A New Menaquinone Series Differing in the Degree of Unsaturation of the Side Chain*

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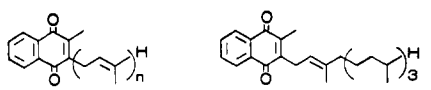
ABSTRACT: A new series of naphthoquinones has been isolated from a member of the *Streptomyces* group. This series contains five quinones which vary from each other in the degree of unsaturation of their C_3 isoprenoid side chain. By thin-layer chromatography, ultraviolet, infrared, and nuclear magnetic resonance spectra, and mass spectroscopy these quinones have been identified as MK_9, MK_9(2-H), MK_9(4-H), MK_9(6-H), and MK_9(8-H).

Two major naphthoquinone types, vitamins K_1 and K_2, have been isolated from tissues. Vitamin K_1 was identified as 2-methyl-3-phytyl-4-naphthoquinone and this structure was verified by synthesis (Mac Corquodale *et al.*, 1939). In the following year the structure of vitamin K_2, 2-methyl-3-solanesyl-1,4-naphthoquinone, was also reported (Bunkley *et al.*, 1940). These two groups of compounds differ only in the length and extent of saturation of the isoprenoid side chain of the quinone; menaquinones (MK series I) being the fully unsaturated members with side chain ranging in length from 20 to 45 carbon atoms and phylloquinone (II) a monounsaturated naphthoquinone with a C_{20} side chain.[1]



$n = 4$–9

I II

Phylloquinone may be regarded as a modified menaquinone-4 since its name could be written as MK_4(6-H). Such a comparison may indeed reflect the possible biochemical origin of K_1 from MK_4 though at the moment there is no evidence for this supposition. Gale *et al.* (1963a) examined the naphthoquinone from *Mycobacterium phlei* and showed this to be a menaquinone with a C_{45} side chain with one of the isoprene units saturated. The position of saturation was shown by Azerad *et al.* (1967a) to be in the second isoprene unit from the ring. Such monosaturated menaquinones were shown to be quite common in the *Mycobacteria* and *Corynebacter* groups (Beau *et al.*, 1966). This novel monosaturation was not restricted to the naphthoquinones since Lavate *et al.* (1962) demonstrated the presence of a monosaturated ubiquinone in *Penicillium stipitatum*. Gale *et al.* (1963b) also demonstrated the presence of a ubiquinone in *Gibberella fujikuroi* which differed from ubiquinone-10 in possessing one saturated isoprene unit which was shown by nuclear magnetic resonance spectroscopy to the ω-terminal unit (*i.e.*, furthest from the ring). Lavate and Bentley (1964) confirmed that this type of quinone was common to a number of molds. Of 18 molds examined 10 were shown to contain Q_{10} (2-H) though in these organisms the position of saturation was not determined. *G. fujikuroi* was of further interest as it contained Q_{10} besides Q_{10} (2-H) and small amounts of Q_8 and Q_9. Lester and Crane (1959) reported finding a "vitamin K" in *Streptomyces griseus*, however, no further structural characterization was made of this quinone.

We wish to report the presence of a new series of menaquinones from an unspecified member of the *Streptomyces* group. This new series of quinones differ from one another in the number of saturated isoprene units.

Materials and Methods

Lipid Extraction and Chromatography. The *Streptomyces* cells were extracted by a modification of the method of Folch *et al.* (1951). The cells (2.6 kg) were homogenized with six volumes of a chloroform–methanol (2:1, v/v) and the resulting liquid was filtered. This procedure was repeated three times. Water was added to the pooled chloroform–methanol extracts and the lower chloroform layer, containing the lipids, was removed and taken to dryness *in vacuo* yielding 31 g of dark yellow lipid material. The resulting lipid was dissolved in petroleum ether (bp 30–60°) and chromatographed on a column of acid-washed silicic acid (100–200 mesh, Clarkson Chemical Co., Inc., Williamsport, Pa), or Permutit, Folin (Fisher Scientific Co). After washing the column with petroleum ether, the quinones were eluted with 3% diethyl ether in petroleum ether from silicic acid or 4% diethyl ether in petroleum ether from Permutit. Half of this

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[1] IUPAC–IUB Nomenclature Commission reported in *Biochem. J. 102*, 15 (1967).

