matschiner and Taggart, 1967), and on thin-layer plates containing silica gel untreated or impregnated with 1% silver nitrate (Matschiner and Amelotti, 1968).[7]

[7] The authors are indebted to Josephine Amelotti for performing the thin-layer separations described in this report.

TABLE IV: Thin-Layer Chromatograms[a] of a Mixture of Radioactive Metabolite and Menaquinone-4.

	Untreated Plate			1% $AgNO_3$	
Band[b]	Tritium (dpm)	MK-4 (μg)	Band[b]	Tritium (dpm)	MK-4 (μg)
Front			Front		
7					
6			5		
5	18,320	344	4		
4	310		3		
3					
2	100		2	3,380	101
1	120		1	8,300	145
Origin	200		Origin	130	

[a] Silica gel G (500 μ) with and without $AgNO_3$; developing solvent benzene–heptane (8:2, v/v). [b] The chromatograms between the origin and solvent front were separated into approximately equal bands. The corresponding silica gel was eluted and examined for the presence of radioactivity and recovered MK-4.

Results

The distribution of radioactivity in various tissues of the rat 18 hr after injection of menadione-6,7-^{3}H is shown in Table II along with urinary and fecal excretion for the same period. Lipid-extractable radioactivity for the same tissues is also shown in Table II. Most of the ^{3}H was present in urine indicating a rapid turnover and elimination of menadione under these conditions. The viscera included intestinal contents so that the contribution from bile and excretory products is included. Skeletal muscle (carcass) contained the next largest pool of radioactivity (nearly 10%) and liver contained approximately 1% of the administered dose. No difference in distribution or concentration of radioactivity was apparent in rats fed Dicumarol. Extraction of radioactivity from the kidneys of Dicumarol-treated rats was low; however, extraction from all other tissues was approximately 50% (range from 35 to 65% of tissue radioactivity). Prothrombin response to the injected menadione and the anticoagulant effect of the fed Dicumarol are shown in the footnote to Table II.

The lipid obtained from each tissue was chromatographed on silicic acid to separate alkylated forms of vitamin K from more polar metabolites. The results of a chromatogram of liver extract is shown in Table III along with the elution of reference compounds. Although a portion of the lipid-soluble radioactivity from each tissue had chromatographic properties on silicic acid resembling menadione, no menadione was detected on further examination of these fractions. The identity of this hydrophilic material has not been determined.

In order to examine the properties of the lipophilic metabolite more carefully, the residue from chromatographic fractions 3–5 from each tissue of one animal per group was purified further by reversed phase partition chromatography. The results of such a chromatogram of hepatic radioactivity containing carrier menaquinone-4[5] is shown in Figure 2. Those fractions containing MK-4 also contained 93% of the chromatographed radioactivity. No evidence of other isoprenologs of vitamin K was obtained in liver or in other tissues. The slight separation of isotope through the zone of elution of menaquinone-4 resembled the data obtained with menadione in earlier experiments.

TABLE V: Identification of Menaquinone-4.

	Sp Act. (dpm/μg)	
Chromatogram	Deficient	Deficient + Dicumarol
Partition	88	52
Thin-layer chromatography	111	53
Thin-layer chromatography with $AgNO_3$	100	48

Identification of menaquinone-4 in liver was supported by further purification of the vitamin on thin-layer chromatograms in the presence and absence of impregnating silver nitrate. The results with reference compounds are shown in Figure 3A,B. Plates without silver nitrate gave similar mobilities for phylloquinone and menaquinone-4 (Figure 3A). In the presence of silver nitrate, the mobility of menaquinone-4 is retarded (Figure 3B). Throughout these separations the radioactive metabolite had chromatographic properties resembling the added menaquinone-4 (Table IV). These data add further support to the identification of menaquinone-4 as the fat-soluble form of vitamin K resulting after the administration of menadione (Martius and

[5] Synthetic menaquinone-4 was donated by Hoffmann-LaRoche, Inc. through the generosity of Dr. O. Isler.

