Separation and identification of menaquinones from microorganisms

PATRICK J. DUNPHY,* PHILIP G. PHILLIPS,‡ and ARNOLD F. BRODIE

Departments of Biochemistry and Microbiology, University of Southern California School of Medicine, Los Angeles, California 90033

ABSTRACT Simple thin-layer chromatographic procedures are outlined for the separation, isolation, and characterization of a complex of lipophilic naphthoquinones. Procedures are also described for the quantitative recovery of naphthoquinones from thin-layer plates. The general usefulness of the described methods is demonstrated by their application in the analysis of menaquinones from several microorganisms. The methods allow distinction between menaquinones varying in side-chain length, degree of saturation, and geometry, as well as in the presence or absence of ring methyl groups.

SUPPLEMENTARY KEY WORDS bacterial quinones · vitamin K_2 separation · TLC of bacterial quinones

THE NATURALLY OCCURRING menaquinones (MK's) comprise a very complex group of molecules in part consisting of the 2-methyl,3-alkyl,1,4-naphthoquinones, where the substitution at the C-3 position can contain 20–45 carbon atoms (4–9 isoprene units) (1). These isoprene units may show *all trans* or mono *cis* geometry (2) and may include various numbers of saturated isoprene double bonds (3). Other modifications such as hydroxylation (4) and perhaps even epoxidation (5) can be found. In addition to these fully substituted compounds, a number of 2-desmethyl compounds also occur naturally (6). The desmethyl naphthoquinones also display side-chain variation as seen in the fully substituted species.

Thin-layer methods have been described for the resolution of homologous series of menaquinones and ubiquinones and a quantitative method has been outlined for the latter group (7–9). It is the purpose of this communication to extend the simple thin-layer chromatographic procedures for the separation of menaquinones and, by a combination of the methods employed, to indicate the basic structural elements of any uncharacterized menaquinone with particular reference to chain length, degree of saturation, geometry, and ring substitution. The menaquinones of a number of different microorganisms have been characterized.

METHODS AND MATERIALS

Thin-layer Chromatography

As principal adsorbents, kieselgel G or kieselguhr G were used or modified by the addition of silver ions or liquid paraffin. Layers ranged in thickness from 250 to 500 μ. Kieselgel G layers were prepared using a water:adsorbent ratio of 2:1. For rhodamine 6G-impregnated layers, 30 mg of rhodamine 6G per 100 ml of solution was used. Activation was achieved by heating the plates at 100–110°C for 35–45 min. Ag^+-impregnated layers were prepared as above except that a solution of 5% aqueous $AgNO_3$ was used. For layers impregnated with Ag^+ and rhodamine 6G, it was found best to add a concentrated solution of the dye to the aqueous $AgNO_3$ solution; this resulted in a final concentration of 5% $AgNO_3$ and 30 mg of rhodamine 6G per 100 ml of solution. The Ag^+ layers were activated at 100–110°C for 40 min and stored thereafter in a dark, moisture-free container. This storage procedure maintained the stability of the plates for up to 1 month.

The incorporation of rhodamine in the above two types of layers permitted the easy visualization of naphthoquinones on the developed chromatograms. In visible light the quinones appeared as red spots on a

Abbreviations: TLC, thin-layer chromatography; MK, menaquinone.

* Present address: Unilever Research Laboratory, Colworth House, Sharnbrook, Bedfordshire, England.

‡ U.S. Public Health Service Predoctoral Fellow (Grant 1-F1-GM-40,893-01).

pink background, while under longwave UV illumination they appeared as purple spots on a yellow-green fluorescent background. For visualization on reversed-phase TLC, the plates were sprayed after development with a 0.1% solution of fluorescin in ethanol. Kieselguhr G plates, prepared as above, were activated at room temperature overnight, or at room temperature for 2 hr followed by 30–40 min at 100°C. For reversed-phase chromatography the plates were immersed in a 5% paraffin in petroleum ether solution, and the petroleum ether was allowed to evaporate. With all chromatographic methods used, the tanks were lined with filter paper to facilitate chamber saturation.

