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Biosynthesis of Ubiquinone in Non-Photosynthetic Gram-Negative Bacteria

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1. The polyprenylphenol and quinone complements of the non-photosynthetic Gram-negative bacteria, *Pseudomonas ovalis* Chester, *Proteus mirabilis* and 'Vibrio O1' (*Moraxella* sp.), were investigated. 2. *Ps. ovalis* Chester and *Prot. mirabilis* were shown to contain 2-polyprenylphenols, 6-methoxy-2-polyprenylphenols, 6-methoxy-2-polyprenyl-1,4-benzoquinones, 5-demethoxyubiquinones, ubiquinones, an unidentified 1,4-benzoquinone [2-polyprenyl-1,4-benzoquinone (?)] and 'epoxyubiquinones'. 'Vibrio O1' was shown to contain only 5-demethoxyubiquinones, ubiquinones and 'epoxyubiquinones'. 3. It was established that in *Ps. ovalis* Chester 2-polyprenylphenols, 6-methoxy-2-polyprenylphenols, 6-methoxy-2-polyprenyl-1,4-benzoquinones, 5-demethoxyubiquinones and 2-polyprenyl-1,4-benzoquinones (?) are precursors of ubiquinones. 4. Intracellular distribution studies showed that in *Ps. ovalis* Chester ubiquinone and its prenylated precursors are localized entirely on the protoplast membrane. 5. Investigations into the oxygen requirements for ubiquinone biosynthesis by *Ps. ovalis* Chester showed that the organism could not convert *p*-hydroxybenzoic acid into ubiquinone in the absence of oxygen, although it could convert a limited amount into 2-polyprenylphenols. 6. Attempts were made to prepare cell-free preparations capable of synthesizing ubiquinone. Purified protoplast membranes of *Ps. ovalis* Chester were found to be incapable of carrying out this synthesis, even when supplemented with cytoplasm. With crushed-cell preparations of *Ps. ovalis* Chester, organism PC4 (*Achromobacter* sp.) and *Escherichia coli*, synthesis was observed, although this was attributable in part to a small number of intact cells present in the preparations.

It is now well established that *p*-hydroxybenzoic acid is a precursor of ubiquinone in both photosynthetic and non-photosynthetic Gram-negative bacteria (Rudney & Raman, 1966; Whistance, Dillon & Threlfall, 1969*b*). In contrast, the pathway(s) by which this acid is incorporated into bacterial ubiquinones is (are) not clearly defined. The greatest advances in respect of the problem have been made by K. Folkers, H. Rudney and their co-workers who detected in (Parson & Rudney, 1965), and subsequently isolated from (Olsen *et al.* 1966; Friis, Daves & Folkers, 1966; Friis, Nilsson, Daves & Folkers, 1967), the lipids of the photosynthetic bacterium, *Rhodospirillum rubrum*, a series of decaprenyl-substituted phenols and quinones which are believed to be intermediates in the biosynthesis of ubiquinone-10 in this organism (Scheme 1). At present, however, there is experimental evidence of such a role for only two of the compounds, 2-decaprenylphenol and 6-methoxy-2-decaprenylphenol (Parson & Rudney, 1965; Olsen *et al.* 1966).

Recently Whistance *et al.* (1969*b*) have examined a number of non-photosynthetic Gram-negative bacteria for the presence of polyprenylphenols of the type known to be precursors of ubiquinone in *R. rubrum*. Of the 22 organisms examined all (ten) of the facultative anaerobes and half (six) of the obligate aerobes contained both 2-polyprenylphenols and 6-methoxy-2-polyprenylphenols, whereas the other six obligate aerobes contained neither. Radiochemical studies with *p*-hydroxy[U-^{14}C]-benzoic acid provided some evidence that in the polyprenylphenol-containing organisms, *Escherichia coli*, *Pseudomonas ovalis* Chester and *Pseudomonas fluorescens* (the remainder were not examined), these compounds are ubiquinone precursors. In addition, it was shown that the latter organisms and the non-prenylphenol-containing organism 'Vibrio O1' (*Moraxella* sp.), contain a number of polyprenylquinones which become highly labelled on administration of *p*-hydroxy[U-^{14}C]benzoic acid.

