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Menaquinone Biosynthesis: Conversion of *o*-Succinylbenzoic Acid to 1,4-Dihydroxy-2-naphthoic Acid and Menaquinones by *Escherichia coli* Extracts†

R. W. Bryant, Jr.,‡ and Ronald Bentley*

ABSTRACT: Two intermediates in the biosynthetic pathway to bacterial menaquinones are *o*-succinylbenzoic acid and 1,4-dihydroxy-2-naphthoic acid. Cell-free extracts have been prepared from *Escherichia coli* which catalyze the conversion of labeled *o*-succinylbenzoic acid to the naphthoic acid and also to menaquinones. The naphthoate synthetase has been partially purified and found to require coenzyme A and ATP. The synthetase has an approximate molecular weight of 45 000. The conversion of *o*-succinylbenzoic acid to menaquinones is stimulated by the presence of farnesyl pyrophosphate. The major menaquinone produced is then MK-3 with the farnesyl side chain.

Experiments with intact organisms have established that *o*-succinylbenzoic acid (OSB)[1] is an important intermediate in the biosynthesis of menaquinones (MK) in bacteria, phylloquinone in plants, and several other naphthoquinones in plants (references in reviews of Bentley, 1975a,b). This benzenoid derivative is accumulated by *menB*$^-$ strains of *Escherichia coli* (Young, 1975) and is believed to cyclize to 1,4-dihydroxy-2-naphthoic acid. The earlier presumptive evidence for a role for the naphthoate in MK biosynthesis in *Bacteroides melaninogenicus* (Robins et al., 1973) has now been supported. The *menA*$^-$ and wild type strains of *E. coli* accumulate 1,4-dihydroxy-2-naphthoic acid in culture supernatants and wild type and *menB*$^-$ strains utilize it as a precursor of MK (Young, 1975). Furthermore, we have reported the conversion of labeled 1,4-dihydroxy-2-naphthoic acid to MK in cell-free extracts of *E. coli* (Bentley, 1975b).

To provide further details of the chemical mechanisms and intermediates involved in the sequence, OSB → 1,4-dihydroxy-2-naphthoic acid → MK, we have investigated cell-free enzyme preparations of *E. coli* in some detail. These preparations contain a cyclizing enzyme which will be termed 1,4-dihydroxy-2-naphthoate synthetase, abbreviated as naphthoate synthetase. This enzyme has been partly purified. In addition, the extracts catalyze the overall synthesis of MK from OSB. Preliminary reports of some of this work have already appeared (Bentley, 1975b; Bryant and Bentley, 1975).

Materials and Methods

Growth of E. coli. A culture of *E. coli* K-12 (nalidixic acid resistant) was kindly provided by Dr. Robert B. Yee and was maintained on 1.5% Nutrient Agar (Difco, Detroit, Mich.). For production of cells, 2.8-l. Fernbach flasks, containing 1300 ml of Soy Broth (BBL, Cockeysville, Md.), were inoculated with 50 ml of a seed culture grown up overnight. Six Fernbach cultures were grown at 37 °C with shaking (3-in. stroke, 240 rpm). Cell growth was measured by the absorbance at 700 nm of one to ten dilutions of the cultures. After 4 h of growth (approximately late-log phase) the cell paste was harvested by centrifugation at 5200 rpm for 10 min in a Sorvall HG-4 rotor. The cells from six flasks were washed by resuspending one time in 0.05 M potassium phosphate buffer (pH 7.0) and yielded 40–50 g of packed cell paste. The paste was usually frozen and stored at −15 °C, and used for enzyme preparations within 1 month.

Preparation of Cell-Free Extracts. The *E. coli* cells were disrupted essentially according to Walker et al. (1973) using the French Press and high concentrations of EDTA. Typically, 25 g of thawed cell paste was suspended in 50 ml of 0.05 M potassium phosphate buffer (pH 6.9) also containing 0.01 M EDTA and 0.2 mM dithiothreitol. The suspension was treated by two passages through the French Pressure Cell (Aminco, Silver Springs, Md.) at 8 000–10 000 psi. After addition of 2 mg of DNase (Worthington) to reduce the viscosity of the lysate, 1 ml of 1 M $MgCl_2$, and 3.5 ml of 1 M Tricine NaOH, the lysate was centrifuged at 17 500 rpm (37 000*g*) for 1 h or, in early experiments, at 10 000 rpm (12 000*g*) for 20 min. These lower speed extracts were checked for viable cells by plating serial dilutions on 1.5% nutrient agar petri dishes and were found to be essentially cell free. The supernatant fraction was used for the cell-free extract.

