Conversion of *o*-Succinylbenzoate to Dihydroxynaphthoate by Extracts of *Micrococcus luteus*†

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ABSTRACT: Cell-free extracts were prepared from either freshly grown or spray-dried cells of *Micrococcus luteus* ATCC 4698 by treatment with deoxyribonuclease and lysozyme. These extracts converted *o*-succinylbenzoic acid (OSB) to 1,4-dihydroxy-2-naphthoic acid (DHNA) as shown by spectrophotofluorometric and radioactivity assays. The conversion required the presence of ATP, CoA, and Mg^{2+}. By use of [2-^{14}C]OSB, the simultaneous production of the spirodilactone form of OSB was also demonstrated. The two products formed from OSB were further characterized by gas chromatography combined with mass spectrometry. The production of the spirodilactone was suppressed by the addition of a preparation of the enzyme DHNA synthase obtained from *Mycobacterium phlei*. (This enzyme catalyzes the conversion of a CoA derivative of OSB to DHNA.) On mild acid treatment, the *M. luteus* extracts retained the ability to produce spirodilactone but lost the ability to form DHNA. These results are interpreted to mean that an OSB-CoA derivative is an intermediate in the conversion of OSB to DHNA by *M. luteus* and that two enzymes are involved, one to form the OSB-CoA derivative and the second to carry out a cyclization reaction.

The first naphthalenoid compound recognized in the menaquinone biosynthetic pathway is 1,4-dihydroxy-2-naphthoic acid, DHNA[1] [see reviews by Bentley (1975a,b)]. DHNA is derived from *o*-succinylbenzoic acid, OSB, by a cyclization which formally involves the elimination of the elements of water. The enzymatic conversion of the benzenoid OSB to the naphthalenoid DHNA has been shown with extracts from either *Escherichia coli* or *Mycobacterium phlei* and with both microorganisms was dependent on the presence of ATP, CoA, and Mg^{2+} (Bryant & Bentley, 1976; McGovern & Bentley, 1978).

Hutson & Threlfall (1978) investigated the utilization of radioactive OSB by extracts from *Micrococcus luteus* but were unable to detect DHNA production. Instead, the spirodilactone derivative of OSB was consistently obtained; spirodilactone formation was dependent on the presence of ATP, CoA, and Mg^{2+}. The occasional formation of the spirodilactone, along with DHNA, had been noted in the earlier work with *E. coli* (Bryant & Bentley, 1976), but conditions for the consistent accumulation of this compound were not established. In a limited number of experiments with *E. coli* extracts, Hutson & Threlfall (1978) failed to detect DHNA formation.

Since all of the previous work has clearly implicated DHNA as an obligatory intermediate in menaquinone biosynthesis and since *M. luteus* contains a relatively high level of menaquinone (about 4 times as much as does *E. coli*; Bishop & King, 1962), the failure to show the conversion OSB → DHNA in *M. luteus* extracts was disturbing. Taken at face value, the negative results of Hutson & Threlfall (1978) implied a biosynthetic pathway to menaquinone without DHNA as an intermediate. We have, therefore, reexamined OSB utilization in *M. luteus* extracts. This was particularly necessary in view of our recent observation (Meganathan & Bentley, 1979) that DHNA formation in *M. phlei* extracts proceeds through an OSB-CoA intermediate and requires two separate enzymes termed OSB-CoA synthetase and DHNA synthase. In fact, the formal elimination of water from OSB is more accurately an elimination of CoA from OSB-CoA. This paper reports the production of both DHNA and OSB spirodilactone from OSB by cell-free extracts from *M. luteus* and presents evidence that two enzymes are involved.