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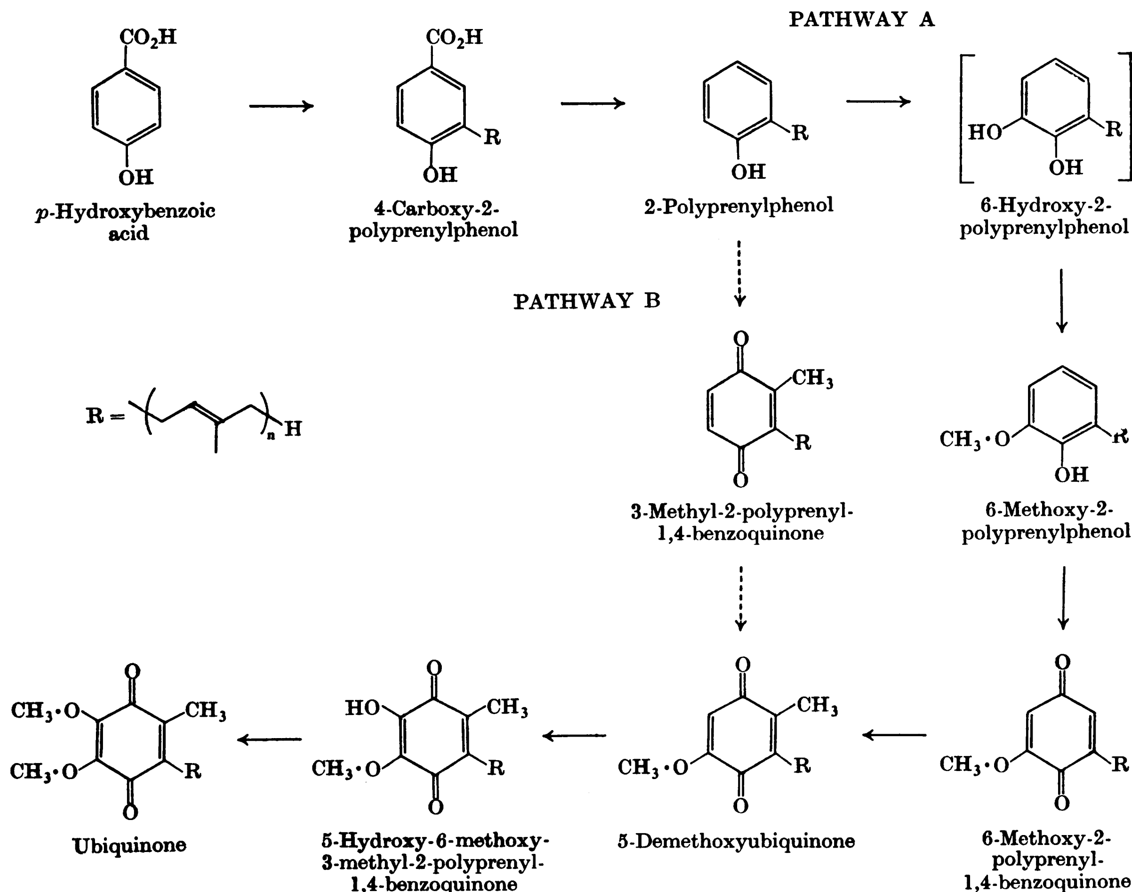


Scheme 1. Proposed pathways for the biosynthesis of ubiquinone from *p*-hydroxybenzoic acid in *R. rubrum* (after Daves, Friis, Olsen & Folkers, 1966; Friis *et al.* 1967).

On the basis of their chromatographic properties some of these quinones were tentatively identified as 5-demethoxyubiquinones, a quinone series the nonaprenyl form of which is known to occur in *Ps. ovalis* (Imamoto & Senoh, 1968) and the decaprenyl form of which is believed to be a ubiquinone-10 precursor in *R. rubrum* (Friis *et al.* 1966).

In the present paper further studies on the biosynthesis of ubiquinone from *p*-hydroxybenzoic acid by the non-photosynthetic Gram-negative bacteria, *Ps. ovalis* Chester, *Proteus mirabilis* and 'Vibrio O1', are described. These investigations were concerned with (*a*) the nature of the intermediates on the biosynthetic pathway, and (*b*) the intracellular location of ubiquinone and its prenylated precursors. A preliminary report on some aspects of this work has appeared previously (Whistance, Brown & Threlfall, 1969*a*).

EXPERIMENTAL

Radiochemicals

L-[*Me*-^{14}C]Methionine (56.8mCi/mmol) and L-[U-^{14}C]-tyrosine hydrochloride (5.5mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. *p*-Hydroxy[U-^{14}C]benzoic acid (4.27mCi/mmol) was prepared by alkaline fusion of L-[U-^{14}C]tyrosine (Parson & Rudney, 1964). The crude product was purified as described by Whistance, Threlfall & Goodwin (1967).

Biological methods

Source, growth and maintenance of organisms. Pseudomonas ovalis Chester (A.T.C.C. 17474) was obtained from the American Type Culture Collection. *Proteus mirabilis* (N.C.I.B. 5887) and 'Vibrio O1' (*Moraxella* sp.; N.C.I.B. 8250) were obtained from the National Collection of Industrial Bacteria.

Cells were grown aerobically on the medium described

by Whistance *et al.* (1969*b*). Cultures of up to 10 litres in volume were grown in a New Brunswick Fermenter (Whistance *et al.* 1969*b*); cultures of greater than 10 litres in volume were grown in the fermenter assembly described by Robinson (1964).

All organisms were maintained by periodic subculture on nutrient agar slopes (Whistance *et al.* 1969*b*).

Harvesting and determination of dry weight of cells. These were accomplished by using the methods described by Whistance *et al.* (1969*b*).

Preparation of protoplast membranes. These were prepared by an adaptation of a method described by Nagata, Mizuno & Maruo (1966). After harvesting and washing by resuspension in 0.05M-tris–HCl buffer, pH 8.0, the cells (from 2 l of growth medium) were suspended in 0.05M-tris–HCl buffer, pH 8.0, containing 1M-sucrose (to give a turbidity reading of 4 in an EEL colorimeter). Lysozyme (40μg/ml) and EDTA (400μg/ml) were then added and the suspension was allowed to stand for 30 min at room temperature. At the end of this time the suspension was centrifuged for 30 min at 29700g in a Sorvall RC-2B refrigerated centrifuge. The resulting protoplast pellet was resuspended in 50 ml of 0.05M-potassium phosphate buffer, pH 7.0, containing 5mM-$MgSO_4,7H_2O$ and then treated with a few crystals of deoxyribonuclease to lower the viscosity. After standing for 30 min the suspension of lysed protoplasts was centrifuged for 1 h at 94000g in a Spinco model L preparative centrifuge; this gave a membrane pellet and a protoplasmic supernatant. Finally, the protoplast membranes were washed a further four times with phosphate buffer to ensure the removal of all traces of protoplasm.

