Isolation of a Naphthaquinone with Partly Hydrogenated Side Chain from *Corynebacterium diphtheriae*

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1. *Corynebacterium diphtheriae* contains relatively large amounts (6.6 μ moles/g. dry wt.) of a naphthaquinone whose ultraviolet-absorption spectrum is that of a typical menaquinone (vitamin K₂), the $E_{1\text{cm}}^{1\%}$ value corresponding with that of MK-8, but on reversed-phase paper chromatograms it runs with MK-9. 2. In the presence of Adams catalyst hydrogen uptake is 2 atoms/mol. less than that calculated for MK-8. 3. Hydrogenated samples of the *Corynebacterium* quinone and the hydrogenation product of authentic MK-8 ran together on reversed-phase chromatograms. 4. Infrared-absorption spectra indicated close relationship with the menaquinone series, and nuclear-magnetic-resonance measurements show that one, non-terminal, double bond of the side chain has been saturated. 5. The compound is thus designated MK-8(2H), indicating a menaquinone with eight isoprene units but only seven double bonds in the side chain.

Most menaquinones obtained from bacterial sources have been characterized as one or other of the isoprenologues MK-6, MK-7 (Isler, Rüegg, Chopard-dit-Jean, Winterstein & Wiss, 1958), MK-8 or MK-9 (Lester & Crane, 1959; Page *et al.* 1960; Bishop, Pandya & King, 1962), but recently microbial menaquinones have been reported differing from the above pattern. These include: naphthaquinones with an isoprenoid side chain but no 2-methyl group, from *Streptococcus faecalis* (Baum & Dolin, 1963) and *Haemophilus parainfluenzae* (Lester, White & Smith, 1964); a 2-methyl-1,4-naphthaquinone with a chain of 34 carbon atoms, i.e. one fewer than in MK-7, at position 3 (Frydman & Rapoport, 1963); and an 'MK-9' in which the side chain contained only eight double bonds (Gale *et al.* 1963). We report the finding, in a Park-Williams strain of *Corynebacterium diphtheriae*, of a menaquinone with a C₄₀ side chain (i.e. eight isoprene units) containing only seven double bonds. A preliminary account of this work has appeared (Scholes & King, 1964).

MATERIALS AND METHODS

Materials. We gratefully acknowledge reference samples of MK-6, MK-7, MK-8 and MK-9 supplied by Hoffmann-La Roche and Co., Basle, Switzerland. We are also grateful to The Wellcome Research Laboratories and Dr D. C. Edwards for growing in bulk the Park-Williams no. 8 *Corynebacterium diphtheriae* strain CN2000, on a medium containing 5 μ g. of iron/ml., i.e. sufficient to suppress toxin formation (Edwards, 1960; Edwards & Seamer, 1960).

Isolation of naphthaquinone. A 100 g. (wet wt.) sample of cells, harvested in late exponential phase, was washed twice

with water and the wet mass was refluxed with 400 ml. of methanol for 1 hr. The solid residue was filtered off and re-extracted by refluxing twice with 150 ml. of methanol. The combined methanol extracts were diluted with 3 vol. of water, and the lipid-soluble material was extracted with five 400 ml. portions of ether. The combined extract was dried with anhydrous Na₂SO₄ and evaporated to dryness under N₂ to give 4.4 g. of crude lipid. This was chromatographed on activated alumina (Bishop *et al.* 1962), and the fraction eluted with diethyl ether-light petroleum (b.p. 40–60°) (1:49, v/v) yielded 150 mg. of material that, from its ultraviolet-absorption spectrum, was a fairly pure menaquinone. It was recrystallized at –20° from ethanol until (after seven recrystallizations) a constant value of $E_{1\text{cm}}^{1\%}$.257 for the peak absorption at 249 m μ in cyclohexane was obtained.

Spectroscopy. The molar concentration of the quinone was determined from the ultraviolet absorption measured in cyclohexane solution (Bishop *et al.* 1962). Infrared-absorption spectra were recorded on a Perkin-Elmer Infracord instrument, by using in most cases films between rock-salt plates; carbon tetrachloride solutions were also examined. Nuclear-magnetic-resonance spectra were obtained, through the courtesy of Dr R. J. Abrahams and Mr J. V. Barkley (Department of Organic Chemistry, University of Liverpool), on a Varian A-60 instrument at 60 Mcyc./sec., in carbon tetrachloride solution; tetramethylsilane was used as reference compound.

Reversed-phase paper chromatography. Whatman no. 1 paper was impregnated by immersion in a 5% (w/v) solution of liquid paraffin, B.P., in light petroleum (b.p. 60–80°). The test material (10–20 μ g. in light petroleum solution) was applied to the paper; chromatograms of the menaquinones were developed with *NN*-dimethylformamide; the perhydro derivatives were developed with redistilled acetone.

Micro-hydrogenation. This was carried out in an apparatus supplied by J. W. Towers and Co., Widnes, Lancs., with an Adams catalyst (platinum oxide) (Morton *et al.* 1958).

