Inhomogeneity of Vitamin K_2 in *Escherichia coli**

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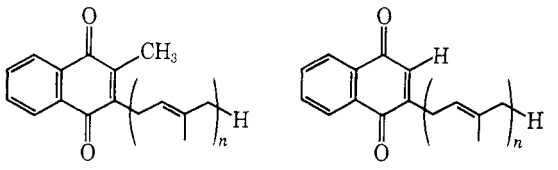
ABSTRACT: Gel filtration has been used to study the menaquinone content of *Escherichia coli*. Menaquinones-6, -7, and -9 have been detected and isolated in addition to the previously encountered menaquinone-8. The principal 2-demethylmenaquinone has been unambiguously characterized as 2-octaprenyl-1,4-naphthoquinone. It is accompanied by the corresponding heptaprenyl derivative. A comparison between the fractionation power of hydroxypropylated Sephadex G-25 and G-50 is made.

There is an increasing body of evidence suggesting that the menaquinone (vitamin K_2) content of bacteria is made up of a mixture of prenylogs rather than of a single, homogeneous entity. Thus, it has been shown that the menaquinone fraction isolated from *Sarcina flava* consists of a mixture of two menaquinone prenylogs (Jacobsen and Dam, 1960), that from *Mycobacterium phlei* and some aerobic *Micrococci* of at least three prenylogs (Campbell and Bentley, 1968; Jeffries *et al.*, 1967), and that of one strain of *Staphylococcus aureus* of no less than five prenylogs (Cawthorne *et al.*, 1967). In a recent survey, Whistance *et al.* (1969) deduce from paper chromatographic evidence that menaquinone multiplicity occurs in seven *Enterobacteria* and two *Pseudomonads*. Inhomogeneity has also been encountered in the 2-demethylmenaquinones of *Hemophilus parainfluenzae* (Lester *et al.*, 1964).

To establish the generality of bacterial menaquinone inhomogeneity we are undertaking the detailed examination of the menaquinone content of organisms currently believed to produce but a single prenylog. The organism discussed herein, *Escherichia coli*, was known to form menaquinone-8 (I, n = 8) (Bishop *et al.*, 1962) together with a 2-demethyl derivative, tentatively attributed structure II, n = 8 (Baum and Dolin, 1965).



I

II

In addition to searching for menaquinone multiplicity, this investigation has assessed the value of hydroxypropylated Sephadex G-50 in the purification of relatively large molecular weight nonpolar lipids such as the menaquinones.

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Materials and Methods

General. All solvents etc. were of reagent grade. The silica gel used in layer chromatography was obtained from Brinkmann, Inc. (Macherey and Nagel, G-HR). Plates of specified thickness were made containing 0.02% Rhodamine 6G. Reversed-phase chromatography was conducted on silica gel "Chromagrams" (Eastman, 6060 with fluorescent indicator) which had been dipped in a 10% solution of paraffin oil in ethyl acetate, and dried. Visualization was by means of ultraviolet light examination (254 mμ) and iodine adsorption. The silicic acid used in column chromatography was Clarkson Chemical Company's "Unisil," mesh 200–325. Hydroxypropylated G-50 was the gift of Pharmacia Inc.

Ultraviolet spectrometry was performed on a Zeiss PMQ II spectrometer and on a Coleman Hitachi 124 recording spectrometer. Mass spectra were obtained by the direct probe method using an LKB 9000 spectrometer working at 70eV with an electron current of 60 μA, an accelerating voltage of 3.5 kV, and an ion source temperature of 270°.

Pure samples of menaquinones-5, -7, and -9 were obtained through the generosity of Professor Isler (Basle).

Growth and Harvest of the Organism. E. coli K 12, the generous gift of Dr. I. R. McManus, was maintained on nutrient agar slopes at 37°. Liquid innocula were prepared by growth for 24 hr at 37° as shake cultures in 250-ml erlenmeyer flasks containing 25 ml of the following culture medium: Bacto-Peptone (Difco), 1.0%; Casamino Acids (Difco), 0.5%; glucose, 0.5%; Beef Extract (Difco), 0.5%; disodium hydrogen phosphate, 0.8%; potassium dihydrogen phosphate, 0.15%; sodium chloride, 0.76%; ferric sulfate·$7H_2O$, 7.0×10^{-4}%.