Solvent Systems

The main systems used were: (*a*) for adsorption layers, *n*-butyl ether–hexane 10:90 or equivalent diethyl ether–pentane mixtures; (*b*) for Ag^+-impregnated layers, methanol–benzene 5:95, or 2-butanone–hexane or acetone–pentane mixtures 10–20% (v/v) depending on the number of isoprene units in the side chain, e.g., for MK_4 10% of the ketone is satisfactory, but for MK_9 20% of the ketone is more suitable; (*c*) for reversed-phase layers, dimethyl formamide–water 97:3 or acetone–water (90–97%), and again, as with the Ag^+ layers, a lower percentage of acetone is more suitable for the shorter-chain menaquinones, while higher concentrations of acetone were used for longer-chain menaquinones. The reversed-phase solvent mixtures were saturated before use with liquid paraffin.

Preparative-layer Chromatography

Loading of layers depended on the complexity of the mixture to be resolved, although as a general rule adsorption plates (500 μ thick) accommodated up to 20 mg/plate (20 × 20 cm), and reversed-phase paraffin-impregnated layers (250 μ thick) accommodated not more than 4 mg/plate (20 × 20 cm). Compounds were visualized under UV light and recovered from the plates by scraping off the adsorbent and extracting it three times with diethyl ether (20 ml/g of adsorbent), acetone, or ethanol, depending on the adsorbent used and on the menaquinone in question (see Results).

Culture and Extraction of Bacteria

Mycobacterium phlei (ATCC 354) was grown according to Brodie and Gray (10). *Corynebacterium createnovorans* was grown by the procedure used for *M. phlei*. Neutral lipid from *Mycobacterium tuberculosis* $H_{37}R_a$ was a gift of Dr. Dexter Goldman, Veterans Administration Hospital, Madison, Wis. *Streptomyces* sp. was given by Calbiochem, Los Angeles, Calif. *Micrococcus lysodeikticus* was grown by the method of Beers (11). *Flavobacterium* sp. was a gift of Dr. G. Britton, Department of Biochemistry, University of Liverpool, Liverpool, England, and *Bacillus stearothermophilis* 1504-R was a gift of Dr. Howard Kuramitzu, Northwestern University Medical School, Chicago, Ill.

Naphthoquinones were isolated from the bacterial sources by extraction of the washed cells with acetone and subsequent partitioning of the acetone with pentene–water (2). The pentane-soluble lipids were chromatographed on silicic acid, acid-washed alumina, or Permutit, Folin, and eluted with 1–4% diethyl ether in pentane. Quantitative estimations were made from UV absorption maxima as previously described (2, 3).

Quinones in general are susceptible to strong acid or alkaline conditions (12). They are also quite rapidly photooxidized in the presence of oxygen and strong light (13). It is necessary to conduct the isolation and subsequent procedures as rapidly as possible, avoiding extremes of pH and sunlight. It is not necessary, however, to work in a nitrogen atmosphere or in very dim light as long as the procedures described are carried out as rapidly as possible. The quantitative recovery experiments (see Results) were carried out under normal laboratory conditions, and high recoveries were achieved.

All solvents used were redistilled before use. Acetone was dried over anhydrous potassium carbonate; diethyl and petroleum ethers were dried over sodium wire; and the diethyl ether was distilled over ferrous sulfate. Menaquinone standards were a gift of Dr. O. Isler, Hoffmann-LaRoche, Basel, Switzerland. Thin layer materials were obtained from Brinkmann Instruments, Westbury, N.Y. Naphthoquinones were named according to the tentative rules recommended by the IUPAC-IUB commission on biochemical nomenclature (14). Thus 2-methyl,3-nonaprenyl,1,4-naphthoquinone is called menaquinone-9 or MK_9.

RESULTS

Recovery of Naphthoquinones from Thin-layer Plates

Quantitative recovery studies were carried out with phylloquinone (vitamin K_1), MK_4, and MK_9 from adsorption, Ag^+, and reversed-phase TLC plates. Diethyl ether was used as the eluent. The results of the experiment are shown in Table 1. The low recovery of MK_9 from the Ag^+ layers was probably due to the strong complexing of the nine double bonds with Ag^+. Substitution of ethanol or acetone for diethyl ether resulted in an 83 and 93% recovery of MK_9, respectively. There was high recovery of all three quinones from the other adsorbents, and of MK_4 and vitamin K_1 from Ag^+ layers. Isolation of MK's from rhodamine-impregnated plates using acetone or ethanol led to the elution of the dye in addition to the

TABLE 1 RECOVERY OF MENAQUINONES FROM THIN LAYERS

Compound	Adsorption	Ag†	Reversed Phase
		% recovery*	
K_1	96	92	91
MK_4	97	94	90
MK_9	97	27	91

* Average of three determinations using 38–62 μg of quinone for each assay. Reproducibility was ±2%. The eluting solvent was diethyl ether.

quinone. Rhodamine was readily removed by partitioning the extract between petroleum ether and water. Using this procedure the quinone was retained in the hydrocarbon solvent while the rhodamine was retained in the aqueous phase. Paraffin from reversed-phase chromatography was removed from the samples by TLC on Silica Gel G in a diethyl ether–petroleum ether solvent system. By this procedure the paraffin hydrocarbons were separated from the quinones.

