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Thiamine Pyrophosphate Requirement for *o*-Succinylbenzoic Acid Synthesis in *Escherichia coli* and Evidence for an Intermediate

R. MEGANATHAN† AND RONALD BENTLEY*

Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260

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Cell-free extracts of various strains of *Escherichia coli* synthesize the menaquinone biosynthetic intermediate *o*-succinylbenzoic acid (OSB) when supplied with chorismic acid, 2-ketoglutaric acid, and thiamine pyrophosphate (TPP). To assay for OSB synthesis, 2-[*U*-^{14}C]ketoglutaric acid was used as substrate, and the synthesized OSB was examined by radiogas chromatography (as the dimethyl ester). [*U*-^{14}C]Shikimic acid also gave rise to radioactive OSB if the cofactors necessary for enzymatic conversion to chorismic acid were added. Use of 2-[1-^{14}C]ketoglutaric acid does not give rise to labeled OSB. In the absence of TPP during the incubations, OSB synthesis was much reduced; these observations are consistent with the proposed role for the succinic semialdehyde-TPP anion as the reagent adding to chorismic acid. Extracts of cells from *menC* and *menD* mutants did not form OSB separately, but did so in combination. There was evidence for formation of a product, *X*, by extracts of a *menC* mutant incubated with chorismic acid, TPP, and 2-ketoglutaric acid; *X* was converted to OSB by extracts of a *menD* mutant. It appears that the intermediate, *X*, is formed by one gene product and converted to OSB by the second gene product.

It has been known since 1964 that menaquinones are produced in bacteria by way of the shikimic acid pathway (1). As with other aromatic compounds, the "branch point" for the biosynthetic pathway is at chorismic acid. The first committed step in menaquinone biosynthesis is the conversion of chorismic acid to the benzenoid aromatic compound *o*-succinylbenzoic acid [OSB; 4-(2′-carboxyphenyl)-4-oxobutyrate]. OSB is then cyclized to the naphthalenoid derivative 1,4-dihydroxy-2-naphthoic acid; the action of a prenyltransferase enzyme on 1,4-dihydroxy-2-naphthoic acid gives rise to a demethylmenaquinone with loss of the carboxyl group.

Up to the present, the area of greatest uncertainty has concerned the synthesis of OSB. Of the 11 carbon atoms in this compound, 7 are known from in vivo tracer experiments to be derived from the nonpyruvoyl carbon atoms of chorismic acid (i.e., the atoms originally present in shikimic acid); the remaining 4 carbon atoms are obtained from 2-ketoglutaric acid, which undergoes a decarboxylation. It was proposed by Campbell (4) that the basic reaction for assembly of OSB was a Michael addition of the anion form of a succinic semialdehyde-thiamine pyrophosphate (TPP) complex to the prearomatic component at C-2 (the numbering used here for chorismic acid is the same as that for the corresponding atoms of shikimic acid). The necessary anion, containing the required four carbon atoms, could be derived from 2-ketoglutaric acid and TPP by the action of a 2-ketoglutarate decarboxylase. It seemed probable that this decarboxylase would be the first enzyme of the 2-ketoglutarate dehydrogenase complex; however, the existence of a separate and distinct decarboxylase could not be ruled out.

Recently, it was possible to obtain enzyme preparations from strains of *Escherichia coli* which catalyze the formation of OSB from chorismic acid and 2-ketoglutaric acid (12). The work reported here shows that these enzyme preparations incorporate atoms from the precursors in the manner just described and provides further information concerning the TPP requirement. In addition, evidence is presented for the involvement of an intermediate in the overall reaction. Until the enzymology of OSB synthesis has been more completely clarified, the cell-free preparations will, for convenience, be described as the "OSB synthase system."

A preliminary report of some of the findings

† Present address: Department of Biological Sciences, Northern Illinois University, DeKalb, IL 60115.