Materials and Methods

Chemicals. OSB, OSB spirodilactone, and DHNA were prepared as previously described (Bryant & Bentley, 1976; McGovern & Bentley, 1978). The trimethyl derivative of DHNA was prepared by treating either DHNA or Me_2DHNA (McGovern & Bentley, 1978) with a large excess of ethereal diazomethane for 60–70 min at room temperature. [2-^{14}C]OSB was synthesized from [2-^{14}C]pyruvate (obtained from CEA, Saclay, France, through Research Products International Corp.) by the method of Dansette (1972); this synthesis involves the reduction of the initial product, 2-carboxybenzylidene pyruvate, with sodium borohydride to form 2-carboxybenzylidene lactate and acid isomerization of the latter (Fittig, 1896) to OSB. Deoxyribonuclease, 1200 units/mg, and lysozyme, 9125 units/mg, were obtained from Worthington Biochemical Corp., ATP and CoA were from Sigma, and all other chemicals were of the highest quality commerically available. All solvents were redistilled prior to use. Reagents for protein determination according to the method of Bradford (1976) were obtained from Bio-Rad. The following buffers were used. Buffer A: 0.02 M potassium phosphate, pH 6.9, containing 5 mM mercaptoethanol. Buffer B: 0.1 M potassium phosphate, pH 8.0, containing 5 mM mercaptoethanol. *M. phlei* DHNA synthase was prepared as described earlier (Meganathan & Bentley, 1979).

Organism and Growth Conditions. M. luteus ATCC 4698 was maintained on slants containing 1% Bactopeptone (Difco), 0.1% yeast extract (Difco), 0.5% NaCl, and 2% agar. Cells used to prepare enzyme extracts were obtained by first inoculating 100-mL portions of the above medium without agar

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[1] Abbreviations used: OSB, *o*-succinylbenzoic acid [4-(2′-carboxyphenyl)-4-oxobutyric acid]; OSB spirodilactone, the spirodilactone of 4-(2′-carboxyphenyl)-4,4′-dihydroxybutyric acid (this material yields OSB on hydrolysis); DHNA, 1,4-dihydroxy-2-naphthoic acid (structures of the preceding three compounds are shown in Figure 7); OSB-CoA, a derivative of OSB with CoA (a possible structure is shown in Figure 7); CoA, coenzyme A; EDTA, ethylenediaminetetraacetic acid.

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and contained in 250-mL Erlenmeyer flasks; the cells were grown, with shaking at 30 °C, for 24 h. These seed cultures were then used to inoculate 900-mL portions of the same medium in 2.8-L Fernbach flasks; growth was continued at 30 °C for a further 24 h. The cells were collected by centrifugation at 4000 rpm for 10 min in a Sorvall HG-4 rotor and were washed once in one-eighth volume of buffer A. The centrifuged cell paste was frozen and stored at −20 °C. Spray-dried cells of the same organism were obtained from Miles Laboratory and were also stored at −20 °C.

Preparation of Cell-Free Extracts from Fresh Cells. Portions of *M. luteus* cell paste (11 g wet weight) were suspended in 22 mL buffer A; following addition of lysozyme (22 mg) and deoxyribonuclease (1 mg), the mixture was incubated at 30 °C for 15 min. A further 11 mL of buffer A was added, and the mixture was then centrifuged at 4 °C and 27000*g* for 15 min. The clear supernatant obtained by this treatment was used directly in most experiments. However, to determine the cofactor requirements for spirodilactone formation, 1-mL portions of the extract were dialyzed against 200 mL of buffer A for 2 h at 4 °C.

Preparation of Cell-Free Extracts from Spray-Dried Cells. Extracts were prepared by one of the following procedures; the procedures are identified in the Results section as method 1, 2, or 3. (1) The cells, 1 g, were suspended in 7 mL of buffer A and were treated with lysozyme, 25 mg, and deoxyribonuclease, 3 mg. After incubation at 30 °C for 30 min, the mixture was centrifuged at 27000*g* for 15 min. The supernatant from this treatment was used directly. (2) Cells were treated exactly as just described, but using 0.2 M potassium phosphate buffer, pH 7.2, containing 5 mM mercaptoethanol. In some cases, the supernatant from the centrifugation was passed through a column of Sephadex G-25 (Hutson & Threlfall, 1978). (3) The cells, 1 g, were suspended in either 0.2 M potassium phosphate buffer, pH 7.2, or 0.2 M sodium phosphate buffer, pH 7.2, conaining 10 mM EDTA and 0.1 mM dithiothreitol, prior to treatment with lysozyme and deoxyribonuclease. The centrifuged extracts were then treated with Sephadex G-25 (Hutson & Threlfall, 1978).