Behavior of Naphthoquinones on TLC

Adsorption TLC was capable of separating the quinones into different classes, each characterized by the presence of a specific functional group(s). The naphthoquinones that were examined consisted of four classes: the *all trans*, 2,3-disubstituted (class I), the 3′-methyl *cis* (class II), the 2-desmethyl (class III), and the 3′-hydroxy (class IV). Table 2 lists the R_F values of a number of naphthoquinones. It can be seen that the extent of separation achieved between individual classes depended on the solvent system used and that there was some resolution between the individual members of class I. Those class I homologues differing by more than three isoprene units were readily separated. The separation of the 3-methyl *cis* (group II) from the corresponding *trans* isomer (group I) was best achieved using *n*-butyl ether in hexane and less readily by diethyl ether–petroleum ether or benzene–hexane mixtures. The geometric isomers were not resolved by either Ag^+ or reversed-phase chromatography.

Ag^+ TLC. The ability of Ag^+ to form loose complexes with olefins has been known for some time (15). The formation of such complexes between unsaturated lipids and Ag^+ permitted the separation of compounds differing in their degrees of unsaturation. With the naphthoquinones, the Ag^+–olefin complexing results principally in the resolution of individual members of classes I, II, and III rather than the resolution between classes. In fact, equivalent members of class I and class II were not resolved. Fig. 1 shows a typical separation of menaquinones in a methyl ethyl ketone–hexane system. The homologous members of class I are readily separated from one another as were the individual

TABLE 2 ADSORPTION TLC OF MENAQUINONES

Compound	10% Butyl Ether in Hexane	25% Hexane in Benzene	Class
		R_F (× 100)	
trans-Phylloquinone	42	47	I
cis-Phylloquinone	51	47	II
Desmethyl phylloquinone	40	43	III
γ-OH Phylloquinone	0	1	IV
MK_3	35	38	I
MK_5	41	44	I
MK_7	46	48	I
trans-MK_9 (II-H)	51	53	I
cis-MK_9 (II-H)	57	53	II
Desmethyl MK_9	47	49	III

members of class II and class III. A decrease in the R_F value was observed with an increase in the number of isoprene units in the side chain. A plot of R_M (16) vs. number of isoprene units showed a linear relationship for MK_2 to MK_8, with a ΔR_M/isoprene unit loss of −0.11.

Using Ag^+ TLC, a number of interesting results were noted. MK_4 was readily separated from vitamin K_1 (MK_4 [6-H]), as was MK_9 (II-H) from MK_9 (mol wt 786 and 784, respectively). It can be seen that on saturation of one double bond a positive shift in R_F value is obtained (ΔR_M/double bond saturated = −0.22). Similar effects were demonstrated in the octaprenyl and lower isoprenologues. From the behavior of the menaquinones on Ag^+ TLC, it appeared that the determining factor in the resolution of these compounds was the number of double bonds in the side chain. It was possible to distinguish between the loss of one isoprene unit, e.g., MK_9 and MK_8, or the saturation of one double bond with no change in isoprene number, e.g., MK_9 and MK_9 (II-H). The difference in migration was utilized in the identification of a number of quinones of bacterial origin. Removal of the C-2 methyl group (class III) resulted in a small but significant change in migration pattern on Ag^+ TLC.

Reversed-phase TLC of a large group of menaquinones resulted in the resolution of compounds of classes I, III, and IV, but not between I and II (Fig. 2). This was similar to the pattern observed with Ag^+ TLC. Classes I and III, e.g., MK_9 and desmethyl MK_9, were better separated by reversed-phase TLC than by Ag^+ TLC, and the direction of separation in reversed-phase TLC was changed, i.e., on reversed-phase TLC desmethyl MK_9 had a higher R_F value than MK_9. As with Ag^+ TLC, fractionation was achieved within classes. The complete separation of compounds MK_2 to MK_{10} was possible. A plot of R_M vs. carbon number was linear for MK_2 to MK_9, the extent of separation

