Biosynthesis of Bacterial Menaquinones: The Membrane-Associated 1,4-Dihydroxy-2-naphthoate Octaprenyltransferase of *Escherichia coli*†

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ABSTRACT: It has been postulated that 1,4-dihydroxy-2-naphthoic acid is the naphthalenic intermediate in the biosynthesis of menaquinone (vitamin K_2) in *Escherichia coli* to which the octaprenyl side chain is attached to form demethylmenaquinone. In the present work the presence of an enzyme, 1,4-dihydroxy-2-naphthoate octaprenyltransferase, which catalyzes the conversion of 1,4-dihydroxy-2-naphthoate to demethylmenaquinone was demonstrated in cell extracts of *E. coli*. Demethylmenaquinone-9 was formed when the naphthoate was incubated with cell extracts and the synthetic substrate, solanesyl pyrophosphate, in the presence of Triton X-100. Solanesyl monophosphate could not substitute for the pyrophosphate in the reaction. The prenylation of 1,4-dihydroxy-2-naphthoate was also studied in a strain of *E. coli* which accumulates octaprenyl pyrophosphate, the natural precursor of the menaquinone side chain. The octaprenyltransferase was shown to be membrane bound and to require magnesium ions for optimal activity. A *menA*$^-$ mutant of *E. coli* was found to lack the octaprenyltransferase activity, suggesting that the *menA* gene is the structural gene for this enzyme. However, this strain had normal levels of 4-hydroxybenzoate octaprenyltransferase, the enzyme catalyzing the analogous prenylation reaction in ubiquinone biosynthesis, providing additional evidence that the two octaprenyltransferases are quite distinct.

E. coli forms two isoprenoid quinones, ubiquinone and menaquinone (Figure 1). In these compounds, a substituted benzoquinone or a substituted naphthoquinone ring is condensed to an all-trans polyisoprenoid side chain, which varies in length but which consists predominantly of eight isoprene units. The isoprenoid side chain apparently serves to locate the quinones in the cytoplasmic membrane, allowing them to function as mobile redox carriers in the various electron transport chains formed by *E. coli*. The prenylation of 4-hydroxybenzoate (Figure 1), the aromatic precursor of the quinone ring of ubiquinone, has been studied in cell extracts of *E. coli* and the position of this reaction in the sequence of steps leading to the biosynthesis of ubiquinone is well understood (Hamilton and Cox, 1971; Young et al., 1972, 1973). The enzyme concerned, 4-hydroxybenzoate octaprenyltransferase, catalyzes the formation of 3-octaprenyl-4-hydroxybenzoate from 4-hydroxybenzoate and a polyisoprenoid substrate. Both the enzyme and the isoprenoid substrate are membrane-bound and the enzyme requires magnesium ions for optimal activity in vitro. The available evidence indicates that the polyisoprenoid substrate in *E. coli* is octaprenyl pyrophosphate since polyisoprenyl pyrophosphates can be used as substrates for the prenylation reaction in assays with cell extracts (Leppik, 1973; El Hachimi et al., 1974). Polyprenyl pyrophosphates have also been found to stimulate the prenylation of 4-hydroxybenzoate in extracts of *Rhodospirillum rubrum* and in rat tissues (Raman et al., 1969; Winrow and Rudney, 1969).

Since ubiquinone and menaquinone have the same polyisoprenoid side chain, it is likely that the prenylation reactions in the two pathways have many features in common. Until recently, however, it has not been possible to examine the octaprenyltransferase reaction in menaquinone biosynthesis, because the naphthalenic precursor which undergoes prenylation has been unknown. In a recent study using *E. coli* mutants defective in menaquinone biosynthesis (Young, 1975), strong evidence has been obtained that 1,4-dihydroxy-2-naphthoate is an intermediate in the biosynthesis of menaquinone. This compound is the last known intermediate prior to the prenylation reaction and appears to be the precursor to which the octaprenyl side chain is attached to form demethylmenaquinol (Figure 1). The role of the naphthoate is supported by the recent experiments of Bentley and co-workers (Bentley, 1975) who have shown that 1,4-dihydroxy-2-naphthoate can be prenylated when incubated with *E. coli* extracts in the presence of farnesyl pyrophosphate and magnesium ions.