Exposure of cells to ^{14}C-labelled substrates. Washed cells were suspended in an appropriate volume of 0.05M-potassium phosphate buffer, pH 7.0, and transferred to a conical flask containing the radioactive substrate. Depending on the nature of the experiment, the suspension was then incubated with either (a) shaking on a Gallenkamp Orbital Shaker (300 rev./min), (b) aeration provided by bubbling compressed air (2 l/min) through a coil of perforated polythene tubing (sealed at one end) lying on the bottom of the flask, or (c) gently purging with O_2-free N_2. Further details are given in the Results section.

Analytical methods

Extraction and preliminary fractionation of lipid. The lipid was extracted from the cells by the procedure of Whistance *et al.* (1969*b*). A preliminary resolution of the lipid extract into various isoprenoid phenol- and quinone-containing fractions was achieved by chromatography on a column of acid-washed alumina (Brockman grade III) (M. Woelm, Eschwege, Germany; anionotropic) developed by stepwise elution with 0.25, 1, 3, 5, 8, 12 and 20% (v/v) E/P.* The elution sequence of the compounds under investigation is given in Table 2.

Purification of quinones and phenols. All the quinones and phenols studied were purified by a combination of adsorptive and reversed-phase chromatography on thin-layers of Kieselgel G (E. Merck A.-G., Darmstadt, Germany). Details of the preparation of the thin layers and of the recovery from them of compounds of this nature have been reported previously (Threlfall & Goodwin, 1967; Whistance *et al.* 1969*b*).

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

Table 1. *Reversed-phase thin-layer system for the identification of 5-demethoxyubiquinones and 6-methoxy-2-polyprenyl-1,4-benzoquinones*

Quinones were isolated from *Ps. ovalis* Chester and *Prot. mirabilis.*

Homologue N	n	R_F values on paraffin-impregnated Kieselgel G layers developed with aq. 90% acetone: 6-Methoxy-2-*n*prenyl-1,4-benzoquinone	Demethoxy-ubiquinone-N
4	tetra	—	0.72
5	penta	—	0.66
6	hexa	—	0.60
7	hepta	—	0.54
8	octa	0.50	0.47
9	nona	0.43	0.38

1. Large-scale analysis. (a) 2-Polyprenylphenols and 6-methoxy-2-polyprenylphenols. Details of the purification of these compounds have been reported by Whistance *et al.* (1969*b*).

(b) 6-Methoxy-2-polyprenyl-1,4-benzoquinones. The 8%-E/P fraction was chromatographed on thin-layers (impregnated with Rhodamine 6G) developed with benzene–chloroform (1:1, v/v). In this system, with low plate loadings, a partial separation of 6-methoxy-2-polyprenyl-1,4-benzoquinones (R_F 0.42) from 5-demethoxyquinones (R_F 0.45) can be achieved. The 6-methoxy-2-polyprenyl-1,4-benzoquinones were separated from remaining traces of 5-demethoxyubiquinones and resolved from each other by chromatography on reversed-phase thin-layers developed with aq. 90% (v/v) acetone (Table 1). Finally, to remove paraffin, each homologue was chromatographed on a thin-layer developed with benzene.

6-Methoxy-2-polyprenyl-1,4-Benzoquinones were characterized on the basis of their u.v. spectra (Fig. 2). The lengths of their side-chains were determined by reversed-phase chromatography (Table 1) against a sample of known chain length (nonaprenyl-; isolated from *Ps. ovalis* Chester in the course of this investigation).

(c) Ubiquinones and 5-demethoxyubiquinones. The 5%-E/P fraction was chromatographed on thin layers (impregnated with Rhodamine 6G) developed with benzene–chloroform (1:1, v/v). With low plate loadings it is possible to obtain a complete separation of ubiquinones (R_F 0.53) from 5-demethoxyubiquinones (R_F 0.45) in this system. The ubiquinones were purified and identified by the procedures described by Whistance *et al.* (1969*b*). The 5-demethoxyubiquinones were combined with those from the 8%-E/P fraction and then resolved by chromatography on reversed-phase thin layers developed with aq. 90% (v/v) acetone (Table 1). Finally, to remove paraffin, each homologue was chromatographed on a thin layer and developed with benzene.

5-Demethoxyubiquinones were characterized on the basis of their u.v. spectra (Fig. 1), their R_F values on reversed-phase thin layers (Table 1) and the u.v. spectra

of their chromenols (Fig. 2) (the method by which the quinones were converted into their chromenols is given below).

2. Radiochemical experiments. The procedures employed were similar to those described above, but because of the small amount of cells used in the incubations with radiochemicals, it was not possible to isolate sufficient 6-methoxy-2-polyprenylphenols, 6-methoxy-2-polyprenyl-1,4-benzoquinones and 5-demethoxyquinones to estimate the amounts present spectroscopically. However, since we were usually interested in only the total incorporation (c.p.m.) of radioactivity into each compound, this did not pose a serious problem.

Chemical conversion of 5-demethoxyubiquinone to 7-demethoxyubichromenol. 5-Demethoxyubiquinone (0.1–3mg) was refluxed for 6h in 1ml of freshly distilled pyridine. At the end of this time the mixture was diluted with saturated NaCl solution and extracted with light petroleum (b.p. 40–60°C). 7-Demethoxyubichromenol (R_F 0.30) was separated from unchanged 5-demethoxyubiquinone (R_F 0.45) by chromatographing the light-petroleum extract on thin layers developed with benzene–chloroform (1:1, v/v). The yield of purified product was about 80%.

