The Vitamin K Content of Beef Liver. Detection of a New Form of Vitamin K*

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ABSTRACT: Preparative extraction of raw beef liver with acetone yielded up to 70% of the vitamin K detected in the original tissue by chick bioassay. Some evidence was obtained of unstable forms of vitamin K in pig and rabbit liver. The fat-soluble vitamin K extracted from beef liver was concentrated by chromatography on hydrated silicic acid to a fraction containing biological activity equivalent to about 1 mg of phylloquinone per g of lipid. Further chromatography of this concentrate on a reversed-phase partition column showed the presence of several forms of vitamin K. Most of the vitamin recovered from beef liver was more lipophilic than menaquinone-10 and gave an ultraviolet absorption spectrum resembling that of the phylloquinone, menaquinone, or partially hydrogenated menaquinone series.

After the characterization of vitamin K from plant and bacterial sources in 1939, there followed a period of intense interest in the structural specificity of vitamin K and the development of synthetic analogs for clinical use. These studies were stimulated in part by the dramatic curative effect of this vitamin in several hemorrhagic conditions. Despite clinical interest, the intervening years yielded little substantial information concerning the biochemistry and nutrition of vitamin K. After more than 25 years of study, the nutritional sources and requirements of the vitamin in mammalian species are unknown or ill defined.

The best evidence available of the structure of vitamin K in animal tissue was provided by Martius (1961a,b) who identified menaquinone-4 as a metabolite of radioactive menadione, phylloquinone, and even other isoprenologs of menaquinone.[1] More recently, Billeter *et al.* (1964) reported that ingested vitamin K is metabolized by intestinal bacteria to menadione which is absorbed and uniformly alkylated at C-3 by an isoprenoic side chain of 20 carbon atoms.

Despite these metabolic studies, the forms of vitamin K in animal tissue have not been isolated for chemical or spectroscopic identification. Dam's early studies on the chemical nature of vitamin K were conducted on hog liver (Dam and Schønheyder, 1936), but this tissue was soon replaced by richer sources of the vitamin. Later, Green *et al.* (1956) fractionated beef liver cells and determined by chick bioassay that this tissue contains about 0.7 μg of menadione activity per gram. They did not detect the vitamin chemically. While our studies were in progress Lev and Milford (1966) presented evidence that pig liver contains a water-soluble factor with vitamin K activity. This factor was destroyed by heat and certain hydrolytic enzymes and was not dissolved by lipid solvents.

Our interest in vitamin K arose during nutritional studies in rats fed irradiated beef (Matschiner and Doisy, 1966b). Lack of information concerning vitamin K necessitated the exploration of several associated problems. The initial phase of a study directed toward the isolation and characterization of vitamin K from animal tissue is described in this report.

Materials and Methods

Samples of fresh liver of the best available grade were obtained from local suppliers. They were either processed immediately or cooked in an autoclave at 120° for 20 min. Solvents were distilled before use and all extracts were prepared in subdued light. The vitamin K content of tissues, extracts, and chromatographic fractions was determined by chick bioassay (Matschiner and Doisy, 1966a) and is expressed as phylloquinone. Groups of ten chicks were used for each assay; the standard error of each determination is approximately 10% of the mean.

Chromatographic separation of extracted lipids was accomplished on silicic acid and finally on reversed-phase partition columns already described (Matschiner and Taggart, 1967). A sample of radioactive phylloquinone (Michael and Elliott, 1961) used as a marker in these studies was generously donated by Dr. W. H. Elliott. Samples of synthetic menaquinone were a gift from Dr. O. Isler. Menaquinone-9(H) was donated by Dr. M. Weber and Dr. C. Coscia. Radioassays were carried out in a Packard Model 314 liquid scintillation spectrometer and absorption spectra were determined with a Cary Model 14 spectrophotometer.

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[1] Reference to specific forms of vitamin K is made in accordance with the recommendation of the Nomenclature Commission of the IUPAC–IUB (*J. Biol. Chem. 241*, 2989 (1966)). The K_2 vitamins are menaquinones, abbreviated MK, and vitamin K_1 is phylloquinone, abbreviated K.

