Biosynthesis of Bacterial Menaquinones. Menaquinone Mutants of *Escherichia coli*†

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ABSTRACT: The isolation of six menaquinone mutants of *Escherichia coli* is described. It was shown that the mutants fall into two genetic classes. The first class carries mutations in a gene designated *menA*, which was located at minute 78 on the *E. coli* chromosome by cotransduction with the *glpK* and *metB* genes. The second class carries mutations in a gene designated *menB*. It was shown that this gene was not cotransducible with the *menA* gene. The biosynthesis of menaquinone in *E. coli* was studied using a variety of mutants blocked in aromatic biosynthesis together with the two classes of menaquinone mutants. It was demonstrated that chorismate is the branch point compound leading to menaquinone, and that 2-succinylbenzoic acid and 1,4-dihydroxy-2-naphthoic acid can serve as menaquinone precursors in *E. coli*. It was also shown that *menA*$^-$ and *menB*$^-$ strains accumulate 1,4-dihydroxy-2-naphthoic acid and 2-succinylbenzoic acid, respectively, in their culture supernatants. The accumulation of the two compounds by the mutants together with their activity as menaquinone precursors provide strong evidence that they are true intermediates in menaquinone biosynthesis. A pathway is proposed for the biosynthesis of bacterial menaquinones in which each intermediate has been adequately characterized.

In recent years, a good deal of information has been gained about the biosynthesis of bacterial menaquinones from precursor studies using isotopically labeled precursors. It has been established that the 2-methyl group is derived from L-methionine (see Threlfall, 1971), and that both quinone oxygens are derived from water and not from molecular oxygen (Snyder and Rapoport, 1970). The aromatic ring and the C-4 quinone carbonyl have been shown to be derived from shikimic acid (Cox and Gibson, 1966; Leistner *et al.*, 1967; Leduc *et al.*, 1970; Campbell *et al.*, 1971; Baldwin *et al.*, 1974) and the remaining three carbon atoms of the naphthalene nucleus from 2-oxoglutaric acid (Campbell *et al.*, 1971).

The contribution from shikimic acid indicates that the specific pathway for the synthesis of menaquinone must branch from the common pathway of aromatic biosynthesis at shikimic acid or later. The only known aromatic precursor which feeds into the specific pathway for menaquinone synthesis is 2-succinylbenzoic acid (Dansette and Azerad, 1970) which provides all the carbon atoms of the naphthalene nucleus of menaquinone (Campbell *et al.*, 1971). A number of possible naphthalenic precursors have been tested in precursor feeding experiments with generally negative results (see Campbell *et al.*, 1971; Baldwin *et al.*, 1974).

In order to gain a clearer understanding of the biosynthesis of menaquinone it is essential to define the branch point in aromatic biosynthesis leading to menaquinone, to verify that 2-succinylbenzoic acid is a true intermediate, and to establish the structure of later naphthalenic intermediates in the pathway.

A new approach to the problem which has not been exploited so far is the study of mutants blocked in various steps in the specific pathway leading to menaquinone. In *Escherichia coli* one such mutant has already been isolated and the mutation it carries approximately located on the *E. coli* chromosome (Newton *et al.*, 1971). The present paper describes the isolation and genetic analysis of six additional menaquinone mutants and the use of these and other aromatic mutants of *E. coli* to help elucidate the pathway for the biosynthesis of bacterial menaquinones.

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Experimental Section

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

Media and Growth of Cells. The minimal medium used and the concentration of supplements have been described previously (Stroobant *et al.*, 1972). Cells were grown in 1-l. quantities in 2-l. flasks shaken at 37° and cultures checked for purity to ensure that no contamination or reversion had occurred.

Isolation of Menaquinone Mutants. Strain AB3311 was treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and mutants able to grow on glucose but unable to grow on succinate as the sole carbon source were isolated (Stroobant *et al.*, 1972). A 1-l. culture of each mutant was grown in shaken flasks, and an extract of the cell lipids prepared and examined for the presence of menaquinone as described below.

Determination of Menaquinones. An extract of cell lipids from 5–10 g (wet weight) of cells was prepared by the Soxhlet procedure as described previously (Young *et al.*, 1973) and chromatographed on silica gel thin-layer plates using chloroform–light petroleum (70:30, v/v) as solvent. The pale yellow menaquinone band (R_F 0.7), which contained any demethylmenaquinone or menaquinone present, was eluted with ethanol and the respective concentrations of demethylmenaquinone and menaquinone estimated spectrophotometrically (Dunphy and Brodie, 1971).

Mapping of the Menaquinone Mutants. Transduction experiments using the generalized transducing phage P1Kc were carried out as described by Pittard (1965). In order to test for cotransduction between the *men*$^-$ alleles and a suitable selective marker a derivative was prepared, which contained the *ubiA420* allele and the particular selective marker, *e.g.*, *glpK*$^-$. The *men*$^-$ strains were then used in turn as

TABLE I: Strains of *E. coli* K12 used.

