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SEPARATION OF VITAMIN K_2 ISOPRENOLOGUES BY REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A simple thin-layer chromatographic system for the separation of the isoprenologues of vitamin K_2 is described. This system employs hexadecane-impregnated Kieselguhr thin-layer plates and an acetone–water solvent system. Using α-[1-^{14}C]-naphthol or 2-[^{14}C]methyl-1,4-naphthoquinone and DL-[5-^{3}H]mevalonate, the separation of the isoprenologues was confirmed. Each isoprenologue was recovered quantitatively and its proportion determined either by ultraviolet spectroscopy or radioactivity in the ring nucleus. *Staphylococcus aureus* synthesizes vitamin K_2 isoprenologues with 0, 5, 10, 15, 20, 25, 30, 35, 40, and 45 carbon atoms in the side chain. Vitamin K_2-0 represents about 6%; K_2-5 through 30, between 1 and 1.5% each; K_2-35, 20%; K_2-40, 60%; K_2-45, 6% of the total. *Haemophilus parainfluenzae* forms 2-demethyl vitamin K_2 isoprenologues with 0, 5, 10, 15, 20, 25, 30, 35, 40 and 45 carbon atoms in the side chain.

INTRODUCTION

Vitamin K_2* is a naphthoquinone with isoprenoid side chains and is found in the respiratory systems of bacteria. When the formation of the membrane bound respiratory system is induced in *Staphylococcus aureus*, there is both a marked increase in the level of vitamin K_2 and a shift in the proportion of the major isoprenologues[1]. To examine how the synthesis of vitamin K_2 isoprenologues could be related to the formation of the electron transport system, a rapid method that would allow determination of the specific activities of the various isoprenologues of vitamin K_2 was needed. Reversed-phase thin-layer chromatography (TLC) or paper chromatographic systems in which the supporting phase was impregnated with paraffin oil, polyamide, silver nitrate or silicone oil have been described[2–4]. Complex impregnating agents often contain components that interfere with the spectral determination of the quinone. When highly purified hexadecane was used to form reversed-phase thin-layer plates, the isoprenologues could be separated. Each isoprenologue could be recovered

* Vitamin K_2-30 indicates 2-methyl-1,4-naphthoquinone with a polyisoprenoid side chain at position 3 containing 30 carbon atoms in the following configuration: $-(CH_2-CH=C(CH_3)-CH_2)_n$.

quantitatively and its concentration determined by the absorbance at 248 mμ without further purification. When necessary the contaminating hexadecane was readily removed.

MATERIALS AND METHODS

Materials

α-[1-^{14}C]naphthol and 2-[^{14}C]methyl-1,4-naphthoquinone were supplied by Amersham/Searle, Des Planes, Ill. Dibenzylethylenediamine-DL-[5-^{3}H]mevalonate was supplied by New England Nuclear Corporation, Boston, Mass. Kieselguhr G and Silica Gel G (Brinkmann Instruments, Inc., Westbury, N. Y.) and hexadecane-99 %, olefin free (Matheson, Coleman and Bell, Cincinnati, Ohio), were used to make the TLC plates. Highest purity commercial solvents were utilized without further purification. Synthetic vitamin K_2 isoprenologues were supplied through the generosity of O. ISLER, Hoffman-La Roche, Basle, Switzerland.

Growth of bacteria

The strain, media, growth and harvesting conditions of *S. aureus* have been described[1]. The bacteria were grown for 14 h in the presence of 50 μCi each of a ^{14}C- and a ^{3}H-labeled substrate per 1500 ml to introduce radioactivity into the vitamin K_2. *Haemophilus parainfluenza* was grown, harvested and DMK_2 extracted as discussed previously[5].

Extraction of the lipids and purification of the vitamin K_2

Bacteria were harvested by centrifugation and the lipids extracted with a modified BLIGH AND DYER procedure[6] as follows: The bacterial pellet was suspended in 30 ml of phosphate buffer and 75 ml of methanol and 37.5 ml of chloroform were added. The one-phase mixture was shaken in a separatory funnel and allowed to stand for at least 2 h. Then 37.5 ml of chloroform and 37.5 ml of water were added, the mixture shaken, and the phases allowed to separate. The lower phase was filtered through about 5 g of anhydrous sodium sulfate. The extract was dried in a stream of nitrogen and the residue was redissolved in a small volume of chloroform. This solution was applied as a series of spots near the bottom of a Silica Gel G thin-layer plate prepared as previously described[1]. The quinone fraction (R_F 0.6) was separated from the phospholipids and polar carotenoids (origin) and non-polar carotenoids (solvent front) by ascending chromatography with a solvent of chloroform–isooctane (2:1). The quinone can be detected as a spot which quenches U.V. light (360 mμ). There was no spectral evidence for contamination of the purified quinone by carotenoids or lipid phosphate. The pure vitamin K_2 was recovered and eluted from the Silica Gel G as described[1].