The liquid innocula so produced were used to seed 2.8-l. Fernbach flasks containing 700 ml of the above culture medium. Growth as shake cultures continued at 37° for a further 24-hr period. Thereafter the cells were harvested by continuous centrifugation and the wet paste washed twice with distilled water. The yield of wet paste was approximately 7.5 g/flask.

Isolation of Nonpolar Lipids. The wet paste was homogenized in acetone and the nonpolar lipids extracted as previously described (Campbell and Bentley, 1968). The yield of nonpolar lipid was *ca.* 60 mg/100 g wet weight of paste. As ever, it must be emphasized that the above operations and all those which follow must be conducted in virtual darkness if

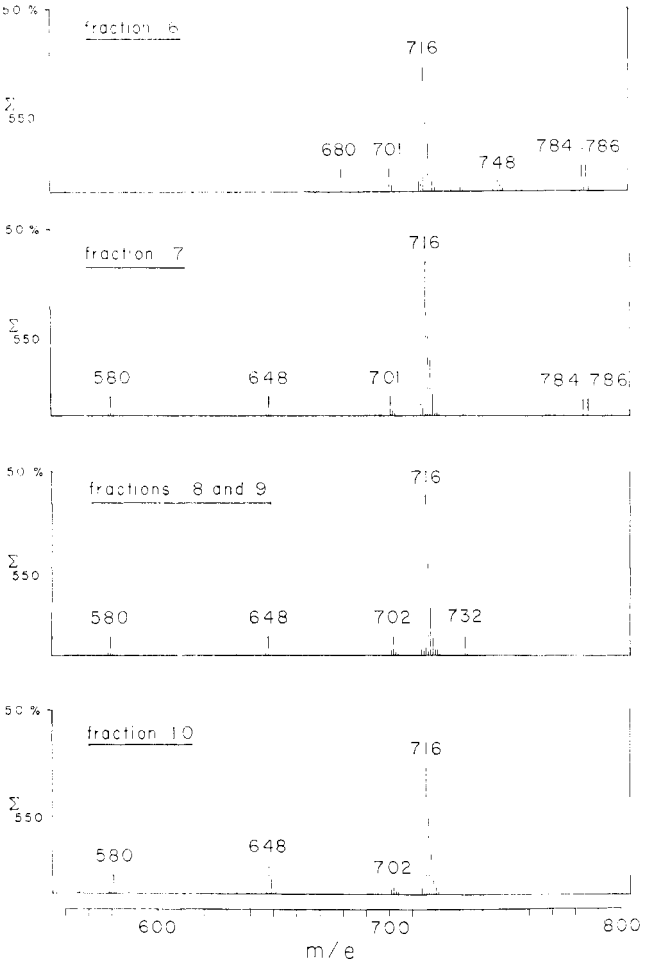


FIGURE 1: Mass spectrometric assay (70 eV) of fractions 6–10 from Sephadex LH-20 column chromatogram of *E. coli* menaquinones.

