VITAMIN K BIOSYNTHESIS IN BACTERIA—PRECURSORS, INTERMEDIATES, ENZYMES, AND GENES[1]

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INTRODUCTION

Although it has been known for almost 50 years that a fat soluble material, vitamin K, is an essential component of the blood clotting process in animals, knowledge of its role at the metabolic level has been gained only slowly. In 1974, a new amino acid, γ-carboxyglutamate (gla), was discovered in prothrombin; this material is now known to be formed by a post-translational carboxylation of glutamate (glu) residues, a reaction which is dependent on the presence of vitamin K (for reviews, see 1,2). The precise mechanism of the "vitamin K dependent carboxylase" enzyme (glu+CO_2→gla) is under intensive investigation at the present time since this is a rather unique reaction. Investigations of vitamin K biosynthesis have also proceeded slowly, especially in comparison with studies of the biosynthesis of other fat soluble vitamins. As with the functional role of this vitamin, however, rather surprising results have now been obtained.

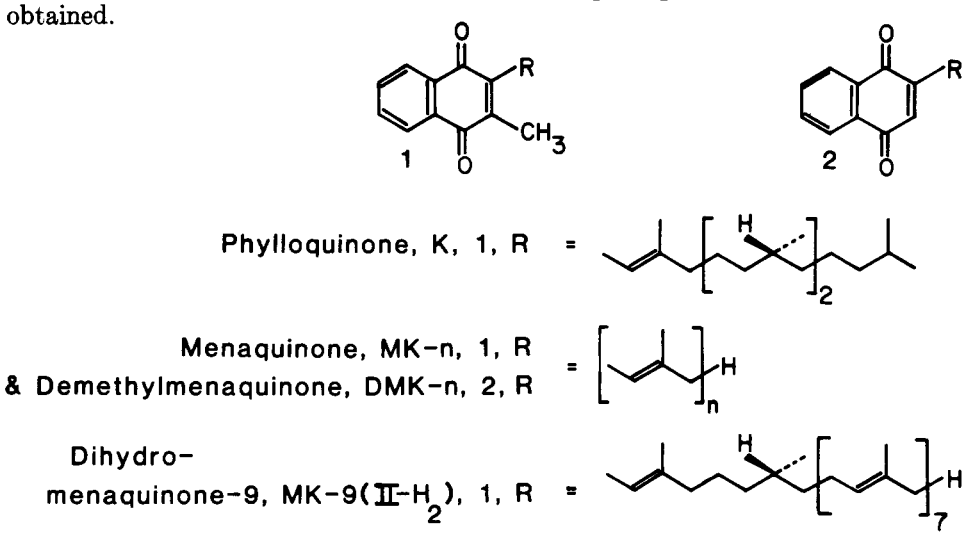

FIGURE 1. The naturally occurring forms of vitamin K. The side chain component in phylloquinone is a phytyl residue. The dihydromenaquinone-9 is used to illustrate one possibility for side chain hydrogenation in a menaquinone structure. Other positions can be hydrogenated, and more than two extra hydrogens are present in some cases.

Vitamin K occurs naturally in three major forms (see fig. 1). In green plants, the material is vitamin K_1, 2-methyl-3-phytyl-1,4-naphthoquinone (**1**, R=phytyl); the recommended name for vitamin K_1 is phylloquinone and the abbreviation, K. Bacteria usually contain a similar methyl 1,4-naphthoquinone, except that the phytyl side chain is replaced by an isoprenoid chain of varying length; these materials, originally termed vitamin K_2, are now called menaquinones (*me*thyl *na*phtho*quinone*) and are abbreviated as MK (**1**, R=polyprenyl). A numeral is

[1]Presented as a plenary lecture at the "Topics in Biotransformation and Metabolism" Symposium of the 23rd Annual Meeting of the American Society of Pharmacognosy at the University of Pittsburgh, Pittsburgh, Pennsylvania, August 1-5, 1982.

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used to indicate the number of isoprene residues; for example, the bacterium *Escherichia coli* contains MK-8, with 8 isoprene residues and a total of 40 carbon atoms in this side chain. Another bacterial form of vitamin K is a demethylmenaquinone, abbreviated as DMK (**2**, R = polyprenyl). The actual quinone pattern in any given bacterium can be quite complex. Many bacteria (*E. coli* is an example) contain benzoquinones (ubiquinones) as well as menaquinones, and some contain only DMK and no MK. The DMK and MK components are rarely homogeneous, and a typical bacterium will contain several prenylogs of varying chain length. An extreme case is *Staphylococcus aureus*, which is stated to contain MK-0 through MK-9 (3). Another possibility is that one or more of the isoprenoid groups in the side chain will have been reduced. For example, the major quinone component in *Mycobacterium phlei* is MK-9 in which the second isoprene unit, counting from the ring, has been reduced. This material is described as MK-9 (II-H_2). *M. phlei*, unlike *E. coli*, contains no ubiquinone component. An extensive compilation of the menaquinone components of bacteria is available (4).