Assays for DHNA Production. DHNA formation was measured by the spectrophotofluorometric method using an excitation wavelength of 370 nm and an emission wavelength of 430 nm (McGovern & Bentley, 1978) in an Aminco Bowman instrument (catalog no. 4-8202). Unless otherwise indicated, incubation mixtures routinely contained the following amounts of reagents, in micromoles, in a total volume of 3.0 mL of buffer B: OSB, 0.25; ATP, 4.8; CoA, 0.5; $MgCl_2$, 20.0. The solutions were incubated for 30 min at 30 °C. At the end of the incubation period, the reaction was terminated by addition of 3 mL of a mixture of acetone–concentrated HCl, 100:1 (v/v). Benzene, 3 mL, was then added, and after thorough shaking (Vortex mixer) the organic phase was separated and used for the spectrophotofluorometric assay (McGovern & Bentley, 1978). In experiments with [2-^{14}C]OSB, the radioactive material (sp act. 25 mCi/mmol, total disintegrations per minute added ~170000) was substituted for the cold material in the above assay. The organic phase obtained after the workup just described (4 mL) was mixed with 100-μg portions of carrier OSB, OSB spirodilactone, and DHNA. The solvent was removed on a rotary evaporator, and the dried residue was redissolved in 500 μL of ethyl acetate prior to thin-layer chromatography on silica gel GF plates (Analtech) in the solvent system chloroform–ethyl acetate–formic acid, 135:20:1.5 (v/v). After development and drying, the plates were scanned for radioactivity by using a Packard Model 7201 radiochromatogram scanner.

Table I: Cofactor Requirements for DHNA Formation in *M. luteus* Extracts

system[a]	enzyme act. (nmol of DHNA formed per tube)
complete	12.7
$-MgCl_2$	6.9
−OSB	<0.1[b]
−CoA	<0.1
−ATP	<0.1
boiled enzyme	<0.1

[a] The control complete system contained 0.2 mL of *M. luteus* cell-free extract from freshly grown cells (6.6 mg of protein), and the amounts of ATP, CoA, $MgCl_2$, and OSB are as described for the routine assay (see Materials and Methods). In the other tubes, one component of the routine mixture was omitted. For the boiled enzyme, the extract was held in a boiling water bath for 5 min. [b] The assay lacks precision at these low levels.

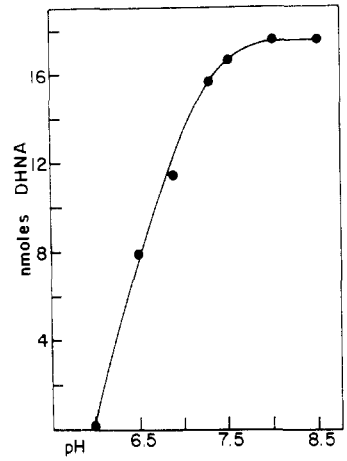

FIGURE 1: Effect of pH on the formation of DHNA by *M. luteus* extracts. The routine assay conditions were used, as described under Materials and Methods, and each tube contained 0.2 mL of extract (6.8 mg of protein) from freshly grown cells. The volume was made up to 3.0 mL with 0.1 M potassium phosphate buffer containing 5 mM mercaptoethanol, with varying pH values as indicated.

Results

Spectrophotofluorometric Evidence for DHNA Production. The enzyme extracts catalyzing the conversion OSB → DHNA which had been examined earlier were obtained from freshly grown cells of *E. coli* or *M. phlei* (or those stored briefly in the frozen state); hence, initial experiments were performed with extracts obtained from freshly grown cells of *M. luteus*. As shown in Table I, the spectrophotofluorometric assay readily revealed the production of DHNA. The formation of this naphthalenoid compound was rigidly dependent on the presence of both CoA and ATP; omission of Mg^{2+} gave an ~50% decline in DHNA production. No DHNA was produced when the incubation mixtures were at pH 6.0; there was a marked increase in enzyme activity from pH 6.5 to 7.5 and a broad plateau of activity between pH 7.5 and 8.5 (see Figure 1). The rate of DHNA formation by the *M. luteus* extracts was linear over a 40-min period (Figure 2A) and was dependent on protein concentration (Figure 2B). The initial *M. luteus* extracts had a specific activity of 1.9 nmol per mg of protein per 30 min, a value comparable to that of 1.78 nmol per mg of protein per 30 min obtained with enzyme extracts from *M. phlei* (Meganathan & Bentley, 1979).