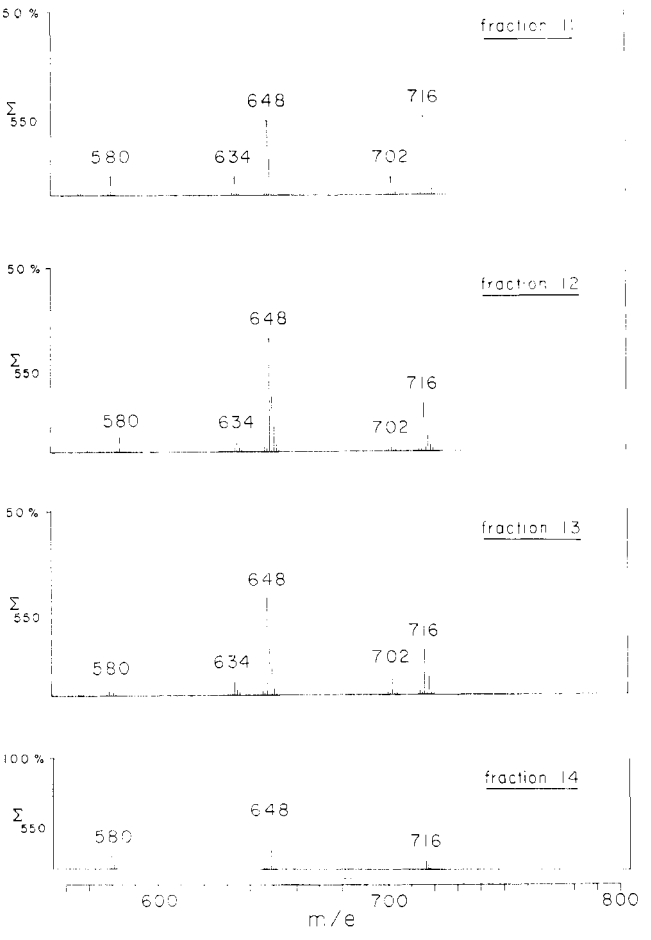


FIGURE 2: Mass spectrometric assay (70 eV) of fractions 11–14 from Sephadex LH-20 column chromatogram of *E. coli* menaquinones.

extensive *trans–cis* isomerism and decomposition of the menaquinones are to be avoided.

Preliminary Separation of Menaquinones from Ubiquinones, Fatty Acids, etc. In a typical run, the nonpolar lipids (396 mg) were applied to a column of silicic acid (18 g; 2.5 × 7 cm; flow rate 1.5 ml/min) established in benzene. After development of the column with 32 ml of this solvent, fractions (14 ml) were taken. Mass spectral, ultraviolet, and thin-layer analysis (solvent cyclohexane–benzene, 2:1; layer thickness 250 μ) indicated that menaquinone was present in fractions 1–4 (60 mg). Similarly, ubiquinone, fatty acids, and some other, as yet unidentified, lipids were detected in fractions 5–20 (89.8 mg).

Fractionation of the Menaquinone Prenylogs on Sephadex LH-20. The combined fractions 1–4 of the silicic acid column (60 mg) were applied to a column of Sephadex LH-20 (133 g; 198 × 1.8 cm; flow rate 10 ml/hr; swelling time, 24 hr) established in the solvent system, isooctane–methanol–chloroform, (2:1:1). After development of the column with 123 ml of the solvent, fractions (2.5 ml) were collected. Ultraviolet spectrometry indicated that menaquinones were present in fractions 6–14. The mass spectral assay of these fractions is shown in Figures 1 and 2; the thin-layer assay in Figure 3A. Yields were fraction 6 (nona- and octaprenylogs), 1 mg; fractions 7–9 (octaprenylogs), 23.6 mg; fractions 10–13 (octa- and heptaprenylogs), 10.1 mg; fraction 14 (octa-, hepta-, and hexaprenylogs), *ca.* 0.5 mg.

Samples of the individual prenylogs MK-9,[1] MK-8, MK-7, and MK-6, were obtained by reversed-phase chromatography of the appropriate fractions on layers of silica gel impregnated with paraffin oil. The solvent system was acetone–water (19:1). After development, the menaquinones were removed from the silica gel by elution with ethyl acetate and were separated from the contaminating paraffin oil by further chromatography on layers (250 μ) of silica gel with the solvent system cyclohexane–benzene (2:1). The identity of the purified materials was established by ultraviolet and mass spectrometry.

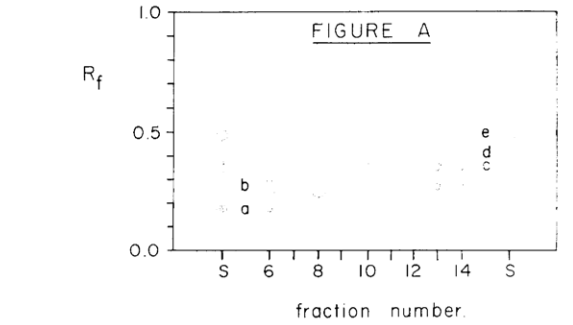
Isolation and Characterization of the 2-Demethylmenaquinones. A. 2-DEMETHYL-MK-8. The contents (23.6 mg) of the octaprenylog containing fractions 7–9 of the Sephadex LH-20 column were combined and crystallized from absolute ethanol (−4°) to give rosettes (13.7 mg), mp 55–57°. Preparative layer chromatography of this material twice on silica gel (layer area, 200 × 200 mm; layer thickness, 0.4 mm) in