Reaction of 6-methoxy-2-polyprenyl-1,4-benzoquinone with cysteine. A purified sample of 6-methoxy-2-polyprenyl-1,4-benzoquinone (0.04μmol) was dissolved in 0.5ml of ethanol. A 1.6μl portion of 0.2M-cysteine in 0.1M-sodium phosphate buffer, pH7.6, was then added to both the sample and reference cell, and the u.v. spectra was determined after 0, 5, 10 and 20 min.

Chemical degradations. ^{14}C radioactivity in the methoxyl group of 6-methoxy-2-nonaprenyl-1,4-benzoquinone was determined by Zeisel degradation (Spiller, Threlfall & Whistance, 1968).

Spectrophotometric estimation of quinones and phenols. (a) Ubiquinones, 5-demethoxyubiquinones and 6-methoxy-2-polyprenyl-1,4-benzoquinones. These were estimated by measuring the fall in extinction at their wavelength of maximum absorption when an ethanolic solution of the quinone was treated with $NaBH_4$ under the conditions described by Threlfall & Goodwin (1967). In all cases the $\epsilon_{oxid.} - \epsilon_{red.}$ value of ubiquinone (12250; Lawson, Threlfall, Glover & Morton, 1961) was used to calculate the amounts present.

(b) 2-Polyprenylphenols and 6-methoxy-2-polyprenylphenols. 2-Polyprenylphenols were assayed in cyclohexane by using a molar extinction coefficient of 2000 ($\lambda_{max.}$ 272nm) (Imamoto & Senoh, 1967). 6-Methoxy-2-polyprenylphenols were assayed in cyclohexane by using the same coefficient ($\lambda_{max.}$ 273.5nm).

Spectrophotometry. All u.v. spectra were determined in a Unicam SP. 800 spectrophotometer. Absorption maxima were determined accurately in a Beckman DK-2A spectrophotometer.

Radioassay. The methods employed have been reported by Threlfall, Whistance & Goodwin (1968) and Whistance *et al.* (1967). All counts were corrected for background and instrument efficiency.

Solvents. Light petroleum (b.p. 40–60°C), 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

Isolation of possible polyprenylphenol and quinone precursors of ubiquinone. The organisms selected for study were the prenylphenol-containing strict aerobe, *Ps. ovalis* Chester, the non-prenylphenol-containing strict aerobe, 'Vibrio O1', and the prenylphenol-containing facultative anaerobe, *Prot. mirabilis*. This choice was determined by the fact that the radiochemical studies had provided some evidence of the presence in these organisms of compounds with properties expected of 2-polyprenyl-1,4-benzoquinones and 5-demethoxyubiquinones (Whistance *et al.* 1969*b*; G. R. Whistance & D. R. Threlfall, unpublished work).

(*a*) *Ps. ovalis* Chester. The wet cell mass (1.6kg) from 310l of aerobically grown culture was extracted by our usual procedure to give 20.5g of lipid. After removal of the more polar lipids by passage through a column of Brockmann grade III acid-washed alumina (500g) developed with ether, the lipid extract (1g) was chromatographed on a column of Brockmann grade III acid-washed alumina (200g) developed with increasing amounts of diethyl ether in light petroleum (b.p. 40–60°C). The distribution of the various phenols and quinones in the fractions obtained from this chromatography, together with details of their purification by adsorptive t.l.c., are given in Table 2. The quantitative aspects of the analysis are given in Table 3.

As in a previous investigation (Whistance *et al.* 1969*b*), the 1%- and 3%-E/P fractions were found to contain 6-methoxy-2-polyprenylphenols and 2-polyprenylphenols respectively. Reversed-phase t.l.c. and staining with Gibbs reagent (Whistance *et al.* 1969*b*) showed that the homologues of both compounds, from tetra- to nona-prenyl, were present. In the cases of 2-nonaprenylphenol, 2-octaprenylphenol and 6-methoxy-2-nonaprenylphenol sufficient amounts were isolated to obtain u.v. spectra; these were identical with those of authentic samples (Imamoto & Senoh, 1967; Daves *et al.* 1967). The identities of 2-nonaprenylphenol and 6-methoxy-2-nonaprenylphenol were confirmed by mass spectrometry, the samples having parent ions at *m/e* 706 and 736 respectively.

The 5%-E/P fraction contained ubiquinone and a quinone complex having a u.v. spectrum similar to that of 5(6)-demethoxyubiquinone. Reversed-phase t.l.c. of the complex showed it to consist of seven components having the R_F values expected of 5(6)-demethoxyubiquinones-4–9 (Table 1). The u.v. spectra (Fig. 1) of these compounds determined in cyclohexane ($\lambda\lambda_{max.}$ 266 and 272nm) and ethanol ($\lambda_{max.}$ 270nm, changing to $\lambda_{max.}$ 292nm after treatment with sodium borohydride) were identical. Further, when determined in the appropriate solvents their absorption maxima corresponded

Table 2. *Isolation of possible polyprenylphenol and quinone precursors of ubiquinone by column and adsorptive t.l.c. of a lipid extract from* Ps. ovalis *Chester*

Lipid extract (1 g) was chromatographed on a column of Brockmann grade III acid-washed alumina (200 g) developed by stepwise elution with increasing amounts of diethyl ether in light petroleum. Polyprenylphenols and quinones were isolated from the column fractions by chromatography on thin layers of Kieselgel G developed with the solvents listed below.

