

## Ubiquinone Biosynthesis in *Escherichia coli* K-12

### ACCUMULATION OF AN OCTAPRENOL, FARNESYLFARNESYLGERANIOL, BY A MULTIPLE AROMATIC AUXOTROPH

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Cell extracts of a multiple aromatic auxotroph of *Escherichia coli* K-12, strain AB2830, grown in the absence of precursors of the quinone rings of the ubiquinone and menaquinone molecules, converted 4-hydroxy[U-<sup>14</sup>C]benzoate into a mixture of 3-octaprenyl-4-hydroxybenzoate and 2-octaprenylphenol. An octaprenol, farnesylfarnesylgeraniol, was isolated from such cell extracts and characterized by n.m.r. and mass spectroscopy. Neither the octaprenol, nor polyprenylation of 4-hydroxy[U-<sup>14</sup>C]benzoate, could be detected in cell extracts of strain AB2830 grown in the presence of 0.1 mM 4-hydroxybenzoate. It was concluded that, in the biosynthesis of ubiquinone, the polyprenyl side chain is added to 4-hydroxybenzoate as a C<sub>40</sub> unit, the active form of which is converted by cell extracts into farnesylfarnesylgeraniol. The multiple aromatic auxotroph, when grown in the absence of 4-hydroxybenzoate but in the presence of 4-aminobenzoate, converted the latter compound into 3-octaprenyl-4-aminobenzoate. This compound was isolated from whole cells and characterized by n.m.r. and mass spectroscopy.

Parson & Rudney (1964) observed that 4-hydroxybenzoate was a direct precursor of the benzoquinone ring of ubiquinone in *Rhodospirillum rubrum*, rat kidney, *Azotobacter vinelandii* and baker's yeast. This observation has been amply confirmed in all species tested thus far from bacteria through to mammals (Rudney & Raman, 1966).

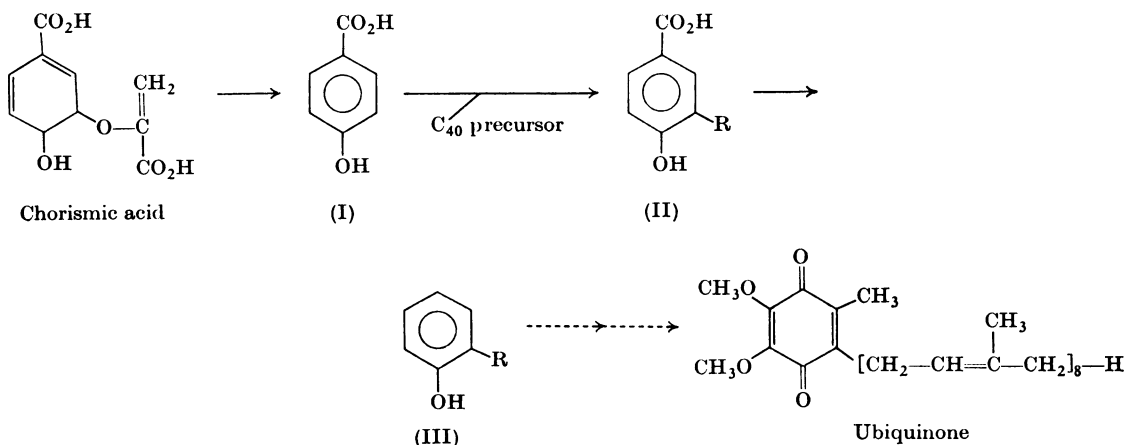
A pathway for the biosynthesis of ubiquinone has been proposed by Friis, Daves & Folkers (1966). Three of the proposed seven intermediates between 4-hydroxybenzoate and ubiquinone have been isolated from apparently 'wild-type' bacterial cells and identified by spectroscopy and chemical synthesis (Daves *et al.* 1967; Imamoto & Senoh, 1967; Olsen *et al.* 1965, 1966*a,b*). However, a methyl multiprenyl-benzoquinone (in this paper 'prenyl' refers to the C<sub>5</sub> isoprene unit) has also been detected, and this compound does not fit into the proposed scheme (Friis, Nilsson, Daves & Folkers, 1967). Cox, Young, McCann & Gibson (1969) have characterized two mutant strains of *Escherichia coli* K-12 unable to form ubiquinone, and these strains accumulate either 3-octaprenyl-4-hydroxybenzoic acid (II) or 2-octaprenylphenol (III) (see Scheme 1). Cell extracts of the ubiquinone-deficient strain accumulating 3-octaprenyl-4-hydroxybenzoic acid lacked the enzymic activity, present in

cell extracts of normal strains of *E. coli* K-12, converting 3-octaprenyl-4-hydroxybenzoic acid into 2-octaprenylphenol, thus establishing that these two compounds are intermediates in ubiquinone biosynthesis. The polyprenyl side chain therefore appears to be added at the 4-hydroxybenzoate level either by the sequential addition of C<sub>5</sub> units or by the addition of a preformed C<sub>40</sub> unit.