TABLE 1. Strains of *E. coli*

Strain	Genotype	Reference
AN154	*thi pro arg phe tyr trp entC aroB*	16
PL2024	*gal trpA trpR iclR rpsL*	8, 11
JRG862	*menC1* (OSB-requiring mutant of PL2024)	8, 9
JRG918	*menD7 gal trpA trpR iclR rpsL gyrA*	8, 9
JRG72	*sucA1 supE iclR*	5

has appeared (R. Meganathan and R. Bentley, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, K138, p. 159).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The various strains and mutants of *E. coli* which were used in this work are listed in Table 1. Aerobic cultures were grown as described by Meganathan (12). For strain AN154, Trypticase soy broth medium (TSB; BBL Microbiology Systems, Cockeysville, Md.) was supplemented in some of the experiments with 2 mg of filter-sterilized thiamine chloride per ml. For anaerobic growth in TSB medium, the glucose concentration was increased from the normal value of 0.25% to 1.0%. Anaerobic seed cultures were grown in completely filled screw-capped tubes overnight at 37°C. For large-scale anaerobic cultures, 2 ml of seed culture was inoculated into 2 liters of TSB–1% glucose medium (completely filling a 2-liter Erlenmeyer flask); growth was continued at 37°C for a further 18 h under stationary conditions. All cultures were harvested by centrifugation. The packed cells were washed with 0.25 volume of 0.02 M potassium phosphate buffer, pH 7, and were then recentrifuged as before. The cell paste was stored at −20°C.

Preparation of cell-free extracts. The cell paste (5 g) was suspended in 7.5 ml of 0.02 M potassium phosphate buffer, pH 7.0, containing 5 mM mercaptoethanol. This suspension was passed through a French pressure cell at 12,000 to 15,000 lb/in^2 and a temperature of 4°C. To reduce the viscosity of the extract, DNase (100 μg) was added; after incubation for 5 min at 30°C, the mixture was centrifuged at 26,000 × *g* for 30 min. The resulting supernatant solution was dialyzed against 1 liter of 0.02 M potassium phosphate buffer, pH 7.0, containing 5 mM mercaptoethanol for 3 h; after changing the buffer, dialysis was continued for a further 3 h.

Enzyme assays. To assay for the synthesis of OSB, ^{14}C-labeled substrates were used in incubations; labeled OSB was isolated and subjected to radiogas chromatography. For assays with 2-[*U*-^{14}C]ketoglutarate or 2-[1-^{14}C]ketoglutarate, 5 μCi of the labeled substrate (0.24 or 0.3 μmol, respectively) was incubated with chorismic acid (1.4 μmol) and TPP chloride (0.11 μmol) in a total volume of 3.0 ml of 0.1 M glycylglycine buffer, pH 8.5, containing 5 mM mercaptoethanol. In all cases, the volume of bacterial extract was 0.5 ml. For work with [*U*-^{14}C]shikimic acid, the substrate contained 5 μCi in 0.2 μmol. Other components with this substrate were as follows (amounts in micromoles): 2-ketoglutarate, 1.4; NADH, 2.7; $MgCl_2$, 15.0; TPP chloride, 0.11; ATP, 12.0; KF, 30.0; phosphoenolpyruvate, 3.0; flavin adenine dinucleotide, 6.0. The total volume was made up to 3.0 ml, using the glycylglycine buffer just described. In all cases, after addition of enzyme extract, incubation was carried out for 30 min at 30°C.

The enzymatic reaction was terminated by addition of 2 drops of concentrated HCl. Nonradioactive "carrier" OSB (100 μg) was then added, and the reaction tubes were cooled on ice for 10 min. To facilitate the precipitation of protein, saturated NaCl (2 ml) was added, and the mixtures were then centrifuged at 12,000 × *g* for 10 min. The supernatant solution from this centrifugation was extracted three times with equal volumes of ethyl acetate, and the combined extracts were evaporated to dryness (rotary evaporator). The dried residue was redissolved in ethyl acetate (500 μl), and the entire amount was subjected to thin-layer chromatography on Anasil OF plates. The solvent used initially was chloroform-methanol-acetic acid, 70:31:1; in the latter stages of the work it was found that increasing the amount of acetic acid fivefold resulted in a more compact spot for OSB. The area of the plate corresponding to standard OSB (R_f = 0.64) was removed, and the silica was suspended in 0.2 N HCl (3.0 ml). This mixture was extracted three times with 3.0-ml portions of ethyl acetate; the combined extracts were evaporated to dryness. The residue from these operations was dissolved in methanol (0.5 ml) and was treated for 15 min with an ethereal solution of diazomethane (2.0 ml) to form dimethyl OSB. The solvent and excess diazomethane were then removed by vacuum evaporation. For analysis by radiogas chromatography, the dried residue was dissolved in ethyl acetate (50 μl), and appropriate volumes were injected onto a packed column (6 ft [ca. 183 cm] by 4 mm) of 3% OV-17 on Gas-Chrom Q, 80 to 100 mesh.