In the present work the prenylation of 1,4-dihydroxy-2-naphthoate is demonstrated in vitro using *E. coli* cell extracts with both synthetic solanesyl pyrophosphate and the natural octaprenyl pyrophosphate as substrates, and the cellular location and properties of the enzyme catalyzing the reaction are examined.

Experimental Section

Bacterial Strains. The strains used were all derivatives of the *E. coli* K12 strain AB3311 and are described in Table I.

Media. The minimal medium used and the concentration of supplements have been described previously (Stroobant et al., 1972). The medium used for the growth of *aroB*$^-$ strains was supplemented with phenylalanine (0.2 mM), tyrosine (0.2 mM), tryptophan (0.2 mM), 2,3-dihydroxybenzoate (10 μM), and 4-aminobenzoate (1 μM).

Preparation of Cell Extracts. Cultures were grown into late exponential phase in glucose-minimal medium containing the appropriate supplements. The cells were centrifuged, washed in 0.05 M potassium phosphate buffer (pH 7.0), and were resuspended in 2 ml of the same buffer for each gram (wet weight) of cells. The cells were disrupted at 20 000 psi in a Sorvall Ribi cell fractionator. The supernatant fluid, after

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TABLE I: Strains of *Escherichia coli* K12 Used.

Strain	Relevant Genetic Loci[a]
AB3311	*metB*$^-$
AN209	*menB404 metB*$^-$
AN211	*menA406 metB*$^-$
AN164	*metB*$^-$, *aroB351*
AN479	*menA406 metB*$^-$, *aroB351*

[a] Genetic nomenclature follows the usage of Taylor and Trotter (1972).

centrifugation at 30 000*g* for 30 min, was used as the cell extract. The centrifugation of the culture and subsequent operations were carried out at 4 °C.

Chemicals. [^{14}C]-1,4-Dihydroxy-2-naphthoic acid was extracted from the supernatants of 1-l. cultures of strain AN479, supplemented with 10 μmol of shikimic acid (U-^{14}C labeled, specific activity 2.07 μCi/μmol), obtained from New England Nuclear, U.S.A. The method of extracting 1,4-dihydroxy-2-naphthoic acid and its subsequent purification by thin-layer chromatography has been described (Young, 1975). The yield estimated by its fluorescence (activation, 380 nm; fluorescence, 440 nm uncorrected) using an Aminco Bowman spectrophotofluorimeter ranged between 20 and 70 nmol per l. of culture.

Tritiated 1,4-dihydroxy-2-naphthoic acid was synthesized from [G-^{3}H]-1-hydroxy-2-naphthoic acid (Radiochemical Centre Ltd., Amersham, tritium labeling service) (Desai and Sethna, 1951; Young, 1975). The specific radioactivity of the product was 2 μCi/μmol.

Solanesyl pyrophosphate and monophosphate were kindly donated by Dr. D. Magrath.

Assay for 1,4-Dihydroxy-2-naphthoate Octaprenyltransferase. When exogenous polyprenyl phosphates were included in assays, the incubation mixtures contained 0.2 ml of cell extract (7 mg of protein), 100 nmol of either solanesyl monophosphate or solanesyl pyrophosphate, Tris-HCl buffer (0.1 M, pH 8.0), $MgSO_4$ (10 mM), dithiothreitol (10 mM), and Triton X-100 (0.4%), in a total volume of 0.5 ml. The reaction was started by the addition of 0.5–1 nmol of [^{14}C]- or [^{3}H]-1,4-dihydroxy-2-naphthoic acid (1200–1500 cpm, approximate specific activity 2 μCi/μmol) in 5 μl of ethanol–diethyl ether (2:1, v/v). The tubes were incubated at 37 °C for 30 min. The polyprenyl phosphates were stored in solution in chloroform saturated with ammonia at −15 °C. Before their use in assays, the solvent was removed under a stream of nitrogen and they were suspended in buffer containing 0.4% Triton X-100.