Column fraction (% E/P)	Compounds eluted	R_F values on Kieselgel G layers: Benzene	Benzene–chloroform (1:1, v/v)	Ethyl acetate–benzene (3:22, v/v)
0.25	—			
1	6-Methoxy-2-polyprenylphenols	0.65		
3	2-Polyprenylphenols	0.52		
5	Ubiquinones		0.53	
	5-Demethoxyubiquinones		0.45	
8	5-Demethoxyubiquinones		0.45	
	6-Methoxy-2-polyprenyl-1,4-benzoquinones		0.4	
12, 20, 100	'Epoxyubiquinones'			0.55 and 0.65

Table 3. *Polyprenylphenols and quinones present in* Ps. ovalis *Chester*

A wet cell mass (equivalent to 314 g dry wt.) from 310 l of aerobic growth medium was examined by the procedures described in the text. N.D.: not detected.

Homologue N	n	Content (μmol/100 g dry wt.): 2-*n*prenyl phenol	6-Methoxy-2-*n*prenylphenol	6-Methoxy-2-*n*prenyl-1,4-benzoquinone	5-Demethoxy-ubiquinone-*N*	Ubiquinone-*N*
4	tetra	+	+	N.D.	+	+
5	penta	++	++	N.D.	++	++
6	hexa	+++	+++	N.D.	+++	+++
7	hepta	++++	++++	N.D.	++++	++++
8	octa	+++++	+++++	Present	+++++	+++++
9	nona	3.20	0.16	0.12	6.27	90
Total		3.82	0.19	0.12	7.28	105

closely to those reported for authentic 5- and 6-demethoxyubiquinones (Imamoto & Senoh, 1968; Daves, Wilczynski, Friis & Folkers, 1968). The mass spectrum of the principal component showed peaks at m/e 766 ($M+2$; due to a dismutation reaction in the spectrometer), 764 (M), 749 ($M-15$; loss of a methyl group), 695 ($M-69$; loss of a terminal isoprene unit), $M-69-68_n$, where n = 1–7 (loss of seven internal isoprene units) and 205 (base peak). These values are identical with those reported for 5(6)-demethoxyubiquinone-9 (Imamoto & Senoh, 1968). To establish which of the two isomers was present the quinones were converted into their corresponding chromenols and the u.v. spectra (Fig. 3) of these determined [7- and 8-methoxyubichromenols can be readily differentiated by their u.v. spectra (Daves *et al.* 1968)]. In all cases the spectra were identical with that reported for 7-demethoxyubichromenol (Daves *et al.* 1968).

The 8%-E/P fraction contained 5-demethoxyubiquinones and small amounts of 5(6)-methoxy-2-nonaprenyl-1,4-benzoquinone. The identity of the latter quinone was established as follows: (1) its u.v. spectra (Fig. 2) in cyclohexane ($\lambda_{max.}$ 264.5 and shoulder at 273 nm) and ethanol ($\lambda_{max.}$ 267 nm, changing to $\lambda_{max.}$ 291 nm after sodium borohydride treatment) were those expected for a 5(6)-methoxy-2-polyprenyl-1,4-benzoquinone. (2) It reacted with cysteine (reaction followed spectrophotometrically), indicating the presence of an unsubstituted position in the ring adjacent to the polyprenyl side chain [compare with 5-demethoxyubiquinone which shows

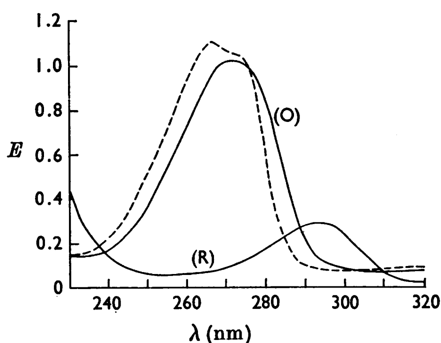


Fig. 1. U.v. spectra of 5-demethoxyubiquinone-9 isolated from *Ps. ovalis* Chester: ----, in cyclohexane; ——, in ethanol [before (O) and after (R) treatment with $NaBH_4$].

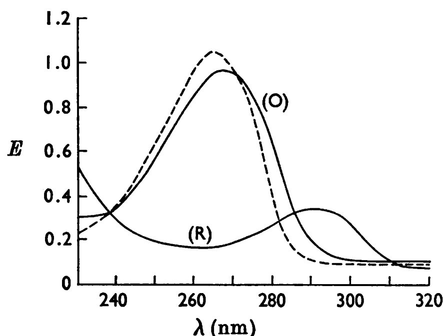


Fig. 2. U.v. spectra of 6-methoxy-2-nonaprenyl-1,4-benzoquinone isolated from *Ps. ovalis* Chester: ----, in cyclohexane; ——, in ethanol [before (O) and after (R) treatment with $NaBH_4$].

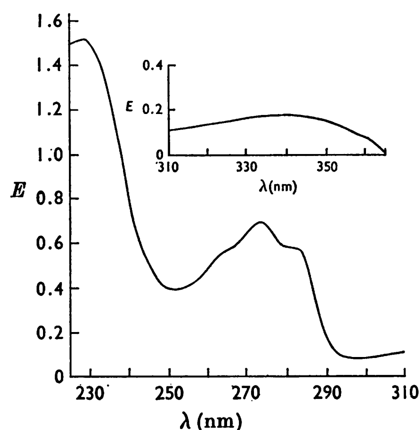
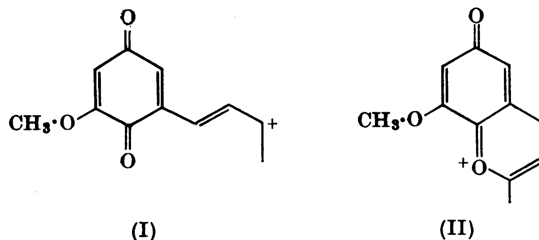