Chemicals. Barium chorismate and TPP chloride were from Sigma Chemical Co., St. Louis, Mo. 2-[*U*-^{14}C]ketoglutaric acid (247 or 249 mCi/mmol), 2-[1-^{14}C]ketoglutaric acid (53.6 mCi/mol), and [*U*-^{14}C]shikimic acid (81.1 mCi/mmol) were products of New England Nuclear, Boston, Mass. Anasil OF thin-layer chromatography plates were obtained from Analabs, North Haven, Conn. Other chemicals were of the highest purity available, and solvents were redistilled from glass before use. An authentic sample of dimethyl OSB was prepared as previously described (13). Diazomethane used to prepare the dimethyl derivative was generated from Diazald supplied by Aldrich Chemical Co., Milwaukee, Wis. Protein was determined by the method of Bradford (2), using reagents supplied by Bio-Rad Laboratories, Richmond, Calif.

RESULTS

Assay for OSB synthase activity in cell-free extracts of *E. coli*. In all of this work, formation of OSB was determined by use of radiogas chromatography, after incubation of extracts with an appropriately labeled substrate. The extracted OSB was actually analyzed as the conveniently prepared dimethyl ester. A typical set of tracings showing output from the flame ionization detector (mass) and proportional

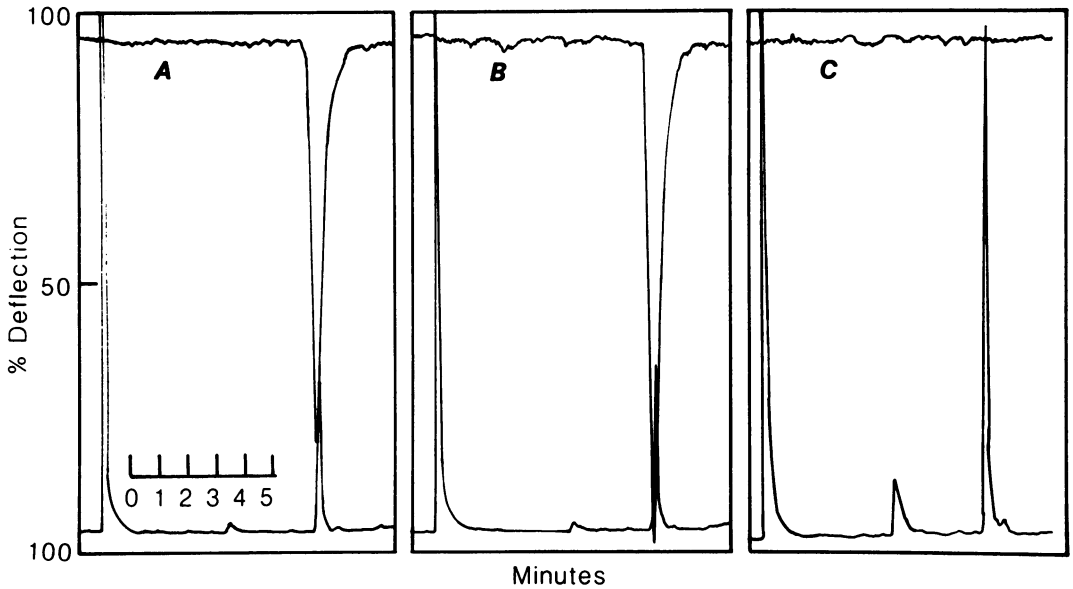


FIG. 1. Radiogas chromatography of OSB samples. The enzyme extracts used in these experiments were prepared from *E. coli* AN154, and the radioactive substrates were as follows: (A) 2-[*U*-^{14}C]ketoglutaric acid; (B) [*U*-^{14}C]-shikimic acid; (C) 2-[1-^{14}C]ketoglutaric acid. Incubations with enzyme extract, chorismic acid, and TPP were carried out as described in the text. The isolated OSB was derivatized to the dimethyl compound and was then subjected to radiogas chromatography. The column temperature of the gas chromatograph was programmed from an initial 1-min hold at 150°C to a final hold at 270°C; the temperature was increased at the rate of 10°C/min. The lower traces record the output from the flame ionization detector (sensitivity setting, 8×10^{-11} amp), and the upper traces record the output from the proportional counter component. For both traces, the ordinate scale is from 0 to 100% of full-scale deflection; for the proportional counter component, 100% full-scale deflection represents 1,000 cpm.