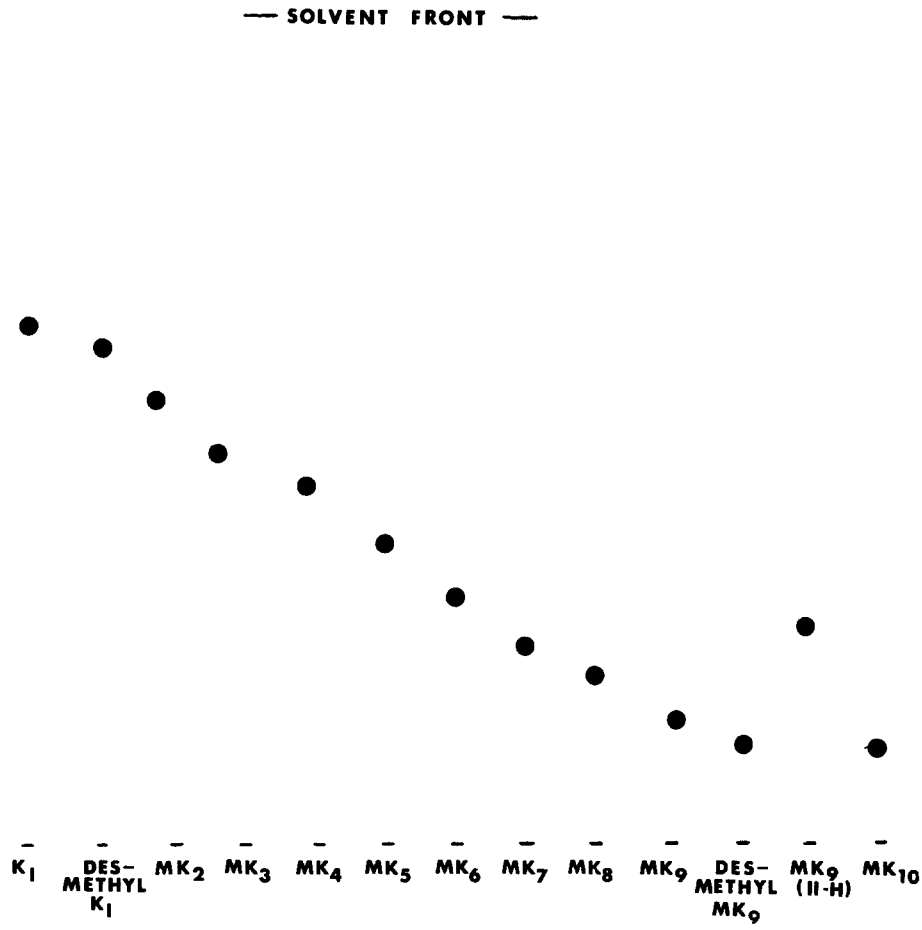

FIG. 1. Ag^+ TLC of menaquinones. Solvent system, 15% methyl ethyl ketone in hexane. 5 μg of each compound was applied to the plate.

of isoprenologues being somewhat greater than for Ag^+ TLC, i.e., ΔR_M/isoprene unit loss = -0.22.

The most significant difference between reversed-phase TLC and Ag^+ TLC was observed with the MK_n/MK_n (2-H) pair. Unlike Ag^+ TLC, where reduction of a double bond resulted in a positive shift in R_F value approximately equivalent to the loss of two isoprene units, on reversed-phase TLC such saturation resulted in a negative shift in R_F value equivalent to one-half of the effect of adding one isoprene unit. Thus, on reversed-phase TLC, saturation of a side-chain double bond resulted in a change of both magnitude and direction of R_F values as compared with those seen with Ag^+ TLC (Figs. 1 and 2). These differences reflected the major dependence on the number of carbon atoms in the side chain with the reversed-phase TLC system, while with Ag^+ TLC the major factor was the number of double bonds in the side chain.

The migratory behavior of the naphthoquinones can readily be used to determine structure. By a combination of Ag^+ and reversed-phase TLC it was thus possible to predict the extent of methylation, length of side chain, and degree of side-chain saturation in any group of isolated naphthoquinones. Thus, with a few suitable standard menaquinones it was possible to purify and determine the basic structure of isolated menaquinones without the use of physical methods such as mass spectrometry.