Normal strains of *E. coli* K-12 form two octaprenyl quinones, ubiquinone and menaquinone, and the precursors of the quinone portions of both molecules are derived from chorismic acid (Cox & Gibson, 1966). The present paper describes the polyprenylation of 4-hydroxybenzoate *in vitro* and the accumulation of an octaprenol and 3-octaprenyl-4-aminobenzoate by a multiple aromatic auxotroph of *E. coli* K-12 grown in the absence of precursors of the quinone rings of the ubiquinone and menaquinone molecules.

## MATERIALS AND METHODS

*Chemicals.* Chemicals generally were of the highest purity obtainable commercially and were not further purified. L-[U-<sup>14</sup>C]Tyrosine (specific radioactivity 468  $\mu$ Ci/ $\mu$ mol) was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). 4-Hydroxy[U-<sup>14</sup>C]benzoic acid was prepared by alkaline fusion of L-[U-<sup>14</sup>C]tyrosine (Parson & Rudney, 1964). 4-Hydroxy[7-<sup>14</sup>C]benzoic acid



Scheme 1. Intermediates in ubiquinone biosynthesis established with mutants or with cell extracts, or both. (I) 4-Hydroxybenzoic acid; (II) 3-octaprenyl-4-hydroxybenzoic acid; (III) 2-octaprenylphenol.

(specific radioactivity  $55 \mu\text{Ci/mol}$ ) was obtained from Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A. Solanesol was a gift from Dr O. Isler of Hoffman-La Roche and Co., Basle, Switzerland, and geraniol was a gift from Professor G. Popjak, School of Medicine, University of California, Los Angeles, Calif., U.S.A. 3-Octaprenyl-4-hydroxybenzoic acid and 2-octaprenylphenol were kindly provided by Dr I. G. Young, Australian National University, Canberra, A.C.T., Australia.

**Bacterial strains.** All the strains used were derived from *E. coli* K-12 and were maintained on nutrient agar and subcultured monthly. Strain AB2826 is a multiple aromatic auxotroph and lacks dehydroquinase synthetase activity (Pittard & Wallace, 1966). Strain AB2830 is also an aromatic auxotroph and lacks chorismate synthetase activity (Pittard & Wallace, 1966). Strain AN62 forms normal amounts of ubiquinone and has been described previously (Cox *et al.* 1969).

**Media and growth of organisms.** The basal medium used was the 56 mineral salts medium described by Monod, Cohen-Bazire & Cohn (1951). For the growth of strains AB2826 and AB2830, L-phenylalanine, L-tyrosine and L-tryptophan, each at a final concentration of 0.2 mM, were autoclaved with the medium. 4-Aminobenzoic acid, 4-hydroxybenzoic acid and 2,3-dihydroxybenzoic acid were autoclaved separately and added to the medium to give final concentrations of  $10 \mu\text{M}$  for 4-aminobenzoic acid and 2,3-dihydroxybenzoic acid and of 0.1 mM for 4-hydroxybenzoic acid. For the growth of strain AN62, L-leucine and L-threonine were autoclaved separately from the basal medium and each was added to a final concentration of 0.2 mM.

For all strains, glucose and thiamin were added as sterile solutions to final concentrations of 30 mM and  $0.02 \mu\text{M}$  respectively.

Strain AB2830 was grown at  $37^\circ\text{C}$  into late exponential phase in 1-litre quantities in 2-litre flasks in a New Brunswick gyrotary shaker, or in 14-litre New Brunswick fermentors with aeration at 10-litres/min and stirring at 600 rev./min, or in a 40-litre capacity New Brunswick Fermacell Fermentor with aeration ( $0.3 \text{ ft}^3/\text{min}$ ) and

stirring (400 rev./min). For the growth of strains AN62 and AB2826 the 2-litre flasks were used.

**Preparation of cell extracts.** Cells were grown as described above, centrifuged, then washed once in cold 0.1 M-potassium phosphate buffer, pH 7.0, and resuspended in fresh buffer (1 ml of buffer/0.5 g wet wt. of cells). The cell extracts were prepared by passing the suspension through a Sorvall Ribi Cell Fractionator at  $20000 \text{ lb/in}^2$ .

**Detection of the polyprenylation of 4-hydroxybenzoate by cell extracts.** Reaction mixtures containing 4 ml of cell extract, 0.3 ml of aq. 4-hydroxy[ $\text{U-}^{14}\text{C}$ ]benzoic acid ( $50000 \text{ c.p.m.}$ , diluted with unlabelled 4-hydroxybenzoic acid to specific radioactivity  $55 \mu\text{Ci}/\mu\text{mol}$ ), and 1.7 ml of 0.1 M-potassium phosphate buffer, pH 7.0, were incubated for 1 h at  $37^\circ\text{C}$ . The reactions were stopped by rapid cooling in liquid  $\text{N}_2$  and the mixtures freeze-dried. 3-Octaprenyl-4-hydroxybenzoate and 2-octaprenylphenol were extracted from the freeze-dried reaction mixtures by stirring with  $2 \times 20 \text{ ml}$  of acidified acetone (pH 1). The volume of the pooled acetone extracts was decreased to about 2 ml on a steam bath, 10 ml of water was added and the products were extracted with  $2 \times 10 \text{ ml}$  of light petroleum (b.p.  $40\text{--}60^\circ\text{C}$ ). The pooled light-petroleum extract was washed twice with water and then assayed for radioactivity in a liquid-scintillation counter or chromatographed on silica-gel plates.