Assays using extracts of the *aroB*$^-$ mutant AN164 did not include exogenous polyisoprenyl pyrophosphates as this strain accumulates octaprenyl pyrophosphate, the natural side chain precursor. The incubation mixture contained 0.2 ml of cell extract and phosphate buffer (0.05 M, pH 7.0) in a total volume of 0.5 ml. The reaction was started by the addition of radioactively labeled 1,4-dihydroxy-2-naphthoic acid as described for the assay with exogenous polyprenyl phosphates. The tubes were incubated at 37 °C for 10 min.

In both types of assay the reaction was stopped by the addition of 0.1 M acetic acid in methanol and extracted with pentane (2 × 3 ml). The combined pentane extract was evaporated to dryness in a scintillation vial, scintillant (10 ml) was added, and the radioactivity was determined.

Assay for 4-Hydroxybenzoate Octaprenyltransferase. Assays were performed according to the procedures described above for the assay of 1,4-dihydroxy-2-naphthoate octaprenyltransferase except that the reactions were started by adding 2 nmol of [^{14}C]-4-hydroxybenzoic acid (10 000 cpm) to the incubation mixtures.

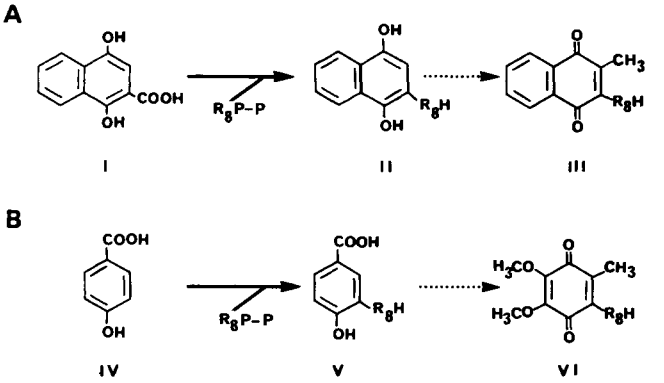

FIGURE 1: Octaprenyltransferase reactions in (A) menaquinone biosynthesis; (B) ubiquinone biosynthesis in *E. coli*. (I) 1,4-Dihydroxy-2-naphthoic acid; (II) demethylmenaquinol; (III) menaquinone; (IV) 4-hydroxybenzoic acid; (V) 3-octaprenyl-4-hydroxybenzoic acid; (VI) ubiquinone; (R_8PP) octaprenyl pyrophosphate; (R_8H) octaprenyl side chain.

Determination of Radioactivity. Radioactivity was determined using a Packard Tri-Carb scintillation spectrometer. The scintillation fluid used for prenylated compounds consisted of 6 g of 2,5-diphenyloxazole and 20 ml of ethanol per l. of toluene. Bray's scintillant (Bray, 1960) was used in determining the radioactivity of solutions of 1-hydroxy-2-naphthoic acid, 1,4-dihydroxy-2-naphthoic acid and 4-hydroxybenzoic acid.

Thin-Layer Chromatography. Pentane extracts were evaporated to dryness on a steam bath, the residue was taken up in ethanol–diethyl ether (2:1, v/v) and applied to paraffin impregnated silica gel plates (Merck F254, 0.25-mm thickness). Plates were prepared by dipping them in a solution of 5% liquid paraffin in light petroleum (60–80 °C) for 15 s and then allowing the solvent to evaporate. The chromatograms were developed in acetone–water (19:1, v/v) saturated with paraffin.

Estimation of Protein. Protein concentrations were determined by the method of Lowry et al. (1951) with bovine serum albumin fraction V as standard.

Results

Demonstration and Assay of 1,4-Dihydroxy-2-naphthoate Octaprenyltransferase in Cell Extracts of E. coli. Since the 4-hydroxybenzoate octaprenyltransferase of *E. coli* can use the nonaprenyl lipid, solanesyl pyrophosphate, as substrate (Leppik, 1973; El Hachimi et al., 1974), an attempt was made to determine whether this lipid could also be used as a substitute for the natural substrate in the prenylation of 1,4-dihydroxy-2-naphthoic acid. Cell extract from *E. coli* was incubated with [^{14}C]-1,4-dihydroxy-2-naphthoic acid and solanesyl pyrophosphate in the presence of 10 mM Mg^{2+} and 0.2% Triton X-100. After incubation, menaquinone-8 and demethylmenaquinone-8 were added as carriers to the assay mixtures, which were then extracted with pentane. The extract was chromatographed on a paraffin impregnated silica gel plate (Merck F254) with acetone–water (19:1, v/v) as solvent. The bulk of the radioactivity migrated in a band just below the demethylmenaquinone-8 marker and approximately coincided with the menaquinone-8 spot (Figure 2a). This chromatographic behavior is consistent with the product of the reaction being demethylmenaquinone-9 which runs with menaqui-