Fig. 3. U.v. spectrum in cyclohexane of 7-demethoxy-ubichromenol-8 formed by base (pyridine)-catalysed cyclization of 5-demethoxyubiquinone-9 isolated from *Ps. ovalis* Chester.



no reaction with cysteine (Whistance *et al.* 1969*a*)]. (3) Its mass spectrum was that expected for 2-nonaprenyl-5(6)-methoxy-1,4-benzoquinone, showing peaks at *m/e* 752 ($M+2$; dismutation reaction in the spectrometer), 750 (M), 735 ($M-15$; loss of a methyl group), 681 ($M-69$; loss of a terminal isoprene unit), $681-68_{1-7}$ ($M-69-68_n$ where n is 1–7; loss of seven internal isoprene units) and 191 (base peak). (The latter can be attributed to the molecular ions I and II.) (4) On Zeisel degradation of [^{14}C]quinone which had been biogenetically labelled from L-[Me-^{14}C]methionine all the radioactivity was recovered in tetramethylammonium iodide. Although these observations clearly establish that the quinone is either 6-methoxy-2-nonaprenyl-1,4-benzoquinone or 5-methoxy-2-nonaprenyl-1,4-benzoquinone, they do not allow a differentiation to be made between the two. However, the finding that its u.v. spectrum in ethanol is similar to that of 3-methoxytoluquinone ($\lambda_{max.}$ 265nm) and differs significantly from that of 4-methoxytoluquinone ($\lambda_{max.}$ 260nm) indicates that it is 6-methoxy-2-nonaprenyl-1,4-benzoquinone.

The 12%-E/P and ether fractions had u.v. spectra similar to that of ubiquinone. Adsorptive t.l.c. with development with ethyl acetate–benzene (3:22, v/v) enabled the isolation from each fraction of two quinones (R_F values 0.55 and 0.65). On reversed-phase chromatography on thin layers developed with aq. 85% (v/v) acetone each quinone was further resolved into three quinones (approximate R_F values 0.20, 0.25 and 0.30). These properties suggested we were dealing with a group of epoxyubiquinones, compounds first reported to occur naturally in *Rhodospirillum rubrum* (Friis, Daves & Folkers, 1967). Support for this was obtained from the mass spectra of the two principal quinones from the 20%-E/P fraction, which in each case showed peaks at *m/e* 812 (M+2), 810 (M) and 235 (base peak), i.e. those expected for epoxy-ubiquinones-9.

(*b*) *Prot. mirabilis* and 'Vibrio O1'. The lipids

Table 4. *Polyprenylphenols and quinones present in 'Vibrio O1' and* Prot. mirabilis

Wet cell masses of 'Vibrio Cl' (equivalent to 32g dry wt.) and *Prot. mirabilis* (equivalent to 70g dry wt.) were examined by the procedures described in the Results section. 2-Polyprenylphenol, 6-methoxy-2-polyprenylphenol and ubiquinone homologues are given in order of decreasing concentrations. In each case the principal homologue was judged to constitute more than 85% of the total amount of homologues present. N.D.: not detected.

	Prot. mirabilis		'Vibrio O1'	
Compound(s)*	Content (μmol/100g dry wt.)	Homologues	Content (μmol/100g dry wt.)	Homologues
2-Polyprenylphenols	9	8, 7, 6, 5, 4	N.D.	
6-Methoxy-2-polyprenylphenols	0.6	8, 7, 6, 5, 4	N.D.	
6-Methoxy-2-polyprenyl-1,4-benzoquinones	0.1	8	N.D.	
5-Demethoxyubiquinones	0.2	8	0.4	9
Ubiquinones	71	8, 7, 6, 5, 4	14	9, 8, 7, 10, 6, 5
'Epoxyubiquinones'†	2	8 (?)	8	9 (?)

* In *Prot. mirabilis* menaquinones and demethylmenaquinones are also present (Whistance *et al.* 1969).

† This term is used to denote a complex mixture of quinones in the 12%-E/P, 20%-E/P and ether fractions that had u.v. spectra similar to that of ubiquinone.

obtained from wet cell masses of 'Vibrio O1' (325g) and *Prot. mirabilis* (350g) from 541 aerobic cultures were examined by the chromatographic and u.v. spectroscopic procedures described above. The results of the analyses are given in Table 4.

Conversion of 2-polyprenylphenols, 6-methoxy-2-polyprenylphenols, 6-methoxy-2-polyprenyl-1,4-benzoquinones and 5-demethoxyubiquinones into ubiquinones in Ps. ovalis *Chester.* Evidence that in *Ps. ovalis* Chester these compounds are precursors of ubiquinones was obtained from a suitable pulse-labelling experiment (Table 5). It was found that on incubating cells previously exposed to *p*-hydroxy-[U-^{14}C]benzoic acid with unlabelled *p*-hydroxybenzoic acid the radioactivity lost from 2-polyprenylphenols, 6-methoxy-2-polyprenylphenols, 2-polyprenyl-1,4-benzoquinone (?), 6-methoxy-2-polyprenyl-1,4-benzoquinones and 5-demethoxyubiquinones could be completely accounted for by the increase in radioactivity in the ubiquinones (Table 5). Further, the loss of radioactivity from these polyprenylphenols and quinones was so marked that there can be little doubt that they are all intermediates in the biosynthesis of ubiquinone. It may be noted that in the second part (and presumably the first part also) of the incubation a marked synthesis of ubiquinone took place.