Isolation of [^{14}C]Naphthoic Acid Derivatives. For identification of the [^{14}C]naphthoic acid produced by the cell-free extracts, two derivatives were used—the methyl ester and methyl 4-acetyl-2-naphthoate. The methyl naphthoate was prepared by adding ethereal diazomethane to naphthoic acid and then removing the ether after 5 min. On gas chromatography (10-ft column of 3% OV-101, 210 °C) the ester gave a single peak with a retention time of 8.1 min. On GC–MS (LKB 9000, 6-ft column of 3% OV-101, 210 °C) the following ions were significant: *m/e* 218 (parent, $C_{12}H_{10}O_4$), 186, and 158 (186 → 158, M* = 134.3). A reference sample of methyl 4-acetylnaphthoate was prepared by adding 0.5 ml of acetic anhydride and 0.1 ml of pyridine to 10 mg of 1,4-dihydroxy-2-naphthoic acid. The mixture was heated 1 h on the steam

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‡ Present address: Biochemistry Department, The George Washington University School of Medicine and Health Sciences, Washington, D.C. 20037.

[1] Abbreviations used are: OSB, *o*-succinylbenzoic acid [4-(2′-carboxyphenyl)-4-oxobutyric acid]; MK, menaquinone (vitamin K_2), and MK-*n*, menaquinone with a side chain of *n* prenyl groups; DMK, desmethylmenaquinone; GC–MS, combined gas chromatography and mass spectrometry; Tricine, *N*-tris(hydroxymethyl)methylglycine; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography; CoA, coenzyme A.

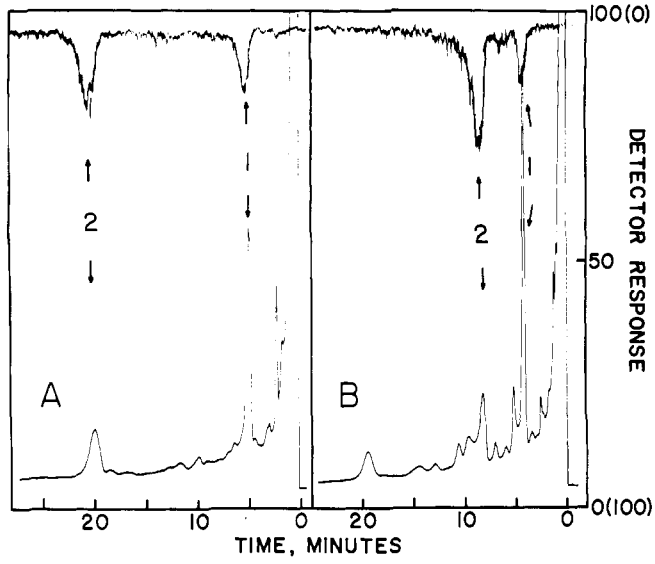


FIGURE 1: Gas chromatographic identification of products obtained on incubation of [2,3-$^{14}C_2$]OSB with *E. coli* cell-free extract. (A) The ether extract was derivatized with ethereal diazomethane. (B) The ether extract was first acetylated and then esterified with ethereal diazomethane. The identifications are based on the observed retention times of authentic reference materials. Radioactivity is indicated by the upper trace; the instrument was set at 1000 cpm for full scale deflection (shown as 0–100%). The lower trace is that from the gas chromatograph flame ionization detector. Peaks indicated by 1 correspond to OSB and its spirolactone; those indicated by 2 correspond to 1,4-dihydroxy-2-naphthoic acid.