RESULTS AND DISCUSSION

C. diphtheriae CN 2000 is rich in naphthaquinone; the batch used contained 6.6 μ moles/g. dry wt., i.e. about 10 times the amount usually found in menaquinone-containing bacteria (Bishop *et al.* 1962). A comparable concentration was reported, however, by Jacobsen & Dam (1960) for *Mycobacterium phlei*. Even higher values were obtained if the organisms were grown well into the stationary phase. The isolated naphthaquinone gave the four sharp absorption peaks at 243, 249, 261 and 270 $m\mu$, with a weaker broad band with $\lambda_{\max}$ at 325 $m\mu$ and an inflexion at 238 $m\mu$, typical of the menaquinones. The $E_{1\text{cm}}^{1\%}$ value of 257 at 249 $m\mu$ may be compared with values of 268 for MK-8 and 243 for MK-9. The compound could not therefore be MK-9, although it behaved similarly on chromatography and was well separated from MK-8 (R_f values: MK-8, 0.49; MK-9, 0.42; unknown, 0.43). The possibility that the cause of the anomaly between the spectroscopic results (suggesting a side chain of not more than eight isoprene units) and the chromatographic data (showing a relative affinity for the non-polar phase) might be saturation of one or more of the isoprenoid double bonds present in the side chain of a typical menaquinone was investigated by hydrogenation. When a menaquinone is treated with the Adams catalyst the double bonds in the side chain and in the benzenoid ring are reduced but not those in the quinonoid ring, though the latter is converted into the quinol form. On reoxidation to the quinone (with ethanolic ferric chloride) the resulting perhydromenaquinone retains the absorption peaks at about 260 and 270 $m\mu$, characteristic of the quinone structure, but loses those at 243, 249 and 325 $m\mu$, which arise from the benzene structure (Ewing, Vanderbelt & Kamm, 1939; Morton & Earlam, 1941). The unknown took up 1.36 μ moles of hydrogen/100 mg., which is consistent with a substance containing one double bond/mol. fewer than a normal menaquinone.

The hydrogenated material was treated with ferric chloride to yield the quinone and compared with hydrogenated samples of authentic MK-8 and MK-9 treated similarly. The three perhydroquinones displayed similar absorption spectra with peaks at 260 and 270 $m\mu$, and when run on a paper chromatogram the *Corynebacterium* perhydroquinone was indistinguishable from perhydro-MK-8 (R_f 0.16) but was sharply separated from perhydro-MK-9 (R_f 0.10). The substance was therefore considered to be MK-8 (2H).

The side chain has little influence on the ultra-violet-absorption spectrum of a menaquinone, but slight effects are detectable in the infrared region. The *Corynebacterium* material showed the

major peaks characteristic of a naphthaquinone (Noll, 1958; Isler & Wiss, 1959) at 1667, 1620, 1600, 1330, 1298, 715 and 690 cm^{-1} , with prominent peaks due to the side chain at 2850–3040, 1450 and 1380 cm^{-1} . The effect of the loss of one double bond will be small. A slight shoulder at 735 cm^{-1} on the strong 715 cm^{-1} band could have been due to the presence of $-\text{CH}_2-\text{CH}_2-\text{CH}_2-$ (Bellamy, 1958, p. 28). This shoulder is seen in the hydrogenated menaquinones and in phyloquinone (vitamin K_1) (Isler & Wiss, 1959); it is also present in perhydrosolanesol (Burgos, Hemming, Pennock & Morton, 1963). Strong similarity to the MK series was shown by a band at 840 cm^{-1} attributed to C–H out-of-plane deformation in the structure $\text{CRR}'=\text{CHR}''$ (Bellamy, 1958, p. 34; Binder, 1963), and by the symmetry of the 1450 cm^{-1} band (Noll, 1960). A point of great significance is the relative intensity of the 1300 and 2925 cm^{-1} absorptions. The former arises from the nucleus whereas the latter is due to CH , CH_2 and CH_3 absorptions. The ratio between the two will thus give an indication of the number of C–H links in the compound. Noll (1960) has pointed out that in MK-9 the 1300 cm^{-1} band is less intense than that at 2925 cm^{-1} , whereas in MK-8 the relationship is reversed. In the *Corynebacterium* compound, the 2925 cm^{-1} band was slightly the more intense. This would imply a side chain with more hydrogen atoms than are present in MK-8.

More conclusive evidence was obtained from the nuclear-magnetic-resonance spectrum (Table 1). We can represent the presumed structure as shown (I). The 6.65–6.7 τ doublet is attributable to the allylic methylenes (*a*) (Gale *et al.* 1963), and the

Table 1. Nuclear-magnetic-resonance spectrum of *Corynebacterium* material

Signal intensities are based on the areas under each peak, that below the 6.7 τ signal being taken as 2. Calculated values are based on MK-8 with one double bond (not proximal or distal) hydrogenated.

Origin	Location (τ)	Intensity	
		Found	Calc.
Adjacent ring protons	2.02, 2.42 multiplet	3.7	4
Olefinic protons	4.92	6.9	7
Allylic methylene protons	6.7 doublet	(2.0)	(2)
Methyl attached to ring	6.78	26.4	{ 3 24
Methylene protons	8.0		
Methyl adjacent to double bond	8.23, 8.32	22.4	24
Saturated side-chain protons	8.75 multiplet	8.6	7
Methyl not adjacent to double bond	9.12 multiplet	3.2	3

area of this signal was assumed to correspond to 2 protons. Its presence, incidentally, shows that the double bond has not been saturated in the isoprenoid unit adjacent to the nucleus. The 4.92τ peak records the olefinic protons (b); it corresponds to 6.9 protons, confirming that only seven out of the eight isoprenoid units contain double bonds. The 8.75τ peak, with a value of 8.6 protons, is accounted for by the protons in the saturated segment of the side chain (c). The value obtained is somewhat in excess of the theoretical value of 7, but should be compared with the 3.2 proton value of the 9.12τ signal, generated by methyl protons not adjacent to a double bond (d). The ratio of (c) to (d) is significant (2.7); were the distal isoprenoid unit saturated, there would be six 'saturated' methyl protons and five methylene, giving a ratio 5:6 or 0.83.

We thus propose the structure MK-8(2H) for the naphthaquinone from *C. diphtheriae*; we cannot identify which isoprenoid unit lacks the double bond, beyond observing that it is neither the unit distal nor that proximal to the nucleus.

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