Results

Bioassay and Extraction of Vitamin K. Initial data on the bioassay and extraction of vitamin K were obtained with liver from several species chosen for their availability and relatively high concentrations of the vitamin (Table I). Earlier studies with ground beef had shown that extraction was facilitated by prior cooking of the meat, but in two of the three samples of liver examined here the activity of vitamin K was diminished by cooking. The accuracy of whole tissue assays was determined by the addition of phylloquinone to diets containing raw and cooked pig liver. In both instances, the added vitamin was detected within the standard error of the assay. Since beef liver was readily available and generally contained the highest concentration of vitamin K, subsequent studies were conducted with this tissue.

Several conditions were evaluated for extraction of the vitamin, but for safety and efficiency in handling large amounts of tissue, acetone appeared to be the most appropriate solvent. After initial dehydration with ethanol and exhaustive extraction with acetone, recovery of the vitamin was low (Table II). Since less than 10% of the original activity was present in other fractions, the low recovery indicated that the vitamin had been lost rather than ineffectively extracted. Sonication or cooking prior to extraction did not have a significant effect on the amount of vitamin recovered.

In later experiments, alcohol–ether (3:1) and ether were used to extract whole tissue and tissue dehydrated with sodium sulfate, respectively (Table III); however, these procedures also yielded low recoveries of vitamin K. In these experiments lyophilized tissue was also assayed and found to contain approximately the same amount of vitamin recovered in extracts of whole tissue. This difference in apparent vitamin K content between whole tissue and lyophilized tissue was confirmed in other experiments at which time lyophilized tissue was also extracted and found to yield about the same amount of vitamin K recovered by extraction of whole tissue.

An evaluation of these and other data led us to the preparative experiments shown in Table IV. The recovery of vitamin K was better from cooked than from raw tissue; however, the final concentration of vitamin K in extracted lipid was similar in both procedures. The concentration of vitamin K was higher in later extracts, indicating that the vitamin was extracted less effectively than other lipids.

Chromatographic Separations. All chromatographic studies on beef liver fat reported here were carried out on aliquots of a sample of lipid extracted from 250 lb of raw tissue essentially as described in Table IV. The concentration of vitamin K in this lipid was approximately 10 μg/g expressed as phylloquinone. Preliminary concentration of the vitamin by molecular distillation or enzymatic hydrolysis of the glycerides was attempted without practical success. Finally, a large adsorption chromatogram was designed to separate the crude lipid. Sharp elution and good recovery of vitamin K

TABLE I: Bioassay of Vitamin K in Raw and Cooked Liver from Several Species.

Tissue	Vitamin K[a] (μg/g)	Tissue	Vitamin K[a] (μg/g)
Pig liver		Rabbit liver	
Raw	0.24	Raw	0.28
Cooked	0.09	Cooked	0.14
Pig liver + K[b]		Beef liver	
Raw	0.67	Raw	0.45
Cooked	0.50	Cooked	0.46

[a] Determined by bioassay and expressed as micrograms of phylloquinone per gram of liver wet weight. [b] Phylloquinone added at 0.33 μg/g of diet; calculated total = 0.57 and 0.42 μg/g of raw and cooked liver, respectively.

TABLE II: Extraction of Vitamin K from Beef Liver with Acetone.

Tissue	Vitamin K[a] (μg/g) Whole Tissue	Alcohol Extract	Acetone Extract	Residue
Raw	0.85	0[b]	0.36	0[b]
Sonicated	0.95	0	0.33	0
Cooked	0.79	0	0.21	0

[a] Determined by bioassay and expressed as micrograms of phylloquinone per gram of liver wet weight. [b] No activity detected; less than 10% of the original vitamin could have been present.

TABLE III: Extraction of Vitamin K from Beef Liver with Ether.

	Vitamin K[a] (μg/g)
Whole tissue	
Raw liver	0.81
Lyophilized liver	0.42
Extracts of raw liver	
Alcohol–ether (3:1)	0.46
Na_2SO_4–ether	0.38

[a] Determined by bioassay and expressed as micrograms of phylloquinone per gram of liver wet weight.

TABLE IV: Preparative Extraction of Vitamin K from Beef Liver.