**Extraction of the octaprenol and 3-octaprenyl-4-aminobenzoic acid.** The octaprenol and 3-octaprenyl-4-aminobenzoic acid were extracted either from wet whole cells or from freeze-dried cell extracts by continuous Soxhlet extraction with acetone for 3 h. The acetone extract was evaporated to dryness under reduced pressure and the residue redissolved in a small volume of methanol. The methanol extract was then examined by t.l.c.

**Chromatography.** The radioactive products from the cell-extract incubations were examined by t.l.c. on silica-gel plates with chloroform-methanol (9:1, v/v) as the developing solvent. The t.l.c. plates were scanned with a Nuclear-Chicago model 1032 Actigraph II chromatogram scanner. Authentic 3-octaprenyl-4-hydroxybenzoic acid and 2-octaprenylphenol were detected after spraying with

diazotized *p*-nitroaniline (Cox *et al.* 1969). The solvent systems used for the co-chromatography of radioactively labelled and authentic 3-octaprenyl-4-hydroxybenzoic acid were chloroform-methanol (19:1, v/v;  $R_F$  0.2), ether-hexane (9:1, v/v;  $R_F$  0.3), ethyl acetate-hexane-methanol (9:3:1, by vol.;  $R_F$  0.5), ether-hexane-methanol (9:1:1, by vol.;  $R_F$  0.6). For 2-octaprenylphenol the solvent systems were ether-hexane (1:9, v/v;  $R_F$  0.2), chloroform-light petroleum (b.p. 60–80°C) (7:3, v/v;  $R_F$  0.5), ether-hexane (9:1, v/v;  $R_F$  0.8), chloroform-methanol (19:1, v/v;  $R_F$  0.8). For the reversed-phase co-chromatography of both compounds, paraffin-impregnated silica-gel plates were developed in a water-acetone (3:97, v/v) solvent system, both compounds giving similar  $R_F$  values ( $R_F$  0.7).

For the detection or isolation of the octaprenol, both from whole cells and from cell-free extracts, pre-poured Merck silica-gel F<sub>254</sub> plates (0.25 mm) were used with chloroform as the developing solvent. The octaprenol was located either with I<sub>2</sub> vapour, or with the anisaldehyde-H<sub>2</sub>SO<sub>4</sub> spray used previously to identify polyprenols (McSweeney, 1965). For the reversed-phase system, paraffin-impregnated silica-gel plates were developed with water-acetone (1:19, v/v) with solanesol (C<sub>45</sub>) and geraniol (C<sub>10</sub>) as markers. For purification of 3-octaprenyl-4-aminobenzoic acid pre-poured silica-gel plates were also used but with chloroform-methanol (50:1, v/v) as the developing solvent. The 3-octaprenyl-4-aminobenzoic acid could be located on the plates by viewing under u.v. light.

**Assays of radioactivity.** <sup>14</sup>C was measured by liquid-scintillation counting in a Packard Tri-Carb model 3320 scintillation spectrometer. The radioactivities of samples dissolved in organic solvents or adsorbed on silica gel were counted in scintillation fluid containing 4g of 2,5-diphenyloxazole and 100mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 1 litre of toluene. The radioactivities of samples in aqueous solution were counted in Bray's (1960) fluid. Counting efficiencies were determined by internal standardization.

**Spectroscopy.** Mass spectra were obtained with an AEI MS9 double-focus mass spectrometer, n.m.r. spectra with a Varian HA100 spectrometer with tetramethylsilane as an internal standard, and u.v. spectra were obtained with a Cary 15 spectrophotometer.

## RESULTS

Strain AB2830 is a multiple aromatic auxotroph derived from *E. coli* K-12, and lacks chorismate synthetase activity. This strain is therefore unable to form the benzoquinone ring of the ubiquinone molecule or the naphthaquinone part of the menaquinone molecule. Strain AB2830 will, however, form ubiquinone if grown in the presence of 4-hydroxybenzoate.

**Polyprenylation of 4-hydroxybenzoate by cell extracts of *E. coli* strain AB2830.** Cell extracts, prepared from strain AB2830 grown in the presence or in the absence of 4-hydroxybenzoate, were incubated with 4-hydroxy[U-<sup>14</sup>C]benzoate and the radioactive products extracted and chromato-