Strain		Relevant Genetic Loci[a]
AB2830		*aroC*$^-$
AB3311		*met B*$^-$
AN1		*his*$^-$, *proA*$^-$, *argE*$^-$, *pheA*$^-$, *tyrA*$^-$, *trpE*$^-$
AN3		*his*$^-$, *proA*$^-$, *argE*$^-$, *pheA*$^-$, *tyrA*$^-$, *trpE*$^-$, *pabA*$^-$
AN4		*argE*$^-$, *ilvC*$^-$, *pheA*$^-$, *tyrA*$^-$, *trpE*$^-$, *pabB*$^-$
AN67	*menA401*	*proA*$^-$, *pheA*$^-$, *tyrA*$^-$, *trpE*$^-$
AN191		*proC*$^-$, *leu*$^-$, *trp*$^-$, *entC*$^-$
AN194		*proC*$^-$, *leu*$^-$, *trp*$^-$
AN195	*menA402*	*metB*$^-$, *ubiD*$^-$
AN208	*menA403*	*metB*$^-$
AN209	*menB404*	*metB*$^-$
AN211	*menA406*	*metB*$^-$
AN213	*menB408*	*metB*$^-$
AN215	*menA410*	*metB*$^-$
AN222	*menA402*	*metB*$^-$, *ubiA420*
AN224	*menA406*	*metB*$^-$, *ubiA420*
AN473		*argE*$^-$, *ubiA420*
AN474		*metB*$^-$, *glpK*$^-$, *ubiA420*
AN475		*glpK*$^-$, *ubiA420*
AN476		*glpK*$^-$, *his*$^-$, *ilv*$^-$
AN477		*glpK*$^-$, *ubiA420*
AN478	*menB404*	*metB*$^-$, *aroB*$^-$
AN479	*menA406*	*metB*$^-$, *aroB*$^-$

[a] Genetic nomenclature is that used by Taylor and Trotter (1972).

donors and used to transduce the recipient strain to *glpK*$^+$. Transductants were selected on media containing 4-hydroxybenzoate (0.1 mM) and then tested for their ability to grow in the absence of 4-hydroxybenzoate. The inability to grow under these conditions indicated that the transductant had received a *men*$^-$ allele (see Results). The system of scoring *men*$^-$ transductants was confirmed by growing a small number of transductants in 1-l. quantities, extracting the cells, and estimating the menaquinone as described above.

Testing of Menaquinone Precursors. Compounds were tested at a final concentration of 0.1 mM except where otherwise stated. The appropriate weight of solid was dissolved in 0.8 ml of ethanol and 0.4 ml was added aseptically to two 1-l. quantities of glucose-minimal medium immediately after inoculation. The cultures were grown into early stationary phase, the cells harvested, and the quantities of demethylmenaquinone and menaquinone in the cells were determined.

Synthesis of 2-Succinylbenzoic Acid. 2-Succinylbenzoic acid was synthesized by the method of Roser (1884). It chromatographed as a single spot on silica gel thin-layer plates in two solvent systems and its identity was confirmed by high-resolution mass spectrometry. The mass spectrum showed the expected molecular ion at *m/e* 222, the accurate mass of which was found to be 222.05391 ($C_{11}H_{10}O_5$ requires 222.05283).

Synthesis of 1,4-Dihydroxy-2-naphthoic acid. 1,4-Dihydroxy-2-naphthoic acid was synthesized by a modification of the method of Desai and Sethna (1951). The method used was as follows. 1-Hydroxy-2-naphthoic acid (3.1 g) was dissolved in NaOH (3.4 g in 34 ml of water) and a solution of potassium persulfate (4.5 g in 90 ml of water) added slowly over 1.5 hr with stirring. The temperature was maintained at 0° throughout the addition using an ice–salt bath. The mixture was just acidified with concentrated HCl and the precipitate filtered off. The filtrate was extracted twice with an equal volume of diethyl ether and the extracts discarded. Concentrated HCl (50 ml) was then added to the filtrate and the mixture heated at 100° until crystals appeared (about 5–10 min), and then cooled. The solid was filtered off, recrystallized from glacial acetic acid, and stored at −15° under vacuum in a desiccator over paraffin wax and P_2O_5. The yield was about 25%. The compound chromatographed as a single spot on cellulose thin-layer plates using benzene–acetic acid–water (62.5:36:1.5, v/v) as solvent and gave a green coloration with alcoholic ferric chloride and its identity was confirmed by high-resolution mass spectrometry. The mass spectrum showed the expected molecular ion at *m/e* 204, the accurate mass of which was found to be 204.04229 ($C_{11}H_8O_4$ requires 204.04226).

Detection of 2-Succinylbenzoic Acid in Culture Supernatants. Two 1-l. cultures of each strain were grown into early stationary phase in glucose-minimal medium, the cells removed by centrifugation, and the culture supernatant adjusted to pH 7 with 10 N NaOH. The supernatant was then passed through a column (2.5 cm × 8 cm) of Dowex 1 Cl^- (X4, 100–200 mesh) and the column washed with 100 ml of phosphate buffer (0.01 M, pH 7.0). The column was then eluted with 1 M NH_4Cl (pH 7.0) and 14-ml fractions were collected. The column chromatography was carried out at 4°. The optical density of the fractions was determined at 272 nm; the peak fractions were pooled, adjusted to pH 1 with concentrated HCl, and extracted with 3 equal volumes of diethyl ether. The ether extracts were dried over anhydrous Na_2SO_4, concentrated by rotary evaporation, and applied to a 20 cm × 20 cm Merck F_{254} silica gel thin-layer plate. The plate was developed in benzene–dioxane–acetic

acid (90:25:4, v/v) and examined under ultraviolet light for the presence of 2-succinylbenzoic acid (R_F, 0.4). The compound, if present, was eluted with 0.2 N HCl and extracted into diethyl ether and its ultraviolet absorption spectrum measured.

The technique described above was used to isolate sufficient 2-succinylbenzoic acid from culture supernatants of AN209 (menA$^-$) to allow its identity to be confirmed. The compound was further purified by rechromatography using the same solvent.

Detection of 1,4-Naphthoquinone in Culture Supernatants. A 1-l. culture of each strain was grown into early stationary phase, the cells removed by centrifugation, and the supernatant immediately adjusted to pH 7 and extracted with 200 ml of diethyl ether. The ether extract was concentrated by rotary evaporation and applied to a 10 cm × 20 cm Merck F_{254} silica gel plate and developed using ethyl acetate–hexane (25:75, v/v) as solvent. The plates were then examined under ultraviolet light for the presence of 1,4-naphthoquinone (R_F 0.7). Naphthoquinol is rapidly oxidized to naphthoquinone during the initial stages of development of the plate. Levels of naphthoquinone of 0.1 μM could be detected by this procedure.