Reversed-phase chromatography

Kieselguhr G was spread to a thickness of 50 μ on glass chromatoplates and impregnated with hexadecane by ascending chromatography with 5 % hexadecane in chloroform. The plates were dried in air for a few minutes and used immediately. The naphthoquinone, after initial purification, was chromatographed by ascending chromatography in a solvent of acetone–water (95:5) (ref. 2) saturated with hexadecane. The chromatography was completed in about 45 min. The plate was allowed

to dry in air for a few minutes. The center of the plate was covered with Saran wrap (Dow Chemical Corp., Midland, Mich.) and the edges of the plate were sprayed first with 1% sodium borohydride in 50% ethanol and then with 0.2% aqueous neotetrazolium. The neotetrazolium was dissolved in 4 ml of 95% ethanol and made up to 200 ml with water. The quinones appeared as red spots without heating. If the quinones were radioactive, they were localized by autoradiography. The separated naphthoquinone isoprenologues were quantitatively recovered from the channels protected by the Saran Wrap by picking up the Kieselguhr in sealing tubes (with reduced ends and a coarse fritt, Corning 39580) with a vacuum, inverting the tube and eluting with 3 ml of chloroform followed by 3 ml of methanol and 3 ml of chloroform. Vitamin K_2 and hexadecane can be separated by TLC in Silica Gel G with solvents of 5% chloroform in hexane or 10% chloroform in methanol. U.V. spectra of the quinones were determined in isooctane using a Cary 15 spectrophotometer[7]. Reversed-phase chromatography of quinone isoprenologues on vaseline-impregnated paper with a solvent of dimethylformamide–water (32:1) was performed as described[8].

Degradation of vitamin K_2

Approximately 180 nmoles of purified vitamin K_2 labeled with ^{14}C from α-naphthol and ^{3}H from mevalonate were degraded by refluxing for 4 h in 10 ml of acetone containing 6.3 mmoles of crystalline potassium permanganate. The mixture was then cooled and filtered through Whatman No. 1 filter paper. The residue was washed with a small volume of acetone to remove unreacted permanganate and then with 150 ml of boiling water to recover the phthalic anhydride. The water extract was adjusted to pH 2.0 with hydrochloric acid and extracted three times with equal volumes of diethyl ether. The ether was washed with water and the solution was concentrated to a small volume and transferred to a silica gel thin-layer plate. The phthalic anhydride (R_F 0.78) and phthalic acid (R_F 0.40) derived from the ring of the naphthoquinone were separated from the degradation products of the side chain by ascending chromatography with a solvent of ethanol–water–12 *N* ammonium hydroxide (25:3:4). The phthalic acid and anhydride were detected as areas that quench U.V. light, and they were recovered from the silica gel as described[1].

Determination of radioactivity

Radioactivity was measured in a Packard Scintillation Spectrometer Model 2311 in a scintillation fluid of 9.25 m*M* 2,5-bis[2-(5-*tert.*-butyl-benzoxazoyl)]-thiophene (BBOT) in toluene. Radioactive samples were dried in the scintillation vials before adding the scintillation fluid. Quinones containing ^{14}C and ^{3}H were counted under conditions such that the ^{14}C channel = 0.017 ^{3}H + 0.542^{14}C and the ^{3}H channel = 0.282 ^{3}H + 0.0002^{14}C in the toluene scintillator. Under these conditions the efficiency of counting was 42% for ^{14}C and 7.6% for ^{3}H. For autoradiography the thin-layer plates were placed on Kodak no-screen X-ray film as described[9].

RESULTS

Separation and recovery by reversed-phase TLC

A purified vitamin K_2 preparation isolated from *S. aureus* grown in the presence of 2-[^{14}C]methyl-1,4-naphthoquinone was chromatographed on a hexadecane-impreg-

nated thin-layer plate. The plate was dried in air and placed in contact with a Kodak no-screen X-ray film for ten days. The film was developed and the silica gel corresponding to each dark spot on the autoradiogram was separated and eluted. A total of 18,500 c.p.m. ^{14}C was applied to the plate. The total ^{14}C recovered from the ten fractions was 18,000 c.p.m. for a 97 % recovery. The autoradiogram of the separation with the total ^{14}C recovered in each isoprenologue is illustrated in Fig. 1.