Solvent[a]	Cooked Liver (10.3 kg, 5450 μg of K)[b]		Raw Liver (22.0 kg, 13000 μg of K)[b]	
	Lipid (g)	Vitamin K (μg)	Lipid (g)	Vitamin K (μg)
Alcohol	—	None	—	None
Acetone	159	400	146	548
Acetone (2)	151	3000	331	3900
Acetone (2)	17	380	74	1480
Hexane	9	100	—	—
Solids		None		792
Total	336	3880 (71%)	551	6720 (52%)
Concentration		11.5 (μg/g)		10.7 (μg/g)

[a] Whole tissue was dehydrated with an equal volume of 95% ethanol and thoroughly extracted with acetone. The number of extracts if more than one are given in parentheses. [b] Activity expressed as phylloquinone.

TABLE V: Chromatography of Beef Liver Lipid on Hydrated Silicic Acid.[a]

Fraction[b]	Column A		Column B	
	Residue (mg)	Vitamin K[c] (μg)	Residue (mg)	Vitamin K[c] (μg)
1	147	None	136	
2	640	None	627	
3	511	290	457	500
4	65		122	
5	—	170	13,200	None
6	24,000		14,000	35
Total		460 (92%)		535 (105%)

[a] Silicic acid AR, 100 mesh, Mallinckrodt Chemicals, St. Louis. Approximately 50 g of lipid on 1250 g of adsorbent; column dimensions 85 × 420 mm. [b] Fraction 1 was eluted with 15% benzene in Skellysolve B, fractions 2, 3, and 4 were eluted with 22.5, 30, and 37.5% benzene, respectively, fraction 5 was eluted with benzene (column B only), and fraction 6 with ethyl ether. [c] Determined by bioassay and expressed as phylloquinone.

were obtained on silicic acid containing about 8% of added water. The results of two early chromatograms are shown in Table V. Under these conditions most of the vitamin was eluted in fractions 3 and 4 before elution of the triglycerides. It was contaminated with part of the residue containing sterol esters. A small amount of vitamin K was retained by the column and eluted with ether. Recovery of chromatographed activity was essentially complete.

In later experiments sieve-graded silicic acids were used. These were helpful since different batches of "100 mesh" silicic acid (Table V) contained varying amounts of fines which caused significant differences in flow rate and in the elution of vitamin K. Results obtained with 100–200 and 200–325 mesh are shown in Figure 1. In these experiments, radioactive phylloquinone was used to estimate the recovery of naphthoquinone. The vitamin was recovered similarly from both adsorbents but on the basis of earlier elution of vitamin K and higher flow rate of eluting solvent 100–200 mesh silicic acid has been used in all subsequent experiments.

The initial chromatographic step on silicic acid was designed to recover vitamin K in a minimum amount of solvent with maximum separation from the large amounts of contaminating lipid which were present. For this purpose it was practical to collect only fractions 3 and 4 for further study since later fractions contained prohibitive amounts of lipid. Figure 2 shows results obtained with pure quinones in separate chromatograms and indicates that some of these quinones may not be recovered in fractions 3 and 4 during chromatog-

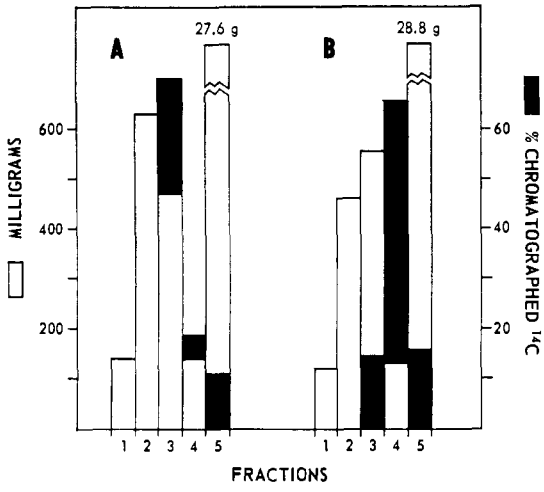

FIGURE 1: Chromatography of beef liver lipids on 100–200 (A) and 200–325 mesh (B) silicic acid (SilicAR CC-4, Mallinckrodt Chemicals, St. Louis) containing 8% of added water. Fifty grams of lipid on 1250 g of adsorbent; column dimensions 85 × 480 mm. The composition of eluting solvents was as described in Table V except that fraction 5 was recovered with acetone. The recovery of added radioactive phylloquinone (4.9×10^5 dpm) is indicated by the dark bars. Open bars indicate milligrams of eluted lipid.