Detection of 1,4-Dihydroxy-2-naphthoic Acid in Culture Supernatants. A 1-l. culture of each strain was grown into early stationary phase, the cells removed by centrifugation, and the supernatant immediately adjusted to pH 1 with concentrated HCl and extracted with 200 ml of diethyl ether. The ether extract was then shaken with 50 ml of phosphate buffer (0.1 M, pH 7.0) and the ether phase discarded. The aqueous phase was adjusted to pH 1 and extracted with 11 ml of diethyl ether and the concentration of 1,4-dihydroxy-2-naphthoic acid in the ether phase measured by its fluorescence (activation, 380 nm; fluorescence, 440 nm uncorrected).

Strain AN211 (*menA*$^-$) was used to accumulate sufficient 1,4-dihydroxy-2-naphthoic acid to allow its identity to be confirmed. After using the extraction procedure described above, the ether extract was concentrated and chromatographed twice on Merck precoated cellulose plates using benzene–acetic acid–water (62.5:36:1.5, v/v) as solvent. The compound ran with an R_F of 0.75 in this solvent and was detected by its pale blue fluorescence under ultraviolet light.

Spectroscopy. Ultraviolet absorption spectra were recorded on a Cary 15 spectrophotometer, activation and fluorescence spectra were measured using an Aminco Bowman spectrophotofluorimeter, and mass spectra were measured using an AEI MS9 double focus mass spectrometer with a direct insertion probe.

Results

Isolation of Mutants Unable to Form Menaquinone. The menaquinone mutants were isolated during a search for ubiquinone mutants. Strain AB3311 was treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and mutants isolated which were able to grow on glucose but unable to grow on succinate as the sole carbon source. These mutants were then examined for the levels of ubiquinone and menaquinone formed. About 1–2% of the mutants unable to grow on succinate were unable to form menaquinone and about 7% formed little or no ubiquinone. Since the estimation of menaquinone in the succinate mutants involved visualization of the pale-yellow menaquinone band after chromatography of a lipid extract of the cells, only those mutants unable to form both demethylmenaquinone and menaquinone were detected by this procedure. Six such menaquinone mutants (strains AN195, AN208, AN209, AN211, AN213, and AN215) were isolated, five of which formed normal levels of ubiquinone and one strain (AN195) which formed low levels of ubiquinone as it also carried a separate mutation in the *ubiD* gene (R. A. Leppik and I. G. Young, unpublished work). The six strains together with strain AN67, a menaquinone mutant derived from strain AB3291 which was isolated previously (Newton *et al.*, 1971), were used in the experiments described below.

Genetic Analysis of the Menaquinone Mutants. In previous genetic studies on strain AB3291 (*menA401*) it was found that the *menA* gene was cotransducible with the *metB* and *argE* genes at frequencies of about 30 and 50%, respectively (Newton *et al.*, 1971). This would place the *menA* gene between *metB* and *argE* on the *E. coli* chromosome (Taylor and Trotter, 1972). At that time there was no known phenotype suitable for scoring *men*$^-$ strains and recombinants had to be analyzed by determination of menaquinone levels. Thus, it was not possible to score sufficient transductants to measure cotransduction frequencies accurately.

In the present work a new procedure was developed for scoring *men*$^-$ strains to enable more accurate location of the *menA* gene and to determine whether all the mutations carried by the *men*$^-$ mutants were closely linked. This procedure made use of the observation that mutant strains blocked in the pathways leading to both ubiquinone and menaquinone are unable to grow aerobically on glucose minimal medium whereas strains blocked in either pathway alone can grow under these conditions (Wallace and Young, 1974). It is therefore possible to distinguish a *ubi*$^-$,*men*$^+$, strain from a *ubi*$^-$,*men*$^-$ strain and this characteristic can be used to score *men*$^-$ strains in transduction experiments. Recipient strains for transduction experiments must carry the *ubiA420* allele as well as the selective marker to be tested for cotransduction. Strains which carry the *ubiA420* allele form no ubiquinone unless high levels of 4-hydroxybenzoate are added to the growth medium (Young *et al.*, 1972). Transductants are selected on media containing 4-hydroxybenzoate and can then be scored to see whether they have received a *men*$^-$ allele by testing for growth on glucose or glycerol-minimal medium in the absence of 4-hydroxybenzoate.

Using the technique described above it was shown that the *men-401* allele was cotransducible with the *metB* and *glpK* genes at frequencies of 60 and 95%, respectively (Table II). Similarly, it was shown that the mutations carried by strains AN195 (*men-402*), AN208 (*men-403*), and AN215 (*men-410*) were cotransducible with the *glpK* gene at frequencies of 90, 95, and 88%, respectively (Table II). Three factor transduction crosses were carried out with the four *men*$^-$ alleles and in each case the data (Table II) were consistent with the gene order *glpK*. . . *men*. . . *metB*.

In experiments with the same recipient (AN475) that was used to map the *men-402*, *men-403*, and *men-410* alleles, no cotransduction was observed between the *glpK* gene and the mutation carried by strain AN211 (*men-406*). Further experiments, however, indicated that this mutation was probably a nonsense mutation and was suppressed in this recipient. A three factor transduction cross with a different recipient strain (AN477) established that the mutation in strain AN211 (*men-406*) was cotransducible with the *glpK* gene at a frequency of 84% and the marker

TABLE II: Transduction Data for the *men-401*, *men-402*, *men-403*, *men-406*, and *men-410* Alleles.