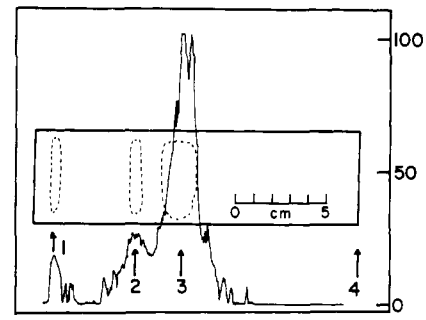


FIGURE 2: Thin-layer chromatography of products obtained on incubation of [2,3-$^{14}C_2$]OSB with *E. coli* cell-free extract. The chromatography was carried out on 250-μm silicic acid plates with fluorescent 254 indicator (Analtech, Newark, N.J.) using benzene–ethyl acetate–acetic acid (150:75:2). A tracing of the thin-layer chromatography is superimposed on the record from the strip scanner. The instrument response was set at 10 000 cpm for full scale deflection (shown as 0–100%); 1 = origin; 2 = OSB; 3 = 1,4-dihydroxy-2-naphthoic acid, 4 = solvent front. Though not indicated on this chromatogram, the spirolactone band travels about 2 cm in front of the naphthoic acid band in this solvent system.

bath, and the acetylation reagents were then evaporated off under nitrogen; the acetyl derivative was then treated with ethereal diazomethane. On gas chromatography as just described, a single peak with retention time of 19.7 min was observed. The mass spectrum showed ions at *m/e* 260 (parent $C_{14}H_{12}O_5$), 218 (minus $COCH_2$), and 186.

Chemicals. A sample of [2,3-$^{14}C_2$]OSB was prepared as described earlier (Campbell et al., 1971) from [2,3-$^{14}C_2$]succinic acid (22 mCi/mmol supplied by Amersham/Searle Corporation). The [2,3-$^{14}C_2$]OSB was added to incubation mixtures as the disodium salt; these solutions contained trace amounts (less than 1%) of the spirolactone. Carrier OSB and its spirolactone were obtained by the method of Roser (1884). 1,4-Dihydroxy-2-naphthoic acid was synthesized as described by Homeyer and Wallingford (1942). Farnesyl pyrophosphate, prepared by the method of Cramer and Böhm (1959), was purified as described by Cornforth and Popják (1969). MK-3 and DMK-3 were prepared according to the procedure of Azerad and Cyrot (1965) and MK-5 and MK-7 were kindly provided by Dr. O. Isler. ATP, coenzyme A, dithiothreitol, glutathione, and Tricine were supplied by Sigma.

Analytical Methods. Radioactivity in samples purified by gas chromatography (Packard Model 7400 gas chromatograph) was determined by proportional gas counting using a Packard gas flow proportional counter (Model 894). For scanning of thin-layer chromatography plates, a Packard radio chromatogram scanner (Model 7201) was used. Samples of the naphthoic acid which were removed from thin-layer chromatography plates were counted in 10-ml portions of Bray's solution (Bray, 1960). The counting efficiency under these conditions was 61%. Protein determinations were carried out by the biuret method (Layne, 1957).

Results

Demonstration of Naphthoate Synthetase Activity. The cell-free extracts from *E. coli*, 0.95 ml containing 39 ml of protein, were incubated for 1 h at 27 °C with [2,3-$^{14}C_2$]OSB (0.006 μmol, 0.13 μCi) and 50 μg of carrier 1,4-dihydroxy-2-naphthoic acid. After 40 min, 1 ml of 0.6 M HCl was added to terminate the reaction and the mixture was then extracted six times with 3-ml portions of ether. After the addition of 50 μg each of nonradioactive OSB, its spirolactone, and 1,4-dihydroxy-2-naphthoic acid to the combined ether extracts, ether was removed in a stream of nitrogen. The formation of radioactive 1,4-dihydroxy-2-naphthoic acid was detected by either gas chromatography (Figure 1) or thin-layer chromatography (Figure 2) as described in Materials and Methods. For gas chromatography, the materials were treated with diazomethane to yield methyl esters (Figure 1A). The resulting chromatogram gave two radioactive peaks which had the same retention times as the mass peaks for dimethyl-OSB and methyl 1,4-dihydroxy-2-naphthoate. Similarly, when the extracts were treated first with pyridine–acetic anhydride, then diazomethane to yield acetate esters (Figure 1B), two radioactive peaks were obtained with retention times corresponding to dimethyl-OSB and methyl 1-hydroxy-4-acetoxy-2-naphthoate.