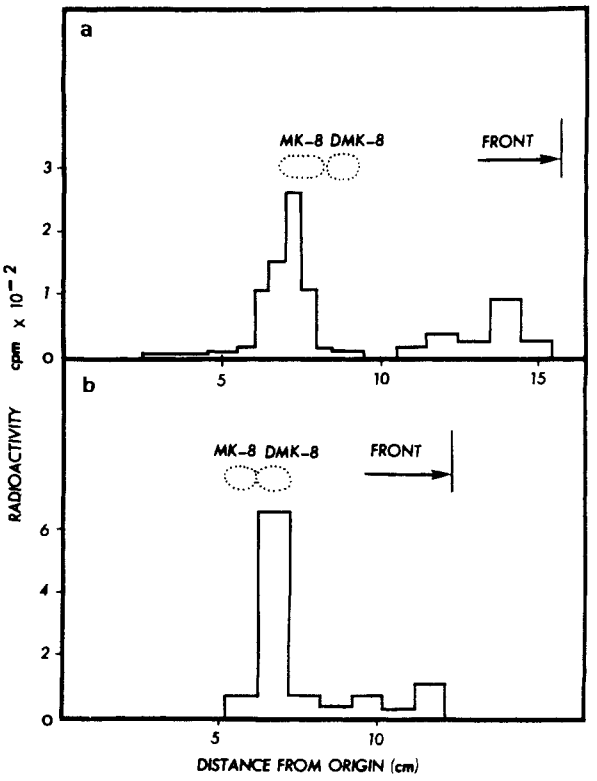

FIGURE 2: Reversed phase thin-layer chromatograms of the radioactive pentane-soluble products of the prenylation of 1,4-dihydroxy-2-naphthoate. Reaction mixtures included: (a) cell extract of strain AB3311 and solanesyl pyrophosphate; (b) cell extract of strain AN164 without added solanesyl pyrophosphate. Details of the reaction mixtures, their extraction with pentane, and their chromatography are given in the Experimental Section. (DMK) Demethylmenaquinone; (MK) menaquinone.

TABLE II: 1,4-Dihydroxy-2-naphthoate Octaprenyltransferase Activities of *Escherichia coli* Cell Extracts Using Exogenous or Endogenous Polyprenyl Phosphates.

Cell Extract from Strain	Polyprenyl Substrate	Octaprenyl-transferase[a] Activity
AB3311 (*men*$^+$)	None	<1
	Solanesyl pyrophosphate	250
	Solanesyl monophosphate	<1
AN164 (*aroB*$^-$, *men*$^+$)	Endogenous	221

[a] Assays were carried out as described in the Experimental Section. Activities are given as picomoles of product formed. Incubation times were: strain AB3311 cell extracts, 30 min; strain AN164 cell extracts, 10 min.

none-8 in this reversed phase system (Dunphy and Brodie, 1971).

In a second demonstration of the prenylation reaction, [^{14}C]-1,4-dihydroxy-2-naphthoic acid was incubated with a cell extract of strain AN164. When grown in the absence of shikimate, this *aroB*$^-$ strain accumulates a membrane-bound octaprenyl side chain precursor which is presumably octaprenyl pyrophosphate (Hamilton and Cox, 1971; Young et al., 1972). In this case most of the radioactivity migrated with demethylmenaquinone-8 in the reversed phase chromatography system (Figure 2b).