Donor Strain	Recipient Strain	Marker Selected	Unselected Marker Frequencies	
			men$^-$	*men*$^+$
AN67 (*men-401*)	AN474 (*metB*$^-$, *glpK*$^-$)	*metB*$^+$	62 *glpK*$^+$, 5 *glpK*$^-$	3 *glpK*$^+$, 42 *glpK*$^-$
AN67 (*men-401*)	AN474 (*metB*$^-$, *glpK*$^-$)	*glpK*$^+$	107 *metB*$^+$, 45 *metB*$^-$	2 *metB*$^+$, 6 *metB*$^-$
AN222 (*metB*$^-$, *men-402*)	AN475 (*glpK*$^-$)	*glpK*$^+$	25 *metB*$^+$, 47 *metB*$^-$	7 *metB*$^+$, 1 *metB*$^-$
AN208 (*metB*$^-$, *men-403*)	AN475 (*glpK*$^-$)	*glpK*$^+$	15 *metB*$^+$, 61 *metB*$^-$	4 *metB*$^+$, 0 *metB*$^-$
AN476 (*glpK*$^-$)	AN224 (*metB*$^-$, *men-406*)	*metB*$^+$	26 *glpK*$^+$, 0 *glpK*$^-$	14 *glpK*$^+$, 37 *glpK*$^-$
AN211 (*metB*$^-$, *men-406*)	AN477 (glpK$^-$)	*glpK*$^+$	17 *metB*$^+$, 48 *metB*$^-$	12 *metB*$^+$, 0 *metB*$^-$
AN215 (*metB*$^-$, *men-410*)	AN475 (*glpK*$^-$)	*glpK*$^+$	20 *metB*$^+$, 50 *metB*$^-$	8 *metB*$^+$, 2 *metB*$^-$

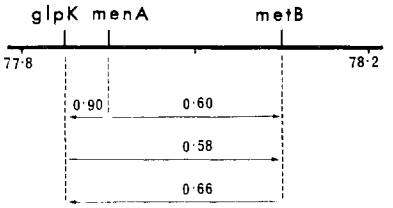

FIGURE 1: Relative positions of the *glpK*, *metB*, and *menA* genes on the *E. coli* chromosome. Cotransduction frequencies from Table II are shown with the selected marker at the head of the arrow and the unselected marker at the tail. The positions of the *glpK* and *metB* genes are taken from the map compiled by Taylor and Trotter (1972).

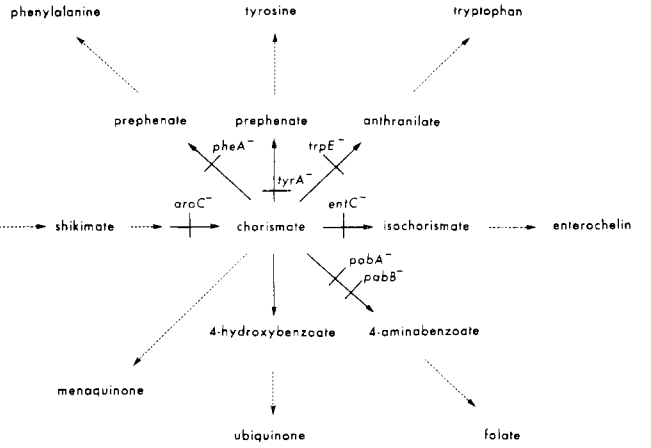

FIGURE 2: An outline of aromatic biosynthesis in *E. coli* showing the metabolic blocks in the mutants used in the present work.

frequencies were again consistent with a gene order of *glpK*. . . *men*. . . *metB* (Table II).

Thus the mutations carried by strains AN67 (*men-401*), AN195 (*men-402*), AN208 (*men-403*), AN211 (*men-406*), and AN215 (*men-410*) were all closely linked. Since these strains are all blocked in the same reaction in the menaquinone biosynthetic pathway (see below) it is likely that these mutations are all carried by the same gene (designated *menA*). The cotransduction data place the *menA* gene between the *glpK* and *metB* genes (Figure 1) at about minute 78 according to the map of the *E. coli* chromosome compiled by Taylor and Trotter (1972).

In contrast to the other *men*$^-$ alleles no cotransduction was detected between the *glpK* gene and the mutations in strains AN209 (*men-404*) and AN213 (*men-408*) irrespective of whether strain AN475 or strain AN477 was used as recipient. Strains AN209 and AN213 are also blocked in the same reaction in the menaquinone biosynthetic pathway (see below) though in a different reaction to that affected in the *menA*$^-$ strains. It is therefore likely that strains AN209 and AN213 are affected in the same gene (designated *menB*). The cotransduction experiments indicate that the *menB* gene is not cotransducible with the *menA* gene.

The Branch Point to Menaquinone. The position of the branch point to menaquinone was reexamined in the present work using a variety of different aromatic mutants (Figure 2). The results are shown in Table III. In agreement with previous studies (Cox and Gibson, 1966; Dansette and Azerad, 1970) it was found that strain AB2830, which is blocked in the common aromatic pathway immediately before chorismate, is unable to form menaquinone and also cannot use 4-hydroxybenzoate, 4-aminobenzoate, or 2,3-dihydroxybenzoate as menaquinone precursors. This places the branch point at chorismate or between chorismate and phenylalanine, tyrosine, tryptophan, 4-aminobenzoate, or 2,3-dihydroxybenzoate. Since strains AN1 (*pheA*$^-$, *tyrA*$^-$, *trpE*$^-$), AN3 (*pabA*$^-$), AN4 (*pabB*$^-$), and AN191 (*entC*$^-$) all form normal levels of menaquinone this indicates that the branch point to menaquinone is at chorismate.