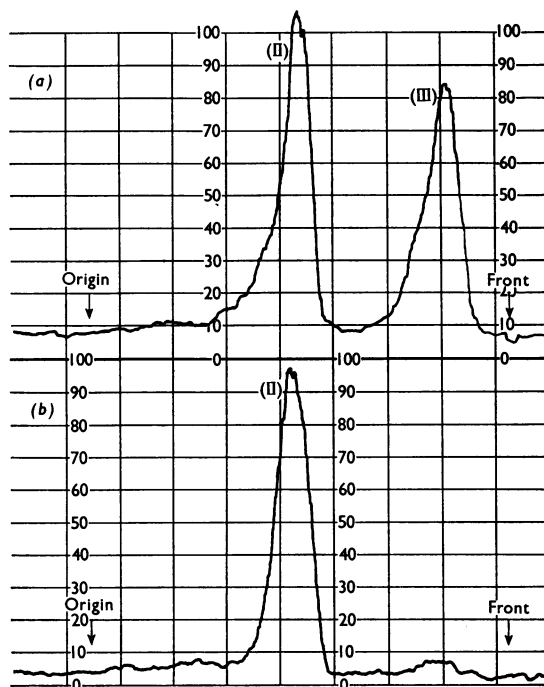


Fig. 1. Formation of 3-octaprenyl-4-hydroxybenzoate (II) and 2-octaprenylphenol (III) from <sup>14</sup>C-labelled 4-hydroxybenzoate by cell extracts. 4-Hydroxy[U-<sup>14</sup>C]benzoate (a) or 4-hydroxy[7-<sup>14</sup>C]benzoate (b) were incubated with cell extracts of strain AB2830, grown in the absence of 4-hydroxybenzoate, as described in the Materials and Methods section. The reaction mixtures were freeze-dried, extracted with acidified acetone, water was added and the aqueous acetone extracted with light petroleum. The light petroleum extract was chromatographed on silica-gel plates that were then scanned for radioactivity at a rate of 12 in/h and sensitivity setting of 1000 c.p.m.

graphed on silica-gel t.l.c. plates, which were then scanned for radioactivity. The two radioactive products formed by the cell extract of strain AB2830 grown in the absence of 4-hydroxybenzoate (Fig. 1a) were shown to be 3-octaprenyl-4-hydroxybenzoic acid (II) and 2-octaprenylphenol (III) by co-chromatography with authentic samples in five solvent systems including one reversed-phase system (see the Materials and Methods section). These assignments were supported by the non-appearance of radioactivity in the band with the higher  $R_F$  value when 4-hydroxy[7-<sup>14</sup>C]benzoate was used as substrate (Fig. 1b). An active precursor of the polyprenyl side chain of the two compounds must therefore be present in cell extracts of strain AB2830 grown in the absence of 4-hydroxybenzoate. Similar results were obtained by using another multiple aromatic auxotroph, strain AB2826.

The corresponding chromatogram for strain AB2830 grown in the presence of 4-hydroxybenzoate indicated that no apparent conversion of 4-hydroxy[U- $^{14}$ C]benzoate into 3-octaprenyl-4-hydroxybenzoate and 2-octaprenylphenol had occurred during incubation of the cell extract. However, it was possible that some unlabelled 4-hydroxybenzoate was retained in the cell extract and consequently could have 'swamped' any incorporation of the 4-hydroxy[U- $^{14}$ C]benzoate. An extract was therefore prepared from *E. coli* strain AN62. This strain is 'wild-type' for the ubiquinone pathway and therefore does not require the addition of 4-hydroxybenzoate to the growth medium. The AN62 cell extract contained no detectable polyprenylation activity and it was presumed that the inactivity of these two extracts was due to the lack of accumulation of an active precursor of the polyprenyl side chain.

*Identification of an octaprenol in cell extracts of strain AB2830.* Cell extracts were prepared from strain AB2830 grown into stationary phase in the presence or in the absence of 4-hydroxybenzoate, the extracts were freeze-dried, and the residue was extracted with acetone. The acetone extracts were chromatographed on a silica-gel t.l.c. plate with chloroform as the developing solvent. The plates were then sprayed with the anisaldehyde spray and heated at 100°C for 10 min. A compound(s) giving a blue-green colour with this spray was present in the extract of cells grown in the absence of 4-hydroxybenzoate, and had an  $R_F$  value (0.4) similar to that of a nonaprenol (solanesol) marker.

Growth in the presence of 4-hydroxybenzoate caused the removal of the compound(s) at  $R_F$  0.4 and the appearance of ubiquinone ( $R_F$  0.7). The chromatography system used does not distinguish clearly between polyprenols with the same basic structure but with different numbers of isoprene units. The area of a chromatogram near  $R_F$  0.4 was therefore scraped off, eluted with ether and the ether solution concentrated and chromatographed in the reversed-phase system with water-acetone (1:19, v/v) as the developing solvent. A single spot was obtained with an  $R_F$  value greater than solanesol but less than geraniol.

Two 40-litre cultures of strain AB2830 were grown in the absence of 4-hydroxybenzoate and a cell extract was prepared, freeze-dried and the residue extracted with acetone. The unknown compound was purified from the acetone extract by repeated t.l.c. on silica-gel plates with chloroform as the developing solvent. The yield of compound, which was an oil at room temperature, was about 5 mg from about 120 g (wet wt.) of cells. The compound was identified as the all-*trans*-octaprenol, farnesylfarnesylgeraniol, by n.m.r. and mass spectroscopy.