Menaquinone Analysis of a Number of Bacterial Systems

Using the above adsorption, Ag^+, and reversed-phase TLC procedures, the naphthoquinone components of a number of bacterial species were readily identified. In some cases the structural identification was verified by mass spectrometry (Table 3). The results of the analysis indicate that the menaquinone pattern in bacteria can be very complex. The organisms studied here contain only menaquinones of classes I and III. The methods of analysis employed for class I apply equally well to the other classes.

Analysis of the menaquinones in all but one microorganism proved to be simple when Ag^+ and reversed-

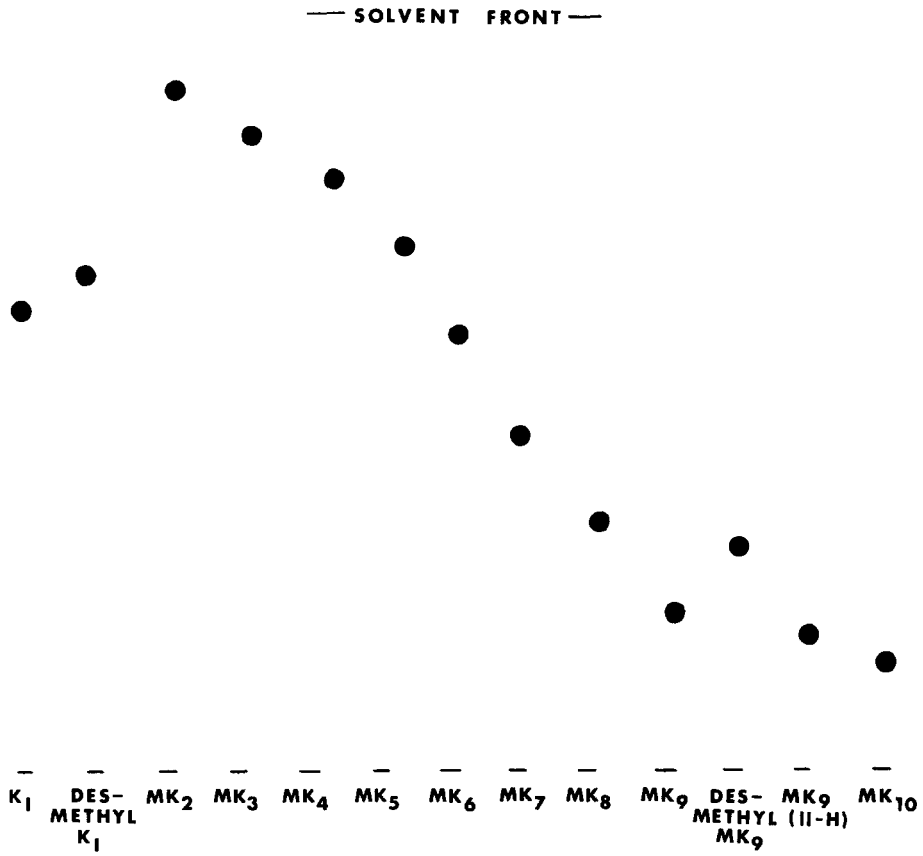

FIG. 2. Reversed-phase TLC of menaquinones. Solvent system, 93% acetone in water. 5 μg of each compound was applied to the plate.

phase TLC behavior was established. The compounds from the *Streptomyces* sp. were found to be of interest. Ultraviolet spectra of the five individual components from this organism confirmed that they were all of the 2,3-disubstituted, 1,4-naphthoquinone type (class I or II). The Ag^+ and reversed-phase TLC patterns of these quinones (Fig. 3) were somewhat unusual. The migrations of all five compounds were completely reversed on changing from Ag^+ to reversed-phase TLC. This pattern has already been clearly demonstrated with structures of the MK_n (X-H)/MK_n types. The migration of components *d* and *e* indicated that they were MK_9 (2-H) and MK_9, respectively. The migration of components *c*, *b*, and *a* on Ag^+ TLC and the small ΔR_F of these components on reversed-phase TLC were more consistent with menaquinones with 9 isoprene units, differing in the extent of side-chain unsaturation. The most plausible structure for *c* was MK_9 (4-H), for *b*, MK_9 (6-H), and for *a*, MK_9 (8-H). These proposals based on chromatographic considerations were verified by mass spectrometry. The five components from *e* to *a* were shown to have molecular weights of 784, 786, 788, 790, and 792 (3). These correspond to MK_9, MK_9 (2-H), MK_9 (4-H), MK_9 (6-H), and MK_9 (8-H), respectively. In addition to the five major compounds, small amounts of MK_8 (2-H) and MK_8 (4-H) were also found (3). Fig. 4 lists the relative mobilities of some of the compounds examined above. These data, in conjunction with adsorption TLC methods, provide a