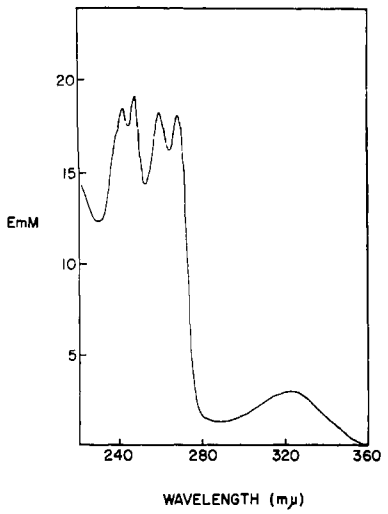


FIGURE 1: Ultraviolet spectrum of compounds taken from preparative thin-layer chromatography. The spectrum was recorded on a Cary recording spectrophotometer, Model 11, in isooctane over a range of 220–360 mμ.

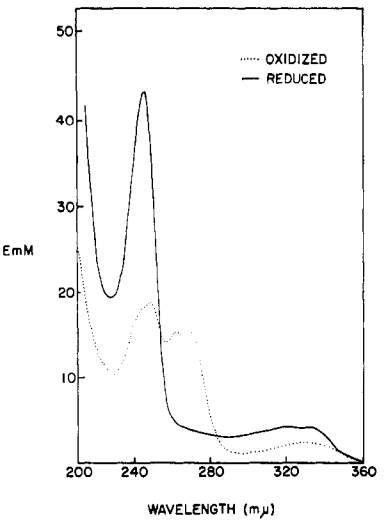


FIGURE 2: Ultraviolet spectrum of the oxidized and reduced forms of the compounds taken from preparative thin-layer chromatography. The spectrum of the oxidized form was taken in buffered enthanol and that of the reduced form in the same solvent after addition of 8 μmoles of freshly prepared aqueous sodium borohydride solution.

lipid material was placed on a 4.5 × 20 cm column packed with 500 g of Permutit; 1 l. of a 3% (diethyl ether–petroleum ether) (fraction I) was collected first, followed by four fractions (II, III, IV, and V) of 500 ml of 4% (diethyl ether in petroleum ether). Fractions III–V were found to contain 558 mg of yellow material. Of these, fraction III (373 mg) displayed only a quinone band on thin-layer chromatographic plates and it was used in all further purification steps. MK_9(II-H) was extracted and purified from *M. phlei* by identical methods. Further purification of the above fraction III was carried out by chromatographic methods described below.

Thin-Layer Chromatography. Adsorption chromatography was carried out on 250- or 500-μ silica gel G plates, impregnated with 0.1% Rhodamine 6G (Avigan *et al.*, 1963). The use of Rhodamine allowed ultraviolet-absorbing lipids to be visualized directly since these appear as a dark red area in normal light or as purple areas on a yellow-green fluorescent background under ultraviolet illumination. Where specified, absorption chromatography was carried out on silica gel plates impregnated with 9% silver nitrate (by weight) according to Beau *et al.* (1966) and with 0.05% Rhodamine 6G. For preparative chromatography silica gel plates (500 μ) and silver nitrate (375 μ) were used. The areas of the chromatographic plates to be recovered were scraped off and the lipids were eluted with diethyl ether. Reversed-phase chromatography was carried out either on cellulose or Kieselguhr plates 250 μ in thickness impregnated with 5% (v/v) liquid paraffin in petroleum ether, following evaporation of the petroleum ether. The following solvents were used: solvent A, *n*-butyl ether–hexane (1:9, v/v); solvent B, 2-butanone–hexane (1:9, v/v); solvent C, water–acetone mixture (3:97, v/v). Solvent C was saturated with liquid paraffin before use and all developing tanks were lined with filter paper to facilitate saturation of the chamber by the solvent. Reversed-phase chromatographic plates were stained for quinones with the borohydride Emmerie Engel reagent (Emmerie and Engel, 1938; Lester and Ramasarma, 1959).