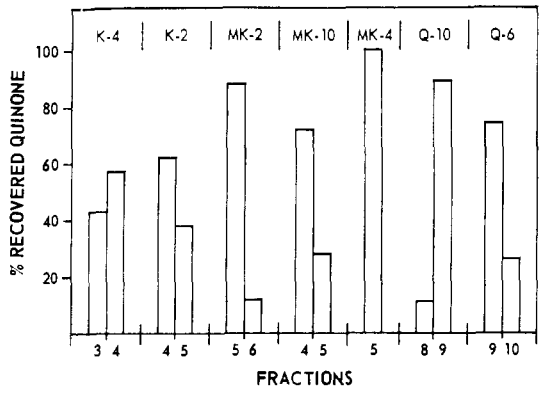

FIGURE 2: Elution of naphtho- and ubiquinones from hydrated silicic acid (SilicAR CC-4, 100–200 mesh). Column dimensions 22 × 130 mm containing 25 g of adsorbent. Approximately 1 mg of quinone was applied to each column. The composition of eluting solvents was as follows: fractions 3, 4, 5, 6, and 8, 30.0, 37.5, 45.0, 52.5, and 67.5% benzene in Skellysolve B, respectively; fraction 9, benzene; and fraction 10, acetone.

raphy of the crude lipid. The ubiquinones are cleanly separated from the naphthoquinones, and among the naphthoquinones studied all were eluted slightly later than phylloquinone. Although nearly all of the natural vitamin from beef liver was recovered in fractions 3 and 4 (Table V), the identification of vitamin K in

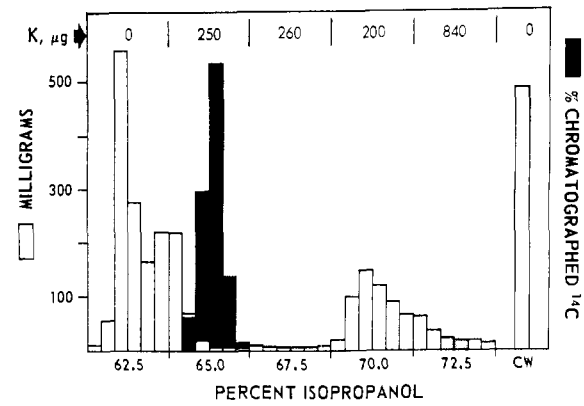

FIGURE 3: Partition chromatography of vitamin K and associated lipids from beef liver. Column dimensions 63 × 330 mm. The recovery of radioactive phylloquinone is shown by the dark bars and eluted lipids are shown by the open bars. The shaded bars indicate those fractions in which spectral evidence of naphthoquinone was obtained. The amount of vitamin K determined by bioassay in each group of six fractions and in the column wash (CW) is expressed as phylloquinone across the top of the figure (K, micrograms). The amount of vitamin shown eluted with 65% isopropyl alcohol has been corrected for the presence of radioactive K and indicates he presence of natural vitamin only.

these fractions must be evaluated in terms of the results shown in Figure 2, *i.e.*, the possible exclusion of some of the natural vitamin present.[2]

After chromatography on silicic acid, the vitamin K rich fractions (3 and 4) were separated on a reversed-phase partition column previously described (Matschiner and Taggart, 1967). The stationary phase was hexane and the movable phases were various mixtures of isopropyl alcohol, acetic acid, and water. The results are shown in Figure 3. Radioactive phylloquinone which had been eluted from silicic acid (Figure 1) continued to serve as an indicator of the effectiveness and reliability of the data obtained. The amount of biological activity recovered was determined by chick bioassay and the results of these assays are shown across the top of Figure 3. Vitamin K was detected in four separate regions of the chromatogram and most of the vitamin (840 μg) was eluted with movable phase containing 72.5% isopropyl alcohol. Although the lipid content of these fractions was low, the absorption of ultraviolet light by contaminating lipids obscured a recognizable spectrum of vitamin K in all but two fractions. These fractions are indicated by shading in Figure 3 and the spectrum of one of them is shown in Figure 4A.