counter (radioactivity) is shown in Fig. 1A. In this case, 2-[*U*-^{14}C]ketoglutaric acid was used as the radioactive component, and the extract was obtained from cells of *E. coli* AN154; chorismic acid and TPP were also present. As can be seen, the mass peak with elution time of 7.8 min, which corresponds exactly to that of authentic dimethyl OSB, also coincides precisely with a radioactivity peak recorded by the proportional counter. Control experiments established that OSB formation did not take place in the absence of either chorismic acid or 2-ketoglutaric acid. In experiments with potassium phosphate and glycylglycine buffers, it was found that maximum OSB synthase activity was obtained at pH 8.5. Slightly higher levels of activity were obtained with glycylglycine, and routine assays were conducted with this buffer at pH 8.5 and 0.1 M.

In most of the work, 2-[*U*-^{14}C]ketoglutaric acid was used as the radioactive substrate, and the area of the radioactive dimethyl OSB peak was determined. Known amounts of ^{14}C present in an "ene-ol" standard were chromatographed under the same instrumental conditions, and the areas of the radioactive peaks were again determined. Radioactivity present in dimethyl OSB could then be obtained in terms of actual disintegrations per minute by direct comparison of areas. From the known specific activity of the 2-[*U*-^{14}C]ketoglutaric acid, it was then possible to determine the yield of OSB. The ene-ol standard is a mixture of components, three of which contain ^{14}C, developed by I. M. Campbell and his colleagues; it is used routinely to check the performance of the radiogas chromatography instrumentation (6). The smallest amount of OSB present in the 3.0-ml volume which could be detected by this assay was approximately 0.5 pmol.

Incorporation of radioactivity from 2-ketoglutaric acid and shikimic acid into OSB. It was important to verify that the OSB synthase system incorporated precursors into OSB following the pattern deduced from in vivo experiments (13). Since labeled chorismic acid is not readily available, the incorporation of activity from [*U*-^{14}C]shikimic acid was investigated. In this incubation, all of the necessary cofactors known to be required for the conversion of shikimic acid to chorismic acid were also present (see Materials and Methods). Radioactive dimethyl OSB was produced from the labeled shikimic acid (Fig. 1B). It was also verified that, as expected, the C-1 carboxyl group of 2-ketoglutaric acid

TABLE 2. Effects of media, growth conditions, and presence of TPP during incubations on OSB synthase activity of *E. coli* strains

Expt	*E. coli* strain	Medium	Condition of growth	OSB formed (pmol/30 min per mg of protein)[a]	
				With TPP	Without TPP
1	AN154	TSB	Aerobic	62.4	<0.5
2	AN154[b]	TSB	Aerobic	21.0	<0.5
3	AN154	TSB + T[c]	Aerobic	66.7	23.6
4	AN154[b]	TSB + T	Aerobic	45.0	18.0
5	PL2024	TSB	Aerobic	37.0	5.0
6	PL2024[b]	TSB	Aerobic	25.5	6.0
7	PL2024	TSB	Anaerobic	79.9	3.5
8	JRG72	TSB	Aerobic	26.6	20.0
9	JRG72	TSB	Anaerobic	46.5	36.4

[a] OSB formation was determined with 2-[*U*-^{14}C]ketoglutarate as substrate and with the radiogas chromatographic assay described in the text.

[b] A different batch of cells was used for this experiment.

[c] T, Thiamine.

was not incorporated into OSB. Figure 1C shows the result of using 2-[1-^{14}C]ketoglutaric acid as the substrate. Unlike the case with 2-[*U*-^{14}C]ketoglutaric acid (Fig. 1A), no radioactivity was present in the dimethyl OSB.

Requirement for TPP. When TPP was omitted from the incubation mixtures, a diminished synthesis of OSB was invariably observed (Table 2). The results in the absence of TPP varied from essentially complete suppression of OSB synthesis to a diminution by as little as 25%; the effects were related to the particular bacterial strains used in preparation of the extracts and, as well, to the cultural conditions. The most complete suppression of OSB synthase activity in the absence of TPP was seen in experiments with *E. coli* AN154; when cultures were grown aerobically, in some cases the OSB synthesis in the presence of TPP was as high as 62.4 pmol/30 min per mg of protein, whereas in the absence of TPP, OSB formation could not be detected (Table 2, experiment 1). This strain had been chosen for the initial work (12) since it is a mutant blocked in all of the aromatic biosynthetic pathways except that for menaquinone biosynthesis (16). Coincidentally, this strain is also a thiamine auxotroph; nevertheless, growth on TSB medium was possible, presumably because a low but sufficient amount of thiamine was present. The results obtained with this strain were rather variable, however; particularly in the later stages of the work, OSB synthesis in the presence of TPP did not reach the level shown in Table 2.