Precursors of Menaquinone. In the work described below the term precursor will be used for compounds which can be converted to menaquinone in feeding experiments but which may or may not be true intermediates. At the commencement of these experiments the only known aromatic precursor of menaquinone was 2-succinylbenzoic acid (Dansette and Azerad, 1970). It was decided to test the ability of 1,4-dihydroxy-2-naphthoic acid, 1,4-naphthoquinone, 2-methyl-1,4-naphthoquinone, and α-naphthol to serve as menaquinone precursors in strain AB2830 (*aroC*$^-$). In order to try and overcome any permeability problems and to minimize the effect of the loss of these compounds due to their instability they were added to cultures immediately after inoculation at a relatively high concentration (0.1 mM).

In agreement with previous studies it was found that 2-succinylbenzoic acid is an efficient precursor of menaquinone (Table IV). *E. coli* strains grown under the partially anaerobic conditions generated in shaken flask cultures form a mixture of demethylmenaquinone and menaquinone. Strain AB2830 (*aroC*$^-$) supplemented with 2-succinylbenzoic acid also formed a mixture of demethylmenaquinone and menaquinone as would be expected for a precursor feeding into the menaquinone biosynthetic pathway. The total level of menaquinone formed in the presence of 2-succinylbenzoic acid is three- to fourfold higher than that of a wild type strain grown under comparable conditions. Elevated levels of menaquinone are typical of strains such as *ubiA*$^-$ strains which are unable to form ubiquinone (Young *et al.*, 1972). Strain AB2830 (*aroC*$^-$) was grown in the ab-

TABLE III: Menaquinone Levels Formed by Various Aromatic Mutants of *E. coli*.[a]

Strain	Level Formed[b]	
	Demethyl-menaquinone	Mena-quinone
AB2830 (*aroC*$^-$)	ND	ND
AN1 (*pheA*$^-$, *tyrA*$^-$, *trpE*$^-$, *pab*$^+$)	40	16
AN3 (*pheA*$^-$, *tyrA*$^-$, *trpE*$^-$, *pabA*$^-$)	51	17
AN4 (*pheA*$^-$, *tyrA*$^-$, *trpE*$^-$, *pabB*$^-$)	31	15
AN191 (*entC*$^-$)	30	45
AN194 (*ent*$^+$)	42	15

[a] The various reactions affected in the mutant strains are shown in Figure 2. [b] Nanomoles per gram wet weight of cells; ND, not detectable.

TABLE IV: Ability of Various Compounds to Serve as Precursors of Menaquinone in Strain AB2830.

Compound[a]	Level Formed[b]	
	Demethyl-menaquinone	Mena-quinone
2-Succinylbenzoic acid	71	140
1,4-Dihydroxy-2-naphthoic acid[c]	45	218
1,4-Naphthoquinone	27	21
2-Methyl-1,4-naphthoquinone	ND	67
α-Naphthol	ND	ND

[a] Compounds were added in 0.4 ml of ethanol to cultures immediately after inoculation to give a final concentration of 0.1 mM. [b] Nanomoles per gram wet weight of cells. The figures given are the average of at least three separate experiments; ND, not detectable. [c] When tested at 0.01 mM, 1,4-dihydroxy-2-naphthoic acid gave levels of demethylmenaquinone and menaquinone of 23 and 86, respectively.

sence of added 4-hydroxybenzoate in these experiments so that only a low level of ubiquinone would be formed enabling the maximum level of menaquinone to be formed when precursors were added.

It was also found (Table IV) that 1,4-dihydroxy-2-naphthoic acid is an efficient precursor of demethylmenaquinone and menaquinone in *E. coli*. This result is of considerable interest as it is the first demonstration that 1,4-dihydroxy-2-naphthoic acid is a precursor of bacterial menaquinone.

1,4-Naphthoquinone and 2-methyl-1,4-naphthoquinone also served as precursors of menaquinone (Table IV) but gave much lower levels than 2-succinylbenzoic acid or 1,4-dihydroxy-2-naphthoic acid (see Discussion). In contrast to the activity of the other four compounds tested, α-naphthol was found to be completely inactive as a menaquinone precursor in agreement with the results of other workers (see Baldwin *et al.*, 1974).

Precursor Studies with the Menaquinone Mutants. The ability of the mutant strains to utilize the four menaquinone precursors was investigated. It was found that none of the *menA*$^-$ strains (AN67, AN195, AN208, AN211, and AN215) was able to convert 2-succinylbenzoic acid or 2-methyl-1,4-naphthoquinone to menaquinone. Similarly neither of the *menB*$^-$ strains (AN209 and AN213) could convert 2-succinylbenzoic acid to menaquinone. Both *menB*$^-$ strains, however, were able to utilize 1,4-dihydroxy-2-naphthoic acid and 2-methyl-1,4-naphthoquinone as precursors forming levels of menaquinone and demethylmenaquinone up to 70% of that produced by the parent strain AB3311.

In order to confirm these results, *aroB*$^-$ derivatives of a *menA*$^-$ and *menB*$^-$ strain were prepared by the method previously described (Young *et al.*, 1972). This enabled the precursor experiments to be repeated with strains unable to form ubiquinone so that high levels of menaquinone could be formed. The results of these experiments (Table V) were in complete agreement with the other findings referred to above. It is therefore clear that the *menA*$^-$ strains are blocked in the attachment of the octaprenyl side chain to the final naphthalenic precursor of demethylmenaquinone and that the *menB*$^-$ strains are blocked immediately after the point at which 2-succinylbenzoic acid feeds into the pathway. The *menA*$^-$ strains would therefore be expected to accumulate the final naphthalenic intermediate in the pathway. On the basis of the results with menaquinone precursors this would be expected to be naphthoquinol or some closely related derivative. The *menB*$^-$ strains, on the other hand, would be expected to accumulate 2-succinylbenzoic acid if this compound is a true intermediate.