Physical Analysis. Ultraviolet spectra were recorded in isooctane, unless otherwise indicated, on either a Cary Model 11 or 14 recording spectrophotometer. Ultraviolet absorption spectra of the reduced quinones were obtained in the following manner: the quinone was dissolved in ethanol containing 0.01 volume of 1 M ammonium acetate buffer (aqueous, pH 5.0) and reduced by the addition of a sufficient amount of freshly prepared sodium borohydride. Total reduction was normally complete within 60 sec. The presence of ammonium acetate minimized the destructive effect of the alkali released by hydrolysis of the borohydride. Infrared spectra were recorded on a Perkin-Elmer Model 21 infrared spectrophotometer fitted with a beam-condensing unit. The samples were applied to potassium bromide disks as oily films. Mass spectra were recorded on a Varian Model M66 mass spectrometer at an ionization potential of 70 V, and functioning at a pressure of 1×10^{-7} torr. The sample was vaporized at the ion source with a heated direct inlet system operating at 250°. Nuclear magnetic resonance spectra were recorded in the frequency sweep made at 100 Mc on a Varian Model HA-100 spectrometer in $CDCl_3$. Tetramethylsilane was used as an internal source of the signal for locking the magnetic field.

Results

Isolation and Separation of Quinones. Both naphtho- and benzoquinones are widely distributed in bacteria. Some organisms have been shown to contain more than one type of quinone or mixtures of benzo- and naphthoquinones. In general the quinones in these organisms differ from one another in the length of the C_3 isoprenoid side chain. Thus it was of particular interest that an examination of a *Streptomyces* organisms revealed the presence of several modified menaquinones. The major series was shown to vary only in the degree of unsaturation of the C_3 isoprenoid side chain, while another minor series of shorter side chain was tentatively identified.

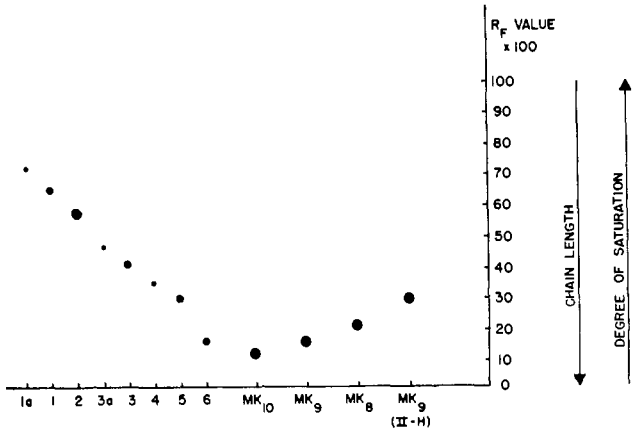


FIGURE 3: Silver nitrate chromatography. Sketch of the quinone bands observed on silver nitrate impregnated thin-layer plate in solvent B.

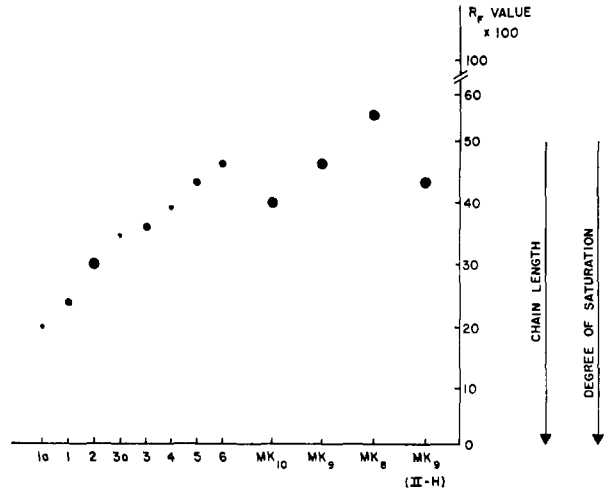


FIGURE 4: Reversed-phase chromatography. Sketch of the quinone bands observed on a reversed-phase system in solvent C.