The growth of *E. coli* AN154 was somewhat variable depending on the particular batch of TSB medium used. Maximal OSB synthase activity was obtained from cells grown in media supplemented with thiamine. Even under these conditions, OSB synthesis was stimulated by the addition of TPP to incubation mixtures (Table 2, experiments 3 and 4).

Stimulation of OSB synthase activity by the presence of TPP during enzyme incubations was also observed with aerobically grown wild-type *E. coli* (strain PL2024; Table 2, experiments 5 and 6); the observed stimulations (4.25- to 7-fold) were similar to those observed with strain AN154 grown with added thiamine (Table 2, experiments 3 and 4). Under anaerobic growth conditions, the wild-type strain gave a high level of OSB synthesis when supplemented with TPP (79.9 pmol/30 min per mg of protein) and a very much diminished synthesis (3.5 pmol/30 min per mg of protein) in its absence (Table 2, experiment 7). This result is comparable to those obtained with strain AN154 grown without added thiamine.

The smallest degree of stimulation of OSB synthase activity by TPP was obtained with a *sucA* mutant, JRG72 (Table 2, experiments 8 and 9). Under aerobic conditions of growth, incubations without TPP produced 25% less OSB than those done with the complete system, and with extracts from cells grown anaerobically the decrease was of the same order.

Enzymatic analysis of *menC* and *menD* mutants. It was known from the work of Guest (7, 8) that the OSB-requiring mutants of *E. coli* could be separated into two groups, namely, *menC* and *menD*. As expected, extracts from these mutants were found not to produce OSB when incubated with 2-[*U*-^{14}C]ketoglutaric acid, chorismic acid, and TPP (Table 3, experiments 1 and 2). However, when extracts from the two mutants were mixed and then incubated as before, OSB synthesis was obtained (Table 3, experiment 3). These experiments provided evidence for the participation of at least two enzyme activities in the formation of OSB, and it was therefore of interest to determine whether one enzyme formed an intermediate, converted by another enzyme to OSB. For this purpose, cell-free extracts from the *menC* and *menD* mutants were incubated separately with all of the materials necessary for OSB synthesis (chorismic acid, 2-ketoglutaric acid, TPP). Reaction was terminated by addition of acid, and the denatured incubation mixtures were each extracted with ethyl acetate. The two residues obtained on removal of solvent by vacuum evaporation were dissolved in pH 8.5 glycylglycine buffer (2.5 ml); the solution containing the residue obtained from the incubation with the *menC*

TABLE 3. Role of the *menC* and *menD* mutations in OSB formation

Expt	Extracts and conditions[a]	Protein (mg)	OSB formed[b] (pmol/30 min)
1	*menC*	31	<0.5
2	*menD*	39	<0.5
3	*menC* + *menD*	31 + 39[c]	460.0
4	*menC* first; *menD* second[d]	31 + 39	790.0
5	*menD* first; *menC* second[e]	39 + 31	<0.5
6	*menC* + chorismate + TPP first; *menD* + 2-ketoglutarate second[f]	31 + 39	<0.5
7	*menC* + 2-ketoglutarate + TPP first; *menD* + chorismate second[g]	31 + 39	<0.5

[a] The *menC* mutant in all cases was *E. coli* JRG862, and the *menD* mutant was *E. coli* JRG918. In experiments 1 to 3, all required components for OSB synthase activity were present (chorismic acid, 2-ketoglutaric acid, TPP). The volumes of extracts used were 0.5 ml in each case.

[b] OSB formation was assayed with 2-[*U*-^{14}C]ketoglutaric acid as described in Table 2, footnote *a*.

[c] When two amounts of protein are recorded, the first refers to the protein content of the first extract named.

[d] The extract from the *menC* mutant (0.5 ml) was incubated with all of the required components for OSB synthase activity. After terminating the reaction, the incubation mixture was extracted with ethyl acetate. Residue obtained on removal of the solvent was dissolved in glycylglycine buffer, pH 8.5 (2.5 ml), and this solution was further incubated with 0.5 ml of the extract from the *menD* mutant without addition of any other substrate.