In view of the fact that Hutson & Threlfall (1978) had used spray-dried rather than freshly grown cells of *M. luteus*, the

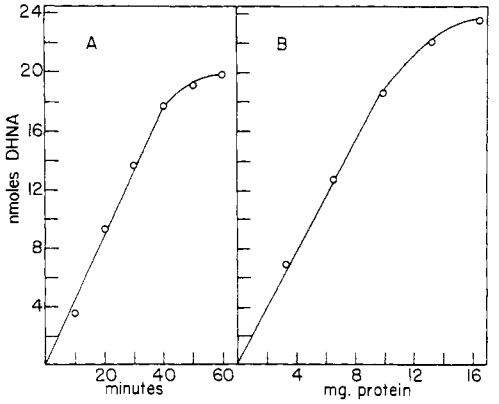

FIGURE 2: Rate of DHNA formation by extracts from *M. luteus* and the relationship of protein concentration to enzymatic activity. (A) The incubation mixture contained 1.4 mL of extract from freshly grown cells (46.2 mg of protein) with addition of the following (amounts in micromoles): OSB, 1.75; ATP, 33.6; CoA, 3.5; $MgCl_2$, 140. The volume was made up to 21 mL with buffer B. At the indicated times, 3-mL aliquots of the incubation mixture were removed and added to 3 mL of a mixture of acetone–concentrated HCl, 100:1 (v/v), held in an ice bath. After all the samples had been taken, benzene was added and workup proceeded in the usual way for spectrophotofluorometric assay. (B) Varying amounts of enzyme obtained from freshly grown cells of *M. luteus* were used in the routine assay.

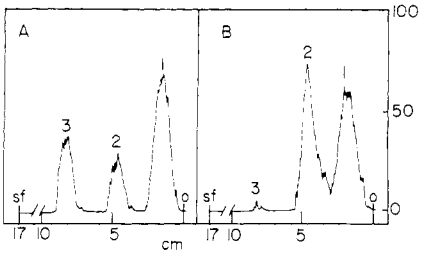

FIGURE 3: Formation of DHNA and spirodilactone by *M. luteus* extracts. The extracts prepared from freshly grown *M. luteus* cells (100 μL, 2.5 mg of protein) were incubated with [2-^{14}C]OSB (total 100 000 cpm) in a final volume of 3 mL, as described under Materials and Methods. Following the standard workup procedure, the thin-layer plates were examined in the radiochromatogram scanner: time constant 10 s; range 3 × 10^3 cpm; speed 1 cm/min. The ordinate of the figure shows the percentage of full-scale deflection. (A) Extract alone. (B) Extract with addition of 11 units of *M. phlei* DHNA synthase (Meganathan & Bentley, 1979). The following identifications are used: o = origin, sf = solvent front, 1 = OSB, 2 = DHNA, 3 = OSB spirodilactone.

preparation of cell-free extracts from such material was investigated under a variety of conditions. When spray-dried cells were treated with the amounts of lysozyme and deoxyribonuclease used by Hutson & Threlfall (1978) under our usual buffer conditions (see method 1, Materials and Methods), DHNA formation was observed by the spectrophotofluorometric assay. The specific activity of such extracts was 1.1 nmol of DHNA formed per mg of protein per 30 min, a value slightly less than that obtained with extracts from freshly grown cells. Since Hutson & Threlfall (1978) used a pH 7.2 buffer and a Sephadex G-25 column, our usual condition was changed to this pH value (see method 2). However, DHNA formation was still observed, a result which was unaffected by inclusion or not of the Sephadex G-25 column treatment. Finally, an attempt was made to duplicate the Hutson & Threlfall (1978) conditions exactly; since they did not specify whether a sodium or potassium phosphate buffer was used, both cations were tried (see method 3). As before, DHNA production was observed; the extracts showed a specific activity of 1.02 nmol of DHNA formed per mg of protein per 30 min in potassium phosphate buffer at pH 7.2 and 0.7 nmol of DHNA per mg of protein per 30 min in sodium phosphate buffer under similar conditions. In summary, DHNA production has been observed consistently with extracts of spray-dried cells prepared under a variety of conditions.

