The Nature, Intergeneric Distribution and Biosynthesis of Isoprenoid Quinones and Phenols in Gram-Negative Bacteria

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1. Twenty-two aerobically grown Gram-negative bacteria were analysed for demethylmenaquinones, menaquinones, 2-polyprenylphenols, 6-methoxy-2-polyprenylphenols and ubiquinones. 2. All the eight enterobacteria and both the two facultative organisms (*Aeromonas punctata* and *Aeromonas hydrophila*) examined contain all the compounds listed above. The principal homologues are octaprenyl; in addition lower (down to tri- or tetra-prenyl for the 2-polyprenylphenols) and sometimes higher homologues are also present. 3. Strict aerobes are of two types, those that contain 2-polyprenylphenols, 6-methoxy-2-polyprenylphenols and ubiquinones, and those that contain ubiquinones only. The principal homologues are generally octa- or nona-prenyl, although one organism (*Agrobacterium tumefaciens*) has ubiquinone-10 as its principal homologue. As in the enterobacteria, lower homologues of these compounds are also present. 4. In *Escherichia coli* W, *Pseudomonas ovalis* Chester and *Pseudomonas fluorescens*, radioactivity from *p*-hydroxy[U-¹⁴C]benzoic acid is incorporated into 2-polyprenylphenols, 6-methoxy-2-polyprenylphenols, 6-methoxy-3-methyl-2-polyprenyl-1,4-benzoquinones, ubiquinones and a compound tentatively identified as 2-polyprenyl-1,4-benzoquinone. The fact that radioactivity is incorporated into the first three compounds suggests that in these organisms, and indeed in all those Gram-negative bacteria that contain 2-polyprenylphenols and 6-methoxy-2-polyprenylphenols, ubiquinones are formed by a biosynthetic sequence similar to that in *Rhodospirillum rubrum*. 5. The finding in 'Vibrio O1' (*Moraxella* sp.) and organism PC4 that 2-polyprenylphenols and 6-methoxy-2-polyprenylphenols are chemically and radiochemically undetectable leads to the conclusion that they are not intermediates in the biosynthesis of ubiquinone by these and by other Gram-negative bacteria that do not contain detectable amounts of 2-polyprenylphenols and 6-methoxy-2-polyprenylphenols. However, 'Vibrio O1' (organism PC4 was not examined) does contain 6-methoxy-3-methyl-2-polyprenyl-1,4-benzoquinone. 6. In *Ps. ovalis* Chester, radioactivity from L-[Me-¹⁴C]methionine is incorporated into the nuclear C-methyl and O-methyl groups of 6-methoxy-3-methyl-2-polyprenyl-1,4-benzoquinones and ubiquinone-9, and into the O-methyl group of 6-methoxy-2-polyprenylphenols.

The distribution of ubiquinones and menaquinones in non-photosynthetic Gram-negative bacteria has been the subject of investigation by several groups of workers (Lester & Crane, 1959; Page *et al.* 1960; Bishop, Pandya & King, 1962; Rebel & Mandel, 1965; Raman, Sharma, Jayaraman & Ramasarma, 1965). It was found that the organisms were of two types, those containing ubiquinone-8, -9 or -10, and those containing ubiquinone-8 and menaquinone-8. It is noteworthy that the only bacteria that fell into the latter group were the three enterobacteria examined (*Escherichia coli*, *Proteus vulgaris* and *Proteus mirabilis*). The

two *Proteus* species contained, in addition to ubiquinone-8, smaller amounts of ubiquinone-7 and -9 (Page *et al.* 1960; Rebel & Mandel, 1965). Dolin & Baum (1965) reported that *Esch. coli* contained both menaquinone-8 and demethylmenaquinone-8 (I).

Studies on the effects of aerobiosis and anaerobiosis on the concentrations of ubiquinone and menaquinone in members of the Enterobacteriaceae have produced conflicting results (Lester & Crane, 1959; Kashket & Brodie, 1960, 1961; Bishop *et al.* 1962; Polglase, Pun & Withaar, 1966; Whistance & Threlfall, 1968). However, apart from the observation by Bishop *et al.* (1962) that these factors had

(I) Demethylmenaquinone-8

(II) 2-Nonaprenylphenol

(III)

6-Methoxy-3-methyl-2-nonaprenyl-1,4-benzoquinone

no effect on the concentrations of ubiquinone and menaquinone, the results are in general agreement that the concentrations of ubiquinone and menaquinone are affected by the growth conditions. Thus high concentrations of ubiquinone and low concentrations of menaquinone are found in aerobically grown cells, and the converse occurs in anaerobic cultures.

The biosynthesis of menaquinone and ubiquinone in non-photosynthetic bacteria has received little attention. Cox & Gibson (1964, 1966) and Campbell, Coscia, Kelsey & Bentley (1967) found that in *Esch. coli* the *p*-benzoquinone nucleus of ubiquinone and the aromatic ring plus one carbonyl carbon atom of menaquinone are formed from shikimic acid. Isotope-competition experiments (Cox & Gibson, 1964) and mutant studies (Jones & Lascelles, 1967) showed that in this organism *p*-hydroxybenzoic acid is an intermediate in the formation of the ubiquinone ring. This acid, which is an effective precursor of the ubiquinone ring in a variety of organisms (see Rudney & Raman, 1966), is also a precursor of the ubiquinone ring in two other non-photosynthetic Gram-negative organisms, namely *Pseudomonas denitrificans* and *Azotobacter vinelandii* (Rudney & Raman, 1966). Jackman, O'Brien, Cox & Gibson (1967) demonstrated that in *Esch. coli* the nuclear *C*-methyl and *O*-methyl groups of ubiquinone and menaquinone are formed by the transfer of intact methyl groups from methionine. Surprisingly, the nature of the specific distal precursor required for the biosynthesis of the polyprenyl units of these compounds remains to be determined.