To provide confirmation for the conversion, OSB → 1,4-dihydroxy-2-naphthoic acid, the incubation samples were also examined by thin-layer chromatography (Figure 2). In some preliminary experiments, evidence had been obtained for an enzymatic conversion of OSB to its spirolactone; this conversion was not routinely observed and efforts to pinpoint the phenomenon were unsuccessful. Although on thin-layer chromatography the best separation of OSB and 1,4-dihydroxy-2-naphthoic acid was obtained in hexane–ether–acetic acid, 105:45:3, the spirolactone and naphthoic acid were poorly resolved in this solvent system. A good separation of spirolactone and naphthoic acid was achieved with benzene–ethyl acetate–acetic acid (150:75:2), and, as well, the naphthoic acid was resolved from OSB. In this system (Figure 2) three peaks of radioactivity were obtained: one remaining at the origin, one corresponding to OSB and one to 1,4-dihydroxy-2-naphthoic acid. In this particular experiment essentially no conversion of radioactivity from OSB to its spirolactone was observed (less than 1%).

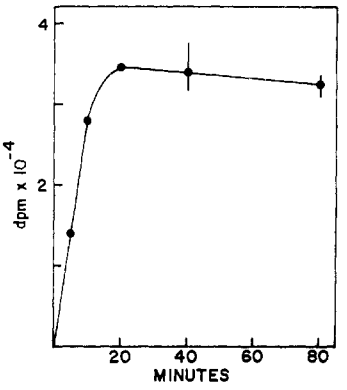


FIGURE 3: Conversion of [2,3-$^{14}C_2$]OSB to naphthoic acid as a function of time. The radioactive OSB (2.66 × 10^6 dpm/μmol, 66 500 dpm) was incubated with 0.8-ml portions of the *E. coli* extract containing approximately 40 mg of protein (centrifuged at 37 000 × *g* for 60 min), 20 μg of naphthoic acid, 30 μmol of potassium phosphate, 40 μmol of Tricine-NaOH, 11 μmol of $MgCl_2$, 6 μmol of EDTA, 0.6 μmol of dithiothreitol, and 5 μmol of ATP in a total volume of 1.0 ml (pH 8.1). The incubation was stopped at various times by addition of 2 ml of 0.6 M hydrochloric acid and 1 ml of methanol. More naphthoic acid (30 μg), OSB (50 μg), and spirolactone (50 μg) were added; the solutions were then extracted with 3 ml of ether three times. The gels which often formed during the extractions were broken by centrifugation. The combined ether extracts were dried under nitrogen and the residues subjected to chromatography on 250-μm silicic acid plates with fluorescent indicator in benzene–ethyl acetate–acetic acid (150:75:2). The naphthoic acid, which separated well from OSB and spirolactone, was removed from the plate and counted as described in Materials and Methods. Points are averages of duplicate assays, and the bars indicate the range of duplicate values.

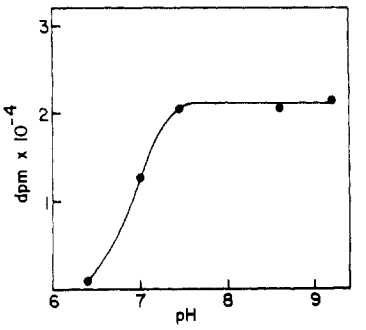


FIGURE 4: Conversion of OSB to naphthoic acid as a function of pH. To 22-ml cell-free extract was added 0.2 mmol of glycine and either 1 M HCl or 1 M NaOH to vary the pH of the extract from 6 to 9 pH units. Aliquots of 0.9 ml were removed at the appropriate pH and assayed using the conditions described in Figure 3, with a 20-min incubation period.