Fraction III was chromatographed in solvent A with a *cis-trans*-phylloquinone marker on 500-μ thin-layer plates of silica gel G impregnated with Rhodamine 6G. A quinone band which comigrated with the *cis*-phylloquinone marker showed a predominant band (R_F 0.35) with a less intense band directly in front. These two bands corresponded closely to the bands seen in the *cis-trans*-phylloquinone which are known to be *cis* and *trans* mixtures (Mayer *et al.*, 1964). The two bands were eluted and rechromatographed using the same system until they were free of mutual contamination. Both of these compounds showed ultraviolet spectra which were characteristic of a 2,3-disubstituted 1,4-naphthoquinone (Morton, 1965) having a shoulder at 238 mμ and absorption maxima at 242, 248, 260, 268, and 326 mμ (Figure 1). The reduced spectra of both of the quinone bands, taken in ethanol, were identical and showed a major absorption peak at 243 mμ and a minor broad maxima between 290 and 340 mμ (Figure 2).

The two separated quinone bands were recombined and chromatographed on silver nitrate impregnated plates in solvent B. Marker spots of authentic $MK_{(8-10)}$, and MK_9(II-H), were run on the same plate. The results, along with R_F values, are shown in Figure 3. The entire fraction III was chromatographed on 20 375-μ silver nitrate plates as above, and the individual bands were eluted and quantitated. The five major bands, 1, 2, 3, 5, and 6, appeared, after elution from the chromatographic plates in ratios of approximately 4:20:4:2:1, respectively. In addition to the five major bands there were four minor bands, which will be referred to as bands 1a, 3a, 4, and 6a which were present in slight amounts. After purification all of these compounds except 6a displayed typical spectra for a 2,3-disubstituted 1,4-naphthoquinone, the only variation being slight changes in relative absorption intensity, with no change in absorption maxima. Band 6a had a shoulder at 268 mμ with a broad absorption maxima from 330 to 350 mμ and was not considered to be a quinone.

The purified compounds were chromatographed in solvent C by reversed-phase chromatography, using 250-μ thin-layer plates. Markers of authentic $MK_{(8-10)}$, and MK_9 (II-H) were used and the results along with R_F values are shown in Figure 4. A more complete comparison of menaquinone chromatographic behavior has been published (Beau *et al.*, 1966). It should be noted here that when silver nitrate and reversed-phase chromatography were carried out on extracts of *Streptomyces olivaceus* qualitatively similar results were seen.

It would be predicted from the results of the silver nitrate chromatography (Figure 3) that the *Streptomyces* quinones varied by having shorter C_3 isoprenoid side chains with decreasing band numbers. That is band 1 being of shorter side-chain length than band 2, etc. It can be seen in Figure 3 from the marker spots that as the quinone side chain loses one isoprene unit the R_F value increases by about 0.07 unit (compare, for example, MK_8 with MK_9). It should be noted also that using this silver nitrate chromatographic system, saturation of one isoprenoid group causes an increase in R_F values by about 0.1 unit (compare MK_9 with MK_9 (II-H) (Figure 3). Using the reversed-phase system (Figure 4), it can be seen that, as with the silver nitrate system a loss of one isoprene unit from the side chain increases the R_F value, but by about 0.1 unit, compare MK_8 with MK_9. However, with reversed-phase chromatography, the saturation of one isoprenoid group results in a slight decrease in the R_F value, compare MK_9 with MK_9 (II-H) (Figure 4). Examination of the results of both silver nitrate and reversed-phase chromatography indicated that there may be a difference in unsaturation between the major bands. This conclusion is drawn from the fact that chain-length difference results in R_F values which are inversely related to the number of isoprenoid units with both solvent systems a situation not seen with these quinones. With an increase of unsaturation in the isoprenoid units, a lowering of R_F values is seen with silver nitrate chromatography while an increase in R_F values is seen using reversed-phase chromatography. The observation with *Streptomyces* quinones (Figures 3 and 4) correspond to this latter pattern.

On both systems band 5 comigrates with MK_9 (II-H) and band 6 comigrates with MK_9. If it is assumed from these results that band 6 is MK_9 and band 5 an MK_9 with one saturated isoprenoid unit, MK_9 (2-H), then it can be seen from subtraction of about 0.1 R_F unit that bands 3, 2, and 1 can be interpreted as being MK_9 (4-H), MK_9 (6-H), and MK_9 (8-H), respectively. Bands 5a, 4, and 3a could, by this same reasoning, be MK_8 (2-H), MK_8 (4-H), and MK_8 (6-H), respectively.