Identification of the isoprenologues

The chromatographic mobility of a purified vitamin K_2 preparation from *S. aureus* was compared with synthetic isoprenologues of vitamin K_2 in two chromatographic systems. The R_M values $\{\log(1/(R_F)-1)\}$ (ref. 10) of isoprenologues in both the reversed-phase TLC system and a reversed-phase paper system agree with those of the synthetic standards. The R_M values are linear for the isoprenologues with 15 to 50 carbon atoms (Fig. 2). *H. parainfluenzae* contains isoprenologues of 2-demethyl

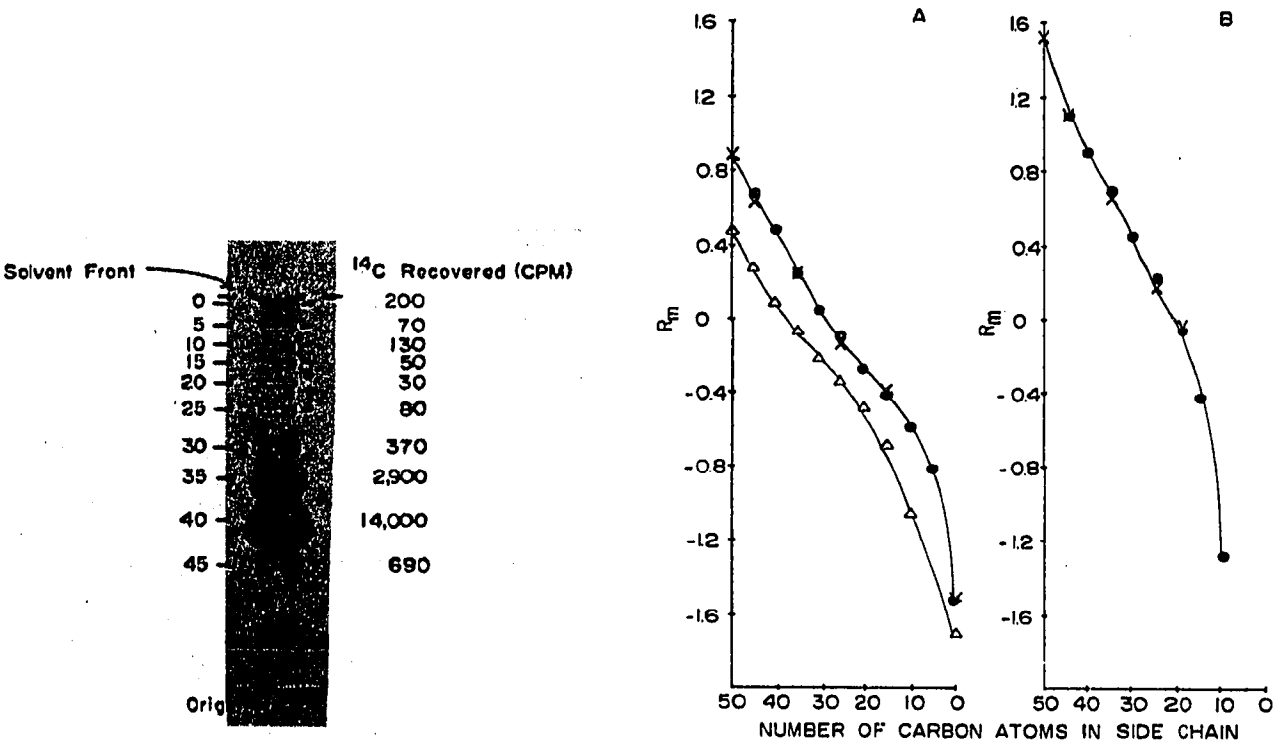

Fig. 1. Autoradiogram of vitamin K_2 isoprenologues. *S. aureus* grown in the presence of 2-[^{14}C]-methyl-1,4-naphthoquinone was harvested, the lipids extracted and the vitamin K_2 isolated and purified. The purified vitamin K_2 was spotted on a hexadecane-impregnated Kieselguhr thin-layer plate and chromatographed using acetone–water (95:5), saturated with hexadecane as solvent. The chromatogram was then placed in contact with Kodak no-screen X-ray film for ten days. The Kieselguhr corresponding to each radioactive spot was removed and the vitamin K_2 assayed for radioactivity as described. The total radioactivity of each isoprenologue is given on the autoradiogram, as counts per minute.