Accumulation of Intermediates by the Menaquinone Mutants. In view of the relatively low level of menaquinone formed by cells one could not expect an intermediate which

TABLE V: Utilization of Menaquinone Precursors by the *menA*$^-$ and *menB*$^-$ Strains.

Strain	Precursor[a]	Level Formed[b]	
		Demethyl-menaquinone	Menaquinone
AN479 (*menA406*, *aroB*$^-$)	2-Succinylbenzoic acid	ND	ND
AN479 (*menA406*, *aroB*$^-$)	1,4-Dihydroxy-2-naphthoic acid	ND	ND
AN479 (*menA406*, *aroB*$^-$)	2-Methyl-1,4-naphthoquinone	ND	ND
AN478 (*menB404*, *aroB*$^-$)	2-Succinylbenzoic acid	ND	ND
AN478 (*menB404*, *aroB*$^-$)	1,4-Dihydroxy-2-naphthoic acid	58	171
AN478 (*menB404*, *aroB*$^-$)	2-Methyl-1,4-naphthoquinone	ND	61

[a] Precursors were tested as described in Table IV except that 2-methyl-1,4-naphthoquinone was used at 0.05 mM. [b] Nanomoles per gram wet weight of cells; ND, not detectable. Each level is the average of two experiments.

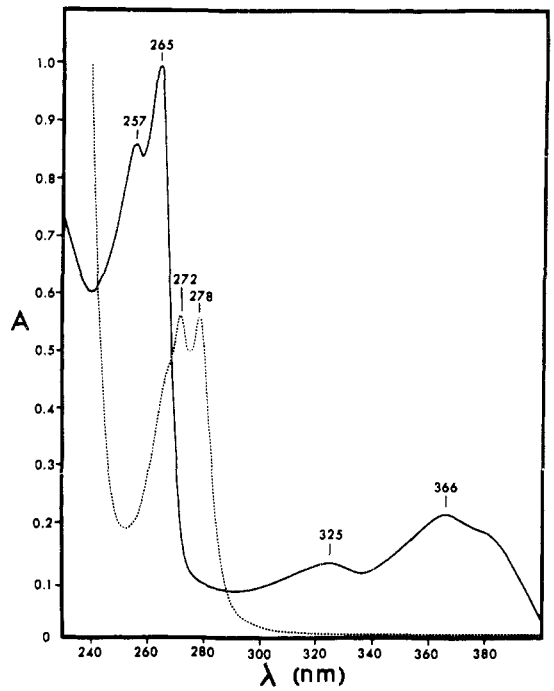

FIGURE 3: Ultraviolet absorption spectra in diethyl ether of 2-succinylbenzoic acid isolated from AN209 (*menB*$^-$) (dotted line) and 1,4-dihydroxy-2-naphthoic acid isolated from AN211 (*menA*$^-$) (solid line).

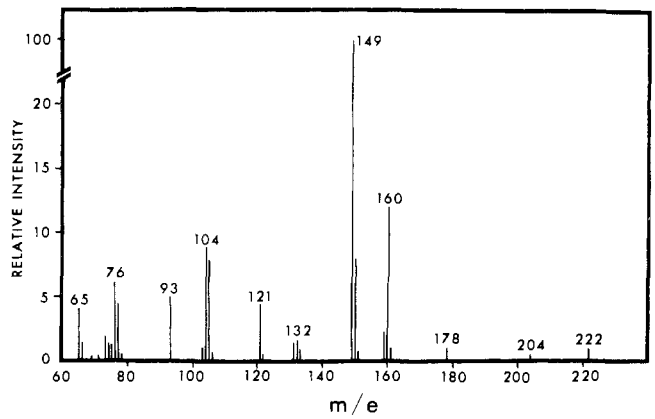

FIGURE 4: Mass spectrum of 2-succinylbenzoic acid isolated from AN209 (*menB*$^-$).

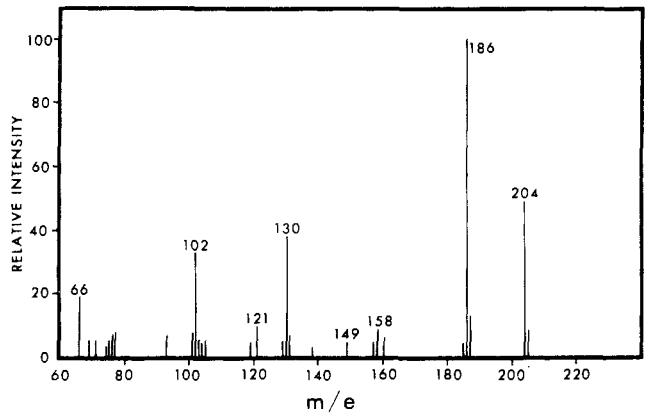

FIGURE 5: Mass spectrum of 1,4-dihydroxy-2-naphthoic acid isolated from AN211 (*menA*$^-$).

accumulated in the culture supernatant to reach a concentration greater than about 1 μM. Sensitive methods for detection of 2-succinylbenzoic acid, 1,4-dihydroxy-2-naphthoic acid, and 1,4-naphthoquinone in culture supernatants were therefore developed (see Experimental Section) and the *men*$^-$ mutants examined to determine if they accumulated any of these compounds.

2-Succinylbenzoic Acid. This compound was regularly detected in supernatants of AN209 (*menB*$^-$) at concentrations of about 0.7–0.9 μM but was not detcted (*i.e.*, less than 0.05 μM) in supernatants of either strain AB3311 (wild type) or strain AN211 (*menA*$^-$). The 2-succinylbenzoic acid was purified and shown to be indistinguishable from authentic 2-succinylbenzoic acid in terms of its chromatographic behavior and its ultraviolet absorption spectrum (Figure 3). Its mass spectrum (Figure 4) showed a molecular ion at *m/e* 222 and the same fragmentation pattern as the authentic compound. This demonstration of the accumulation of 2-succinylbenzoic acid by the *menB*$^-$ strain together with the ability of this compound to serve as a menaquinone precursor provide strong evidence that this compound is a true intermediate in the biosynthesis of menaquinone.

