# Characterization of vitamin K from bovine liver

JOHN T. MATSCHINER and JOSEPHINE M. AMELOTTI

Department of Biochemistry, St. Louis University School of Medicine, St. Louis, Missouri 63104

ABSTRACT Concentrated fractions of vitamin K from bovine liver were purified by thin-layer chromatography and fractions were analyzed by UV spectroscopy and mass spectrometry. The chromatographic behavior of the purified vitamins was compared with that of known compounds on thin layers of silica gel, either untreated or impregnated with silver nitrate or paraffin.

The principal forms of vitamin K recovered from bovine liver were highly lipophilic. Two fractions were obtained which collectively gave identifiable mass spectra of menaquinone-10, menaquinone-11, and menaquinone-12.

KEY WORDS vitamin K · bovine liver · mass spectrometry · lipophilic naphthoquinones · thin-layer chromatography · menaquinones-10, 11, 12

THE PREPARATION OF vitamin K from bovine liver reported recently (1) required large-scale chromatographic procedures to remove accompanying lipids from the small amounts of vitamin present in that tissue. On the basis of partition chromatograms and UV absorption spectra, the principal form of vitamin K appeared to be a highly lipophilic member of the vitamin K family, i.e., a 2,3-disubstituted 1,4-naphthoquinone more lipophilic than menaquinone-10. With relatively concentrated preparations of the vitamin at hand, efforts toward final purification have centered about the application of suitable thin-layer techniques, which have recently been reviewed by Sommer and Kofler (2). The present report is concerned with these studies and with the presentation of additional evidence regarding the structure and lipophilic character of vitamin K from bovine liver.

## MATERIALS AND METHODS

Thin-layer chromatographic plates were prepared by spreading a slurry of Silica Gel G–water 1:2. All plates were allowed to dry at room temperature. Plates impregnated with silver nitrate were prepared according to Morris (3). Paraffin-impregnated plates were obtained by dipping prepared plates in a tank containing 5% paraffin (mineral oil, extra heavy, U.S.P.) in hexane. Samples for preparative TLC were applied with a Radin-Pelick streaker (Applied Science Laboratories Inc., State College, Pa.) using either a 250 or 500 μl syringe. All plates were developed in a Sandwich chamber (Desaga); development time varied with the system but was generally between 30 min and 1 hr. Compounds were made visible by brief exposure to UV light if the plates were untreated or impregnated with paraffin, or by sprays of 2′, 7′-dichlorofluorescein (4) or of mixtures containing Rhodamine B (5) if the plates were impregnated with silver nitrate. Spots were photographed after charring with concentrated sulfuric acid at 110°C for 30 min. Alternatively, samples were recovered by elution from zones scraped from the plate (6) for further examination.

Concentrated samples of vitamin K from bovine liver were those obtained earlier by chromatography of portions of lipid extracted from 250 lb of tissue (1). Synthetic menaquinones were made available through the generosity of Dr. O. Isler (Hoffmann-La Roche, Inc., Basel, Switzerland). Menaquinone-9(H) was donated by Dr. M. Weber and Dr. C. Coscia (St. Louis University).

Absorption spectra were determined with a Cary model 14 spectrophotometer. Mass spectra were obtained on an LKB-9000 mass spectrometer with a direct inlet probe.

Abbreviations: TLC, thin-layer chromatography. Reference to specific forms of vitamin K is made in accordance with the recommendation of the Nomenclature Commission of the IUPAC–IUB (1966 *J. Biol. Chem.* 241: 2989). The $K_2$ vitamins are menaquinones, abbreviated MK, and vitamin $K_1$ is phylloquinone, abbreviated K-4.

TABLE 1 MOBILITY OF VARIOUS FORMS OF VITAMIN K IN SEVERAL THIN-LAYER SYSTEMS

| Plates* | Solvent | $R_f$ Values | | | | | |
|---|---|---|---|---|---|---|---|
| | | K-4 | MK-4 | MK-6 | MK-9 | MK-9(H) | MK-10 |
| Untreated | Heptane–benzene 1:1 | 0.43 | 0.35 | 0.39 | 0.46 | 0.46 | 0.45 |
| Untreated | Heptane–benzene 2:8 | 0.70 | 0.60 | 0.66 | 0.80 | 0.81 | 0.82 |
| Paraffin | Acetone–water 96:4 | 0.71 | 0.85 | 0.73 | 0.40 | 0.36 | 0.32 |
| 0.5% $AgNO_3$ | Benzene | — | 0.37 | 0.35 | 0.33 | 0.40 | 0.26 |
| 0.5% $AgNO_3$ | Heptane–benzene 2:8 | — | 0.25 | 0.22 | 0.21 | 0.29 | 0.19 |
| 1% $AgNO_3$ | Benzene | 0.94 | 0.33 | 0.25 | 0.13 | 0.25 | 0.12 |
| 1% $AgNO_3$ | Heptane–benzene 2:8 | 0.72 | 0.25 | 0.19 | 0.12 | 0.18 | 0.09 |

* Thin layers of Silica Gel G (500 $\mu$) treated as indicated.