TABLE 3 MENAQUINONE* COMPOSITION OF VARIOUS BACTERIA

Microorganisms	Menaquinone Present
Mycobacterium phlei ATCC 354	MK_8 (2-H), MK_9 (2-H)†‡
Corynebacterium createnovorans	MK_8 (2-H), MK_9 (2-H),† MK_{10} (2-H)
Mycobacterium tuberculosis $H_{37}R_a$	MK_7, MK_8, MK_9,‡ MK_8 (2-H), MK_9 (2-H)†‡
Streptomyces sp.	MK_9, MK_9 (2-H)‡, MK_9 (4-H),‡ MK_9 (6-H),†‡ MK_9 (8-H)‡
Flavobacterium sp.	MK_5, MK_6†‡
Bacillus stearothermophilis 1504-R	MK_7
Micrococcus lysodeikticus	MK_3,† MK_4,†‡ MK_5, MK_8, MK_8 (2-H)

* Resolution into classes I and II not attempted in order to simplify the analysis.
† Major component.
‡ Molecular weight verified by mass spectrometry.

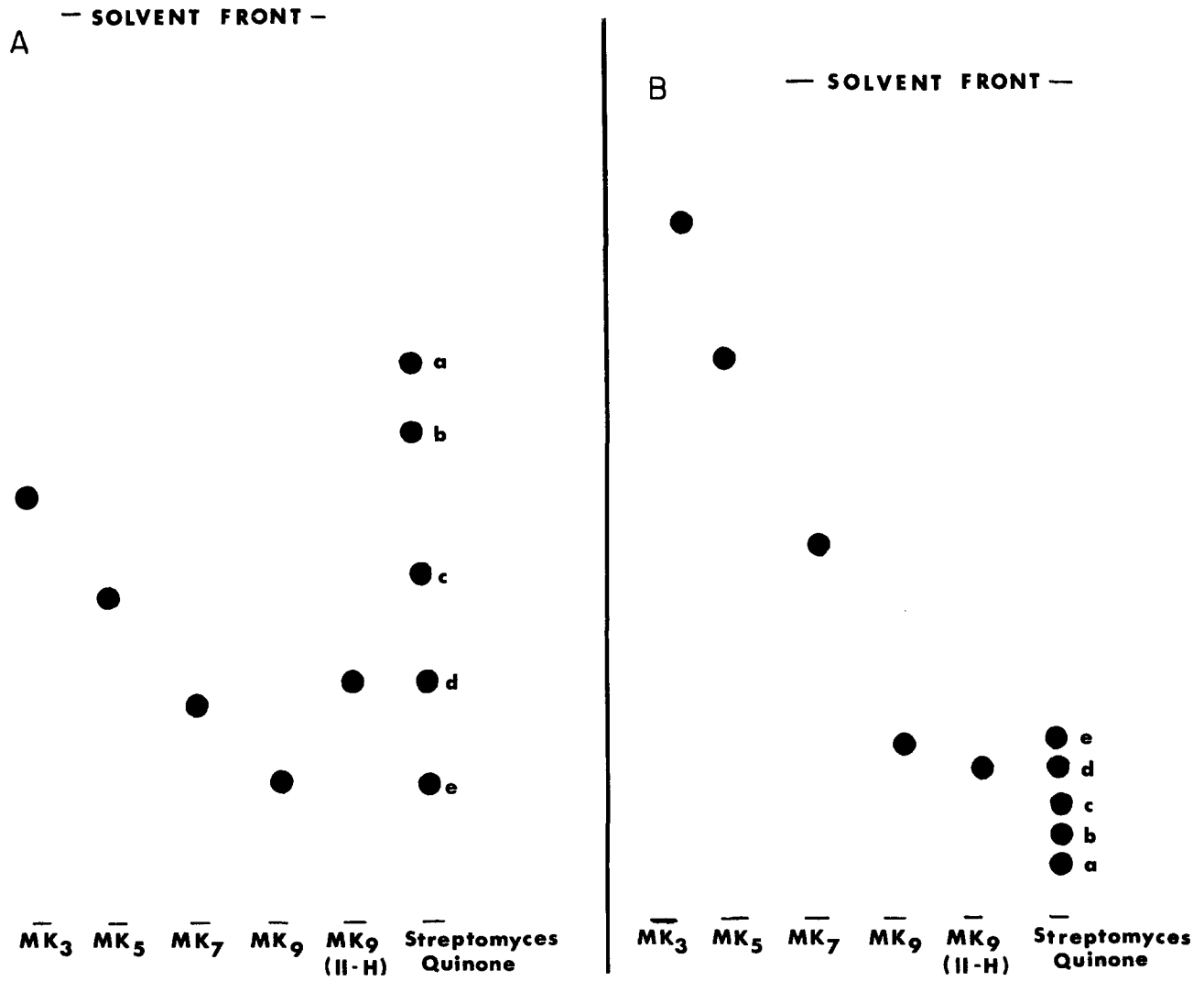

FIG. 3. Ag^+ and reversed-phase TLC of *Streptomyces* menaquinones. *A*, Ag^+ system, 15% acetone in hexane. *B*, reversed-phase system, 93% acetone in water. 5 μg of each marker and 25 μg of mixtures were applied to the plate.

simple method for the identification of an extensive group of naphthoquinones.

DISCUSSION

The methods described above provide a simple and unambiguous procedure of analyzing naphthoquinones. The methods permit identification and fractionation of such complexes, and isolation of individual components on a microgram or milligram basis. Recovery experiments indicated that the menaquinones were stable under the conditions described, provided that manipulations were rapidly carried out and exposure to direct sunlight and extremes of pH were avoided. Using suitable eluting solvents such as diethyl ether, acetone, or ethanol, almost quantitative amounts of quinones can be regained from all three chromatographic systems.