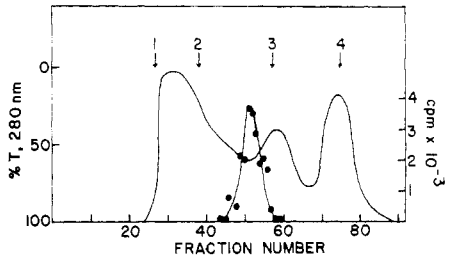


FIGURE 5: Partial purification of naphthoate synthetase from *E. coli* by gel filtration. The *E. coli* extract (20 ml) was eluted over a 2.5 × 90 cm column of Sephadex G-150 in 0.05 M Tricine-NaOH, 0.1 M KCl, 0.001 M dithiothreitol, and 0.001 M EDTA at pH 8. Fractions (6 ml) were collected and 0.8-ml aliquots of the fractions were assayed for naphthoate synthetase. Each assay contained 10 μmol of ATP, 0.5 μmol of CoA, 20 μmol of $MgCl_2$, 160 000 dpm (30 nmol) of [^{14}C]OSB, and 100 nmol of carrier naphthoic acid in a total volume of 1 ml. The mixtures were incubated 20 min at 37 °C and the [^{14}C]naphthoic acid synthesized was isolated and counted as described in the legend of Figure 2. The elution of protein was monitored at 280 nm with a Uvicord-II using a 1.0-mm path length cell. This record is shown as the continuous line. The elution volume of appropriate molecular weight markers, determined in other runs, is indicated with arrows as follows: 1 = blue dextran; 2 = aldolase; 3 = α-chymotrypsinogen; 4 = K_2CrO_4. The specific activity of the synthetase increased from 141 dpm per mg per 20 min to 692 dpm per mg per 20 min, and there was an essentially complete recovery of the activity originally applied. The radioactivity is shown as ●—●.

An assay system based on the conversion of [2,3-$^{14}C_2$]OSB to radioactive 1,4-dihydroxy-2-naphthoate was devised. The appearance of radioactivity in the naphthalene derivative was linear for 10 min (Figure 3). The initial rate of the reaction corresponded to formation of 0.3 nmol of product per 10 min per mg of protein. In this assay system there was a linear relationship between radioactivity finally obtained in the naphthoic acid spot (thin-layer chromatography) and protein concentration over the range 5–40 mg of protein.

The effect of pH on the naphthoate synthetase is shown in Figure 4. As the pH of the assay was raised from 6.4 to 7.4 there was a rapid increase in activity. This strong pH dependence between 6.4 and 7.4 was probably a catalytic effect and not due to pH inactivation of the enzyme because enzyme solutions brought to pH 5 then reassayed at pH 7 retained most of their activity. Between pH 7.5 and pH 9 the assay was fairly independent of pH effects.

An approximately five- to eightfold purification of the naphthoate synthetase was achieved by gel filtration on Sephadex G-150 (Figure 5). The synthetase activity eluted as a single peak well after the high molecular weight nucleic acids and proteins in the crude extract. The activity was not associated with a marked protein peak. An approximate molecular weight of 45 000 was calculated by comparison of the elution volume of naphthoate synthetase (307 ml) on a Sephadex G-150 column (blue dextran 2000 elution volume = 186 ml) with the elution volumes of the molecular weight markers aldolase (230 ml; mol wt 147 000) and α-chymotrypsinogen (347 ml; mol wt 25 000).

Attempts to purify the synthetase on a preparative scale have not yet been successful. Ammonium sulfate precipitation, acetone or ethanol fractionation, and heat treatment resulted in at least a 90% loss in the activity originally present in crude extract.

The partially purified enzyme was found to require the presence of ATP, Mg^{2+}, and coenzyme A. Omission of either ATP or coenzyme A resulted in an almost complete loss of activity (Table I). The absence of $MgCl_2$ gave a decreased conversion of OSB to the naphthoate. Neither cysteine nor glutathione could replace the coenzyme A requirement.

While conversion of OSB to naphthoic acid was very efficient in *E. coli* extracts supplemented with ATP and coenzyme A, significant synthesis of menaquinones was not generally observed. However, addition of a side chain precursor, farnesyl pyrophosphate, greatly stimulated conversion of [2,3-$^{14}C_2$]OSB into menaquinones. For these experiments, the MK components were purified first by thin-layer chromatography and then by high-pressure liquid chromatography (Figure 6). A peak of radioactivity coincident with MK-3 was observed; there was very little activity associated with DMK-3. The effect of farnesyl pyrophosphate addition was also to decrease the incorporation of radioactivity into naphthoic acid. Thus, in comparable experiments, the percent incorporations of radioactivity into naphthoic acid and menaquinones were 54.3 and 0.7 in the absence of farnesyl pyrophosphate. In presence of farnesyl pyrophosphate, the percent incorporations were 9.2 into naphthoic acid and 28.4 into menaquinones.

TABLE I: Cofactor Requirements of *E. coli* Naphthoate Synthetase.