## RESULTS AND DISCUSSION

### *Chromatography of Reference Compounds*

Data obtained by TLC of reference compounds are shown in Table 1. The effect of $AgNO_3$ on the mobility of the menaquinones shown here is greater than that reported by others (7, 8), presumably because of the different solvents used for development of the plates. The use of $AgNO_3$ is particularly valuable in studies with vitamin K since the degree of unsaturation is often an important characteristic distinguishing different forms of the vitamin.

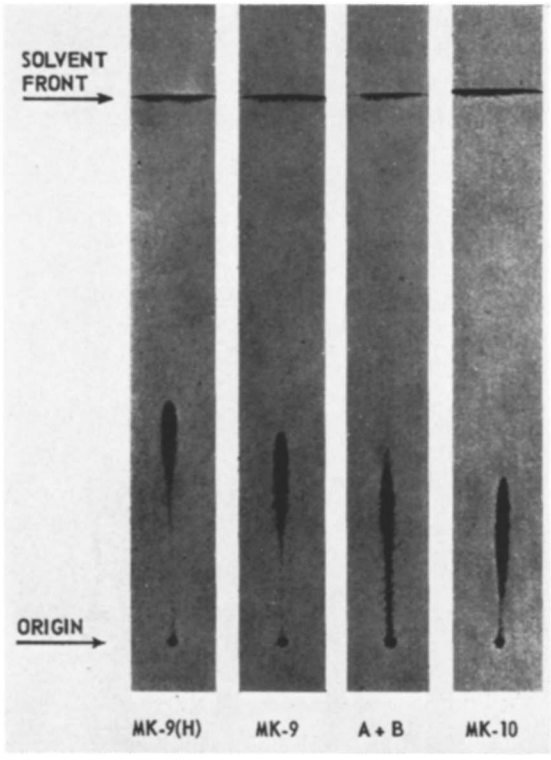


FIG. 1. Thin-layer chromatogram of reference compounds and of two fractions of vitamin K from bovine liver (A + B) on Silica Gel G impregnated with 0.5% $AgNO_3$. Developing solvent, benzene–heptane 4:1.

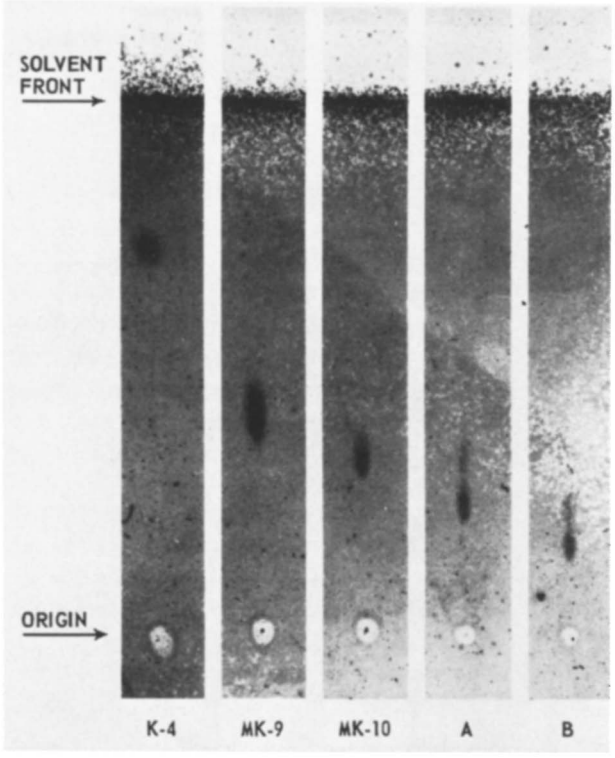


FIG. 2. Thin-layer chromatogram of reference compounds and two fractions of vitamin K from bovine liver (A + B) on Silica Gel G impregnated with paraffin. Developing solvent, acetone–water 96:4.