Infrared spectra taken of bands 1, 2, 3, and 5 were all iden-

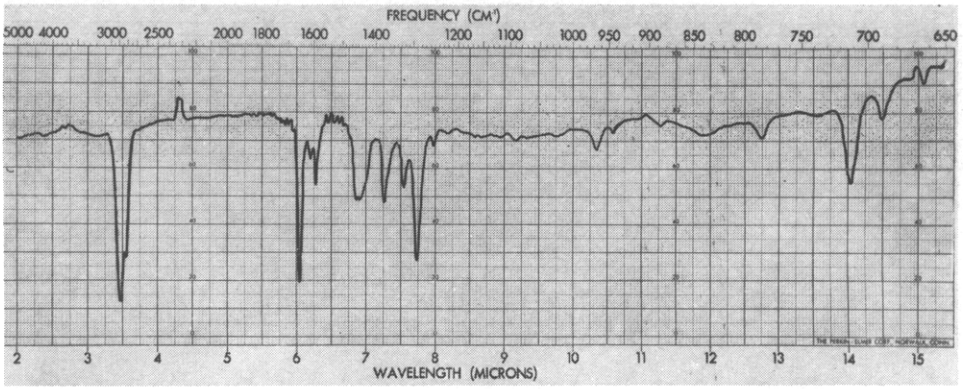
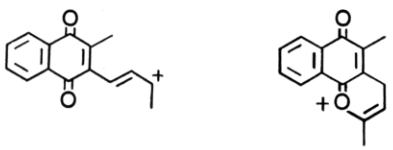


FIGURE 5: Infrared absorption spectrum of band 2 taken on a Perkin-Elmer Model 21 infrared spectrophotometer. The sample was in the form of an oily film and placed directly on the crystal.

tical and typical of absorption spectra of a naphthoquinone with an isoprenoid side chain (Figure 5). The large band at 2900 cm^{-1} and shoulder at 2860 cm^{-1} are associated with CH stretching, while bands appearing at 1445 and 1379 cm^{-1} are associated with CCH_2 and the CCH_3 stretching, respectively. The bands appearing in the 1250–1350- and 1550–1700-cm^{-1} regions are associated with the aromatic quinone ring, the most intense being at 1660 cm^{-1} (C=O stretching of the quinone) and the less intense 1620-cm^{-1} band being due to the C=C stretch of the ring. The band at 1598 cm^{-1} was due to the C=C skeletal in plane vibrations of the conjugated aromatic ring (Bellamy, 1958) and the band at 712 cm^{-1} is associated with the CH out of plane deformation of the four adjacent ring hydrogens of the naphthoquinone nucleus. The bands at 1250–1350 cm^{-1} which are associated with all vitamin K compounds have not been given definite assignments (Pennock, 1965). The main band in the 840-cm^{-1} region which appears at 838 cm^{-1}, along with the shoulder at 812 cm^{-1} and small band at 888 cm^{-1} are all representative of the isoprenoid side chain of the naphthoquinone. The weak band at 1740 cm^{-1} was believed due to the presence of slight carbonyl impurity.

Mass spectra of bands 1, 2, 3, and 5 were recorded. The molecular ion of the four compounds appeared at *m/e* 792, 790, 788, and 786, respectively. The *m/e* for authentic MK_9 (II-H) appears at 786 (Beau *et al.*, 1966). All of the spectra showed an intense peak at *m/e* 225 which might represent fragments corresponding to one of the two structures shown below



(Di Mari, *et al.*, 1966). This peak is the base peak of a dialkylated 1,4-naphthoquinone, characteristic of vitamin K type compounds. The peak at *m/e* 225 is evidence of a compound with a dialkylated 1,4-naphthoquinone nucleus with at least one isoprenoid unit adjacent to the ring. The finding of a molecular ion at *m/e* 786 for band 5 suggested as did its chromatographic properties on all the systems tested, that this compound was MK_9 (2-H). The other compounds, bands 3, 2, and 1, with molecular ions at *m/e* 788, 790, and 792, respectively, suggests, as to their chromatographic properties, that they are MK_9 (4-H), MK_9 (6-H), and MK_9(8-H), respectively. Because of the low sensitivity of the mass spectrometer, it was impossible to detect ions corresponding to the loss of consecutive isoprenoid units (68, 69, and 70 mass units depending upon location and saturation). Such sequential isoprenoid loss in the cracking pattern has been observed for MK_9 (II-H) by Lederer (1964) and by Dunphy *et al.* (1968).