Parson (1965), on incubating cells of *Esch. coli* with *p*-hydroxy[^{14}C]benzoic acid in the absence of oxygen, found that radioactivity was incorporated into a compound with properties similar to those of a polyprenylphenol (2-octaprenylphenol?). On aeration of the cells the radioactivity present in this compound was incorporated into ubiquinone. Imamoto & Senoh (1967) isolated from the lipids of *Pseudomonas ovalis* 2-nonaprenylphenol (II) and 6-methoxy-3-methyl-2-nonaprenyl-1,4-benzoquinone (III). The knowledge that in the anaerobic photosynthetic bacterium *Rhodospirillum rubrum* these two compounds are involved in the

biosynthesis of ubiquinone (Daves, Friis, Olsen & Folkers, 1966) suggests that in *Esch. coli* and *Ps. ovalis* ubiquinone is biosynthesized by a similar route.

In the investigations described in this paper we were concerned with (a) determining the distribution of menaquinones, demethylmenaquinones, ubiquinones, 6-methoxy-2-polyprenylphenols and 2-polyprenylphenols in 22 Gram-negative bacteria, and (b) examining, through the use of *p*-hydroxy-[^{14}C]benzoic acid and L-[^{14}C]methionine, some aspects of ubiquinone biosynthesis in these organisms.

EXPERIMENTAL

Radiochemicals

L-[^{14}C]Tyrosine hydrochloride (5.5 mc/m-mole) and L-[^{14}C]methionine (56.8 mc/m-mole) were purchased from The Radiochemical Centre, Amersham, Bucks.

p-Hydroxy[^{14}C]benzoic acid of high specific radioactivity (4.27 mc/m-mole) was prepared by alkaline fusion of L-[^{14}C]tyrosine hydrochloride (Parson & Rudney, 1964). The product was purified as described by Whistance, Threlfall & Goodwin (1967).

Biological methods

Organisms. Organisms were obtained from the American Type Culture Collection (A.T.C.C.), the National Collection of Type Cultures (N.C.T.C.), the National Collection of Industrial Bacteria (N.C.I.B.), the Northern Regional Research Laboratory (N.R.R.L.) and Dr Muriel E. Rhodes, Department of Botany, University College of Wales, Aberystwyth. Others were stock strains of this Laboratory.

All organisms were maintained by periodic subculture on nutrient-agar slopes containing 2% of Ionagar no. 2 (Oxoid Ltd., London, E.C. 4), 1% of bacto-peptone (Oxoid), 0.5% of yeast extract (Difco Laboratories, Detroit, Mich., U.S.A.) and 0.5% of NaCl, in tap water.

Aerobic culture. The following medium (final pH 7.1) was used in all cases: 0.7% of K_2HPO_4 , 0.3% of KH_2PO_4 , 0.1% of $(\text{NH}_4)_2\text{SO}_4$, 0.1% of NaCl, 0.04% of MgSO_4 , 0.5%

of glucose, 0.2% of sodium glutamate, 0.1% of Bacto-peptone (Oxoid), 0.1% of yeast extract (Difco) and 0.2% of casein hydrolysate (Oxoid), in tap water. Glucose and MgSO_4 , sterilized separately as concentrated solutions, were added to the medium just before inoculation.

The organisms were grown in 10l. batches at 30° (or in some cases 28°, 33° or 37°; see Table 4) with constant aeration (9l./min.) and agitation (180 rev./min.) in a Microform Laboratory Fermenter (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.). The culture vessel was inoculated by adding 1l. (in two lots of 500 ml.) of a 12 hr. aerobic broth culture (Nutrient Broth no. 2; Oxo Ltd., London, S.E. 1). Foaming was controlled by adding a few drops of MS Antifoam Emulsion RD (Midland Silicones Ltd., Barry, Glam.).

Harvesting and determination of dry weight. Organisms were harvested in the late exponential phase of growth by centrifugation in a Sharples Super centrifuge. The wet cell mass was transferred to a weighed 250 ml. plastic centrifuge bottle and washed twice by resuspension in water followed by recentrifugation. The centrifuge bottle with the washed cells was then reweighed and, after the cell mass had been stirred thoroughly, a sample was taken for freeze-drying. From these values the total dry weight of cells was calculated.

Exposure of cells to ^{14}C -labelled substrates. Harvested cells were suspended in 70 ml. of 0.05 M-sodium phosphate buffer, pH 7.0, and transferred to a 250 ml. flask containing the labelled substrate. The cell suspension was then incubated with vigorous shaking at 30° for 3 hr.

Analytical methods

Extraction of lipid. The cell mass, in the centrifuge bottle, was extracted twice with boiling ethanol (two lots of 100 ml.) followed by an extraction with hot ethanol-diethyl ether (1:3, v/v) (100 ml.). The cell debris was separated from the extracts by centrifugation at 5000 rev./min. in a Sorvall Superspeed RC2-B refrigerated centrifuge at 0° and the supernatants were decanted into a 2l. separating funnel containing 500 ml. of diethyl ether. After vigorous shaking the ethanol was removed by careful washing with water (Threlfall & Goodwin, 1967). The ethereal extract was then dried over anhydrous Na_2SO_4 and the solvent removed (Threlfall & Goodwin, 1967).

Preliminary purification of lipid. This was carried out by column chromatography on acid-washed alumina (Brockmann grade III) (M. Woelm, Eschwege, Germany: anionotropic) developed by stepwise elution with 0.25, 1, 3, 5, 12 and 20% (v/v) E/P* as described in detail by Threlfall & Goodwin (1967). Under these conditions demethylmenaquinones, menaquinones and 6-methoxy-2-polyprenylphenols are eluted by 1% E/P, 2-polyprenylphenols by 3% E/P and ubiquinones by 5% E/P. In quantitative experiments ubiquinones and demethylmenaquinones plus menaquinones were determined directly from the column fractions by using the procedures outlined below: occasionally the u.v. spectra of the 1%-E/P fractions would show high end absorption; in these cases the fractions were purified further before quantitative determinations were performed. The presence of 6-methoxy-2-polyprenylphenols

in the 1%-E/P fractions did not interfere with the determinations of the naphthaquinones.

Purification and characterization procedures. The compounds under investigation were purified by thin-layer chromatography. The practical details for the preparation of plates and the recovery of compounds were reported by Whistance *et al.* (1967).