Other methods of analysis, particularly column methods using lipophilic Sephadex (LH-20) (17, 18), have achieved fractionation of isoprenologues within class I. These methods suffer from the disadvantage of the overlapping of components and the necessity of monitoring the column fractions. Lipophilic gel fractionation has so far been applied only to the separation of isoprenologues, and it remains to be seen if the separation of complex mixtures of the type found in *M. tuberculosis* or *Streptomyces* sp. can be achieved.

Fractionation and positive identification were readily achieved using the Ag^+ and reversed-phase TLC methods, and by reference to a scheme such as that shown in Fig. 4. With the use of standard menaquinone markers (MK_{1-10}, MK_9 [II-H], and desmethyl MK_9) it was possible to determine the chain length, degree of saturation, and extent of methylation of a series of naphthoquinones. Such methods might also be readily

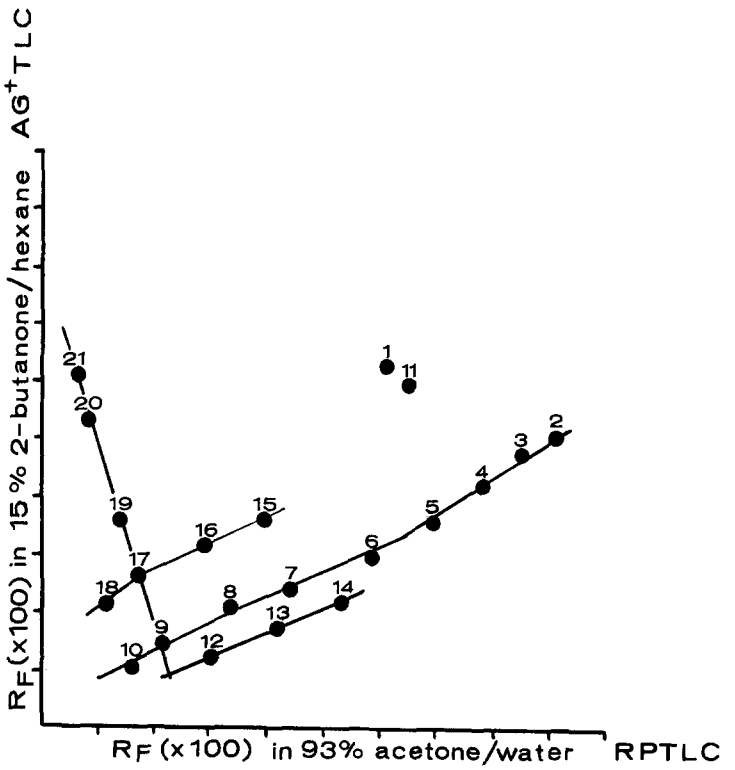

FIG. 4. Ag^+ vs. reversed-phase properties of menaquinones. 5 μg of each compound was applied to the plate. Key: *1*, K_1; *2*, MK_2; *3*, MK_3; *4*, MK_4; *5*, MK_5; *6*, MK_6; *7*, MK_7; *8*, MK_8; *9*, MK_9; *10*, MK_{10}; *11*, desmethyl (K_1); *12*, desmethyl (MK_9); *13*, desmethyl (MK_8); *14*, desmethyl (MK_7); *15*, MK_7 (II-H); *16*, MK_8 (II-H); *17*, MK_9 (II-H); *18*, MK_{10} (II-H); *19*, MK_9 (4-H); *20*, MK_9 (6-H); *21*, MK_9 (8-H).