TABLE VI: Distribution of Menaquinone-4 in Various Tissues 18 hr after Administration of Menadione-6, 7-^{3}H.

Tissue	Menaquinone-4 (pmoles/g)	
	Deficient	Deficient + Dicumarol
Liver	9.9[a]	3.8
Heart	15.7	0.8
Kidneys	48.4	8.2
Carcass	7.3	7.6
Viscera	2.4	1.1

[a] These data, expressed as picomoles per gram of tissue, were calculated on the assumption that the specific activity of MK-4 present in tissue was the same as that of the administered menadione.

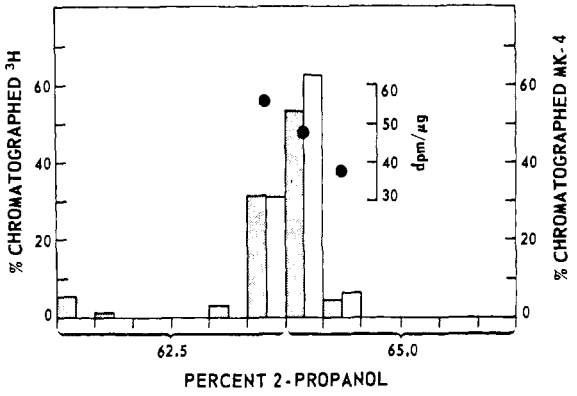

FIGURE 2: Chromatographic identification of menaquinone-4 from rat liver after the administration of menadione-6, 7-^{3}H. The sample was mixed with authentic MK-4 and chromatographed on a reversed-phase column (stationary phase, hexane; movable phase, mixtures of 2-propanol in water containing 5% acetic acid). Recovered vitamin is shown by the open bars, recovered radioactivity by the shaded bars and the specific activity of eluted vitamin (disintegrations per minute per microgram) by the dark circles.

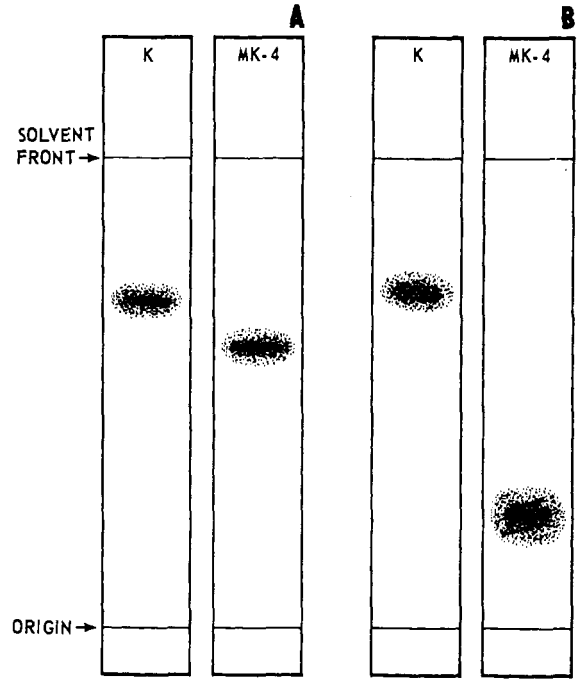

FIGURE 3: Thin-layer chromatograms of phylloquinone (K) and menaquinone-4 (MK-4) on silica gel G in the absence (A) and presence (B) of 1% $AgNO_3$. Developing solvent was benzene–heptane (8:2, v/v). The depicted spots were made visible by heating the developed plate after spraying with concentrated H_2SO_4.

Esser, 1958; Griminger and Brubacher, 1966; Horth *et al.*, 1966). A summary of the identification based on the specific activities through each step of the purification procedure is shown in Table V.

The distribution of menaquinone-4 in tissues following the administration of menadione is shown in Table VI. These values were determined after extraction and silicic acid chromatography followed by partition chromatography of appropriate fractions from each tissue of at least one animal in each experimental group. At this point in the investigation, marked differences in the metabolism of menadione in rats fed Dicumarol were observed. Although menaquinone-4 was recovered from each of the tissues examined, the level of vitamin was lower in nearly all tissues from animals fed the anticoagulant drug.