[e] The protocol was similar to that of experiment 4 except that the *menD* extract was used for the first incubation and the *menC* extract was used in the second incubation.

[f] The *menC* extract, 0.5 ml, was incubated with the standard amounts of chorismic acid and TPP under the usual conditions. After termination of the reaction and extraction with ethyl acetate, the residue obtained on solvent removal was dissolved in 2.5 ml of glycylglycine buffer, pH 8.5. This solution was then incubated with 0.5 ml of the *menD* extract for a further 30-min period.

[g] This protocol was similar to that for experiment 6. In the first incubation, the components were *menC* extract, 2-ketoglutaric acid, and TPP; in the second incubation, *menD* extract and chorismic acid were used.

extract was then further incubated with the *menD* extract (no additions of substrates or cofactors were made). Similarly, the solution from the initial *menD* extract incubation was further treated with the *menC* extract. When the materials from the second incubation were examined by radiogas chromatography in the usual way, it was found that the sequence *menC* extract first and then *menD* extract produced OSB (Table 3, experiment 4); the reverse order, *menD* extract first and then *menC* extract, did not (Table 3, experiment 5).

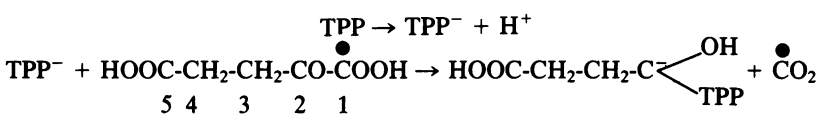
To verify that the formation of the intermediate required the presence of both chorismic acid and 2-ketoglutaric acid, the following experiments were carried out. The extract from cells of the *menC* mutant was incubated with either chorismic acid or 2-ketoglutaric acid in the presence of TPP. The reactions were terminated and an ethyl acetate extraction was carried out as previously described. The residues were then subjected to a second incubation with extract from cells of the *menD* mutant. If chorismic acid had been present during the first incubation with the *menC* extract, 2-ketoglutaric acid was added during the second incubation with the *menD* extract. Likewise, if 2-ketoglutaric acid had been present during the first incubation with the *menC* extract, chorismic acid was added during the second incubation with the *menD* extract. It was found that neither of these sequences resulted in the formation of OSB (Table 3, experiments 6 and 7). Hence, it appears most likely that both chorismic acid and 2-ketoglutaric acid, as well as TPP, are required for the formation of the intermediate, *X*, by the extract from the *menC* mutant.

DISCUSSION

With the demonstration of the enzymatic synthesis of OSB (12), all of the steps of menaquinone biosynthesis have been observed in cell-free systems (1). Detailed studies of the enzymes and of the regulation of the menaquinone biosynthetic pathway will now be possible. In this latter connection, it is of considerable interest that OSB synthase activities of extracts from wild-type and JRG72 cells grown under anaerobic conditions are approximately twice as high as those obtained with extracts of aerobically grown cells (Table 2, cf. experiments 6 and 7 and experiments 8 and 9). It is known that under anaerobic growth conditions, menaquinone levels of bacteria increase; if the bacteria also contain ubiquinones, the ubiquinone levels decrease (1). The increased formation of menaquinones under anaerobic conditions is apparently related to the role of these quinones as obligatory hydrogen carriers for the oxidation of dihydroorotate coupled to fumarate reduction.

The increases in menaquinone concentration are usually in the range of two- to threefold; these values compare with those reported here for the increase in OSB synthase activity. The response of overall menaquinone levels and of OSB synthase activities to anaerobiosis suggests that the OSB synthase system may represent an important control point for menaquinone biosynthesis; this would certainly be in line with its role as the first committed step of the biosynthetic pathway.

The overall stoichiometry of the OSB synthase reaction is represented by the following equation: chorismic acid + 2-ketoglutaric acid → OSB + CO_2 + pyruvic acid + water. Although this is a straightforward equation, the chemistry involved in OSB synthesis is complex and novel. It will be discussed first with reference to a cofactor requirement which does not appear from the overall statement of stoichiometry. Campbell had originally proposed (4) a role for the succinic semialdehyde-TPP complex as a reactant in a Michael addition. Formation of the anion was believed to be by way of the 2-ketoglutarate decarboxylase enzyme; by analogy with the pyruvate dehydrogenase complex sequence, this enzyme is believed to carry out the decarboxylation of 2-ketoglutaric acid by reaction with TPP in its anion form (TPP^-):



The observed loss of C-1 of 2-ketoglutaric acid during OSB synthase action and the fact that all of the OSB synthase preparations so far examined by us have been found to be stimulated to some extent by the addition of TPP during the incubation strongly support Campbell's original hypothesis.