extended for the study of other lipophilic quinones containing the same basic terpenoid structure.

We wish to acknowledge the technical assistance of Mrs. Hiroko Sakamoto and Miss Patricia Brodle.

This work was supported by grants from the National Institutes of Health (AI 05637), the National Science Foundation (GB 6257X), and the Hastings Foundation of the University of Southern California School of Medicine.

Manuscript received 25 September 1970; accepted 24 February 1971.

REFERENCES

1. Pennock, J. F. 1966. Occurrence of vitamins K and related quinones. *Vitamins Hormones*. **24:** 307–329.
2. Dunphy, P. J., D. L. Gutnick, P. G. Phillips, and A. F. Brodie. 1968. A new natural naphthoquinone in *Mycobacterium phei*. *Cis*-dihydromenaquinone-9, structure and function. *J. Biol. Chem.* **243:** 398–407.
3. Phillips, P. G., P. J. Dunphy, and A. F. Brodie. 1969. A new menaquinone series differing in the degree of unsaturation of the side chain. *Biochemistry*. **8:** 2856–2186.
4. Allen, C. F., H. Franke, and O. Hirayama. 1967. Identification of a plastoquinone and two naphthoquinones in *Anacystis nidulans* by NMR and mass spectroscopy. *Biochem. Biophys. Res. Commun.* **26:** 562–568.
5. Friis, P., G. D. Daves, Jr. and K. Folkers. 1967. New epoxyubiquinones. *Biochemistry*. **6:** 3618–3624.
6. Dolin, M. I., and R. H. Baum. 1965. Evidence for thioether formation between 2-solanesyl-1,4-naphthoquinone and 2-mercaptoethanol. *Biochem. Biophys. Res. Commun.* **18:** 202–205.
7. Wagner, H., L. Hörhammer, and B. Dengler. 1962. Thin layer chromatography of ubiquinones. *J. Chromatogr.* **7:** 211–215.
8. Bolliger, H. R., and A. König. 1969. Thin Layer Chromatography. 2nd ed. Springer-Verlag, Berlin, Heidelberg, and New York. 253–302.
9. Beau, S., R. Azerad, and E. Lederer. 1966. Isolation and characterization of dihydromenaquinones from myco and corynebacteria. *Bull. Soc. Chim. Biol.* **48:** 569–581.
10. Brodie, A. F., and C. T. Gray. 1956. Phosphorylation coupled to oxidation in bacterial extracts. *J. Biol. Chem.* **219:** 853–862.
11. Beers, R. F. 1955. Submerged culture of *Micrococcus lysodeikticus* for large scale production of cells. *Science*. **122:** 1016.
12. Links, J. 1960. The conversion of ubiquinone to ubichromenol. *Biochim. Biophys. Acta*. **38:** 193–194.
13. Synder, C. D., and H. Rapoport. 1969. Photooxygenation of phylloquinone and menaquinones. *J. Amer. Chem. Soc.* **91:** 731–737.
14. IUPAC-IUB Commission on Biochemical Nomenclature. 1967. Nomenclature of vitamins, coenzymes, and related compounds. *Biochem. J.* **102:** 15–22.
15. Morris, L. J. 1964. Specific separations by chromatography. *In* New Biochemical Separations. A. T. James and L. J.

Morris, editors. D. Van Nostrand Co. Ltd., London. 295–314.
16. Martin, A. J. P. 1950. Some theoretical aspects of partition chromatography. *Biochem. Soc. Symp.* **3:** 4–20.
17. Nyström, E., and J. Sjövall. 1966. Separation of vitamins K_2 on capillary columns of methylated Sephadex. *J. Chromatogr.* **24:** 212–214.
18. Campbell, I. M., and R. Bentley. 1968. Inhomogeneity of vitamin K_2 in *Mycobacterium phlei*. *Biochemistry.* **7:** 3323–3327.