1,4-Naphthoquinone. Culture supernatants of strain AN211 (*menA*$^-$) were examined for the presence of 1,4-naphthoquinone, 1,4-naphthoquinol, and related compounds but none of these compounds was detected. The cells of this strain were also extracted and the lipid extracts examined for the presence of naphthoquinone or naphthoquinol but neither compound was detected.

1,4-Dihydroxy-2-naphthoic Acid. This compound was regularly detected in culture supernatants of strain AN211 (*menA*$^-$) at a concentration of about 1.0 μM but was not detectable in supernatants of strain AN209 (*menB*$^-$). Supernatants of AB3311 (wild type) also contained 1,4-dihydroxy-2-naphthoic acid at a level of about 0.75 μM. The compound formed by AN211 was purified and shown to be indistinguishable from authentic 1,4-dihydroxy-2-naphthoic acid in terms of its activation and fluorescence spectra, its ultraviolet absorption spectrum (Figure 3), and its chromatographic behavior. Its mass spectrum (Figure 5) showed a molecular ion at *m/e* 204 and the same fragmentation pattern as the authentic compound. The accumulation of 1,4-dihydroxy-2-naphthoic acid by strain AN211 (*menA*$^-$) but not by strain AN209 (*menB*$^-$) together with the demonstration that it is an efficient precursor of menaquinone provides strong evidence that it is an intermediate in menaquinone biosynthesis.

Discussion

The menaquinone mutants described above were all isolated from among mutants unable to grow on succinate as the sole carbon source. The isolation of *men*$^-$ mutants in this way proved to be fortuitous since genetic analysis established that none of the *men*$^-$ alleles affected growth on succinate and that the original *men*$^-$ strains carried other mutations preventing growth on succinate. This is in agreement with recent work (Wallace and Young, 1974) in which it was demonstrated that it is ubiquinone and not menaquinone or demethylmenaquinone which functions in the succinoxidase system in *E. coli*. Newton *et al.* (1971) have shown that in *E. coli* menaquinone is required under anaerobic conditions for the synthesis of orotate and that a strain deficient in ubiquinone and menaquinone had an absolute requirement for uracil anaerobically. This finding provides the basis for a more rational approach to the isolation of menaquinone mutants of *E. coli* and it has been verified

that menaquinone mutants can be isolated in this way (Andrews, 1974). Alternatively, it should also be possible to isolate menaquinone mutants by using a strain carrying the *ubiA420* allele and isolating mutants unable to grow aerobically on glucose-minimal medium in the absence of 4-hydroxybenzoate.

Of the seven *men*$^-$ mutants studied, five were shown to carry mutations in the *menA* gene and two were affected in the *menB* gene. The *menA* gene was located on the *E. coli* chromosome approximately 0.05 min clockwise from the *glpK* gene (Figure 1) by cotransduction with the *glpK* and *metB* genes. This represents a revision of the approximate location of the *menA* gene near *argE* made by Newton *et al.* (1971). The *menB* gene was shown to be not cotransducible with *menA* so that at the present time there is no evidence for clustering of genes concerned with menaquinone biosynthesis. Inspection of the proposed pathway (Figure 6) indicates that there are probably at least two genes concerned with menaquinone biosynthesis yet to be identified.

The results of the biosynthetic studies with the *menA*$^-$ and *menB*$^-$ mutants together with the evidence provided above that chorismic acid is the branch point compound allow a sequence to be formulated for the biosynthesis of menaquinone in *E. coli* (Figure 6). The first step of the pathway is the condensation of chorismic acid with the succinyl precursor to form 2-succinylbenzoic acid. Detailed studies using cell-free extracts will probably be required in order to learn the biochemical details of this reaction. At the present time it is known that the succinyl group of 2-succinylbenzoic acid is derived from 2-oxoglutaric acid (Campbell *et al.*, 1971) but the nature of the immediate succinyl precursor is unknown. It is possible that a cyclohexadiene intermediate exists between chorismic acid and 2-succinylbenzoic acid and a mechanism involving such an intermediate has been proposed (Dansette and Azerad, 1970).

The second reaction of the pathway is the cyclization of 2-succinylbenzoic acid to form 1,4-dihydroxy-2-naphthoic acid. Apparently 1,4-dihydroxy-2-naphthoic acid is produced in excess since the wild-type strain AB3311, in contrast to the *menB*$^-$ strain, produces only slightly lower levels of this compound than the *menA*$^-$ mutant. A similar situation occurs in *E. coli* in the ubiquinone pathway where 4-hydroxybenzoate, the intermediate to which the octaprenyl side chain is transferred, is also produced in excess (Young *et al.*, 1972).

The third reaction involves transfer of the octaprenyl grouping, presumably from octaprenyl pyrophosphate, to 1,4-dihydroxy-2-naphthoic acid with simultaneous decarboxylation. The product of this reaction would be demethylmenaquinol which is shown in Figure 6 in the quinone form since it would be oxidized to this form *via* the electron transport chain (Wallace and Young, 1974).

The final reaction of the pathway is the methylation of demethylmenaquinone to form menaquinone using *S*-adenosylmethionine as the methyl donor. This reaction presumably also involves both compounds in their quinol forms but they have been depicted as quinones for the reason outlined above.