Nuclear Magnetic Resonance Spectroscopy. Nuclear magnetic resonance spectra (given in τ values) of bands 2 and 5 were taken, that of band 2 is shown in Figure 6. The spectra was typical of an MK and band assignments were made as follows. The rather complex pattern from 2.0 to 2.5 is due to the four protons adjacent in the benzoid ring. The peaks at 4.95, 8.18, and 8.28 all result from effects of the β,γ double bond. The triplet at 4.95 is due to the methyl group on the C_2' atom. The doublet at 6.69 is due to the ring adjacent isoprenoid unit and reflects the presence of the methylene group adjacent to the ring. The fact that there is a doublet at 6.69 indicates that the ring-terminal isoprenoid unit is unsaturated. If the ring-terminal isoprenoid unit is saturated this is reflected by a shift in the 6.69 doublet to an upfield triplet appearing around 6.95 (Feeney and Hemming, 1967). The singlet at 7.88 is due to the ring methyl group at the C_2 position of the quinone ring.

The large peak at 8.04 is attributed to the methylene groups in the C_3 side chain. The large peak at 8.46 is due to the *trans*-methyl groups on internal and ω-terminal isoprenoid units. Of particular interest is the absence of a peak at 8.34–8.40 which is seen in MK_9 (II-H) Figure 7 at 8.34 and is attributed to the contribution of either *cis* or *trans* ω-terminal isoprenoid units. This finding tends to indicate that one of the three saturated isoprenoid units of the band 2 compound is the ω-terminal unit. It is interesting to note here that the nuclear magnetic resonance spectra of band 5 which was tentively identified as MK_9 (2-H) by mass spectra and chromatographic methods does in fact shown an absorption peak at 8.40, indicating that its ω-terminal isoprenoid unit is unsaturated. The remaining two major peaks, the singlet at 8.8 and the doublet at 9.18 are due to the C_3 side chain CH_2 and CH_3 groups, respectively. It should be noted that these peaks at 8.8 and 9.18 only appear when there is saturation of one or more double bonds in the side chain (Lavate and Bentley, 1964). The small peak at 6.44 was believed to be contributed by impurities often