Further Evidence for DHNA Production and Identification of OSB Spirodilactone as a Coproduct. Since Hutson & Threlfall (1978) were unable to detect DHNA formation in *M. luteus* extracts, it was important to confirm the spectrophotofluorometric results by independent means. For this purpose, identical incubation mixtures were prepared, but with [2-^{14}C]OSB replacing the nonradioactive material. Following incubation, extraction, and thin-layer chromatography (see Materials and Methods), scanning for radioactivity revealed the presence of three prominent peaks (see Figure 3A). Of these, the slowest moving component with R_f 0.09 (peak no. 1 of Figure 3A) was clearly unchanged OSB, while the next with R_f 0.29 (peak no. 2) had the correct characteristics for DHNA. The most rapidly moving peak, R_f 0.49 (peak no. 3), corresponded to the known position of the spirodilactone form of OSB [although named for convenience as the spirodilactone form of OSB, this material is more correctly described as the spirodilactone of 4-(2′-carboxyphenyl)-4,4′-dihydroxybutyric acid]. This pattern of three peaks was repeatedly observed with several batches of *M. luteus* extracts prepared at different times; in addition, an identical pattern was observed with extracts prepared from spray-dried cells by method 3. The areas of the spirodilactone and DHNA peaks were in the ratio of 2.3:1.

For a further check on the identity of the products, gas chromatography combined with mass spectrometry was employed. Following incubation of OSB with M. *luteus* extract from freshly grown cells under the routine assay conditions, the mixture was extracted with chloroform. This treatment is known to remove OSB spirodilactone (Hutson & Threlfall, 1978); 3 times the volume of chloroform, methanol, and water used by these workers was employed. From a separate incubation, DHNA was recovered by treatment with acetone–concentrated HCl and benzene extraction as normally used for the spectrophotofluorometric assay. The residue obtained on solvent removal was treated with a large excess of ethereal dizaomethane for 1 h at room temperature; the excess diazomethane and the solvent were then removed by vacuum evaporation. The materials from the two extraction procedures were examined separately by combined gas chromatography–mass spectrometry. The recorded spectra (see Figure 4) fully confirm the identifications of the two products as OSB spirodilactone and DHNA.

Evidence That DHNA Formation in M. luteus Extracts Requires Two Enzymes. In *M. phlei* extracts, the following sequence has been observed (Meganathan & Bentley, 1979): (a) conversion of OSB to an OSB-CoA derivative in an ATP-dependent reaction catalyzed by OSB-CoA synthetase and (b) cyclization of the OSB-CoA derivative to DHNA, with release of CoA, catalyzed by DHNA synthase. With the unfractionated *M. phlei* extracts, spirodilactone formation was never observed; however, when the separated OSB-CoA synthetase component was incubated with [2-^{14}C]OSB in the standard incubation mixture, OSB spirodilactone was the only product formed. Hence, it appeared likely that the OSB-CoA derivative could undergo nonenzymatic decomposition to spirodilactone. If this were the case, spirodilactone formation in the *M. luteus* extracts could have resulted from a relatively low level of DHNA synthase compared to the level of OSB-CoA synthetase. A sample of DHNA synthase, obtained from *M. phlei* extracts by protamine sulfate precipitation (Mega-