[2] In seven recent chromatograms of beef liver fat of the proportions shown in Figure 1, a fifth fraction was eluted with 45% benzene in Skellysolve B. Each of these fractions contained 2.7 ± 0.3 g of lipid and had to be kept separate for subsequent studies.

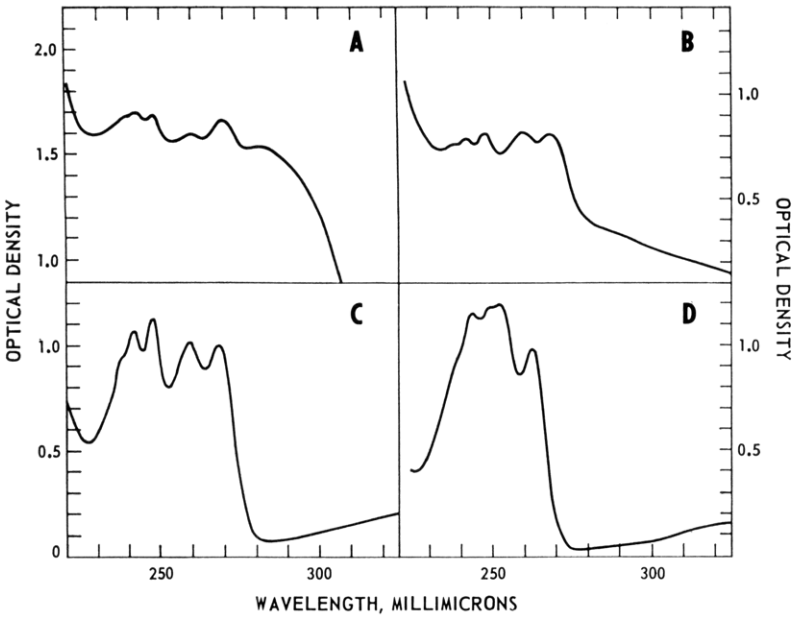

FIGURE 4: Ultraviolet absorption spectra in hexane of samples of naphthoquinone from beef liver (A and B) and of authentic samples of menaquinone (C) and menadione (D). See text for preparation of samples from beef liver.

The elution of vitamin K with 72.5% isopropyl alcohol was unexpected since all forms of the vitamin previously studied were less lipophilic. The relative mobility of this new vitamin was determined more precisely in subsequent experiments. The fractions eluted with 72.5% isopropyl alcohol (Figure 3) were combined and chromatographed on silicic acid as described above. The vitamin was eluted in fractions 3 and 4 and gave the spectrum shown in Figure 4B. Chromatography of the residue from these fractions on a partition column containing isooctane as the stationary phase gave the mobility shown in Figure 5. By comparison with the mobility of MK-9, MK-9(H), and MK-10 also shown in Figure 5 for the same solvent system, the naphthoquinone from beef liver is appreciably more lipophilic.

From earlier data with this partition system (Matschiner and Taggart, 1967) and from the data shown in Figures 4 and 5 some evidence of the structure of the new vitamin may be obtained. According to existing data with the chromatographic system, each isoprene unit contributes about four fractions to the volume required for elution of the vitamin, and each saturated double bond delays elution by approximately an additional fraction. If the new vitamin is a 2-methyl-3-multiprenylnaphthoquinone, its chromatographic mobility suggests that the side chain contains 11 or 12 isoprene units. If the side chain is saturated this estimate may range down to nine isoprene units. It must be emphasized that the elution of naphthoquinone from beef liver shown in Figure 5 is also consistent with the presence of two or more closely associated forms of vitamin K. By comparison with the spectra of other naphthoquinones (*e.g.*, Figure 4C,D) the new vitamin is probably a 2,3-disubstituted naphthoquinone with a conjugated double bond system identical with the phylloquinone, menaquinone, or partially hydrogenated menaquinone series.