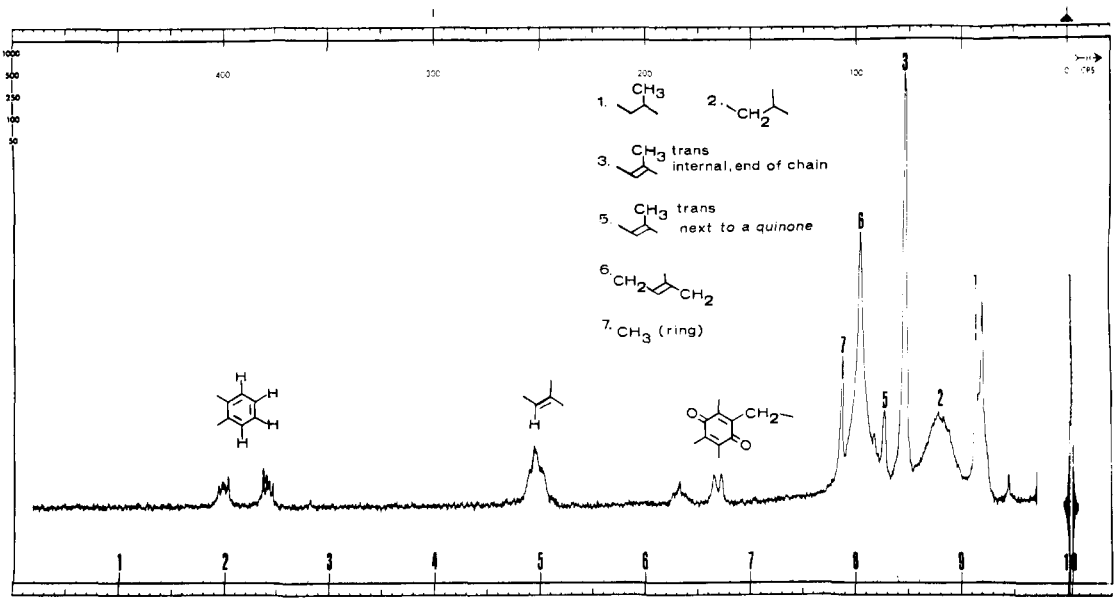


FIGURE 6: Nuclear magnetic resonance spectra of band 2 taken on a Varian Model HA-100 spectrometer in $CDCl_3$.

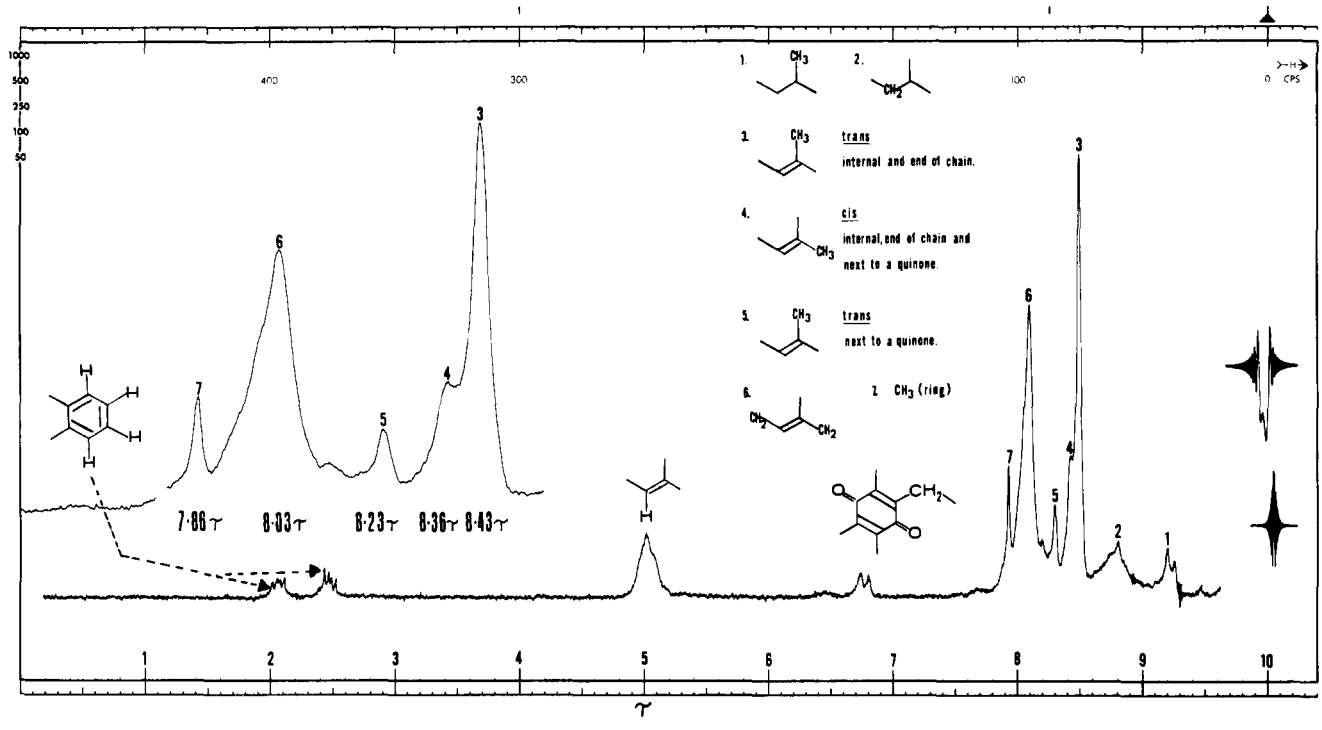


FIGURE 7: Nuclear magnetic resonance spectra of MK_9 (II-H) taken on a Varian Model HA-100 spectrometer in $CDCl_3$.

seen in naphthoquinones purified by thin-layer chromatographic methods. A more detailed analysis of nuclear magnetic resonance spectra of all of the compounds will appear in a subsequent publication.