[1] In this paper the abbreviation MK-*n* signifies a menaquinone with a 3 substituent of *n* prenyl units. Thus MK-9 = menaquinone-9 = 2-methyl-3-nonaprenyl-1,4-naphthoquinone.

the solvent system hexane–*n*-butyl ether (9:1) led to complete separation of MK-8 from 2-demethyl-MK-8. The 2-demethyl derivative was removed from the silica gel by chloroform elution and was obtained as an oil (*ca.* 0.4 mg): λ_{max} (isooctane), 239 (s), 243.5, 248.5, 254.5, 264, and 327 mμ; λ_{max} (ethanol–sodium acetate) 241.5, 247, 250.5, 264.5, and *ca.* 332 mμ; on treatment with sodium borohydride, λ_{max} 245.5 mμ. Parent molecular ion *m/e* 702 and base peak *m/e* 211.

B. 2-DEMETHYL-MK-7. Similar layer chromatography of the heptaprenylog fraction (2.1 mg) in the solvent system hexane–*n*-butyl ether (9:1) yielded 2-demethyl-MK-7 (*ca.* 20 μg); λ_{max} (isooctane) 243.5, 249, 254.5, and 264 mμ. Parent molecular ion *m/e* 634, base peak *m/e* 211.

Fractionation of the Menaquinone Prenylogs on Hydroxypropylated Sephadex G-50. A further sample of the *E. coli* nonpolar lipids was chromatographed on a silicic acid column as described above. The menaquinone containing fraction (58.9 mg) was applied to a column of hydroxypropylated Sephadex G-50 (25 g; 35 × 2.5 cm; flow rate 6.6 ml/hr; swelling time 72 hr) established in the solvent system, isooctane–methanol–chloroform (2:2:1). After development of the column with 73.2 ml of the solvent, fractions (1.4 ml) were collected. By ultraviolet spectrometry it was shown that menaquinones were present in fractions 5–25. The reversed-phase thin-layer chromatogram of these fractions is shown in Figure 3B. As before the presence of MK-6–MK-9 was firmly established by isolation, and mass and ultraviolet spectrometry. Demethyl-MK-8 and dimethyl-MK-7 were detected in the pure octa- and heptaprenylog fractions of this second preparation.



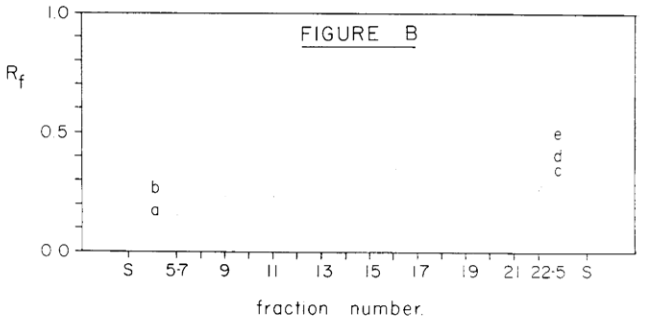


FIGURE 3: Reversed-phase thin-layer chromatographic assay of menaquinone containing fractions from Sephadex columns. (A) Sephadex LH-20 [column dimensions, 133 g, 198 × 1.8 cm; sample size, 60 mg; four fractions (2.5 ml) collected per hour]. (B) Hydroxypropylated Sephadex G-50 [column dimensions, 25 g, 35 × 2.5 cm; sample size, 58.9 mg; 4.7 fractions (1.2 ml) collected per hour]. R_f values and coden; a, MK-9, 0.17; b, MK-8, 0.28; c, MK-7, 0.35; d, MK-6, 0.41; e, MK-5, 0.50; S, standard mixture of MK-5, MK-7, and MK-9.