The general outline of the pathway for vitamin K biosynthesis is now well understood, particularly for menaquinone formation in bacteria. The pathway for phylloquinone biosynthesis in green plants appears to be generally similar to that for menaquinone formation but has received less attention. The work described here will be concerned exclusively with menaquinone biosynthesis in bacteria, and most attention will be given to our own studies of the formation of the naphthoquinone nucleus. Other work will be mentioned briefly to give as complete a picture as possible. A comprehensive review of menaquinone biosynthesis is available (5).

THE FUNDAMENTAL PRECURSORS

Conventional isotope tracer studies were used to determine the fundamental precursors used in menaquinone biosynthesis. From the beginning, it had always appeared, on comparative grounds, that the two side chains of the menaquinone structure would be derived from *S*-adenosylmethionine (the methyl group) and from mevalonate (the isoprenoid side chain). Feeding experiments with labeled methionine confirmed that this material was the immediate precursor of the methyl group in a variety of bacteria (5). Similarly, the utilization of [2-^{14}C]- and [5-^{3}H]mevalonate, predominantly (about 90%) for the isoprenoid side chains of menaquinone components of *S. aureus*, has been demonstrated (3).

The major question that had to be solved by tracer studies was the origin of the aromatic naphthoquinonoid nucleus. Although many simple naphthoquinone components, particularly in fungi, are known to be formed by "acetate+polymalonate" pathways, this pathway is relatively rare in bacteria (6). In 1964, Cox and Gibson administered [G-^{14}C]shikimate to *E. coli* cultures and found that both ubiquinones and menaquinones became radioactive (7). Shortly thereafter, they also demonstrated that the shikimate radioactivity was incorporated predominantly into the naphthoquinone ring system of the menaquinone (8). To obtain more precise information on the role of the shikimate carbons, we devised a chemical degradation for labeled menaquinone samples which enabled identification of the atoms of ring B separately and gave ring A atoms collectively (9,10). The details of this degradation are illustrated in fig. 2.

In our experiments, [G-^{14}C]shikimate, **3**, was administered to various bacteria, and the menaquinones were extracted and carefully purified. Incorporation (I%) and dilution values (D) were determined; these terms have the usual meanings in tracer work (11). With *E. coli*, the incorporation of radioactivity from shikimate to menaquinone (predominantly MK-8) ranged from 0.1 to 1.6%, and the D values from 290 to 5.3. For *M. phlei* and *Streptomyces albus*, lower incorporations (0.02 and 0.007%, respectively) and higher dilutions (3300 and

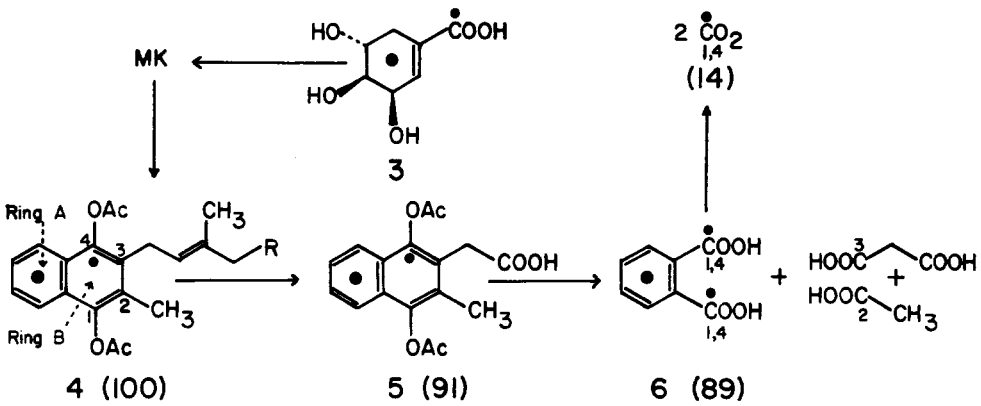


FIGURE 2. Chemical degradation of radioactive menaquinone. Following administration of [G-^{14}C]shikimate, **3**, to the bacteria, MK was isolated, carefully purified, and converted to **4** by reductive acetylation. (Only the first isoprene residue is indicated in this structure, with R representing the remainder of the polyprenyl side chain). Treatment of **4** with OsO_4/HIO_4 yielded the naphthaleneacetate derivative, **5**. Phthalate, **6**, was obtained from **5** by reaction with H_2O_2; in this reaetion, malonate and acetate were also produced. Phthalate was decarboxylated by the Schmidt degradation. Radioactivity is indicated by ● and this same symbol within a six-membered ring implies that all ring positions are labeled. The relative specific activity of **4** is set at 100; this value, and those of other degradation products are given in parentheses. The value of 14 given for the CO_2 relative specific activity is for two molecules of this product. The numbered atoms in the final degradation products refer to the original numbers of the naphthoquinone ring system.

16,000, respectively) were obtained. For *E. coli*, at least, shikimate would be considered an excellent precursor by the usual criteria.