(a) Menaquinones, demethylmenaquinones and 6-methoxy-2-polyprenylphenols. These were purified, but not resolved from each other, by chromatography of the 1%-E/P fraction on thin layers of Kieselgel G impregnated with Rhodamine 6G, developed with benzene. The naphthaquinone-polyprenylphenol band (R_F 0.61) was recovered, and the total naphthaquinone content of the fraction was determined spectroscopically (see below).

Four-fifths of the semi-purified sample was run quantitatively on a reversed-phase thin-layer plate of Kieselgel G impregnated with paraffin; after development with aq. 95% (v/v) acetone the quinones were located by spraying the plate with 0.002% (w/v) sodium fluorescein in ethanol and examination under u.v. light (Whittle, Dunphy & Pennock, 1966) (see Table 1 for R_F values). The principal demethylmenaquinone and principal menaquinone were recovered, and the demethylmenaquinone/menaquinone ratio was determined spectroscopically. (The paraffin present in these samples did not interfere with the determinations.) From this ratio and the value obtained for the total naphthaquinone content of the cells, the contents of menaquinone and demethylmenaquinone ($\mu\text{moles/g. dry wt. of cells}$) were determined.

Menaquinones were characterized on the basis of their u.v. spectra (λ_{max} . 243.5, 248.5, 260, 269 and 326 m μ in cyclohexane), and the lengths of the side chains were determined by reversed-phase chromatography of a portion of the semi-purified sample against menaquinones of known chain length. In all cases the principal homologue ran as menaquinone-8. One complication is that on reversed-phase chromatography the principal demethylmenaquinone homologue (demethylmenaquinone-8) behaves as menaquinone-7, and so it was not possible to ascertain whether any menaquinone-7 was present. However, the observation that the u.v. spectra of purified demethylmenaquinone-8 contained no absorption bands characteristic of menaquinones suggested that the amounts of any lower menaquinone homologues were very small. Finally, as a check that the menaquinone-8 was not a dihydromenaquinone, a purified sample was run on a thin layer of Kieselgel G impregnated with AgNO_3 (Goad & Goodwin, 1966) with acetone-butan-2-one (1:19, v/v) as developing solvent (see Table 1 for R_F values). In this system, which is known to resolve menaquinones from dihydromenaquinones (Jeffries *et al.* 1967), all our samples migrated as menaquinone-8.

Demethylmenaquinones were characterized on the basis of their u.v. spectra (λ_{max} . 243.5, 248.5, 255, 264 and 326 m μ in cyclohexane; see Fig. 1) and their reaction with cysteine. A purified sample of demethylmenaquinone (about 0.15 μmole) was dissolved in 3 ml. of ethanol. Then 10 $\mu\text{l.}$ of 0.2 M-cysteine in 0.1 M-sodium phosphate buffer, pH 7.6, was added to the sample and to the reference cell, and the u.v. spectra were determined after 0, 5, 10 and 20 min. (Fig. 2).

It was to be expected that for any one organism the principal demethylmenaquinone would have the same chain length as the principal menaquinone, i.e. octaprenyl.

* Abbreviation: E/P, solution of diethyl ether in light petroleum (b.p. 40–60°).

Table 1. *Chromatographic systems used for the identification of ubiquinones and menaquinones*

<i>R_F</i> values					
Compound	Kieselgel G plates Water-acetone (1:19, v/v) (reversed phase)	Kieselgel G plates impregnated with AgNO ₃		Compound	<i>R_F</i> values Kieselgel G plates Water-acetone (3:17, v/v) (reversed phase)
		Acetone- butan-2-one (1:19, v/v)	Acetone- butan-2-one (1:4, v/v)		
6-Methoxy-2-octaprenylphenol	0.5*	—	—	Ubiquinol-4	0.78
			—	Ubiquinol-5	0.74
Demethylmenaquinone-8	0.4	0.56	—	Ubiquinol-6	0.70
Menaquinone-8	0.3	0.56	—	Ubiquinol-7	0.65
Ubiquinone-8	—	—	0.52	Ubiquinol-8	0.60
Ubiquinone-9	—	—	0.50	Ubiquinol-9	0.55
Ubiquinone-10	—	—	0.48	Ubiquinol-10	0.49

* *R_F* values for other homologues were given by Spiller *et al.* (1968).

This was verified for *Prot. mirabilis* by mass spectrometry. Thus the molecular ions for the principal menaquinone and principal demethylmenaquinone from this bacterium were found to be at *m/e* 716 and *m/e* 702. Reversed-phase chromatography showed that in all organisms possessing demethylmenaquinones the principal homologue was the same as that occurring in *Prot. mirabilis*.

The remaining one-fifth of the semi-purified 1%-E/P fraction was examined for the presence of 6-methoxy-2-polyprenylphenols by chromatography on a reversed-phase thin-layer plate developed with aq. 95% acetone. Authentic samples of known chain lengths [isolated from *R. rubrum* (deca- and nona-) and *Chromatium* strain D (octa-, hepta- and hexa-)] were also run at the same time. After development the plate was sprayed with a modified Gibbs reagent (Whistance *et al.* 1967), when 6-methoxy-2-polyprenylphenols appeared as blue-violet spots (for *R_F* values see Spiller, Threlfall & Whistance, 1968).

(b) 2-Polyprenylphenols. The 3%-E/P fraction was chromatographed quantitatively on thin layers of Kieselgel G impregnated with Rhodamine 6G, with benzene as developing solvent. On examination of the plate under u.v. light, 2-polyprenylphenols appeared as a pink band (*R_F* 0.52) against a yellow background. After recovery the u.v. spectrum was determined in cyclohexane ($\lambda_{\max}$ 272 and 278.5 m μ) (see Fig. 3) and the amount present determined (see below). The lengths of the side chains were determined by reversed-phase chromatography against samples of known chain length (isolated from the same sources as the 6-methoxy-2-polyprenylphenols) on thin layers developed with aq. 95% acetone; the prenylphenols were located by spraying with Gibbs reagent (for *R_F* values see Spiller *et al.* 1968). The limit of detection was found to be 0.5 μ g. of 2-polyprenylphenol applied as a spot.