Recovery of vitamin K from thin layers is shown in Table 2. Menaquinones, particularly the higher isoprenologs, were recovered with difficulty from plates containing $AgNO_3$, but phylloquinone was easily recovered from these plates. Recovery of all compounds from other plates was satisfactory.

*Studies of Vitamin K from Bovine Liver*

Concentrated fractions of the principal form of vitamin K from liver (1) were purified on preparative plates (20 × 40 cm) containing 2-mm layers of adsorbent. Up to 1 g of lipid was separated on each plate. The vitamin was purified on untreated plates and then on plates containing $AgNO_3$ to give the product shown in Fig. 1. Further separation was accomplished on plates impregnated with paraffin. The chromatographic properties of these fractions, shown in Fig. 2, confirm the earlier estimate that the principal form of vitamin K in bovine liver is more lipophilic than any previously observed. In fact, both fractions of vitamin K obtained from paraffin plates (A and B, Fig. 2) were more lipophilic than menaquinone-10. The mobility of these vitamins on plates containing $AgNO_3$ would be expected from a menaquinone of high molecular weight and, therefore, a high degree of unsaturation. Finally, the UV absorption spectrum of purified vitamin K from bovine liver (Fig. 3) supported earlier evidence that it is a 2,3-disubstituted 1,4-naphthoquinone.

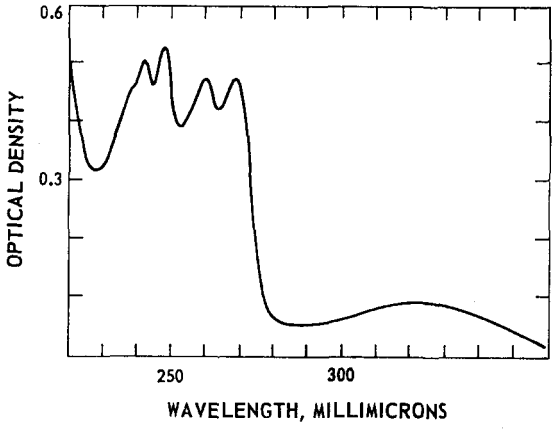


FIG. 3. UV absorption spectrum of purified vitamin K from bovine liver.

Based on these chromatographic and spectral data and the known forms of naturally occurring vitamin K, the structure most likely for the vitamins in samples A and B from bovine liver (Fig. 2) is that of a 2-methyl-3-multiprenyl-1,4-naphthoquinone. The mobility of these vitamins in the presence of $AgNO_3$ suggests that they may be partially hydrogenated, but mass spectral evidence does not support this view. Rather, preliminary data from the