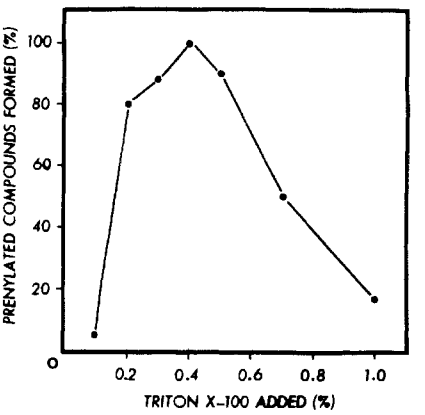

FIGURE 3: Effect of Triton X-100 concentration on the prenylation of 1,4-dihydroxy-2-naphthoate in assays (see Experimental Section) containing cell extracts of strain AB3311 and solanesyl pyrophosphate. Activities are expressed as percentages of the activity of extracts incubated in the presence of 0.4% Triton X-100 (250 pmol of product per 30 min).

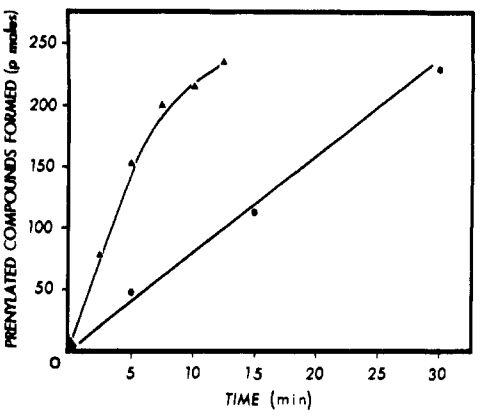

FIGURE 4: Effect of incubation time on the prenylation of 1,4-dihydroxy-2-naphthoate. The assays were carried out as described in the Experimental Section. (●) Reaction mixture containing cell extract of strain AB3311 and solanesyl pyrophosphate; (▲) reaction mixture containing cell extract of strain AN164.

As well as the radioactivity associated with the demethylmenaquinone spots in these chromatograms, a proportion of the radioactivity (about 30%) was located close to the solvent front. Since this material regularly appeared when the demethylmenaquinone spots were eluted from the chromatograms and rechromatographed under the same conditions, it was assumed to arise from the degradation of demethylmenaquinone.

The above results indicated the presence of an enzyme in cell extracts of *E. coli* which catalyzed the transfer of the polyprenyl group from polyprenyl pyrophosphate to 1,4-dihydroxy-2-naphthoate. This enzyme will be referred to throughout this paper by the trivial name 1,4-dihydroxy-2-naphthoate octaprenyltransferase.

A convenient quantitative assay was developed (see Experimental Section) to enable the properties of the transferase to be examined in more detail. In assays using cell extracts of strain AB3311, the formation of demethylmenaquinone depended on the addition of solanesyl pyrophosphate to the reaction mixture (Table II). Solanesyl monophosphate was not active as substrate when substituted for the pyrophosphate. The reaction was stimulated by the addition of Triton X-100 at an optimal concentration of 0.4% (Figure 3). Under these conditions the rate of the reaction was linear over 30 min. Solanesyl pyrophosphate was not required in assays in which

TABLE III: 1,4-Dihydroxy-2-naphthoate Octaprenyltransferase Activities in *menA*$^-$ and *menB*$^-$ Mutants.

Cell Extract from Strain	Octaprenyltransferase[a] (pmol of Product/30 min)
AB3311 (*men*$^+$)	260
AN211 (*menA*$^-$)	<1
AN209 (*menB*$^-$)	240

[a] Assays were carried out as described in the Experimental Section, using exogenous solanesyl pyrophosphate as substrate.

TABLE IV: Sedimentation Properties of 1,4-Dihydroxy-2-naphthoate Octaprenyltransferase and Effect of Metal Ions on Enzymatic Activity.