(c) Ubiquinones. These were purified and identified by procedures described by Spiller *et al.* (1968). An additional method used to identify the homologues was as follows. The purified ubiquinones were dissolved in a few drops of ethanol and reduced to their corresponding quinols by the addition of a small crystal of NaBH₄. Samples were then chromatographed on reversed-phase thin layers developed with aq. 85% acetone (see Table 1 for *R_F* values). The quinols were detected by spraying with an aq. 50% ethanolic solution containing 0.1% (w/v) FeCl₃ and 0.05% (w/v) $\alpha\alpha'$ -bipyridyl.

As a check on the possible occurrence of dihydro-ubiquinones, ubiquinone samples were chromatographed on AgNO₃-impregnated plates developed with acetone-butan-2-one (1:4, v/v) (see Table 1 for *R_F* values). In this system, dihydroubiquinones have higher *R_F* values than the corresponding ubiquinones; for example, dihydro-ubiquinone-8 co-chromatographs with ubiquinone-7. In all cases the principal homologues plus the next lower homologues behaved as ubiquinones; other homologues were present in such small amounts that they could not be checked.

Chemical degradations. ¹⁴C radioactivity in the polyprenyl side chain of ubiquinone was determined by ozonolytic degradation (Whistance *et al.* 1967).

¹⁴C radioactivity in the methoxyl and nuclear methyl groups of ubiquinone and an unknown compound were determined as described by Threlfall, Whistance & Goodwin (1968).

Spectrophotometry. Ultraviolet spectra were determined in the Unicam SP.800 spectrophotometer. Ubiquinones were measured by a procedure described by Threlfall & Goodwin (1967). Menaquinones, demethylmenaquinones and 2-polyprenylphenols were assayed in cyclohexane, the extinction coefficients used being 19000 ($\lambda_{\max}$ 249 m μ) (Bishop *et al.* 1962), 19000 ($\lambda_{\max}$ 249 m μ) and 2000 ($\lambda_{\max}$ 272 m μ) (Imamoto & Senoh, 1967) respectively.

Radioassay. The methods employed were described by Threlfall *et al.* (1968).

Solvents. Light petroleum (b.p. 40–60°), benzene (A.R.) and diethyl ether were dried over sodium wire and redistilled; ether was also distilled over reduced iron immediately before use. Spectroscopic solvents were of the appropriate grade; all other solvents were of A.R. or equivalent grade.

RESULTS

Distribution and concentrations of demethylmenaquinones, menaquinones, ubiquinones and polyprenylphenols in Gram-positive bacteria. During studies of the biosynthesis of ubiquinones and menaquinone by *Escherichia freundii* and *Prot. mirabilis* we found that they contained 2-poly-

Table 2. *Gram-negative bacteria containing only ubiquinones*

Each analysis was carried out on the cells obtained from 10l. of aerobic culture medium. The ubiquinone homologues are given in order of decreasing concentration. In each case the principal homologue was judged to constitute more than 85% of the total ubiquinone content.

Organism	Dry wt. (g.)	Lipid (mg.)	Ubiquinones	
			Content (μ moles/g. dry wt.)	Homologues
Achromobacteraceae				
<i>Alcaligenes viscolactis</i> (from milk, 1950)*	4.6	284	1.38	9, 8, 7
Organism PC 4*	8	336	0.78	9, 8, 7, 6, 5
Pseudomonadaceae				
<i>Comamonas percolans</i> (A.T.C.C. 8461)	7	487	1.70	8, 7, 6, 4, 5
<i>Xanthomonas campestris</i> (N.C.T.C. 528)	5.8	363	0.85	8, 7, 6, 5, 4
Rhizobiaceae				
<i>Agrobacterium tumefaciens</i> (N.C.T.C. 397)	8	373	0.71	10, 9, 8
Spirillaceae				
' <i>Vibrio O1</i> ' (<i>Moraxella</i> sp.) (N.C.I.B. 8250)	6	244	1.47	9, 8, 7, 10, 6

* Provided by Dr Muriel E. Rhodes.

prenylphenols, 6-methoxy-2-polyprenylphenols and demethylmenaquinones. In the light of these observations it was decided to determine the distribution and concentration of these compounds in a variety of non-photosynthetic Gram-negative bacteria. The investigations showed that for the organisms examined it was possible to recognize three types of distribution pattern: (a) bacteria containing only ubiquinones (Table 2); (b) bacteria containing 2-polyprenylphenols, 6-methoxy-2-polyprenylphenols and ubiquinones (Table 3); (c) bacteria containing demethylmenaquinones, menaquinones, 2-polyprenylphenols, 6-methoxy-2-polyprenylphenols and ubiquinones (Table 4).

The u.v. spectra of the 1%-E/P fractions obtained from lipid extracts of bacteria in group (c) fell into three groups: those similar to menaquinone (Fig. 1) (e.g. *Prot. vulgaris*); those similar to mixtures of various proportions of demethylmenaquinone and menaquinone (e.g. *Prot. mirabilis*, approx. 1:1 mixture; Fig. 1); and those similar to demethylmenaquinone (Fig. 1) (e.g. *Aeromonas punctata*). Indeed, from the nature of the u.v. spectra in the 255–275 m μ region it was possible to obtain rough estimates of the naphthaquinone compositions of the fractions. The purified demethylmenaquinone samples all showed u.v. absorption maxima at 243.5, 248.5, 255, 264 and 326 m μ in cyclohexane (Fig. 1). Further, when determined in the appropriate solvents the wavelengths of absorption were found to be identical with the values quoted for demethylmenaquinone-9 isolated from *Streptococcus faecalis* (Lester, White & Smith, 1964) and synthetic demethylmenaquinone-9 (Misiti, Moore & Folkers, 1965).

Redfearn (1965) reported that *p*-benzoquinones

possessing unsubstituted positions next to electron-attracting groups react with cysteine in a manner that can be followed spectrophotometrically (Scheme 1). We found that a similar reaction takes place between demethylmenaquinones and cysteine (Fig. 2), and this was used as an additional aid in the characterization of these compounds. With menaquinone, as expected, no spectral changes were observed.