Fig. 2. R_M values of vitamin K_2 and DMK_2 isoprenologues. (A) vitamin K_2 isoprenologues isolated from *S. aureus* (●), synthetic vitamin K_2 isoprenologues (×) and DMK_2 isolated from *H. parainfluenzae* (△) were separated on hexadecane-impregnated thin-layer plates as in Fig. 1. (B) vitamin K_2 isoprenologues isolated from *S. aureus* (●) and synthetic vitamin K_2 isoprenologues (×) were separated on vaseline-impregnated papers.

vitamin K_2 (DMK_2) (ref. 5) that can also be separated by the reversed-phase TLC system (Fig. 2).

Labeled isoprenologues

Mevalonic acid is preferentially incorporated into the carbon side chain of vitamin K_2 isoprenologues in *S. aureus*. The vitamin K_2 was isolated from cells grown for about 13 divisions with 25 μCi each of α-[1-^{14}C]naphthol and DL-[5-^{3}H]-mevalonate per 1500 ml. About 180 nmoles of quinone containing 15,000 c.p.m. ^{14}C and 46,000 c.p.m. ^{3}H were oxidized with permanganate and the phthalic acid and phthalic anhydride recovered from the products. Approximately 87 % of the ^{14}C and 13 % of the ^{3}H were recovered in the phthalic acid and phthalic anhydride. Presumably, the 13 % of the ring ^{14}C not recovered in the phthalic derivatives could be found in levulinic acid derived partly from the ring or in other products of ring degradation. LEISTNER *et al.*[11] found that all the ^{14}C in vitamin K_2 formed from α-[1-^{14}C]naphthol in *Bacillus megaterium* was recovered in the ring after permanganate oxidation. In *S. aureus* vitamin K_2-0, about 10–14 % of the ^{3}H can be recovered from cells grown