The total recovery of vitamin K from all fractions shown in Figure 3 was 1550 μg, which is 77% of the biological activity originally present in the crude lipid used for these studies. More than one-half of this

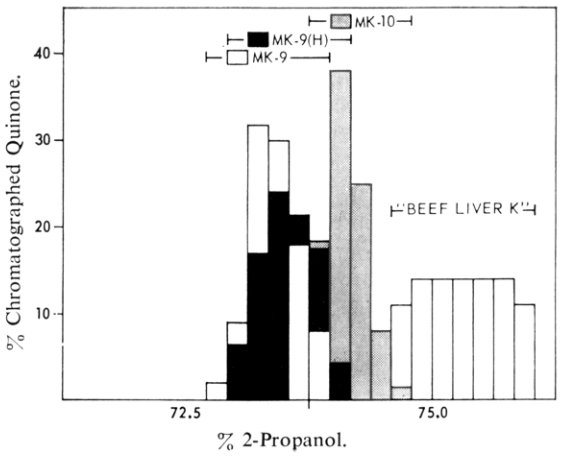

FIGURE 5: Elution of authentic samples of vitamin K and a sample of highly lipophilic naphthoquinone from beef liver. This figure is a composite of the results of individual columns as described in the text. The elution of MK-9(H) and MK-10 is shown in dark and shaded bars, respectively, to show their overlap with the elution of MK-9 and the quinone from beef liver.

activity was present in the lipophilic naphthoquinone described above. Studies on other fractions which contain vitamin K have not been completed, but menaquinone-4 has not been detected in any fraction studied thus far.[3]

Discussion

There is little precedent on which to design a single inclusive approach to the isolation of vitamin K from animal tissue. Dam's studies on pig liver fat early confirmed that the fat-soluble form of vitamin K is heat stable and resistant to oxidation even by ether peroxides; however, Lev and Milford (1966) recently discovered a water-soluble form of vitamin K in pig liver which could be destroyed by heat and enzymes. In the studies reported here, vitamin K in beef liver clearly did not diminish on heating, but the activity of vitamin K in pig liver and possibly in rabbit liver was lower in cooked tissue.

It is not clear whether the incomplete recovery of vitamin K detected by whole tissue assays is due to the presence of destructible forms of vitamin K or to a nutritional anomaly between the assay of whole tissues and extracts. Among possible explanations of this loss is the presence of menadione which would be sufficiently volatile to be depleted *in vacuo*. Recovery of menadione from beef liver would be unexpected since this quinone reacts rapidly and irreversibly with blood and tissue constituents (Canady and Roe, 1956; Taylor *et al.*, 1957), but the prospect of its natural occurrence has been resurrected by Martius who proposed that it is an intermediate in the metabolism of fat-soluble vitamin K. Support for the natural occurrence of menadione is also provided by the report of its isolation from a sample of crude lactulose (Glick *et al.*, 1959).

Despite the uncertainty regarding a small amount of the vitamin in beef liver, 50–70% of the original activity of the whole tissue was extracted in a stable, fat-soluble form and this provided the basis for continued studies on the characterization of vitamin K in animal tissue. The silicic acid column devised to make the initial separation returned most of the biological activity of the crude extract. Of the initial vitamin, 77% was carried through subsequent partition chromatography and identified in four separate regions of the column. More than one-half of the activity was accounted for by a naphthoquinone more lipophilic than menaquinone-10.

The detection of such a highly lipophilic form of vitamin K was unexpected but appears to have had a precedent in earlier metabolic experiments. Martius and co-workers (Martius and Esser, 1958; Schiefer and Martius, 1960; Stoffel and Martius, 1960) observed that a lipophilic metabolite of vitamin K resembling MK-10 was formed *in vitro*, but the identity of this substance was not clearly established and little attention has been paid to its occurrence. More recently Martius *et al.* (1965) found that a similar highly lipophilic metabolite was formed from menadione in invertebrates but only by the action of intestinal flora. They concluded that this metabolite is not formed in tissues. Their contention that menaquinone-4 is the only isomer of vitamin K formed in animal tissue leaves the conclusion in this study that the vitamin K content of bovine liver is determined more by dietary sources than by metabolic events. On this basis the vitamin discovered here may have originated in the rumen during bacterial digestion and been absorbed directly from the bowel. Further studies are underway to ascertain the structure of this vitamin and to determine the relationship between the occurrence of vitamin K and its functions in animal systems.

[3] Although menaquinone-4 may not occur as an endogenous component of beef liver, confirming evidence of its occurrence as a metabolite of menadione in the rat has been obtained (Taggart and Matschiner, Abstracts, 152nd National Meeting of the American Chemical Society, New York, N. Y., 1966).

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