The results obtained on addition of TPP to OSB synthase incubation mixtures were markedly dependent on the *E. coli* strain used and on the cultural conditions. In particular, considerable variation was observed with strain AN154. The OSB synthase activity obtained with the complete incubation system fell by a factor of about three in the later stages of the work; it is possible that a mutation in this multiple auxotroph reverted and may have influenced OSB synthesis. However, despite these difficulties, the levels of OSB synthase activity were always considerably higher in the presence of TPP than in its absence (Table 2, experiments 1 to 4). When *E. coli* AN154 was grown on thiamine-supplemented medium, there was a significant OSB synthase activity on incubation of the extracts without TPP. Under these conditions, sufficient TPP may have remained after dialysis. Alternatively, TPP may have been firmly bound to a protein (3).

With the wild-type *E. coli*, the results were clear-cut in showing marked stimulation of OSB synthase activity by the presence of TPP. The values obtained with anaerobically grown cells were particularly significant: 79.9 pmol/30 min per mg of protein for incubation with TPP compared to 3.5 pmol/30 min per mg of protein in its absence.

To explore the possible role of the first enzyme of the 2-ketoglutarate dehydrogenase complex, some experiments were carried out with the 2-ketoglutarate decarboxylase-negative *sucA* mutant JRG72. Whereas there was some tendency for extracts of this mutant to show diminished levels of OSB synthase in comparison to extracts from wild-type cells, the effects were small (the largest decrease, about 42%, was seen under anaerobic conditions). The interpretation of these results is complicated by the known leakiness of this *sucA* mutation (12). The 2-ketoglutaric acid decarboxylase activity was determined by Herbert and Guest (10) to be below 50 nmol/60 min per mg of protein. However, this mutant can be grown without added 5-aminolevulinic acid if lysine and methionine are present (succinyl coenzyme A is required for the biosynthesis of all of these components). Furthermore, if succinate utilization by succinate dehydrogenase is prevented in a *sucA sdh* double mutant, sufficient succinate is available to satisfy the requirement for the synthesis of lysine, methionine, and 5-aminolevulinic acid. Since the level of 2-ketoglutarate decarboxylase required for menaquinone biosynthesis is certainly small and is probably comparable to that required for 5-aminolevulinic acid synthesis, it appears that the *sucA* mutant still has sufficient decarboxylase activity to account for our observed levels of OSB synthase. The levels of OSB synthase activity are in the picomolar range rather than the nanomolar range of the 2-ketoglutaric acid decarboxylase activity. In a few preliminary experiments, we have observed that the 2-ketoglutaric acid decarboxylase activity of *E. coli* strains AN154 and PL2024 is actually stimulated by the presence of TPP in the enzyme incubations. These results, and those reported here, provide strong evidence for a role for the succinic semialdehyde-TPP complex in