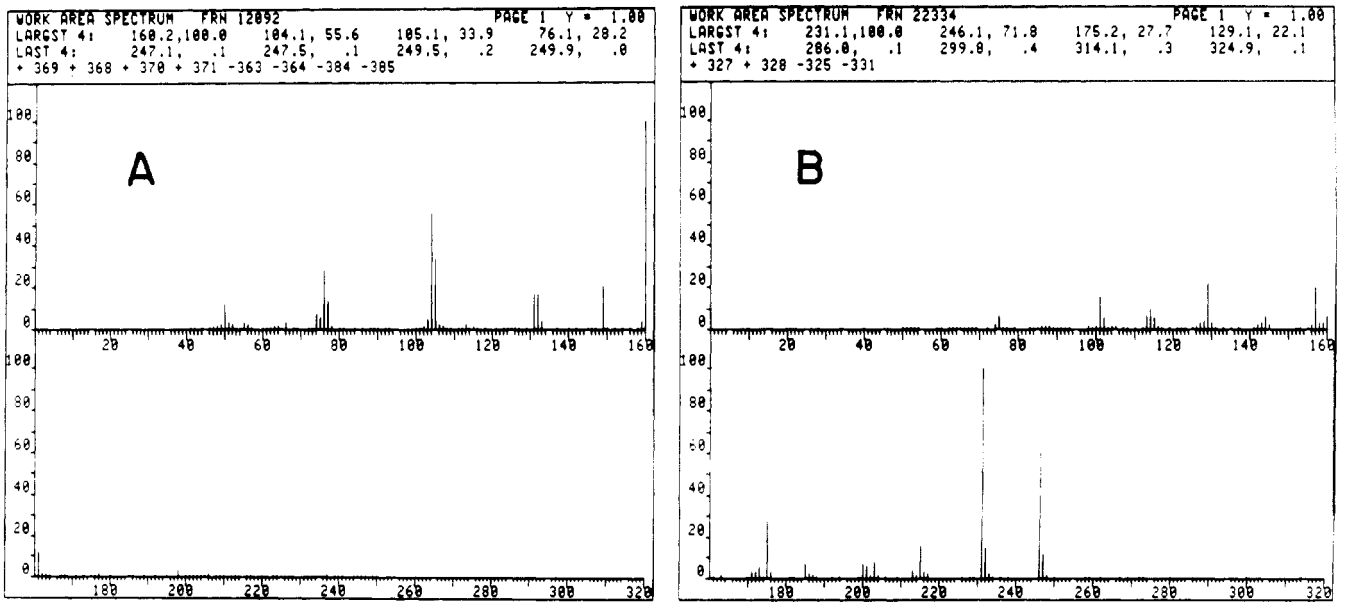

FIGURE 4: Identification of OSB spirodilactone and DHNA by gas chromatography combined with mass spectrometry. All of this work was carried out on the Hewlett Packard instrument, 5985B. (A) Mass spectrum of OSB spirodilactone. The experiment used 0.2 mL of cell-free extract (5.4 mg); incubation and chloroform extraction were performed as described in the text. A sample of the dried chloroform extract was injected in ethyl acetate onto a column of 3% OV 17 (6 ft × 2 mm); temperature programming was from 150 to 270 °C at 10 °C/min. Although the parent molecular ion (*m/e* 204) is very small, the following peaks correspond to the predicted fragmentation pattern: successive loss of CO (*m/e* 160), CH_2CH_2 (*m/e* 132), CO (*m/e* 104), and CO (*m/e* 76). The small peak at *m/e* 198 is probably derived from a small amount of contaminant. Identical mass spectra were obtained on authentic OSB spirodilactone samples, and the pattern is very similar to that recorded on another instrument (Grotzinger, 1974). (B) Mass spectrum of DHNA as the trimethyl derivative. A sample of the material from the benzene extraction was derivatized with diazomethane and was then injected onto a column of 3% SP 2250 (6 ft × 2 mm); temperature programming was from 150 to 300 °C at 10 °C/min. The fragmentation pattern shows a pronounced parent molecular ion (*m/e* 246) and a characteristic loss of two methyl groups and one methoxyl group (*m/e* 231, 215, 201). Identical mass spectra were obtained on samples of Me_3DHNA prepared as described in the text.

nathan & Bentley, 1979), was added to an extract from *M. luteus* to test this supposition. In the presence of this protein, the scanning of the thin-layer chromatograms showed only two peaks of radioactivity, unchanged OSB and DHNA (see Figure 3B); the spirodilactone peak of Figure 3A (extract alone) had disappeared, and at the same time the area of the DHNA peak had increased substantially, in harmony with our expectations.

A further test of the hypothesis would be possible if the product ratio could be manipulated in the other direction (more spirodilactone and less or no DHNA). This would require a selective inactivation of the putative second enzymatic component (DHNA synthase). Since our previous experience indicated that OSB-CoA synthetase from *M. phlei* could withstand acid conditions (0.1 N HCl, 5 min) that brought about complete inactivation of DHNA synthase, the following experiments were performed on the *M. luteus* extracts. Portions of the extract (0.1 mL) were cooled in ice, and 3 N HCl (3.3 μL) was added so that the final concentration was 0.1 N. The acid was added along the sides of the tubes, and the contents were then quickly swirled around. This treatment resulted in the separation of a whitish precipitate. After standing in the ice bath for 30 s, buffer B was added to a final volume of 3.0 mL. When such acid-treated extracts were incubated with [2-^{14}C]OSB, CoA, ATP, and Mg^{2+}, exactly as described earlier, scanning of the thin-layer chromatograms for radioactivity revealed only the presence of spirodilactone and unchanged OSB (see Figure 5A). When a preparation of *M. phlei* DHNA synthase was added to the acid-treated *M. luteus* extracts, DHNA production was restored (see Figure 5B).