Requirement of oxygen for ubiquinone biosynthesis in Ps. ovalis *Chester.* Whistance *et al.* (1967) and Spiller *et al.* (1968) found that in the absence of oxygen maize shoots and baker's yeast were unable to synthesize ubiquinone from *p*-hydroxybenzoic acid. Parson (1965) observed a similar effect with *E. coli.* It was decided therefore, for comparative purposes, to investigate the effect of the presence or absence of oxygen on the biosynthesis of ubiquinone in *Ps. ovalis* Chester. It was found that in the absence of oxygen the incorporation of radioactivity into the compounds examined was markedly decreased, the total incorporation falling from 33.5% to 1.3% (Table 6). The ratios of total radioactivity incorporated into each compound under aerobic and anaerobic conditions show that the fall in incorporation is greater the more oxygenated the compound. Thus the greatest fall is seen for ubiquinone, followed in order by 6-methoxy-2-polyprenyl-1,4-benzoquinones plus 5-demethoxyubiquinones, 2-polyprenylphenols and 6-methoxy-2-polyprenylphenols. This inhibition of ubiquinone biosynthesis can also be seen in the amounts of ubiquinone present; thus the cells from the aerobic incubation contained 3.71 μmol as opposed to 2.85 μmol in the cells from the anaerobic incubation.

Intracellular distribution of ubiquinone and its prenylated precursors in Ps. ovalis *Chester.* Previous investigations had provided evidence that in non-photosynthetic Gram-negative bacteria ubiquinone is associated entirely with the protoplast membrane (Bishop & King, 1962; Rebel, Sensenbrenner & Mandel, 1964). It seemed likely, therefore, that the prenylated precursors of ubiquinone would be located at the same site. To investigate this, cells of *Ps. ovalis* Chester (9g wet wt.) were suspended in 100ml of 0.05M-potassium phosphate buffer, pH 7.0, containing 2 μCi of *p*-hydroxy[U-^{14}C]benzoic acid and incubated for 4h at 30°C with constant shaking. At the end of this time the cells were harvested and fractionated into protoplast membranes and cytoplasm. Examination of the lipids from these two components

Table 5. *Conversion of 2-polyprenylphenols, 6-methoxy-2-polyprenylphenols, 6-methoxy-2-polyprenyl-1,4-benzoquinones and 5-demethoxyubiquinones into ubiquinones in* Ps. ovalis *Chester*

Cells from 20 l of aerobic growth medium were suspended in 150 ml of 0.05 M-potassium phosphate buffer, pH 7.0, containing 5 μCi of *p*-hydroxy[U-^{14}C]benzoic acid. The suspension was then incubated in a 500 ml conical flask for 3 h at 32°C in the Orbital shaker. At the end of this period half the cells were taken for analysis. After washing with buffer the remaining cells were resuspended in 500 ml of 0.05 M-potassium phosphate buffer, pH 7.0, containing 500 μmol of *p*-hydroxybenzoic acid and aerated for 3 h.

	Radioactivity (c.p.m.)	
Compounds	Cells incubated with *p*-hydroxy-[U-^{14}C]benzoic acid	Cells incubated with *p*-hydroxy[U-^{14}C]benzoic acid followed by *p*-hydroxybenzoic acid
2-Polyprenylphenols	131200 (sp. radioactivity 468000 c.p.m./μmol)	12100 (sp. radioactivity 41200 c.p.m./μmol)
6-Methoxy-2-polyprenylphenols	1640	0
2-Polyprenyl-1,4-benzoquinones (?)*	41000	1500
6-Methoxy-2-polyprenyl-1,4-benzoquinones	22350	2540
5-Demethoxyubiquinones	19750	8040
Ubiquinones	58000 (sp. radioactivity 13840 c.p.m./μmol)	249700 (sp. radioactivity 42300 c.p.m./μmol)
Total	273940	273880

* It should be noted that in a previous investigation (Whistance *et al.* 1969*b*) this quinone was incorrectly referred to as Compound D; the correct reference should have been Compound A. Similarly, 5-demethoxyubiquinone-9 was referred to as Compound C (now known to be 6-methoxy-2-nonaprenyl-1,4-benzoquinone) instead of Compound D.

Table 6. *Requirement of oxygen for ubiquinone biosynthesis in* Ps. ovalis *Chester*

Cells obtained from 5 l of aerobic growth medium were suspended in 100 ml of 0.05 M-potassium phosphate buffer, pH 7.0. Half the suspension was then incubated with 1 μCi of *p*-hydroxy[U-^{14}C]benzoic acid for 4 h with aeration. The other half was incubated with 1 μCi of *p*-hydroxy[U-^{14}C]benzoic acid for 4 h with gentle purging with O_2-free N_2.

	Radioactivity (c.p.m.)		
Compound(s)	Cells (equivalent to 2.9 g dry wt.) incubated in the presence of oxygen (air)	Cells (equivalent to 3.0 g dry wt.) incubated in the absence of oxygen	Ratio of radioactivity (c.p.m.) incorporated (aerobic/anaerobic)
2-Polyprenylphenols	55000	19900	1:0.36
6-Methoxy-2-polyprenylphenols	3300	1650	1:0.50
6-Methoxy-2-polyprenyl-1,4-benzoquinones plus 5-demethoxyubiquinones	578514	16530	1:0.03
Ubiquinones	98500 (sp. radioactivity 8970 c.p.m./μmol)	20 (sp. radioactivity 7 c.p.m./μmol)	1:0.00
Incorporation (% of radioactivity administered)	33.5	1.3	

showed that radioactivity was present mainly in the lipids of the protoplast membranes, where it was associated almost entirely with 2-polyprenylphenols (102150 c.p.m.), 6-methoxy-2-polyprenylphenols (2720 c.p.m.), 2-polyprenyl-1,4-benzoquinones (?) (11000 c.p.m.), 6-methoxy-2-polyprenyl-1,4-benzoquinones (12100 c.p.m.), 5-demethoxyubiquinones (22500 c.p.m.) and ubiquinones (51850 c.p.m.). A small amount of radioactivity (2200 c.p.m.) was present in the cytoplasmic lipid fraction, where it appeared to be associated with the above compounds; however, these were present in amounts too small to be detected by chemical means.