Cofactors Present[a]	Naphthoic Acid Synthesis[b] (dpm)
Complete	1190
−CoASH	18
−ATP	2
$-MgCl_2$	442
−CoASH + cysteine	10
−CoASH + glutathione	28

[a] The complete system contained in a total volume of 1 ml: 5 mg of protein purified by Sephadex G-150 chromatography; 160 000 dpm of [2,3-$^{14}C_2$]OSB; 10 μmol of ATP; 0.5 μmol of CoA; 20 μmol of $MgCl_2$; 80 μmol of KCl; 0.8 μmol of EDTA; 0.8 μmol of dithiothreitol; and 40 μmol of Tricine–NaOH at pH 8. When added, 2.5 μmol of cysteine and 0.5 μmol of glutathione were present. [b] The naphthoic acid was extracted and assayed as described in Materials and Methods. The synthesis is reported as total dpm incorporated for the 1-ml volume. Each incubation was run in duplicate; the time of the incubation was 20 min.

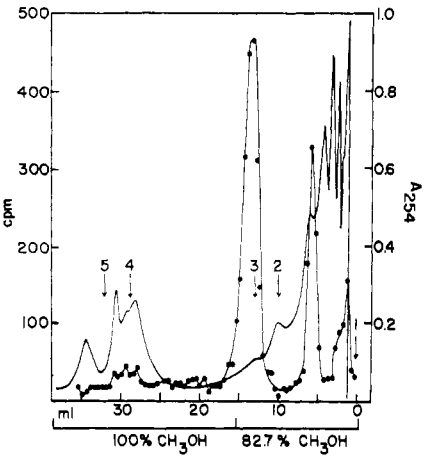


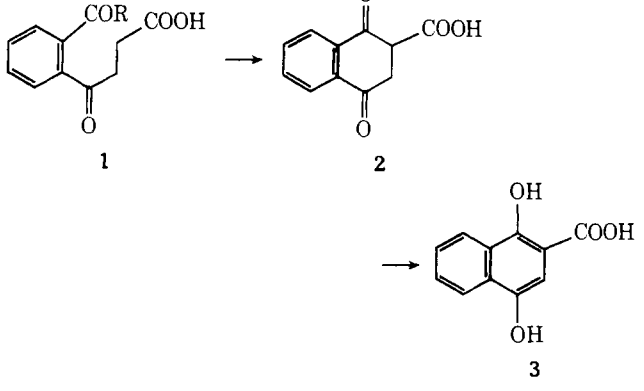
FIGURE 6: HPLC purification of [^{14}C]menaquinone-3 (MK-3) formed by incubation of an *E. coli* extract with [2,3-$^{14}C_2$]OSB and farnesyl pyrophosphate. A control incubation with 4 ml of crude cell-free extract contained approximately 200 mg of protein, 120 μmol of potassium phosphate buffer, pH 8, 28 μmol of EDTA, 2.8 μmol of dithiothreitol, 56 μmol of $MgCl_2$, 50 μmol of ATP, 2 μmol of CoA, and 0.008 μmol of [^{14}C]OSB (0.18 μCi). The experimental incubation contained all of the above plus 0.7 μmol of farnesyl pyrophosphate. The total volume was 5.0 ml. The mixtures were shaken for 1 h at 37 °C, and then 20 ml of acetone–concentrated hydrochloric acid, (100:1) was added. The protein was pelleted by centrifugation and the acetone solution was poured into 20 ml of benzene. The water and organic phases were separated by centrifugation and the organic phase was removed and dried under nitrogen. Carrier MK-3 and DMK-3, 20 μg each, were added to the organic extract which was then chromatographed on a 1000-μm silica gel GF 254 plate in benzene–ethyl acetate–acetic acid (150:75:2). The menaquinone band (R_f 0.7) was removed and eluted with acetone. Aliquots (½) of the menaquinone fractions were further purified on a Waters high-pressure liquid chromatograph equipped with a 440 Absorbance Detector. The sample in 25 μl was injected onto a C18-Corasil column (⅛ × 24 in.) and was eluted with methanol–water (82.5:17.5 v/v) at 1500 psi and a flow rate of 1 ml/min. The menaquinones were monitored at 254 nm and the eluant was collected in scintillation vials. One-half-milliliter fractions were collected and, after addition of 5 ml of Bray's solution, the radioactivity of each fraction was measured by liquid scintillation counting. The counts are uncorrected for quenching. The solvent was switched to 100% methanol after about 15 ml, in order to elute the more lipophilic menaquinones. The absorbance is shown as the continuous line, and radioactivity as ●—●. The control experiment (not shown) showed no radioactive peaks. An unknown radioactive peak eluted after about 5 ml of solvent had been recovered. The arrows (2–5) show the elution position of known standards and 1 indicates the point of sample injection: 2 = DMK-3; 3 = MK-3; 4 = MK-5; 5 = MK-7.