Although in the untreated initial extracts DHNA and spirodilactone formation were both equally dependent on the presence of ATP, CoA, and Mg^{2+}, it was important to demonstrate that the formation of spirodilactone, by itself, was dependent on the presence of these cofactors. For this purpose, the intitial *M. luteus* extract was dialyzed as described under Materials and Methods prior to an acid treatment to inactivate DHNA synthase. When such dialyzed and acid-treated extracts were incubated with [2-^{14}C]OSB, using a complete system (ATP, CoA, and Mg^{2+}), the radiochromatographic scan showed the presence of OSB spirodilactone and unchanged OSB (see Figure 6A); in the absence of either ATP or CoA, no products were detected except unchanged OSB itself (see Figure 6B).

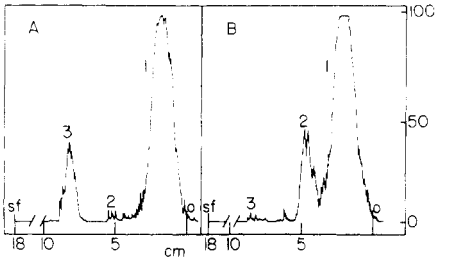

FIGURE 5: Effect of acid on enzyme activities of *M. luteus* extracts. The extract from freshly grown *M. luteus* cells (100 μL, 2.5 mg of protein) was treated with 3 N HCl to yield a final concentration of 0.1 N (Meganathan & Bentley, 1979). Incubation was carried out in the presence of [2-^{14}C]OSB (total 100 000 cpm) as described in the legend to Figure 3. (A) Acid-treated extract. (B) Acid-treated extract with addition of 11 units of *M. phlei* DHNA synthase. Instrument settings for radioactivity scan were the following: time constant 10 s; range 1 × 10^3 cpm; speed 1 cm/min. The ordinate of the figure shows the percentage of full-scale deflection. Identifications are the same as those of Figure 3.

Discussion

Although Hutson & Threlfall (1978) failed to demonstrate the conversion of radioactive OSB to DHNA using cell-free

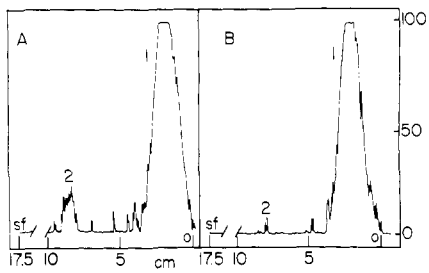

FIGURE 6: Cofactor requirements for spirodilactone formation by *M. luteus* extracts. A portion of *M. luteus* extract was dialyzed as described under Materials and Methods. This dialyzed extract (200 μL, 3.9 mg of protein) was treated with acid as described in the legend for Figure 5 and was then incubated with [2-^{14}C]OSB (total 100000 cpm); the products were examined for radioactivity as described in the legend for Figure 3. Instrument settings for radioactivity scan were the following: time constant 10 s; range 1 × 10^3 cpm; speed 1 cm/min. The ordinate shows the percentage of full-scale deflection. (A) Complete system, including ATP, CoA, OSB, and Mg^{2+}. (B) Complete system minus ATP. A similar scan to that of (B) was obtained with the complete system minus CoA. The following identifications are used: o = origin, sf = solvent front, 1 = OSB, 2 = OSB spirodilactone.

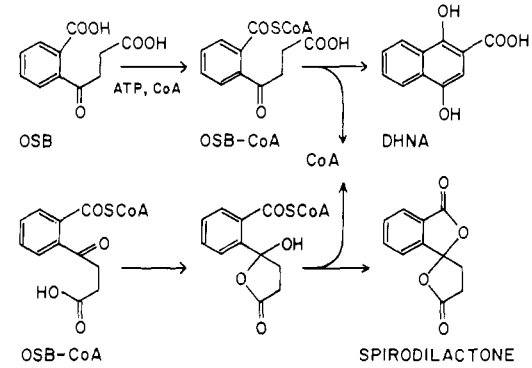

FIGURE 7: Role of OSB-CoA in formation of OSB spirodilactone and DHNA. The structure shown for OSB-CoA is that proposed earlier (Bryant & Bentley, 1976; McGovern & Bentley, 1978). In the bottom line, the same OSB-CoA structure has been redrawn to emphasize the relationship to OSB spirodilactone. The conversion OSB-CoA → DHNA requires the enzyme DHNA synthase, and this step is mandatory for vitamin K biosynthesis. Breakdown of OSB-CoA to OSB spirodilactone is presumed to be nonenzymatic.