Results

The menaquinone composition of *E. coli* was examined by the gel filtration–mass spectrometric methods which previously had proved effective in demonstrating menaquinone multiplicity in *M. phlei* (Campbell and Bentley, 1968). However, since *E. coli* also produces a series of ubiquinones of comparable molecular size (Daves *et al.*, 1967) steps had to be taken to separate the menaquinones from the ubiquinones prior to gel filtration. Column chromatography of the crude nonpolar lipids on silicic acid in benzene proved effective for this purpose. After this preliminary purification the menaquinone containing portion of the nonpolar lipids was chromatographed on Sephadex LH-20. The column effluent was monitored for the characteristic menaquinone ultraviolet absorption chromophore; the latter was found in nine consecutive fractions. Bar graphs of the higher mass region of the mass spectra of the contents of these nine fractions are shown in Figures 1 and 2. Evidence for menaquinone multiplicity was present. While the expected MK-8 (*m/e* 716) was virtually the sole constituent of fractions 7–9, it was accompanied by MK-7 (*m/e* 648) to an ever increasing extent in fractions 10–13. Proportions of MK-6 (*m/e* 580) appeared in fractions 13–14, while ions at *m/e* 784 and 786 in the spectra of fractions 6 and 7 were attributable to traces of MK-9.[2] These findings were confirmed, firstly by reversed-phase chromatography of the contents of the fractions on layers of paraffin oil impregnated silica gel (Figure 3A), and secondly by mass and ultraviolet spectrometry of pure samples of the individual prenylogs obtained by preparative reversed-phase layer chromatography of the contents of appropriate fractions. Authentic samples of MK-5, MK-7, and MK-9 were available for comparison.

Detailed examination of the partial mass spectra shown in Figures 1 and 2 revealed that ions other than those due to menaquinones were present, albeit in trace amounts. As yet the nature of the materials giving rise to ions of *m/e* ratio 748 and 732 is unknown although it appears probable that both have polyprenyl side chains. The possibility of their being oxygenated menaquinones is being considered.

A greater level of success was achieved in elucidating the structure of the substances giving rise to the parent molecular ions, *m/e* 702 and 634. Layer chromatography of the purified octaprenylog fraction on silica gel in the *n*-butyl ether–hexane system used to separate menaquinone geometrical isomers (Mayer *et al.*, 1964) revealed that the major *trans*-menaquinone-8 band was accompanied by a small satellite band of lower R_F value. Preparative layer chromatography in the

[2] The occurrence of an ion of *m/e* ratio 786 in the mass spectra of fractions 6 and 7 suggested initially that a dihydro-MK-9 was present in the organism. To date, this point has not been completely settled. It has been shown, however, that under the conditions used, MK-9 and to a somewhat lesser extent the lower prenylogs, pick up two hydrogen atoms to form the corresponding hydroquinone. This behaviour is typical of quinones (Ukai *et al.*, 1967; I. M. Campbell, unpublished observations) and is responsible for at least a portion of the *m/e* 786 intensity in the spectra of fractions 6 and 7.