Mass spectrometry confirmed the identity of the principal demethylmenaquinone isolated from *Prot. mirabilis*, and showed that the polyprenyl side chain contained eight isoprene units. Thus the sample showed peaks at *M* (*m/e* 702), *M* – 15 (loss of a methyl group), *M* – 69 (loss of terminal isoprene unit), *M* – 69 – (68)_{*n*}, where *n* = 1–6 (sequential loss of six internal isoprene units), and a base peak at *m/e* 211. By analogy with menaquinone (Dimari, Supple & Rapoport, 1966) the latter can be attributed to the molecular ions (IV) and (V).

Although only the principal menaquinone and demethylmenaquinone present in each organism was unequivocally identified, reversed-phase thin-layer chromatography showed the presence of lower homologues of both compounds.

6-Methoxy-2-polyprenylphenols in most cases were present in amounts that could only be detected through the use of our extremely sensitive spray reagent. In *Prot. mirabilis* sufficient of this compound was isolated for us to determine the u.v. spectrum (Fig. 3). This was identical with that of the sample isolated from *R. rubrum* (λ_{max} , 273.5 and 280 m μ).

2-Polyprenylphenols could only be assayed spectrophotometrically after purification by thin-layer chromatography. The u.v. spectra of the

Table 3. *Gram-negative bacteria containing ubiquinones, 2-polyprenylphenols and 6-methoxy-2-polyprenylphenols*

Each analysis was carried out on the cells obtained from 10 l. of aerobic culture medium. The ubiquinone, 2-polyprenylphenol and 6-methoxy-2-polyprenylphenol homologues are given in order of decreasing concentration. In each case the principal homologue was judged to constitute more than 85% of the total amount of the homologues present.

Organism	Dry wt. (g.)	Lipid (mg.)	Ubiquinones		2-Polyprenylphenols		6-Methoxy-2-polyprenylphenols	
			Content (μ mole/g. dry wt.)	Homologues	Content (μ mole/g. dry wt.)	Homologues	Content (μ mole/g. dry wt.)	Homologues
<i>Pseudomonadaceae</i>								
<i>Pseudomonas fluorescens</i> (stock strain)	7	338	0.85	9, 8, 7, 6	0.04	9, 8, 7, 6, 5, 4	0.04	9, 8, 7
<i>Pseudomonas fragi</i> (N.R.L. B25)	10.8	510	0.78	9, 8, 7, 6	0.04	9, 8, 7, 5, 6	0.04	9, 8
<i>Pseudomonas ovalis</i> Chester (A.T.C.C. 17474)	7	369	0.94	9, 8, 7, 6	0.04	9, 8, 7, 6, 5, 4	0.04	9, 8
<i>Pseudomonas putida</i> (stock strain)	10.5	493	0.70	9, 8, 7, 6	0.06	9, 8, 7, 6, 10	0.06	9, 8
<i>Rhizobiaceae</i>								
<i>Chromobacterium violaceum</i> (from milk, 1962)*	12	668	0.85	8, 7, 9	0.06	8, 7, 9, 6	0.06	8, 7, 9, 6
<i>Spirillaceae</i>								
<i>Spirillum serpens</i> (from London, Ont., Canada, 1959)*	5	263	0.91	8, 7, 6, 5	0.09	8, 7, 6, 5	0.09	8, 7, 6

* Provided by Dr Muriel E. Rhodes.

purified samples (Fig. 3) were qualitatively identical with that of authentic 2-nonaprenylphenol (λ_{max} . 272 and 278.5 m μ ; shoulder at 260 m μ). Reversed-phase chromatography showed that in addition to the principal homologue (judged to be 85% of the total sample) smaller amounts of lower homologues and in some cases a higher homologue were present (Tables 3 and 4).

The nature and relative amounts of the ubiquinone homologues present in any given organism followed the same pattern as that found for the 2-polyprenylphenols (Tables 3 and 4).

Radiochemical studies. Chemical analyses showed that, although most of the Gram-negative bacteria examined contained 2-polyprenylphenols and 6-methoxy-2-polyprenylphenols, many of the strict aerobes did not (Table 2). These observations suggested that in the latter organisms 2-polyprenylphenols and 6-methoxy-2-polyprenylphenols are not involved in the biosynthesis of ubiquinone. To investigate this possibility further, the incorporation of radioactivity from *p*-hydroxy[U-¹⁴C]benzoic acid into the lipids of 'Vibrio O 1' and organism PC 4 was examined. At the same time, experiments were carried out in which L-[Me-¹⁴C]methionine and *p*-hydroxy[U-¹⁴C]benzoic acid were administered to *Ps. ovalis* Chester, *Pseudomonas fluorescens* and *Esch. coli* W. The results of these experiments are summarized in Table 5.

The finding that in *Esch. coli* W, *Ps. ovalis* Chester and *Ps. fluorescens* the specific radioactivities of the principal 2-polyprenylphenols were much higher than those of the principal ubiquinones was consistent with a precursor-product relationship.

The distribution of radioactivity between the fractions obtained by column chromatography from the total lipid extracts isolated from cells of *Ps. ovalis* Chester that had been incubated with *p*-hydroxy[U-¹⁴C]benzoic acid or L-[Me-¹⁴C]methionine, and of 'Vibrio O 1' that had been incubated with *p*-hydroxy[U-¹⁴C]benzoic acid, are shown in Fig. 4. The radioactivity-distribution patterns of *Ps. fluorescens* and *Esch. coli* W were similar to that of *Ps. ovalis* Chester, and that of organism PC 4 was similar to that of 'Vibrio O 1'.

In experiments with *p*-hydroxy[U-¹⁴C]benzoic acid all the radioactivity (when present) in the 1% and 3% E/P fractions was associated with 6-methoxy-2-polyprenylphenols and 2-polyprenylphenols respectively (Fig. 4). 'Vibrio O 1' and organism PC 4 contained no radioactivity in these fractions (Fig. 4). Radioactivity from L-[Me-¹⁴C]methionine was incorporated, as expected, into the 6-methoxy-2-polyprenylphenol-containing fraction.