Although both 1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone can serve as menaquinone precursors for *aroB*$^-$ and *aroC*$^-$ strains of *E. coli* (Tables IV and V), they have not been included in the postulated pathway since neither compound appears to be a true intermediate. The evidence in support of this proposal is that 2-methyl-1,4-

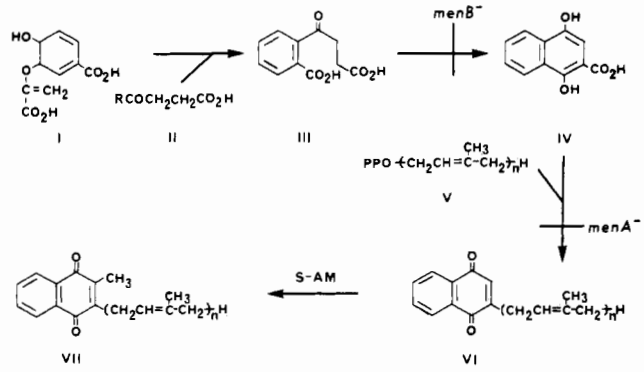

FIGURE 6: Pathway for the biosynthesis of bacterial menaquinones. Intermediates are: (I) chorismic acid; (II) unknown precursor of succinyl group; (III) 2-succinylbenzoic acid; (IV) 1,4-dihydroxy-2-naphthoic acid; (V) *all-trans*-polyprenyl pyrophosphate; (VI) demethylmenaquinone; (S-AM) *S*-adenosyl-L-methionine; (VII) menaquinone. Intermediates VI and VII are shown in the quinone form although it is probable that they are initially formed as quinols. The reactions affected in the *menA*$^-$ and *menB*$^-$ strains are also shown.

naphthoquinone is unable to serve as a precursor of demethylmenaquinone (Table IV), no accumulation of either compound by *menA*$^-$ strains was detected, and both are less efficient precursors of menaquinone than either 2-succinylbenzoic acid or 1,4-dihydroxy-2-naphthoic acid (Table IV). Since the *menA*$^-$ strains are unable to utilize 1,4-naphthoquinone (unpublished data) or 2-methyl-1,4-naphthoquinone (Table V) as menaquinone precursors this indicates that the prenylation of both compounds is catalyzed by the octaprenyltransferase of the menaquinone pathway. It appears therefore that neither compound is an intermediate but that both can be prenylated by the octaprenyltransferase when supplied at relatively high concentrations to mutant strains unable to synthesize 1,4-dihydroxy-2-naphthoic acid.

The proposed pathway which is based on data obtained using *E. coli* is likely to be generally applicable to all organisms which svnthesize menaquinone from chorismate. It is in good agreement with the data from isotopic tracer experiments carried out with a variety of different organisms (see introductory statement). In particular the recent finding by Baldwin *et al.* (1974) that the carboxyl carbon of shikimate is exclusively found in the C-4 quinone carbonyl of menaquinone indicates that no symmetrical intermediate such as 1,4-naphthoquinol can be involved in the pathway. In addition, the postulated transmethylation reaction has been demonstrated using cell extracts of *Mycobacterium phlei* (Samuel and Azerad, 1969).

The demonstration of the role of 1,4-dihydroxy-2-naphthoic acid as an intermediate in the biosynthesis of bacterial menaquinones lends support to the postulate (Robins *et al.*, 1970); Baldwin *et al.*, 1974) that this compound may be the key intermediate for the synthesis of other shikimate-derived naphthoquinones and anthraquinones such as alizarin, juglone, and lawsone.

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Metabolic Properties of Substrate-Attached Glycoproteins from Normal and Virus-Transformed Cells†

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ABSTRACT: Balb/c 3T3, SV40-transformed 3T3 (SVT2), and Con A revertant variants of transformed cells leave a layer of glycoprotein on the culture substrate upon EGTA mediated removal of cells. The metabolic properties of this substrate-attached material (glycoprotein) have been examined. Pulse and cumulative radiolabeling experiments with glucosamine and leucine precursors established that this substrate-attached material accumulates on the substrate in growing cultures until cells have completely covered the substrate. The synthesis and/or deposition of the material diminished dramatically in cultures whose substrates had been completely covered with cells as observed microscopically, even though the contact-inhibited cell lines continued to make cell-associated and medium-secreted glycoproteins and transformed cells continued to divide and form multilayered cultures. Pulse-chase analysis using long periods of pulsing with radioactive leucine demonstrated that these glycoproteins are deposited directly on the substrate by cells and not subsequent to secretion into the medium. The substrate-attached material accumulated during long pulses was stably adherent to the substrate and displayed little appreciable turnover during 3 days of chasing of either sparse or dense cultures. Short-term pulse-chase analysis with leucine revealed two metabolically different pools of material—one which turns over very rapidly with a half-life of 2–3 hr (observed in both low-density and high-density cultures) and a second pool which is stably deposited on the substrate and whose proportion increased with the length of the radiolabeling period. No appreciable differences in the metabolic properties of substrate-attached material were observed in the three cell types studied during growth on a plastic substrate. These results are discussed with regard to the implicated roles of these glycoproteins in mediating adhesion of normal and virus-transformed cells to the substrate.

A class of glycoproteins has been found at the surface of normal and virus-transformed fibroblasts which appears to mediate adhesion of cells to the culture substrate (Culp, 1974)—so-called substrate-attached material which remains on the substrate subsequent to EGTA[1] mediated removal of cells. The more adherent Balb/c 3T3 and Con A revertant variants (Culp and Black, 1972a) of transformed 3T3 cells deposit more of this material than SV40-trans-

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[1] Abbreviations used are: Con A, concanavalin A; CPC, cetylpyridinium chloride; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; MEM X4, Eagle's minimal essential medium supplemented with four times the concentration of vitamins and amino acids; SV40, Simian virus 40.