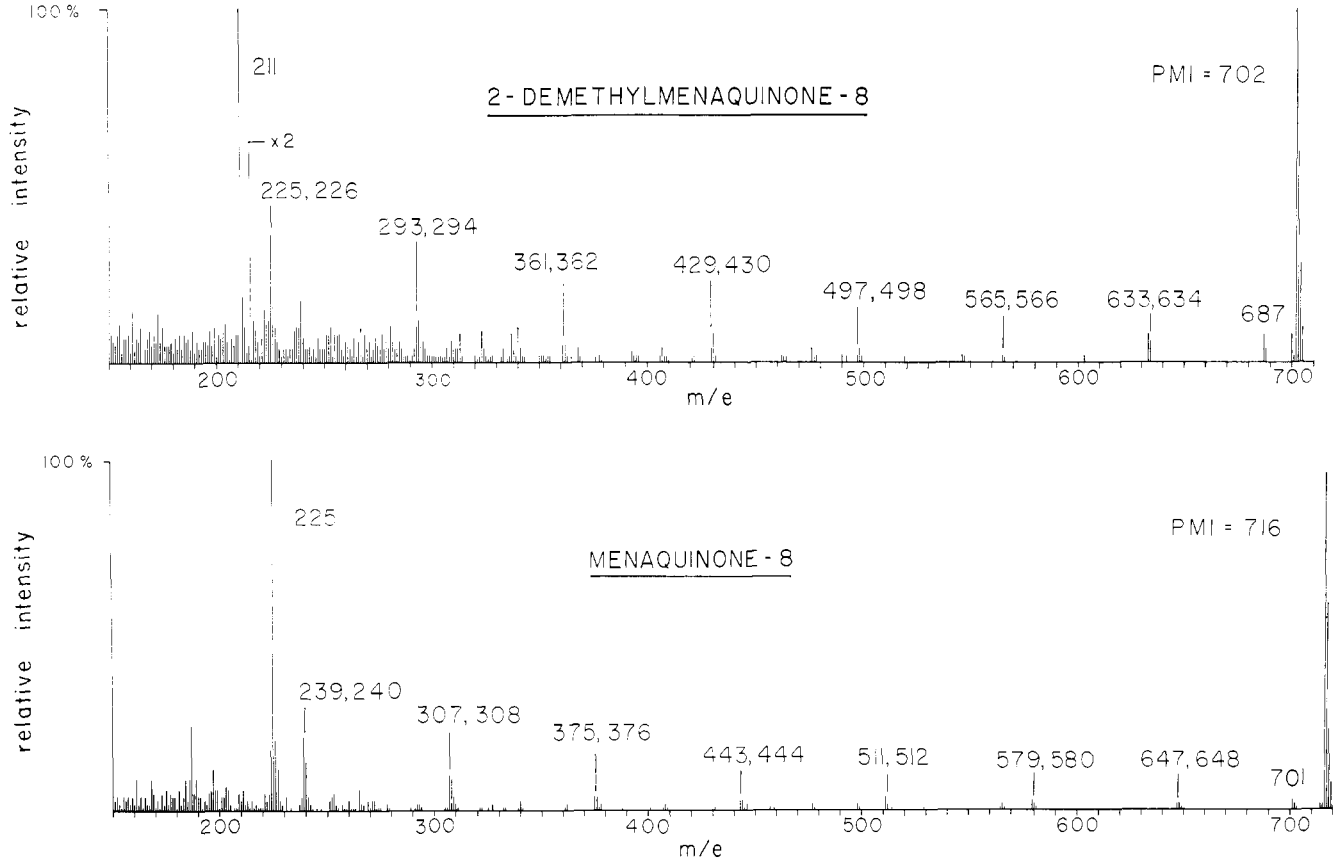
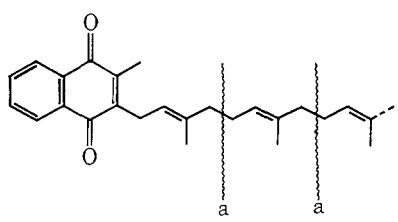


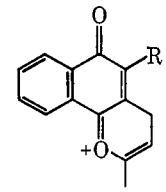
FIGURE 4: Partial mass spectra (70 eV) of menaquinone-8 and 2-demethylmenaquinone-8.

same solvent system led to the isolation of this latter substance in a pure form. As detailed in Methods, its ultraviolet spectrum, recorded in buffered ethanol, altered on treatment with sodium borohydride in the manner characteristic of quinones (Lester *et al.*, 1964). Furthermore, when the spectrum was recorded in isooctane the chromophore was easily recognized as that of a 2-alkyl-1,4-naphthoquinone (Baum and Dolin, 1965).

Final proof that the material was 2-octaprenyl-1,4-naphthoquinone (II, n = 8) followed comparison of its mass spectrum with that of menaquinone-8 (I, n = 8) (Figure 4). In addition to the parent molecular ion (P) at *m/e* 716, the principal features of the 70-eV mass spectrum of menaquinone-8 were a P-15 ion at *m/e* 701, a base peak at *m/e* 225, and a series of fragment ions of general formula *m/e* 716–68*n* and 716–69*n*, *viz.* 648, 580, 512, 444, 376, 308, 240 and 647, 579, 511, 443, 375, 307, and 239. Mass measurement has shown that these latter series of ions are formed by cleavage through side-chain diallylic bonds such as "a" in III (I. M. Campbell, unpublished observations). The first series involves cleavage with hydrogen transfer, the second series involves simple bond fission. The base peak at *m/e* 225 has been accorded structure IVa by Di Mari *et al.* (1966).



III



IVa, R = CH_3, *m/e* 225
b, R = H, *m/e* 211

Examination of the complete 70-eV mass spectrum of the new quinone revealed all the above mentioned features of the menaquinone-8 spectrum albeit shifted 14 units to lower mass. Thus the parent molecular ion occurred at *m/e* 702, the base peak at *m/e* 211. A P-15 ion appeared at *m/e* 687 and was accompanied by series of fragment ions of general formula *m/e* 702–68*n* and 702–69*n*, *viz.* 634, 566, 498, 430, 362, 294, 226 and 633, 565, 497, 429, 361, 293, and 225 (Figure 4). From this and the ultraviolet data it was deduced that the material was a menaquinone-8 lacking the methyl group on the naphthoquinone ring system, *i.e.*, 2-octaprenyl-1,4-naphthoquinone (II, n = 8). The *m/e* 211 mass spectral base peak presumably has structure IVb.