Reversed-phase chromatography coupled with radioautography showed that *Ps. ovalis* Chester, *Ps. fluorescens* and *Esch. coli* W contained all the

Table 4. *Gram-negative bacteria containing menaquinones, demethylmenaquinones, ubiquinones, 2-polyprenylphenols and 6-methoxy-2-polyprenylphenols*

Each analysis was carried out on the cells obtained from 10 l. of aerobic culture medium. The ubiquinone, 2-polyprenylphenol and 6-methoxy-2-polyprenylphenol homologues are given in order of decreasing concentration. In each case the principal homologue was judged to constitute more than 85% of the total amount of the homologues present. Lower homologues of menaquinone and demethylmenaquinone were present. However, for the reasons given in the Experimental section, they could not be positively identified.

Organism	Dry wt. (g.)	Lipid (mg.)	Ubiquinones		2-Polyprenylphenols		6-Methoxy-2-polyprenylphenols Homologues	Demethylmenaquinone-8 Content ($\mu\text{mole/g.}$ dry wt.)	Menaquinone-8 Content ($\mu\text{mole/g.}$ dry wt.)
			Content ($\mu\text{mole/g.}$ dry wt.)	Homologues	Content ($\mu\text{mole/g.}$ dry wt.)	Homologues			
Enterobacteriaceae*									
<i>Erwinia carolinovora</i> (N.C.T.C. 312)	11.3	737	0.73	8, 7	0.05	8, 7, 6, 5	8, 7, 9, 6	0.03	0.06
<i>Escherichia aureescens</i> (N.C.I.B. 8714)	16	573	0.32	8, 7, 6, 4, 5	0.10	8, 7, 6, 9, 5	8, 7, 6, 9	0.28	0.14
<i>Escherichia coli</i> Crookes (N.C.I.B. 8545)	12.5	618	0.47	8, 7, 6, 5	0.06	8, 7, 5, 6, 9	8, 7	0.34	0.13
<i>Escherichia coli</i> W (N.C.I.B. 8666)	13	900	0.66	8, 7, 6, 5, 4	0.13	8, 7, 6, 9, 5	8, 7, 6, 5	0.13	0.06
<i>Escherichia freundii</i> (N.C.I.B. 3735)	23	1070	0.43	8, 7, 9, 6, 5, 4	0.16	8, 7, 9, 6, 5, 4	8, 7, 9, 6, 5	0.16	0.08
<i>Klebsiella aerogenes</i> (N.C.T.C. 418)	6	161	0.49	8, 7, 6, 4, 5	0.08	8, 7, 9, 6	8, 7	0.07	0.03
<i>Proteus mirabilis</i> (N.C.I.B. 5887)	16	831	0.76	8, 7, 6, 5, 4	0.10	8, 7, 9, 6, 5, 4	8, 7	0.24	0.32
<i>Proteus vulgaris</i> (Stock strain)	15.5	550	0.55	8, 7, 6	0.12	8, 7, 6, 5	8, 7, 9	0.07	0.35
Pseudomonadaceae*									
<i>Aeromonas hydrophila</i> (N.C.M.B. 7810)	14	1000	0.58	8, 7, 6, 4, 5	0.07	8, 7, 6	8, 7, 6, 9	0.07	0.05
<i>Aeromonas punctata</i> (N.C.M.B. 640)	18	1126	0.49	8, 7, 6, 4, 5	0.10	8, 9, 7, 6	8, 9, 7	0.13	0.01

* *Aeromonas* spp. were grown at 33°; enterobacteria, with the exception of *Erw. carolinovora*, which was grown at 28°, were grown at 37°.

2-polyprenylphenol homologues from tri- to nona-prenyl. In radiochemical terms the dominant homologues were 2-octaprenylphenol in *Esch. coli* W, and 2-nona- and 2-tetra-prenylphenol in *Ps. ovalis* Chester and *Ps. fluorescens* (Fig. 5a). One noteworthy observation was that, on adsorptive thin-layer chromatography of the 3%-E/P fractions (the first step in the purification of 2-polyprenylphenols) from *Ps. ovalis* Chester and *Ps. fluorescens*, a partial separation of the 2-polyprenylphenols into lower and higher homologues took place (Figs. 5b, 5c and 6). The low radioactivity in the 6-methoxy-2-polyprenylphenols meant that the presence of only the principal homologue (nona- in *Ps. ovalis* Chester and *Ps. fluorescens*; octa- in *Esch. coli* W) plus the next lower homologue could be shown.

In the 5%-E/P fractions isolated from organisms that had been incubated with *p*-hydroxy[U-¹⁴C]-benzoic acid it was found that about 22% (*Ps. ovalis* Chester) to 48% ('*Vibrio* O1') of the radioactivity

was associated with ubiquinone (Fig. 4). Adsorptive thin-layer chromatography of the fractions from *Ps. ovalis* Chester, *Ps. fluorescens*, *Esch. coli* W and '*Vibrio* O1' showed that the radioactivity was distributed between ubiquinone (R_F 0.4) and an unknown compound(s) (R_F 0.33) (Fig. 6). On reversed-phase chromatography the unknown compound(s) gave four major radioactive bands (*A*, *B*, *C* and *D*) (Fig. 5d). The finding that on treatment with sodium borohydride compounds *A*, *B*, *C* and *D* gave polar Emmerie-Engel-staining derivatives established that these compounds were quinonoid.

Fig. 1. Ultraviolet spectra in cyclohexane of various naphthaquinone fractions isolated from *Prot. mirabilis*. —, Demethylmenaquinone-8; ----, menaquinone-8; -.-.-, 1%-E/P fraction.

Fig. 2. Ultraviolet spectra obtained on treatment of demethylmenaquinone-8 (in ethanol) with cysteine (for details see the Experimental section). —, At 0 min.; - - - -, after 5 min.; - - - -, after 10 min.; —, after 20 min.

$R'\cdot SH$ = cysteine; R = nonaprenyl side chain.

Scheme 1. Reaction of plastoquinone with cysteine (after Redfearn, 1965).

In the experiments with 'Vibrio O 1' and *Ps. ovalis* Chester, semi-purified ubiquinones plus compounds A, B, C and D were subjected to ozonolytic degradation, when it was found (as expected) that no radioactivity was present in the polyprenyl portions of these molecules.