Discussion

We have reported in this paper the finding of a group of menaquinones which are closely related to each other, the major ones varying only in the extent of unsaturation of the C_3 isoprenoid side chain. Ultraviolet and infrared spectra indicated that all of these compounds are of the 2,3-disubstituted 1,4-naphthoquinone type. Further determination by nuclear magnetic resonance spectroscopy indicated the quinones of bands 2 and 5 were also of this type. It has shown by mass spectroscopy that the four major compounds, bands 1, 2, 3, and 5, have *m/e* at 792, 790, 788, and 786, respectively, which corresponds to the calculated molecular weights of MK_9 (8-H), MK_9 (6-H), MK_9 (4-H), and MK_9(2-H), respectively. In addition to this evidence, the use of thin-layer chro-

matography has shown that band 5, MK_9 (2-H), comigrated on all systems tested with authentic markers of MK_9 (II-H) while band 6 comigrated with all systems tested with authentic markers of MK_9. In addition by use of authentic menaquinone homolog as markers (MK_9 (II-H), MK_8, MK_9, and MK_{10}) it has been shown that bands 1, 2, 3, 5, and 6 migrate with R_F values corresponding to those expected for MK_9 (8-H), MK_9 (6-H), MK_9 (4-H), MK_9 (2-H), and MK_9, respectively. This evidence suggests the partial structures of these quinones, that is they are all menaquinones with nine isoprenoid units at carbon atom 3 and differ from each other only in the degree of unsaturation of the side chain. While a more complete study to determine the points of saturation is now in progress, it is of interest that in light-inactivated preparations of *M. phlei*, band 2, (MK_9 (6-H)), was capable of restoring coupled phosphorylation, a process known to be dependent upon a 1,4-naphthoquinone nucleus with a C_2 methyl group and a C_3 isoprenoid side chain in which the ring-terminal unit is saturated and the second unit from the ring is saturated (Brodie and Ballantine, 1960). It has also tentatively been shown by nuclear magnetic resonance spectroscopy that band 2, (MK_9 (6-H), contains a ω-terminal saturation. Nuclear magnetic resonance spectroscopy has also shown that bands 2 and 5 possess an unsaturated ring-terminal isoprenoid unit. The exact location of all saturations for these compounds has not been determined.

The finding of a series of menaquinones differing from each other only by the addition of two protons in the C_3 side chain may suggest a pathway for the biosynthesis of these compounds. It may well be that the fully unsaturated compound is produced and then the others are formed from sequential hydrogenations. Azerad *et al.* (1967b) have presented evidence with *M. phlei* using desmethyl MK_9, [^{14}C]methionine, and NADH or NADPH that 10–20% of the label from methionine was present in isolated MK_9 (II-H). When cell-free extracts of the *Streptomyces* organism were incubated with T_2O or tritiated NADPH, no incorporation of H_3 was found in any of the isolated quinones.

It is of particular interest that this organism possesses, as do many others, a series of related quinones. In addition some organisms contain unrelated quinones, as for example, MK_8, and coenzyme Q_8 in *Escherichia* (Bishop *et al.*, 1962). The presence of a series of quinones varying only in the number of isoprenoid units, degree of saturation of the C_3 side chain, or even unrelated types of quinones raises the question of the function of so many closely related compounds in tissues. In this connection it is interesting to note that while electron transport and coupled phosphorylation in *M. phlei* have been shown to use MK_9 (II-H) (Brodie and Ballantine, 1960) it has been shown that the organism also contains MK_8 (2-H) (P. J. Dunphy, 1967, unpublished data; Campbell and Bentley, 1968). It seems unlikely that all these related compounds are mistakes of biosynthesis or even biosynthetic or degradative intermediates. Thus an investigation of the roles played by the different quinones in multiquinone systems is necessary for an understanding of the function of quinones in biological systems.

Acknowledgments

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