The n.m.r. spectrum of the octaprenol in benzene is shown in Fig. 2. The chemical shifts and couplings of the various protons are identical with those found for the all-*trans*-solanesol (Feeney & Hemming, 1967) and integration of the various peaks produced values similar to those for a standard sample of solanesol, due allowance being made for the extra isoprene residue (the molecular weight of the

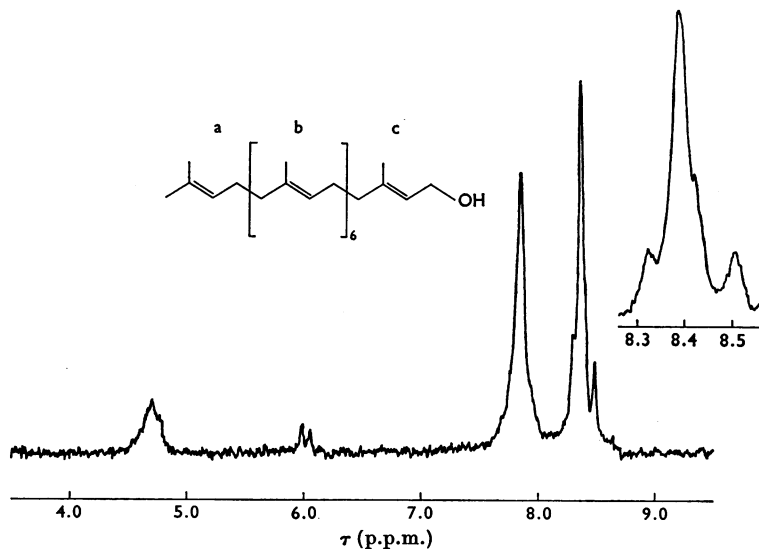


Fig. 2. N.m.r. spectrum of farnesylfarnesylgeraniol, at 100 MHz, in benzene. Other details are given in the text.

octaprenol was obtained from the mass spectrum described below). The resonances at  $\tau=8.32$  and  $8.43$  are assigned to one *cis*-methyl and one *trans*-methyl group from the  $\omega$ -terminal isoprene residue (labelled 'a' in Fig. 2), that at  $\tau=8.38$  to six *trans*-methyl groups from the six internal isoprene residues (labelled 'b' in Fig. 2), and that at  $\tau=8.51$  to one *trans*-methyl group from the 'OH-terminal' isoprene residue (labelled 'c' in Fig. 2). Feeney & Hemming (1967) have described in detail the assignments of the methyl groups for polyprenols with different stereochemical configurations.

The complex peak centred at  $\tau=7.88$  is assigned to the methylene protons of isoprene residues ( $-\text{CH}_2-\text{C}_2-$ ), except for the methylene protons adjacent to the olefinic proton of the 'OH-terminal' isoprene residue ( $=\text{CH}-\text{CH}_2-\text{OH}$ ), which gave a doublet at  $\tau=6.06$ . Also present is a broad peak centred at  $\tau=4.73$  assigned to the olefinic protons of the  $\omega$ -terminal and internal isoprene residues, and there appeared to be a weak absorption centred at about  $\tau=4.6$  due to the olefinic proton of the 'OH-terminal' residue.

In the mass spectrum (Fig. 3) the polyprenyl nature of the unknown compound was evident from the regular series of fragment ions separated by 68 mass units, representing cleavage between individual isoprene units in a chain (Wright, Dankert, Fennessey & Robbins, 1967). The strong ion at  $m/e$  544, resulting from the loss of 18 mass units from the molecular ion, indicated that the compound was composed of eight isoprene units and an alcoholic functional group. The accurate mass of the molecular ion was 562.5109 ( $\text{C}_{40}\text{H}_{66}\text{O}$  requires 562.5114). The positions of the major fragment ions obtained on cleavage between isoprene units were exactly analogous to those observed in the mass spectrum of a standard sample of a nonaprenol

(solanol). The base peak in the mass spectrum given by the octaprenol, as well as by standard samples of geranylgeraniol and solanols (Allen, Alworth, Macrae & Bloch, 1967), is at  $m/e$  69. Von Sydow (1963) has examined the fragmentation pattern for diprenols and has found the base peak at  $m/e$  69 to be characteristic of acrylic alcohols bearing a terminal isopropylidene grouping. As with known prenols (Higashi, Strominger & Sweeley, 1967) the peak at  $(\text{C}_5\text{H}_8)_n$  is somewhat lower in intensity than the companion peak at one lower  $m/e$  value. This suggests that there is no significant contribution from lower-molecular-weight polyprenols. There was no evidence in the mass spectrum for any saturated isoprene units.

*Isolation and identification of 3-octaprenyl-4-aminobenzoic acid.* The octaprenol could be obtained from extracts that had been frozen immediately after the disruption of the cells and was not removed by incubation of the extracts with 4-hydroxybenzoate. This observation suggested that the active form of the  $\text{C}_{40}$  unit was rapidly degraded to the octaprenol after preparation of the cell extract and prompted an examination of acetone extracts of whole cells.