Enzyme Preparation[a]	Metal Added	Octaprenyl-transferase[b]
Cell extract		100
Supernatant (150 000*g*)		<1
Pellet (150 000*g*)		63
Pellet + supernatant		77
Pellet + EDTA (1 mM)		11
Pellet + EDTA (1 mM)	$MgSO_4$ (2 mM)	106
Pellet (150 000*g*)	$MgSO_4$ (1 mM)	108
	$NiSO_4$ (1 mM)	90
Cell extract	$MgSO_4$ (1 mM)	113

[a] A 10-ml amount of AN164 cell extract (35 mg/ml) was centrifuged at 150 000*g* for 3 h. The pellet was resuspended in 0.05 M potassium phosphate buffer (pH 7.0) and centrifuged again under the same conditions. The resulting pellet was resuspended in 10 ml of the same buffer, the final protein concentration being 8 mg/ml, and 0.2 ml of the suspension was used in the assay. [b] Activity is expressed as a percentage of the activity of the cell extract in absence of metal. The activity of this preparation was 130 pmol of product per 5 min.

cell extracts from the *aroB*$^-$ strain, AN164, were used (Table II). In these assays the reaction was more rapid and began to plateau before 10 min (Figure 4), owing probably to the exhaustion of the available endogenous substrate.

Examination of menA$^-$ *and menB*$^-$ *Mutants for 1,4-Dihydroxy-2-naphthoate Octaprenyltransferase Activity.* Two classes of menaquinone-deficient mutants of *E. coli* have been characterized (Young, 1975). It has been proposed that the *menB*$^-$ mutants are blocked in the conversion of 2-succinylbenzoate to 1,4-dihydroxy-2-naphthoate and that the *menA*$^-$ mutants are affected in the prenylation of the latter compound. The octaprenyltransferase activities of these mutants are shown in Table III. There was no detectable 1,4-dihydroxy-2-naphthoate octaprenyltransferase activity in extracts of the *menA*$^-$ mutant, AN211. On the other hand, the activity of extract from the *menB*$^-$ mutant, AN209, was equal to that of the parental strain, AB3311.

The 4-hydroxybenzoate octaprenyltransferase of the *menA*$^-$ mutant measured using exogenous solanesyl pyrophosphate as described in the Experimental Section was normal. The activity obtained was 490 pmol of product/30 min for strain AN211 (*menA*$^-$) compared with 430 pmol of product/30 min for strain AB3311 (*menA*$^+$).

Sedimentation Properties and Magnesium Requirement of 1,4-Dihydroxy-2-naphthoate Octaprenyltransferase. Table IV lists the activities of the supernatant and pellet fractions obtained after a cell extract of strain AN164 was centrifuged at 150 000*g* for 3 h. There was no detectable octaprenyltransferase activity in the supernatant fraction. The washed pellet had 63% of the activity present in cell extracts. When supernatant fluid and pellet were mixed, the original activity found in cell extract was partially regained. The activity was fully regained by the addition of magnesium ions (1 mM) to the pellet, indicating a requirement for a divalent metal ion. In addition, it was found that EDTA[1] (1 mM) strongly inhibited the activity of the washed pellet and that this effect could be overcome by the addition of excess magnesium. Nickel ions were also found to be strongly stimulatory. Of a variety of other divalent metals tested, only cobalt and manganese were effective in restoring the activity of EDTA inhibited preparations above the original activity of the washed pellet. Neither was as effective as magnesium or nickel. Since the washed 150 000*g* pellet plus magnesium ions catalyzed the prenylation of 1,4-dihydroxy-2-naphthoate as efficiently as cell extract, it appears that both the enzyme and its natural polyprenyl substrate (octaprenyl pyrophosphate) are predominantly membrane bound.

TABLE V: Sedimentation Properties of 1,4-Dihydroxy-2-naphthoate Octaprenyltransferase in Cell Extracts of Strain AB3311 Using Solanesyl Pyrophosphate as Substrate.[a]

Enzyme Preparation	Octaprenyltransferase (pmol of Product/30 min)
Cell extract	250
Supernatant (150 000*g*)	1
Pellet (150 000*g*)	75
Supernatant (150 000*g*) + crude lipid extract[b]	44
Pellet (150 000*g*) + crude lipid extract	290

[a] Fractions were prepared according to the procedure described in Table IV. Assays were made as described in the Experimental Section. [b] In the presence of the crude lipid extract, octaprenyltransferase activity was still absolutely dependent on the addition of solanesyl pyrophosphate. The lipid extract was prepared from 20 g of cells (wet weight) by homogenization in 60 ml of chloroform–methanol (1:2, v/v), and was extracted by the method of Bligh and Dyer (1959). Extract from an equivalent of 0.1 g of wet weight of cells was included in each assay.