Discussion

This paper describes the first enzymatic system for the conversion of a benzenoid (**1**, R = OH) to a naphthalenoid compound (**3**). The naphthoate synthetase behaved as a soluble protein with an approximate molecular weight of 45 000.

The experimental evidence for this enzyme was the conversion of [2,3-$^{14}C_2$]OSB to a radioactive product with the same chromatographic properties as authentic 1,4-dihydroxy-2-naphthoate. This coincidence of radioactivity and mass was demonstrated with two derivatives (ester and acetyl ester) on gas chromatography and also by thin-layer chromatography of the acid itself.

The basic chemical operation is an elimination of water between a carboxyl and methylene group, essentially a Claisen condensation (or more specifically a Dieckmann reaction), which presumably leads to the ketotetralone **2**, and by isomerization to **3**. The stimulatory effect observed with CoA and

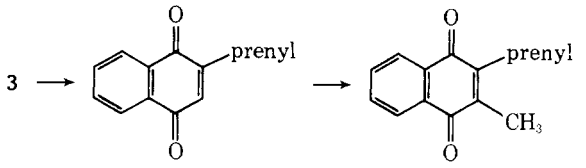


ATP suggests the cyclization may require formation of a CoA derivative of one (or perhaps even both) carboxyls. As a first approximation, the formation of **1**, R = CoA, appears likely but derivatization of the aliphatic carboxyl to activate the adjacent methylene cannot be completely ruled out. The effect was apparently restricted to CoA since neither cysteine nor glutathione could be substituted for CoA.

The enzyme extracts, when supplemented with farnesyl pyrophosphate, also converted [2,3-$^{14}C_2$]OSB to a menaquinone which was identified as MK-3 (**5**) on the basis of thin-layer chromatography and high-performance liquid chromatography, implying the presence of both a methylating system as well as a prenyl transferase. Little or no radioactive DMK-3 (**4**) was observed in these experiments. A related situation had been noted in the earlier work of Ellis and Glover (1968). They had added [1,6-$^{14}C_2$]shikimic acid to *E. coli* E 106 and found incorporation of activity into both MK-8 and DMK-8. The observed molar specific activity of DMK-8 was much lower than that of MK-8, possibly implying that DMK was not a direct precursor to MK. On the other hand, the methylation of DMK-9 to MK-9 has been studied in a particulate membrane fraction from *Mycobacterium phlei* (Samuel and Azerad, 1972). Similarly, we have observed the conversion of DMK-3 to radioactive MK-3 when [$^{14}CH_3$]-*S*-adenosylmethionine was added to our extracts.

Labeled 1,4-dihydroxy-2-naphthoic acid is converted to MK by extracts from *E. coli* (Bentley, 1975b). In a recent paper, Shineberg and Young (1976) have described a membrane-associated 1,4-dihydroxy-2-naphthoate octaprenyltransferase from *E. coli* which specifically converts 1,4-dihydroxy-2-

naphthoic acid to DMK. While the reason for low radioactivity in DMK in the tracer experiments is not clear, it appears most likely that the final stages of menaquinone biosynthesis are **3** → **4** → **5**.



Despite some overall similarities, the biosynthesis of ubiquinones and menaquinones is markedly different. In both cases the substrate for prenylation is an aromatic carboxylic acid. For formation of ubiquinone, this acid is 4-hydroxybenzoate which is prenylated without decarboxylation; the next enzyme then catalyzes a simple decarboxylation so that a 2-prenylphenol is formed. In menaquinone biosynthesis, the carboxyl of 1,4-dihydroxy-2-naphthoic acid is replaced by the prenyl side chain. Decarboxylation and prenylation in MK biosynthesis may be concerted since the bulk of the experimental evidence eliminates symmetrical intermediates such as naphthoquinone or its quinol.

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