A culture of strain AB2830 was grown into stationary phase in the presence or in the absence of 4-hydroxybenzoate; the cells were harvested and continuously extracted with acetone. A sample of the acetone extract was then chromatographed on silica gel with chloroform as the developing solvent. The chromatogram was sprayed with the anisaldehyde spray and heated at  $100^\circ\text{C}$  for 10 min. Apart from the difference in the ubiquinone content of the two extracts, the chromatogram indicated the presence of a compound at  $R_f$  0.2 in the acetone extract of cells of strain AB2830, grown without 4-hydroxybenzoate, which was not present when

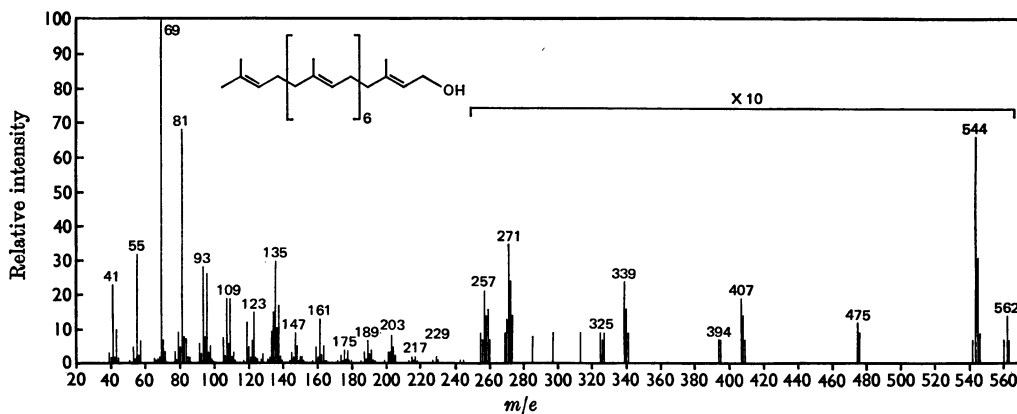


Fig. 3. Mass spectrum of farnesylfarnesylgeraniol. Details are given in the text.

the cells were grown with 4-hydroxybenzoate. (The octaprenol could not be detected and the compounds running with similar  $R_F$  values to the octaprenol could be distinguished by the different colours given with the anisaldehyde spray.) The new compound was purified by repeated chromatography on silica-gel plates with chloroform-methanol (50:1, v/v) as the developing solvent. The u.v. spectrum in ether showed an asymmetric peak of absorption at 275nm. This spectrum resembled closely that of 4-aminobenzoic acid. The compound was isolated from two 40 litre cultures of strain AB2830, grown without 4-hydroxybenzoate, and identified as 3-octaprenyl-4-aminobenzoic acid by n.m.r. and by mass spectrometry; 7mg of compound, which was an oil at room temperature, was obtained from 100g wet wt. of cells.

The n.m.r. spectrum for 3-octaprenyl-4-aminobenzoic acid is presented in Fig. 4. This spectrum is very similar to that given for 3-octaprenyl-4-hydroxybenzoic acid by Cox *et al.* (1969). In the aromatic region of the spectrum, signals from three protons were observed. The signals from two of these protons occurred at low field strength for benzenoid protons ( $\tau$  about 2.2) and are therefore assigned to H-2 and H-6, which would be expected to be deshielded by the carboxyl group. The chemical shifts of the three aromatic protons are very similar to those of 3-octaprenyl-4-hydroxybenzoic acid, indicating that the amino group is present on C-4. In addition, the splitting of the doublet from H-5 ( $\tau=3.5$ ) is approximately 8Hz, this value being characteristic of coupling between protons *ortho* to each other. This coupling, together

with the chemical shift data, places the side chain on C-3, even though the couplings within the aromatic region at low field strength are not resolved in the spectrum obtained. The remaining peaks in the spectrum correspond very closely to those assigned to the protons of the polyprenyl side chain in the spectra for 3-octaprenyl-4-hydroxybenzoic acid and 2-octaprenylphenol (Cox *et al.* 1969). In the olefinic region of the spectrum, signals equivalent to a total of eight methine protons were observed ( $\tau=4.7-5.2$ ), indicating a side chain of eight isoprene units. The methylene protons of the isoprene residue adjacent to the ring appear as a doublet ( $\tau=6.76$ ) and the methylene protons of the other isoprene residues appear as a multiplet centred at approx.  $\tau=7.98$ . The chemical shifts of the methyl groups of the side chain indicate that they are all *trans* to their respective methine protons, except for the *cis*-methyl group at the end of the side chain (Pennock, 1965) ( $\tau=8.24$  for the *trans*-methyl group of the first isoprene unit,  $\tau=8.41$  for the *trans*-methyl groups of the side chain and  $\tau=8.35$  for the *cis*-methyl group at the end of the side chain). No signals were observed for either the amino protons or for the carboxyl proton, presumably because of exchange.

The mass spectrum of 3-octaprenyl-4-aminobenzoic acid (Fig. 5) shows the expected molecular ion at  $m/e$  681, the accurate mass of which was 681.5487 ( $C_{47}H_{71}NO_2$  requires 681.5485). This spectrum also shows marked similarities to that of 3-octaprenyl-4-hydroxybenzoic acid (Cox *et al.* 1969). The same sequential loss of seven isoprene units was found and the peak at  $m/e$  69 may be