extracts from *M. luteus* and *E. coli*, the results reported here and earlier (Bryant & Bentley, 1976) provide evidence for this conversion in both of these organisms. In the present work, DHNA formation has been demonstrated by spectrophotofluorometric assay, by the use of radioactive substrate, and by examination of products in the mass spectrometer. The work of Hutson & Threlfall (1978) with *M. luteus* relied entirely on the use of spray-dried cells; when we tried to duplicate their experimental conditions as accurately as possible (method 3), we found no difficulty in demonstrating the OSB → DHNA conversion, even though the spray-dried cells we used had been stored in the frozen state for over 5 years. Since one possible explanation for the differences might have been a pH variation, extractions of spray-dried cells were carried out at pH 7.2 as well as at pH 6.9. In both cases, DHNA formation was observed, and to the same degree. Although Hutson & Threlfall (1978) routinely passed their extracts through a Sephadex G-25 column, our extracts produced DHNA with or without this treatment. Similarly, addition of the metal chelator EDTA or of dithiothreitol gave the same result in our hands. Thus, in a number of experiments in which some variation was made in the extraction technique from spray-dried cells, the production of DHNA was always observed without difficulty. It appears likely that there is a minor unrecognized, technical difference in the work of Hutson & Threlfall (1978), especially since they were also unable to show DHNA formation in *E. coli* extracts. The conversion of OSB to DHNA and spirodilactone was dependent on the presence of ATP, CoA, and Mg^{2+}; as was the case with *M. phlei*, the experimental observations were consistent with the intermediate formation of an OSB-CoA derivative and the participation of two enzymes, OSB-CoA synthetase and DHNA synthase. Addition of a preparation of the second enzyme, DHNA synthase obtained from *M. phlei*, to the *M. luteus* extracts prevented formation of OSB spirodilactone and at the same time substantially increased DHNA formation. Furthermore, acid treatment of the *M. luteus* extracts apparently inactivated the second enzyme, DHNA synthase, since such preparations formed only the spirodilactone. On addition of a preparation of DHNA synthase to acid-treated preparations, DHNA formation was restored and spirodilactone production was diminished. These are the observations to be expected if the spirodilactone and DHNA both originate from the same intermediate (see Figure 7) and if with diminishing amounts of DHNA synthase there is a spontaneous breakdown of the OSB-CoA intermediate to spirodilactone.

These results imply that in any given extract, the product ratio DHNA/spirodilactone will vary with the actual ratio of the two enzymatic activities (OSB-CoA synthetase/DHNA synthase). The results obtained with three different microorganisms bear out this prediction. With *M. phlei* extracts, spirodilactone formation was never observed, with *E. coli* extracts, spirodilactone was sometimes observed, and in the present work, spirodilactone formation was noted in all of the preparations from *M. luteus*.

The formation of DHNA by *M. luteus* extracts strongly supports the presently proposed pathway for menaquinone biosynthesis. Although all of the evidence implicates an OSB-CoA intermediate, the isolation of the material has not yet been possible and the structure shown in Figure 7 is still speculative.

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References

Bentley, R. (1975a) *Pure Appl. Chem. 41*, 47.
Bentley, R. (1975b) *Biosynthesis 3*, 181.
Bishop, D. H. L., & King, H. K. (1962) *Biochem. J. 85*, 550.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.
Bryant, R. W., & Bentley, R. (1976) *Biochemistry 15*, 4792.
Dansette, P. (1972) Ph.D. Thesis, Université de Paris-Sud, p 131.
Fittig, R. (1896) *Ber. Dtsch. Chem. Ges. 29*, 2582.
Grotzinger, E. W. (1974) Ph.D. Thesis, University of Pittsburgh, p 43.
Hutson, K. G., & Threlfall, D. R. (1978) *Biochim. Biophys. Acta 530*, 1.
Meganathan, R., & Bentley, R. (1979) *J. Bacteriol. 140*, 92.
McGovern, E. P., & Bentley, R. (1978) *Arch. Biochem. Biophys. 188*, 56.