The particulate nature of the octaprenyltransferase was also examined using solanesyl pyrophosphate as substrate in the presence of Mg^{2+} (10 mM). After centrifugation of cell extracts of strain AB3311, the majority of the enzyme was found to be associated with the membrane fraction (Table V). However, a small amount of the enzyme could be detected in the supernatant fraction provided that a crude lipid extract prepared from strain AB3311 was also added. In the absence of added lipid, this fraction was completely inactive. The addition of lipid also stimulated the activity of the enzyme present in the membrane fraction. It therefore appears that the octaprenyltransferase has a lipid requirement, which may have been accentuated by the presence of Triton X-100 in the assays.

Discussion

The evidence given above indicates that cell extracts of *E. coli* contain an enzyme that catalyzes the condensation of octaprenyl pyrophosphate with 1,4-dihydroxy-2-naphthoate to form demethylmenaquinone. This enzyme has been given

[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

the trivial name of 1,4-dihydroxy-2-naphthoate octaprenyltransferase. It is essential for menaquinone biosynthesis since it is absent in *menA*$^-$ mutants. Like the analogous reaction in the synthesis of ubiquinone, the octaprenyltransferase reaction in menaquinone biosynthesis marks the point at which the pathway becomes associated with the membrane.

Because the conversion of 1,4-dihydroxy-2-naphthoate to demethylmenaquinone requires the decarboxylation as well as the prenylation of the substrate, the question arises whether one or two enzymes are involved. This question cannot be answered unequivocally at present, but the available evidence suggests that only one enzyme is concerned. Isotopic tracer experiments using whole cells have shown that a symmetrical compound such as 1,4-naphthoquinol is not an intermediate in the reaction and that the polyprenyl side chain is inserted in the position previously occupied by the carboxyl group of the naphthoate molecule (Baldwin et al., 1974; see also Young, 1975). Moreover, *menA*$^-$ mutants of *E. coli,* which are defective in the prenylation reaction, accumulate 1,4-dihydroxy-2-naphthoate rather than 1,4-naphthoquinone (Young, 1975).

As may be expected from the fact that the menaquinone and ubiquinone formed by *E. coli* possess the same polyisoprenoid side chain, the prenylation reactions in both pathways have important features in common. Like the 4-hydroxybenzoate octaprenyltransferase, the 1,4-dihydroxy-2-naphthoate octaprenyltransferase is membrane bound and requires magnesium ions for optimal activity. Both enzymes appear to utilize a common pool of membrane-bound octaprenyl pyrophosphate as their natural polyisoprenoid substrate (Hamilton and Cox, 1971; Young et al., 1972), and both can use synthetic polyprenyl pyrophosphates such as solanesyl pyrophosphate as alternative substrates (Leppik, 1973; El Hachimi et al., 1974; Table II, this paper). Polyprenyl monophosphates, however, are inactive (Leppik, 1973; Table II, this paper). Since octaprenyl pyrophosphate, in contrast to 1,4-dihydroxy-2-naphthoate, appears not to be formed in excess by wild type *E. coli* cells, competition for it by the two octaprenyltransferases may be a key factor in determining the level of menaquinones formed by the cell.

In spite of their similarities, the genetic evidence indicates that the two octaprenyltransferases are quite distinct. The *menA* and *ubiA* genes, which appear to be the structural genes for the two transferases, map separately on the *E. coli* chromosome at minutes 78 and 81, respectively (Young et al., 1972; Young, 1975), and a mutation in one of the genes does not affect the activity of the octaprenyltransferase coded for by the other gene. Thus *ubiA*$^-$ mutants are able to form menaquinone (Young et al., 1972), while *menA*$^-$ mutants form ubiquinone (Young, 1975) and have a normal 4-hydroxybenzoate octaprenyltransferase activity in vitro. Moreover, the types of reaction catalyzed by the two octaprenyltransferases appear to differ. Although both involve a transfer of the octaprenyl group to their respective substrates, the prenylation reaction of the menaquinone pathway apparently involves a decarboxylation.

Acknowledgments

The authors thank Allen Le Lievre and June MacDonald for skilled technical assistance.

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