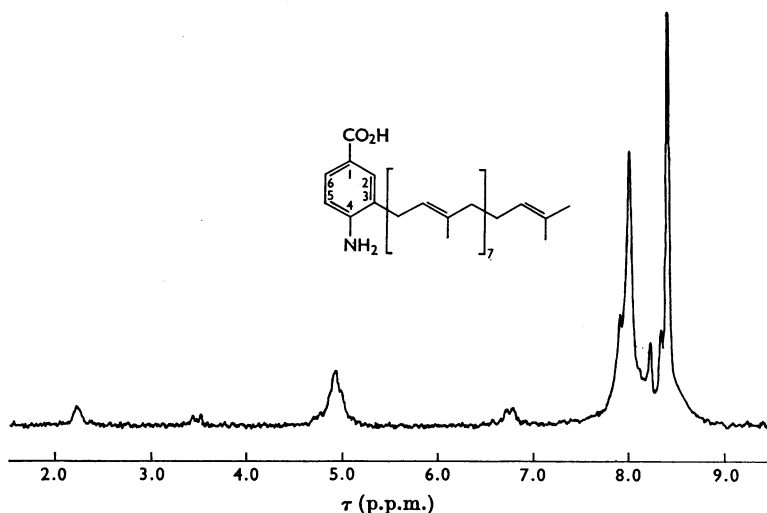


Fig. 4. N.m.r. spectrum of 3-octaprenyl-4-aminobenzoic acid at 100MHz in  $CCl_4$ . Other details are given in the text.

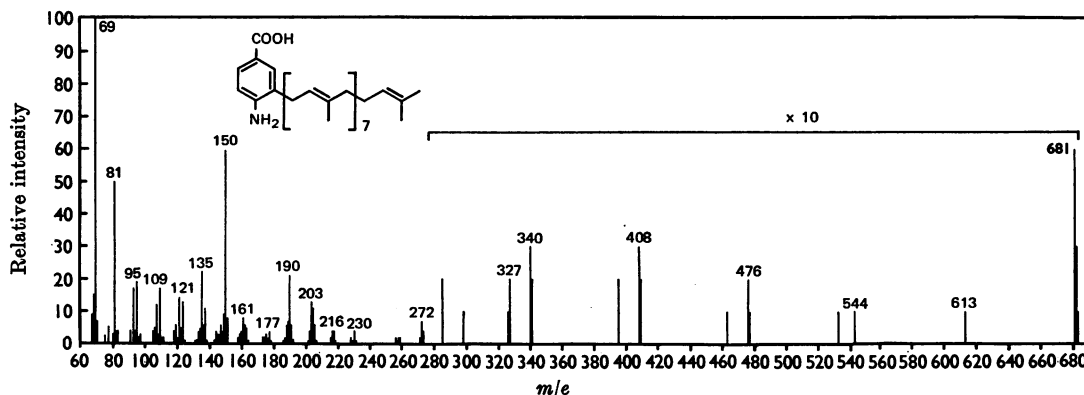


Fig. 5. Mass spectrum of 3-octaprenyl-4-aminobenzoic acid. Details are given in the text.

attributed to the allylic cation  $C_5H_9^+$  formed during the breakdown of the polyprenyl side chain. A prominent peak at  $m/e$  150 can probably be attributed to the formation of a carboxyamino-tropylium ion.

*Isolation of the octaprenol from whole cells and the isoprenylation activity of cell extracts of strain AB2830 grown in the presence of low concentrations of 4-aminobenzoate.* The octaprenyl side chain can be attached to the 4-aminobenzoate nucleus when strain AB2830 is grown in the absence of 4-hydroxybenzoate, and this presumably would cause a decreased accumulation of the octaprenol and its activated form. Therefore the final concentration of 4-aminobenzoate in the growth medium for strain AB2830 was lowered from 10 to  $1\mu M$ . The octaprenol could now be detected in the acetone extract of the whole cells, with a concomitant decrease in the concentration of 3-octaprenyl-4-aminobenzoic acid. The yields of octaprenol and 3-octaprenyl-4-aminobenzoic acid, after purification by chromatography as before, were 3 mg and 1 mg respectively from 120 g wet wt. of cells.

The increase in the content of the octaprenol in acetone extracts of whole cells may be reflected in an increase in both the rate and the amount of polyprenylation of 4-hydroxybenzoate in cell extracts. Strain AB2830 was grown in the absence of 4-hydroxybenzoate and in the presence of  $1\mu M$ - or  $10\mu M$ -4-aminobenzoate; the cells were harvested and extracts prepared as described above. The extracts were incubated with 4-hydroxy[ $U$ - $^{14}C$ ]-benzoate at  $37^\circ C$ , samples removed at time-intervals and the samples assayed for incorporation of 4-hydroxybenzoate into 3-octaprenyl-4-hydroxybenzoate and 2-octaprenylphenol by counting their radioactivity in the liquid-scintillation counter. The decrease in the concentration of 4-aminobenzoate in the growth medium by a factor of ten