In *Ps. ovalis* Chester radioactivity from L-[Me-¹⁴C]methionine was incorporated only into ubiquinones and compounds A and C. Zeisel degradation established that in the ubiquinone-9 molecule 76% of the radioactivity was in the methoxyl groups; Kuhn-Roth oxidation coupled with a Schmidt degradation established that the remaining 24% was in the nuclear methyl group. In compounds A and C (degraded together) 61%

of the radioactivity was in the methoxyl groups and 37% was in nuclear methyl groups. The latter finding coupled with the other known properties (see above) of compounds A and C led us to conclude that in *Ps. ovalis* Chester, *Ps. fluorescens* and 'Vibrio O 1' compound C was 6-methoxy-3-methyl-2-nonaprenyl-1,4-benzoquinone (a compound previously isolated from *Ps. ovalis* Chester by Imamoto & Senoh, 1967), and that compound A was a lower homologue of this compound. In *Esch. coli* W compound C appears to be the corresponding 2-octaprenyl derivative. The observation that in *Ps. ovalis* Chester the quinones B and D were not labelled from L-[Me-¹⁴C]methionine suggested that compound D might be 2-nonaprenyl-1,4-benzoquinone.

The 12%-E/P fractions contained compounds B and D and three other radioactive more polar quinonoid compounds.

DISCUSSION

The results of the survey show that of the 22 Gram-negative bacteria examined all the eight enterobacteria and both the two facultative bacteria (*Aero. punctata* and *Aeromonas hydrophila*) contain demethylmenaquinones, menaquinones, 2-polyprenylphenols, 6-methoxy-2-polyprenylphenols and ubiquinones (Table 4). The strict aerobes fall into two categories: those that contain 2-polyprenylphenols, 6-methoxy-2-polyprenylphenols and ubiquinones (Table 3), and those that contain only ubiquinones (Table 2).

The intergeneric distribution and amounts of menaquinones and ubiquinones in *Ps. fluorescens*, *Pseudomonas fragi*, *Pseudomonas putida*, *Prot. vulgaris* and *Esch. coli* are in agreement with the results obtained by other workers (Page *et al.* 1960;

Fig. 3. Ultraviolet spectra in cyclohexane of 2-polyprenylphenols (----) isolated from *Esch. coli* W and 6-methoxy-2-polyprenylphenols (—) isolated from *Prot. mirabilis*.

Table 5. Incorporation of p-hydroxy[U-¹⁴C]benzoic acid and L-[Me-¹⁴C]methionine into ubiquinones and 2-polyprenylphenols

Cells obtained from 10l. of aerobic growth medium were suspended in 70 ml. of 0.05 M-sodium phosphate buffer, pH 7.0, and transferred to a 250 ml. flask containing the labelled substrate. The cell suspension was then incubated with vigorous shaking at 30° for 3 hr. The amounts of the various components present were similar to those given in Tables 2, 3 and 4. The specific radioactivities of only the principal homologues were determined.

Organism	Substrate	Amount of label given (μc)	Incorporation (% of dose)	Sp. radioactivity (counts/min./μmole)	
				Ubiquinone-8 or -9	2-Octa- or 2-nona-prenylphenol
<i>Esch. coli</i> W	p-Hydroxy[U- ¹⁴ C]benzoic acid	2	3.4	10109	80000
<i>Ps. fluorescens</i>	p-Hydroxy[U- ¹⁴ C]benzoic acid	3	5.9	3155	540600
<i>Ps. ovalis</i>	p-Hydroxy[U- ¹⁴ C]benzoic acid	4	6.7	11800	323600
<i>Ps. ovalis</i> Chester	L-[Me- ¹⁴ C]Methionine	20	1.9	33450	0
'Vibrio O 1' (<i>Morazella</i> sp.)	p-Hydroxy[U- ¹⁴ C]benzoic acid	3	4.8	14170	0
Organism PC 4	p-Hydroxy[U- ¹⁴ C]benzoic acid	2	19.8	29200	0

Fig. 4. Incorporation of radioactivity into fractions obtained from column chromatography of lipid extracts isolated from cells of *Ps. ovalis* Chester and 'Vibrio O1' (*Moraxella* sp.) that had been incubated with *p*-hydroxy-[U-¹⁴C]benzoic acid or L-[Me-¹⁴C]methionine. ----, Total radioactivity; ■, radioactivity in 6-methoxy-2-polyprenylphenols; ▨, radioactivity in 2-polyprenylphenols; ▤, radioactivity in ubiquinones. (a) *Ps. ovalis*, *p*-hydroxy[U-¹⁴C]benzoic acid; (b) *Ps. ovalis*, L-[Me-¹⁴C]methionine; (c) 'Vibrio O1', *p*-hydroxy[U-¹⁴C]benzoic acid.

Fig. 5. Radioautographs prepared from reversed-phase thin-layer chromatograms of 2-polyprenylphenol and 5%-E/P fractions isolated from cells of *Ps. ovalis* Chester that had been incubated with *p*-hydroxy[U-¹⁴C]benzoic acid. (a) 2-Polyprenylphenols after preliminary purification of the 3%-E/P fraction by adsorptive thin-layer chromatography; (b) higher and (c) lower 2-polyprenylphenol homologues obtained from the 3%-E/P fraction by adsorptive thin-layer chromatography (see the text and Fig. 6); (d) compounds resolved from ubiquinone by adsorptive thin-layer chromatography of the 5%-E/P fraction (Fig. 6). The developing solvents used were aq. 95% acetone (a, b and c) and aq. 85% acetone (d). The radioactive bands shown in (a), (b) and (c) corresponded exactly to the 2-polyprenylphenol bands detected by spraying with Gibbs reagent.

Fig. 6. Radiochromatogram scans of adsorptive thin-layer chromatograms (developed with benzene) of the 3%- and 5%-E/P fractions obtained from cells of *Ps. ovalis* Chester that had been incubated with *p*-hydroxy[U-¹⁴C]benzoic acid. (a) 3%-E/P fraction; (b) 5%-E/P fraction. The scanning conditions were similar to those described by Whistance *et al.* (1967).

homologues, lower and in some cases higher homologues of these compounds. In view of the demonstration that *Esch. coli* contains ubiquinone-1 to ubiquinone-8 (Daves, Muraca, Whittick, Friis & Folders, 1967), these results are not unexpected.