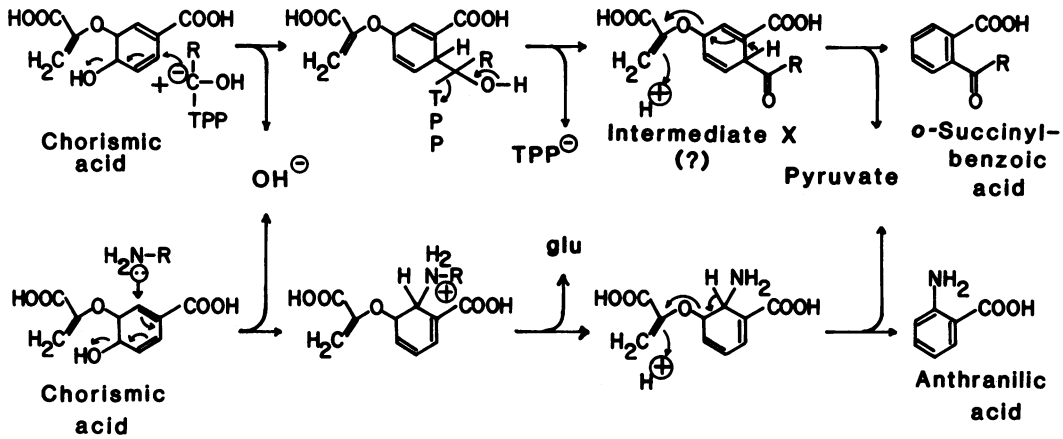


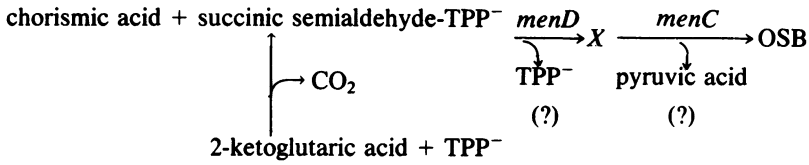
FIG. 2. Comparison of the proposed concerted addition reactions to chorismic acid involved in the biosynthesis of OSB and anthranilic acid. The addition in OSB biosynthesis is at C-2, and in anthranilic acid biosynthesis it is at C-6. The numbering used for chorismic acid identifies the carboxyl bearing carbon atom as C-1 and proceeds clockwise around the ring. This is not a standard nomenclature but is used here to provide identification with the same positions in shikimic acid. The structures in the upper line show the mechanism proposed for OSB biosynthesis; in these structures, R = HOOC—CH_2—CH_2—. A possible structure for the intermediate, *X*, assuming that it does not contain TPP, is the third structure of the upper line. In the mechanism proposed for anthranilic acid biosynthesis (14, 15) the attacking reagent is glutamine and R = HOOC—$CH(NH_2)$—CH_2—CH_2—CO—; not all of the steps involved in the removal of glutamate are shown here.

menaquinone biosynthesis. Since a coenzyme A role is known for the conversion of OSB to 1,4-dihydroxy-2-naphthoic acid, at least two cofactors are required for the biosynthesis of this important fat-soluble vitamin.

Further information concerning the chemistry of the OSB synthase system has come from our work with two groups of mutants, described as *menC* and *menD*. These groups of mutants were identified by Guest as being concerned in OSB biosynthesis (7, 8). It appears, therefore, that a minimum of two separate enzymes are required for OSB synthesis (in addition to the 2-ketoglutarate decarboxylase activity). The results reported here indicate that the gene $menD^+$ codes for an enzyme which forms a thus far unidentified intermediate, *X*, from the reaction of chorismic acid, 2-ketoglutaric acid, and TPP. Furthermore, the enzyme coded by the gene $menC^+$ converts *X* into OSB. Several distinct operations are involved in the overall conversion: chorismic acid + succinic semialdehyde-TPP^- → OSB + TPP^- + pyruvic acid + water. They include the initial anion addition, removal of TPP^-, removal of the pyruvoyl portion of chorismic acid, and removal of the hydroxyl group of chorismic acid. If only two enzymes are involved, some of these steps must be concerted (or spontaneous). One attractive possibility is to postulate attack of the anion on chorismic acid concerted with the loss of the OH group. This reaction is illustrated in Fig. 2. After anion addition, loss of TPP^- and removal of the pyruvoyl group are needed. At present we have no evidence concerning the sequence in which any of the proposed steps is carried out. In other TPP reactions, the TPP is generally regenerated at an early stage so we are inclined to think that this process may precede pyruvate elimination. As to the structure of *X*, the extractability with ethyl acetate suggests that it does not contain TPP. One possible structure for *X* is identified in Fig. 2. An alternative for OSB synthesis, with pyruvate elimination preceding OH group removal, has been diagrammed (12). It will be necessary to obtain considerable information concerning the structure of *X* before definite conclusions concerning the enzymology are possible. It is, however, of considerable interest that a rather similar concerted reaction mechanism was proposed for the addition of glutamine to chorismic acid, a process which provides a branch point for the formation of anthranilic acid by the action of anthranilate synthetase (14, 15). In this case, the addition is at position 6 of the chorismic acid molecule and there is, apparently, no evidence as yet for the actual existence of the postulated intermediate.

From the results reported in this paper, it is apparent that the general outline of the synthesis of OSB, and hence the beginning of the menaquinone biosynthetic pathway, can be represent-

ed as shown below:



Most of the present uncertainty attaches to the release of TPP and pyruvic acid.

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