By similar methods it was found that the isolated hepta-

prenylog fraction of the organism contained 2-heptaprenyl-1,4-naphthoquinone (II, $n = 7$).

So far in this study of the menaquinones of *E. coli* and in the previous one of those of *M. phlei* (Campbell and Bentley, 1968), Sephadex LH-20 was used in the critical fractionation step. This latter material is a hydroxypropylated Sephadex G-25 and, in our hands, has given reasonable separations of nonpolar materials in the molecular weight range 100–800. Some overlapping, however, does occur in the higher regions of the range even when large gel/substrate ratios and recycling techniques were used. To combat these disadvantages, we have investigated the possibility of using hydroxypropylated Sephadex G-50, a lipophilic Sephadex derivative with a lesser degree of cross-linking than Sephadex LH-20. Using the same solvent system, comparable sample size and fraction collection rate, *but a bed volume only 34% the size used in the Sephadex LH-20 experiments*, there was obtained the fractionation of the *E. coli* menaquinones shown in Figure 3B.

Comparing the two fractionations (Figure 3), it is clear that the considerably smaller hydroxypropylated Sephadex G-50 column was at least as efficient as the Sephadex LH-20 column. Thus both systems spread the menaquinone band over 22–25 ml of solvent; both systems gave pure menaquinone-8 in a third of this spread volume. On the basis of this evidence it is concluded that hydroxypropylated Sephadex G-50 is superior to Sephadex LH-20 in fractionating large molecular weight nonpolar lipids such as menaquinone prenylogs.

Discussion

It has been clearly demonstrated that the menaquinone content of *E. coli* is inhomogeneous with respect to length of the side chain. Materials with hexa-, octa-, and nonaprenyl side chains have been isolated and unambiguously identified. A similar situation prevails in the 2-demethylmenaquinones of the organism. The substance previously presumed to be 2-octaprenyl-1,4-naphthoquinone has been isolated and characterized. Additionally, the corresponding heptaprenylog has been isolated and characterized. Further support is therefore given to the hypothesis that inhomogeneity in the bacterial menaquinone content is general. Whether this phenomenon represents a functional necessity or results from enzyme partial specificity is as yet undetermined. The occurrence of side-chain multiplicity in the menaquinone content of *E. coli* is in keeping with similar multiplicity in its ubiquinone content (Daves *et al.*, 1967).

Acknowledgments

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References

Baum, R. H., and Dolin, M. I. (1965), *J. Biol. Chem. 240*, 3425.

Bishop, D. H. L., Pandya, K. P., and King, H. K. (1962), *Biochem. J. 83*, 606.

Campbell, I. M., and Bentley, R. (1968), *Biochemistry 7*, 3323.

Cawthorne, M. A., Jeffries, L. R., Harris, M., Price, S. A., Diplock, A. T., and Green, J. (1967), *Biochem. J. 104*, 35C.

Daves, G. D., Muraca, R. F., Whittick, J. S., Friis, P., and Folkers, K. (1967), *Biochemistry 6*, 2861.

Di Mari, S. J., Supple, J. H., and Rapoport, H. (1966), *J. Am. Chem. Soc. 88*, 1226.

Jacobsen, B. K., and Dam, H. (1960), *Biochim. Biophys. Acta 40*, 211.

Jeffries, L., Cawthorne, M. A., Harris, M., Diplock, A. T., Green, J., and Price, S. A. (1967), *Nature 215*, 257.

Lester, R. L., White, D. C., and Smith, S. L. (1964), *Biochemistry 3*, 949.

Mayer, H., Gloor, U., Isler, O., Ruegg, R., and Wiss, O. (1964), *Helv. Chim. Acta 47*, 221.

Ukai, S., Hirose, K., Tatematsu, A., and Goto, T. (1967), *Tetrahedron Letters*, 4999.

Whistance, G. R., Dillon, J. F., and Threlfall, D. R. (1969), *Biochem. J. 111*, 461.