In Gram-negative bacteria demethylmenaquinone had previously only been reported to occur in *Esch. coli* (Dolin & Baum, 1965). However, in our investigations demethylmenaquinones are found in all ten organisms that contain menaquinones, i.e.

Bishop *et al.* 1962; Raman *et al.* 1965). In contrast with these workers, we found that all 22 organisms examined contain, in addition to the major

those organisms capable of both aerobic and anaerobic growth (Table 4). Previous studies showed that in anaerobically grown cells of *Esch. freundii*, *Prot. mirabilis* and *Aero. punctata* the amounts of menaquinones relative to demethylmenaquinones are appreciably higher than in aerobically grown cells (Whistance & Threlfall, 1968). These findings led us to propose that menaquinones are only required for anaerobic growth (a contention supported by the finding that in *Esch. coli* B/r very little menaquinone is present in cultures grown under forced aeration; Polglase *et al.* 1966) and that the aerobic forms, though not requiring menaquinone, can only partially inhibit its synthesis, which leads to an accumulation of the penultimate precursor demethylmenaquinone (Whistance & Threlfall, 1968). However, the possibility that the latter quinone has a metabolic role in these organisms should not be ignored.

In agreement with Imamoto & Senoh (1967), we found that *Ps. ovalis* Chester contains 2-nonaprenylphenol, and, as suggested by Parson (1965), that *Esch. coli* contains 2-octaprenylphenol. Indeed, 2-polyprenylphenols and 6-methoxy-2-polyprenylphenols are present in most of the bacteria examined; the exceptions are some of the strict aerobes (Tables 2, 3 and 4). Depending on the organism examined, 2-polyprenylphenols are present in amounts that vary from 5 to 37% of the ubiquinone content; the homologues present range from 2-tri-, 2-tetra-, 2-penta- or 2-hexa-prenylphenol through to 2-octa- or 2-nona-prenylphenol, the predominant form being 2-octaprenylphenol or 2-nonaprenylphenol. 6-Methoxy-2-polyprenylphenols are present in such small amounts that they cannot be quantitatively determined. As with the ubiquinones and 2-polyprenylphenols the main homologue is found to be the octa- or nona- form. In addition lower homologues are also present.

It is noteworthy that for a given bacterium the length of the polyprenyl side chain of the principal homologue of each homologous series of compounds is the same.

In the experiments in which *p*-hydroxy[U-¹⁴C]-benzoic acid is administered to *Ps. ovalis* Chester, *Ps. fluorescens* and *Esch. coli* W, radioactivity is incorporated into the nuclei of 2-polyprenylphenols, 6-methoxy-2-polyprenylphenols, 6-methoxy-3-methyl-2-polyprenyl-1,4-benzoquinones, ubiquinones and a compound tentatively identified as 2-polyprenyl-1,4-benzoquinone (Table 5 and Figs. 4, 5 and 6). The fact that radioactivity is incorporated into the first three compounds suggests that in these organisms ubiquinones are biosynthesized by a sequence involving the same intermediates as those that participate in the biosynthesis of ubiquinones by *R. rubrum* (Daves *et al.* 1966). This proposal is supported by the observation that the

specific radioactivities of the principal 2-polyprenylphenols are appreciably higher than those of the principal ubiquinones. Again, although the specific radioactivities of the 6-methoxy-2-polyprenylphenols and 6-methoxy-3-methyl-2-polyprenyl-1,4-benzoquinones were not determined directly, it was apparent from the small amounts of these compounds present and their high radioactivity content that they too had specific radioactivities greater than those of the ubiquinones. However, the presence of compounds with properties similar to those of 2-polyprenyl-1,4-benzoquinones suggests that within the same organism alternative pathways of ubiquinone biosynthesis can exist. This proposal was previously put forward by Friis, Nilsson, Daves & Folkers (1967) to account for the occurrence in *R. rubrum* of 3-methyl-2-polyprenyl-1,4-benzoquinone.

The finding that in 'Vibrio O 1' and organism PC 4 2-polyprenylphenols and 6-methoxy-2-polyprenylphenols are chemically (see above) and radiochemically (Table 2 and Fig. 4) undetectable suggests that they are not present and therefore not involved in the biosynthesis of ubiquinone in these bacteria. However, in 'Vibrio O 1' (organism PC 4 was not examined) labelled compounds with properties similar to those of 6-methoxy-3-methyl-2-polyprenyl-1,4-benzoquinones and 2-polyprenyl-1,4-benzoquinones are present.

In *Ps. ovalis* Chester, radioactivity from L-[Me-¹⁴C]methionine is incorporated into 6-methoxy-2-polyprenylphenols, 6-methoxy-3-methyl-2-polyprenyl-1,4-benzoquinones and ubiquinones (Table 5 and Fig. 4). The appropriate chemical degradations (see the Results section) confirmed that in the latter two compounds the radioactivity was distributed among the nuclear *C*-methyl and *O*-methyl groups. This suggests that these groups are formed by transmethylation from *S*-adenosylmethionine.

The results of the radiochemical studies and of the survey suggest that, in those Gram-negative bacteria containing 2-polyprenylphenols and 6-methoxy-2-polyprenylphenols, these compounds are involved in the biosynthesis of ubiquinone, the converse situation holding for those organisms from which these compounds are absent. In any attempt to arrive at an understanding of how ubiquinone is biosynthesized in Gram-negative bacteria, account must be taken of the plurality shown by the postulated intermediates and the terminal product itself. If it is assumed that the bacterium has a requirement for only its principal ubiquinone homologue, then it appears that the enzymes concerned with the biosynthesis of this homologue are not completely specific for the length of the side chains of the substrates. The most interesting aspect of this problem is whether the 2-polyprenylphenols arise as a result of the action of a relatively

non-specific prenylating enzyme or whether they are formed from 2-monoprenylphenol by sequential C₅ additions. At the present time the first proposal seems better supported, since biosynthetically it involves mostly known reactions.

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