Discussion

In earlier studies Solvonuk *et al.* (1952) followed the distribution of radioactivity in mice and dogs following intramuscular injection of menadione-^{14}C with a specific activity of 4.1×10^4 cpm/mg. Detection of ^{14}C in tissues was difficult since the concentration of radioactivity was generally low. Radioactivity was rapidly excreted in the urine reaching a total of 30–40% of the administered dose. Taylor *et al.* (1957) using labeled menadione of slightly higher specific activity injected rats with 5 mg/kg intravenously and found low concentrations of radioactivity in various tissues 24 hr after administration of the labeled vitamin. Recovery was approximately 30%, with 0.8% in liver and 8% in skeletal muscle. In the present studies, menadione was administered intracardially at 30 μg/kg and was followed by high recovery in urine after 18 hr. Levels in liver and muscle were similar to those reported by Taylor and coworkers (1957).

Further work on the metabolism of menadione followed the synthesis of several homologs of phylloquinone and menaquinone by Isler *et al.* (1958). Martius and Esser (1958) discovered the lipophilic metabolite, menaquinone-4, in rats and chicks following the intraperitoneal administration of several hundred micrograms of labeled menadione. Menaquinone-4 became identified as a ubiquitous form of vitamin K in animals resulting not only from the metabolism of menadione

but also from alkylated forms of vitamin K as well (Billeter and Martius, 1961). While our studies were in progress, supporting evidence of the formation of menaquinone-4 from menadione was presented by Griminger and Brubacher (1966) and by Horth and coworkers (1966).

The discovery by Martius of a lipophilic metabolite of menadione strengthened the early view that the biological activity of menadione is due to its conversion into a fat-soluble form. The present study indicates that the amount of menaquinone-4 present in the liver 18 hr after an effective dose of menadione is very small, approximately 100 pmoles. Furthermore, earlier data (Matschiner and Taggart, 1968) showed that prothrombin response following this dose of menadione in the rat continued for at least 54 hr. The estimate of effective hepatic concentration of menaquinone-4 made from these data (<10 pmoles/g) is much smaller than the minimum normalizing dose of MK-4 determined by intracardial administration to depleted adult male rats (35 μg; Matschiner and Taggart, 1968). Since most of the administered MK-4 appears rapidly in the liver (Gloor and Wiss, 1966) these data suggest that menaquinone-4 synthesized in the liver from menadione may be more efficiently used in maintaining prothrombin concentration than the same vitamin provided from external sources. Further studies are presently under way to elucidate the relationship between hepatic occurrence of vitamin K and concentration of coagulation proteins. A preliminary report of these studies has already appeared (Taggart, 1968).

The present data do not distinguish between several possible explanations for the effect of Dicumarol on the concentration of menaquinone-4: (1) a decrease in the amount of menadione available for alkylation, (2) inhibition of the alkylation reaction, and (3) alteration of the metabolism of menaquinone-4 following its formation. In any event, present data do not indicate that the depression of menaquinone-4 in rats fed Dicumarol is the basis for the failure of menadione to act as an effective antidote against the coumarin drugs. Rather the data are consistent with Griminger's view (Griminger, 1965) (also expressed by Martius, 1967) that menadione is ineffective under conditions of stress because it is normally converted into menaquinone-4 only in limited amounts and cannot provide enough active vitamin to meet increased requirements.

The use of highly labeled vitamin K coupled with improved techniques for the analysis of tissues has provided an opportunity for a number of new studies. The first experiments reported here confirm the localization of menaquinone-4 in every tissue studied following the administration of menadione and give a quantitative estimate of its occurrence. These experiments also provide preliminary data on the correlation of biological response to the concentration of hepatic vitamin K. Subsequent experiments utilizing higher specific activities and extending these studies to fat-soluble forms of the vitamin are under way (Taggart, 1968; Bell *et al.*, 1968).

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