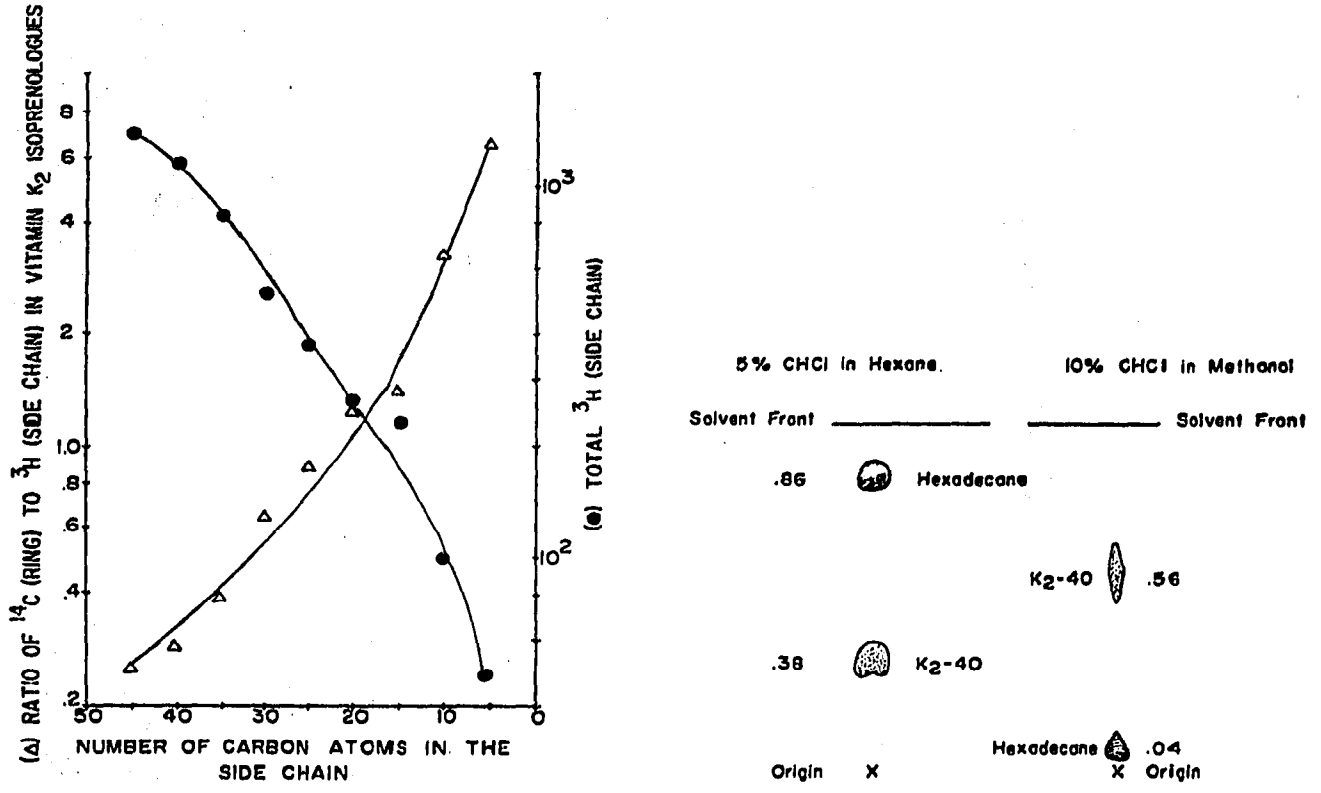

Fig. 3. Ratio of ^{14}C to ^{3}H in the ring and side chain of vitamin K_2 isoprenologues. The isoprenologues of vitamin K_2 labeled with ^{14}C in the ring and ^{3}H in the side chain were separated and assayed for radioactivity as described in Fig. 1. △ indicates the ratio of ^{14}C in the ring to ^{3}H in the side chain; ● indicates the total specific activity of ^{3}H in the side chain of each isoprenologue.

Fig. 4. Separation of vitamin K_2-40 and hexadecane. TLC using the solvents illustrated allowed separation of the quinone from the hexadecane. The quinone was recovered quantitatively from the silica gel. The quinone was detected as a dark spot with U.V. light. The hexadecane can be seen as an oily spot on the plate after drying.

with DL-[5-^{3}H]mevalonate (unpublished data). Thus, it is reasonable to expect that 10–14 % of the ^{3}H from DL-[5-^{3}H]mevalonate and all the ^{14}C from α-[1-^{14}C]naphthol be found in the ring of vitamin K_2.

The total specific activity of ^{3}H in the side chain of each isoprenologue of vitamin K_2 should increase with the increase in number of isoprenoid units in the side chain. The ratio of ring ^{14}C to side chain ^{3}H should decrease with increasing number of isoprenoid units in the side chain. In fact both these suppositions are true as illustrated in Fig. 3. To calculate the ^{3}H in the side chain, the proportion of each isoprenologue was calculated from the ^{14}C and the calculated proportion then used to calculate a specific activity for ^{3}H and ^{14}C in the isoprenologues. From the data on vitamin K_2-o, the specific activity of ^{3}H in the ring could be measured directly. The ^{3}H specific activity of vitamin K_2-o was then used to correct the total specific activity in each of the other isoprenologues for the ^{3}H in the ring. The assumption is made that all the isoprenologues have the same specific activity of ^{3}H and ^{14}C in the ring.

Isolation of isoprenologues

The isoprenologues can be recovered quantitatively from the hexadecane-impregnated thin-layer plates (Fig. 1). The hexadecane recovered in this operation does not interfere with the spectral determination of the isoprenologues but would interfere with mass spectral or nuclear magnetic resonance studies in which pure isoprenologue is required. The hexadecane can be readily separated from the isoprenologue by TLC with a solvent of 5 % chloroform in hexane or 10 % chloroform in methanol (Fig. 4). The isoprenologues can be recovered from the Silica Gel G with the sealing tubes, described above, eluted quantitatively and the solvents removed by evaporation *in vacuo*.

DISCUSSION

The principal advantage of hexadecane-impregnated TLC for the separation of vitamin K_2 isoprenologues is that the separated isoprenologues can be recovered quantitatively and assayed by U.V. spectroscopy without further purification. The hexadecane can be easily removed by TLC yielding pure isoprenologues of vitamin K_2. The separation is complete, rapid and the recovery quantitative using materials that are available commercially. Previous methods[2–4] of reversed-phase chromatography using complex mixtures of hydrocarbons or silicone oils contain contaminants which interfere with the determination of U.V. spectra or do not give as good a separation. Use of highly purified tetradecane, octadecane and eicosane as the impregnating agent gave poor separation of the vitamin K_2 isoprenologues. Perhaps these hydrocarbons would be useful with terpenes of a different type.

The study of the isoprenologues of vitamin K_2 is useful in that the isoprenologue proportions vary with pathogenicity[12] or in the membrane modification concommitant with formation of the electron transport system in *S. aureus*[1]. Multiple isoprenologues of vitamin K_2 and DMK_2 have been shown in other bacteria[7,13–15]. A detailed study of the synthesis by *S. aureus* of the isoprenoid side chain of vitamin K_2 will be presented elsewhere.

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