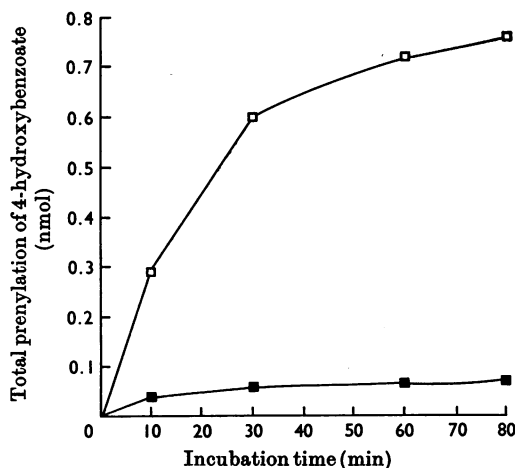


Fig. 6. Polyprenylation of 4-hydroxy[ $U$ - $^{14}C$ ]benzoate by cell extracts of strain AB2830 grown in the presence of different concentrations of 4-aminobenzoate. Reaction mixtures containing 4-hydroxy[ $U$ - $^{14}C$ ]benzoic acid (400000 c.p.m. diluted with unlabelled 4-hydroxybenzoic acid to specific radioactivity  $70\mu Ci/\mu mol$ ) in 4.4 ml of 0.1M-potassium phosphate buffer, pH 7.0, and 0.6 ml of cell extract of strain AB2830 grown in either  $10\mu M$ -4-aminobenzoate ( $\blacksquare$ ) or  $1\mu M$ -4-aminobenzoate ( $\square$ ) were incubated at  $37^\circ C$  and samples taken at time-intervals. The samples were treated as in Fig. 1 except that the light-petroleum extracts were assayed for radioactivity in a liquid-scintillation counter.

results in an approximately parallel increase in the initial rate of incorporation of the 4-hydroxy[ $U$ - $^{14}C$ ]benzoate (Fig. 6). The combined amount of 3-octaprenyl-4-hydroxybenzoate and 2-octaprenylphenol produced after 80 min by the more active cell extract (equivalent to 0.3 g wet wt. of cells) was about 0.8 nmol. Approximately 70 nmol of

ubiquinone is produced by 0.3g wet wt. of cells of strain AB2830, when grown in the presence of 4-hydroxybenzoate.

### DISCUSSION

Hammond & White (1969) have shown that in *Staphylococcus aureus* the menaquinones with various side-chain lengths present in this organism are stable once formed, and they concluded that the side chains are not formed by a sequential addition of isoprene units. They suggested that the different menaquinones may have different functions or that the alkylating enzyme is relatively non-specific to side-chain length. In addition, Allen *et al.* (1967) have partially purified an enzyme from *Micrococcus lysodeikticus* that catalyses the elongation of polyprenyl pyrophosphates by isopentenyl pyrophosphate. A C<sub>55</sub> alcohol involved in cell-wall synthesis has been isolated from *M. lysodeikticus* (Scher, Lennarz & Sweeley, 1968), but it is not known whether the enzyme partially purified by Allen *et al.* (1967) is involved in the biosynthesis of both the C<sub>55</sub> alcohol and menaquinone. The accumulation of an octaprenol by a multiple aromatic auxotroph of *E. coli*, grown under conditions where the quinone portions of the ubiquinone and menaquinone molecules cannot be formed, provides additional evidence that the side chains are added as the C<sub>40</sub> unit. The lack of accumulation of lower-molecular-weight polyprenols would support the idea that the small amounts of ubiquinones and menaquinones with various side-chain lengths are formed because the alkylating enzyme is relatively non-specific rather than that they have different functions.

The observation that the octaprenol could be detected in acetone extracts of cell extracts of strain AB2830, grown in the presence of 10 μM-4-aminobenzoate, but not in acetone extracts of the whole cells, suggested that in whole cells the octaprenol was present in some other form that was rapidly broken down when the cell extract was prepared. This rapid breakdown of the presumed active form would account for the comparatively low concentration of 3-octaprenyl-4-hydroxybenzoate and 2-octaprenylphenol formed from 4-hydroxybenzoate by cell extracts, even when the cells had been grown in the presence of low concentrations of 4-aminobenzoate.

Davis (1951) observed that concentrations of 4-aminobenzoate of the order of 5mM inhibited the growth of *E. coli* strains, including K-12. This inhibition of growth could be reversed by the addition of 4-hydroxybenzoate at a concentration of 50 μM. A similar type of competition between 4-hydroxybenzoate and 4-aminobenzoate has been shown to occur in rickettsiae (Snyder & Davis,

1951). The formation of 3-octaprenyl-4-aminobenzoate by a multiple aromatic auxotroph grown in the absence of 4-hydroxybenzoate, but in the presence of 10 μM-4-aminobenzoate, is probably directly related to this reversible growth inhibition. Thus the enzyme involved in the formation of 3-octaprenyl-4-hydroxybenzoate on the pathway of ubiquinone biosynthesis is able to use 4-aminobenzoate as substrate in the absence of 4-hydroxybenzoate.

The first three reactions involved in ubiquinone biosynthesis, namely the conversion of chorismate into 4-hydroxybenzoate (Gibson & Gibson, 1964), the conversion of 4-hydroxybenzoate into 3-octaprenyl-4-hydroxybenzoate (this paper) and the conversion of 3-octaprenyl-4-hydroxybenzoate into 2-octaprenylphenol (Cox *et al.* 1969) have now been demonstrated in cell extracts of *E. coli*. Although the detailed examination of these three enzymic activities has not yet been carried out, the evidence obtained with cell extracts, plus the accumulation of 3-octaprenyl-4-hydroxybenzoate and 2-octaprenylphenol by the appropriate ubiquinone-deficient mutants (Cox *et al.* 1969), firmly establishes 4-hydroxybenzoate, 3-octaprenyl-4-hydroxybenzoate and 2-octaprenylphenol as intermediates in the synthesis of ubiquinone from chorismic acid in *E. coli*.

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