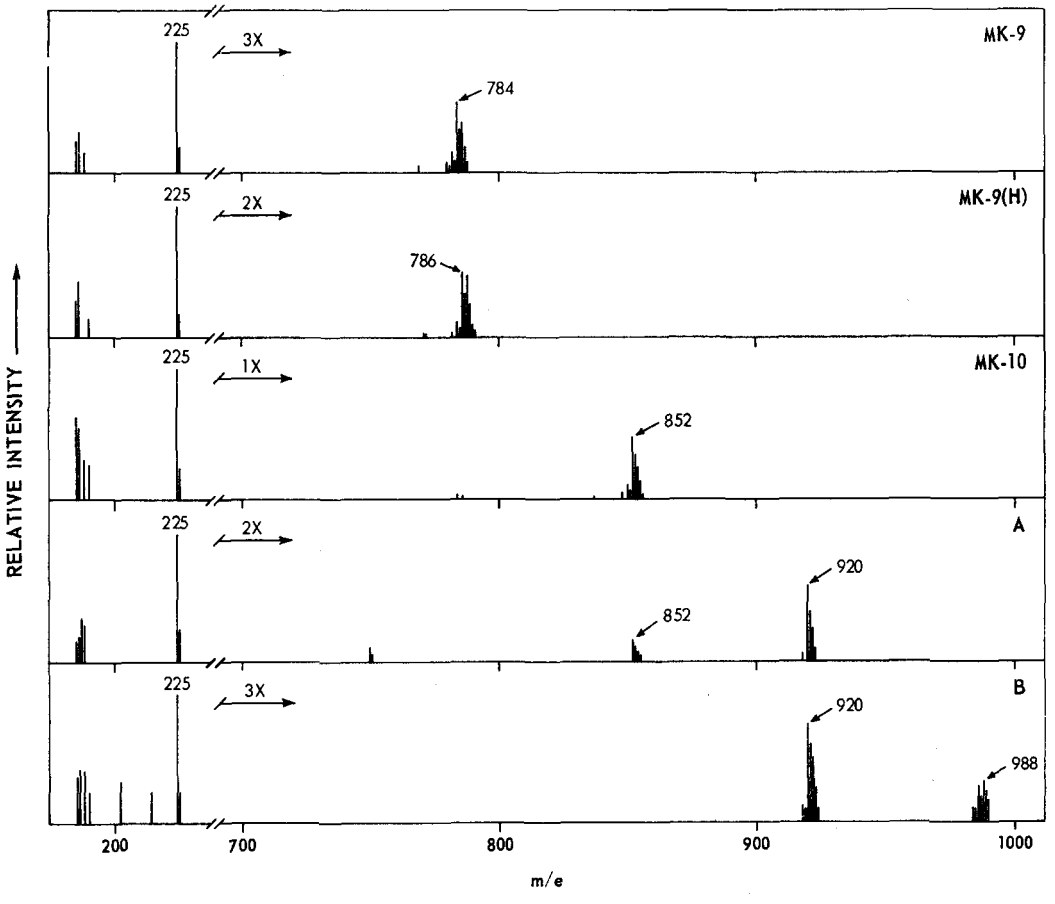


FIG. 4. Mass spectra of reference compounds and of two fractions of vitamin K from bovine liver (A + B). Accelerating voltage, 3.0 kv; ion source, 70 ev, 310°C.

TABLE 2 RECOVERY OF VITAMIN K FROM THIN-LAYER CHROMATOGRAMS

| Plate* | Eluting Solvent | Vitamin | Recovery† |
|---|---|---|---|
| | | | % |
| Untreated | Hexane–benzene 1:1 | K-4 | 94 (3) |
| | Hexane–benzene 1:1 | MK-4 | 91 (2) |
| 1% $AgNO_3$ | Ether | K-4 | 90 (2) |
| | Ether | MK-4 | 73 (2) |
| | Ether | MK-9(H) | 67 (3) |
| Paraffin | Acetone | K-4 | 90 (2) |
| | Acetone | MK-9(H) | 88 (2) |

* Thin layers of Silica Gel G (500 μ) treated as indicated.
† Average recovery; number of experiments shown in parentheses.

mass spectrometer (Fig. 4) indicate that *three* molecular species of vitamin K are present in samples A and B, namely menaquinone-10 (m/e 852), menaquinone-11 (m/e 920), and menaquinone = 12 (m/e 988).[1] The total amount of vitamin K in these fractions, based on UV absorption and expressed as MK-11, was about 160 μg.

In the study of bovine liver previously reported (1), several fractions of vitamin K were recovered. The fractions identified in this report contained more than half of the recovered vitamin. From other fractions we have partially purified two samples with the chromatographic mobilities of menaquinone-9 and menaquinone-9(H). Although vitamins with the mobility of lower isoprenologs are also present, bovine liver clearly contains vitamin K principally in highly lipophilic form. Efforts are now being made to relate these data to the function of vitamin K and to obtain the vitamin from other tissues in amounts adequate for satisfactory characterization.

We are indebted to Mr. Ronald Cambron for capable technical assistance.

This study was supported in part by grants AM 09909 and AM 09992 from the National Institutes of Health.

*Manuscript received 5 September 1967; accepted 24 October 1967.*

[1] For mass spectroscopic studies of vitamin K see Di Mari, Supple, and Rapoport (8). Mass spectra of higher isoprenologs of vitamin K have been reported by Beau, Azerad, and Lederer (9).

## REFERENCES

1. Matschiner, J. T., W. V. Taggart, and J. M. Amelotti. 1967. *Biochemistry*. **6:** 1243.
2. Sommer, P., and M. Kofler. 1966. *Vitamins Hormones*. **24:** 349.
3. Morris, L. J. 1963. *J. Lipid Res*. **4:** 357.
4. Malins, D. C., and H. K. Mangold. 1960. *J. Am. Oil Chemists' Soc*. **37:** 576.
5. Jones, D., D. E. Bowyer, G. A. Gresham, and A. N. Howard. 1966. *J. Chromatog*. **23:** 172.
6. Ruchelman, M. 1967. *J. Chem. Educ*. **44:** 110.
7. Griminger, P., and G. Brubacher. 1966. *Poultry Sci*. **45:** 512.
8. Di Mari, S. J., J. H. Supple, and H. Rapoport. 1966. *J. Am. Chem. Soc*. **88:** 1226.
9. Beau, S., R. Azerad, and E. Lederer. 1966. *Bull. Soc. Chim. Biol*. **48:** 569.