Attempts to prepare cell-free systems capable of synthesizing ubiquinone. Attempts were made to prepare cell-free systems of *E. coli*, organism PC4 and *Ps. ovalis* Chester capable of incorporating *p*-hydroxy[U-^{14}C]benzoic acid into ubiquinone or

its prenylated intermediates. It was found that highly purified membranes of *Ps. ovalis* Chester prepared by a combination of the action of lysozyme and osmotic shock did not incorporate *p*-hydroxy-[U-^{14}C]benzoic acid into ubiquinone or its prenylated precursors. Furthermore, no incorporation was obtained when the membranes were supplemented with a fraction from the cytoplasm.

Cell-free systems prepared by using the French pressure cell were found to carry out incorporations in the order of 0.1% for *Ps. ovalis* Chester, 1% for organism PC4 and 4% for *E. coli*. Although these incorporations could be attributed in part to intact cells in the preparations (2–5% at the end of the incubation), the number of cells present was not sufficient to account for the total.

DISCUSSION

Investigations into the polyprenylphenol and quinone complements of *Ps. ovalis* Chester and *Prot. mirabilis* established that they contain 2-polyprenylphenols, 6-methoxy-2-polyprenylphenols, 6-methoxy-2-polyprenyl-1,4-benzoquinones [a family of quinones the existence of which in Nature has only previously been indicated by mass-spectroscopic studies on a complex lipid fraction from *R. rubrum* (Friis *et al.* 1966)], 5-demethoxyubiquinones, ubiquinones, 'epoxyubiquinones' and an unidentified 1,4-benzoquinone and 2-polyprenyl-1,4-benzoquinone(?)] (Tables 1, 2, 3 and 5 and Whistance *et al.* 1969*b*). In *Ps. ovalis* Chester 2-polyprenylphenols, 6-methoxy-2-polyprenylphenols, 6-methoxy-2-polyprenyl-1,4-benzoquinones,5-demethoxyubiquinones and the unidentified 1,4-benzoquinones were shown to be precursors of ubiquinones (Table 5). More recently, these compounds have also been shown to be ubiquinone precursors in *Prot. mirabilis* (G. R. Whistance & D. R. Threlfall, unpublished work). Although no evidence could be found of the presence of 5-hydroxy-6-methoxy-3-methyl-2-polyprenyl-1,4-benzoquinone, the above findings suggest that in *Ps. ovalis* Chester and *Prot. mirabilis* one pathway of ubiquinone biosynthesis is similar to pathway A outlined in Scheme 1. The detection in both organisms of another 1,4-benzoquinone precursor of ubiquinone, possibly 2-polyprenyl-1,4-benzoquinone, indicates that they also possess at least one other pathway.

Studies on the polyprenyl and quinone complement of 'Vibrio 01' showed it to contain detectable amounts of only 5-demethoxyubiquinones, ubiquinones and 'epoxyubiquinones' (Table 4; Whistance *et al.* 1969*b*). The apparent absence of 2-polyprenylphenols, 6-methoxy-2-polyprenylphenols and 6-methoxy-2-polyprenyl-1,4-benzoquinones suggests that in this organism, and indeed all other organisms not possessing the aforementioned compounds, prenylation is a late step in ubiquinone biosynthesis.

It was established that in *Ps. ovalis* Chester ubiquinone and its prenylated precursors are localized entirely on the protoplast membrane. This is not unexpected in view of the lipophilic nature of the compounds under investigation and indicates that the overall synthesis of ubiquinone from *p*-hydroxybenzoic acid takes place at this site.

In an investigation into the oxygen requirement for ubiquinone biosynthesis by *Ps. ovalis* Chester it was found that the absence of oxygen almost completely inhibited the incorporation of ^{14}C radioactivity into ubiquinone from *p*-hydroxy[U-^{14}C]-benzoic acid, although some radioactivity was incorporated into 2-polyprenylphenols (Table 6). A similar observation has been made by Parson (1965) working with *E. coli*. These findings, together with the fact that maize shoots (Whistance *et al.* 1967) and baker's yeast (Spiller *et al.* 1968) are also unable to synthesize ubiquinone from *p*-hydroxybenzoic acid under anaerobic conditions, indicate an obligatory requirement for atmospheric oxygen for ubiquinone biosynthesis in aerobic organisms.

Attempts to prepare cell-free preparations capable of synthesizing ubiquinone from *p*-hydroxybenzoic acid met with limited success. Purified protoplast membranes of *Ps. ovalis* Chester were found to be incapable of synthesizing ubiquinone or any of its prenylated precursors, even when supplemented with a fraction from the cytoplasm. This suggests that irreparable damage to the synthesizing centres occurred during preparation of the membranes. Crude crushed-cell preparations, on the other hand, were found to synthesize ubiquinone and its precursors, although a part of the synthesis can probably be attributed to small numbers of intact cells (2–5%) in the preparations. Raman, Rudney & Buzzelli (1969) encountered similar difficulties in their studies with *R. rubrum*. However, they succeeded in overcoming them and obtained a cell-free preparation capable of carrying out the prenylation of *p*-hydroxybenzoic acid, i.e. the first step in the conversion of this acid to ubiquinone (Scheme 1).

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