In a typical degradation of the menaquinone from such an incorporation experiment with *E. coli*, 91% of the total radioactivity present in the menaquinol diacetate, **4**, was found in the 1,4-diacetoxy-3-methyl-2-naphthaleneacetate, **5**. Essentially the same activity was present on further degradation to phthalate, **6**, which contains ring A and C–1 and C–4 of ring B; malonate and acetate, containing C–3 and C–2 of ring B, were inactive. Decarboxylation of the phthalate showed that 14% of the ^{14}C was located in the two carboxyl groups. Thus, in the phthalate degradation product, the ratio of activity, ring:carboxyl was 86:14 (6.1%). In the precursor material, [G-^{14}C]shikimate, this same ratio was found to be 5.9 or 6.6% by two different decarboxylation methods. Hence, it could be concluded that shikimate was utilized as a C_6-C_1 unit and provided the whole of the menaquinone A ring and one of the quinonoid carbon atoms.

Similar results were obtained in tracer experiments with other bacteria. In some cases, however, a surprising amount of ^{14}C from [G-^{14}C]shikimate was actually located in the isoprenoid side chain. With *M. phlei*, for example, the naphthaleneacetate derivative, **5**, contained only 71% of the menaquinol diacetate activity (cf. 91% for *E. coli*). With this bacterium and MK–9 (II–H_2) for dilution purposes, it was possible to obtain levulinic acid (7 moles per mole MK–9 (II–H_2)) as a product of the chemical degradation. The levulinate contained in total about 25% of the menaquinol diacetate activity; the actual activity in the side chain is probably somewhat higher, since a ten carbon unit and the terminal acetone unit were not recovered in the degradation. It seems probable that in this organism there must be some breakdown of shikimate to acetate; activity from acetate is then incorporated into the side chain by way of mevalonate. Despite the substantial level of ^{14}C in the isoprenoid side chain in the MK–9 (II–H_2) from *M. phlei*, the radioactivity in the naphthaleneacetate degradation product was distributed exactly as in the case of *E. coli* (9,10).

Our work established the utilization of all seven carbon atoms of shikimate in MK biosynthesis but did not pinpoint whether the shikimate carboxyl became C_1 or C_4 (or both) of the B ring of the naphthoquinone nucleus (the degradation product, phthalate, is a symmetrical compound). Rapoport and his colleagues devised an ingenious chemical degradation so that C_1 and C_4 of the naphthoquinone ring could be obtained as separate chemical entities. They established unequivocally that carboxyl labeled shikimate, when administered to *M. phlei*, provided only C_4 of the menaquinone (12). Another refinement was the demonstration that the *pro-R* hydrogen at position 6 of shikimate was eliminated in MK biosynthesis (13). As a result of these studies, the utilization of shikimate can be represented as shown in fig. 3.

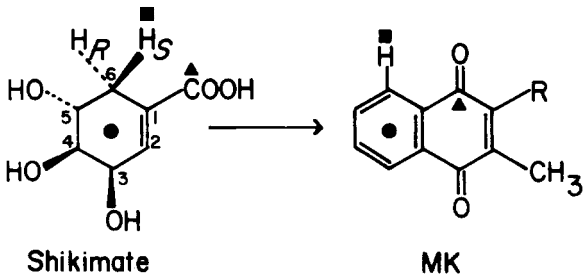


FIGURE 3. The utilization of shikimate for menaquinone biosynthesis. The symbol, ●, within the six membered ring indicates that all positions are labeled. "Chemical numbering" is used for the shikimate molecule. R indicates a polyprenyl residue.

Since seven atoms of the naphthoquinone nucleus were derived from shikimate, we undertook a search for the origin of the "missing three carbon unit". Likely candidates such as pyruvate, malonate, and glycerol were only poorly utilized by *E. coli* and *M. phlei* (I values ranged from 0.0001 to 0.003%). The amino acids with three carbon atoms, alanine and serine, were also incorporated into menaquinones with rather low efficiency (I values from 0.005 to 0.01%) but with rather low dilution (D values from 1.1 to 1.4). Experiments with the two-carbon unit, acetate, showed as expected that activity was incorporated into the isoprenoid side chain. There was also some tendency for [1-^{14}C]acetate to label C_1 and/or C_4 of ring B, and for [2-^{14}C]acetate to label C_2 and/or C_3.

Although inconclusive, these results tended to indicate a tricarboxylic acid cycle intermediate as the source of the three carbon unit. To test this possibility, Campbell used a simpler system than that for MK biosynthesis (14). It was known that in the biosynthesis of lawsone, 2-hydroxy-1,4-naphthoquinone (**8**), by the plant *Impatiens balsamina*, shikimate was utilized exactly as for menaquinone biosynthesis. Campbell therefore administered [1-^{14}C]- and [U-^{14}C]alanine, [U-^{14}C]aspartate, and [2-^{14}C]glutamate to shoots of this plant, isolated lawsone, and degraded it to phthalate (fig. 4). It was assumed that the amino acids would be deaminated, respectively, to pyruvate, oxaloacetate, and 2-ketoglutarate.

The first three of these precursors showed I values from 0.03 to 0.33%, while the incorporation with [2-^{14}C]glutamate was substantially higher (1.36%). In the chemical degradation it was found that [1-^{14}C]alanine gave a rather random distribution of radioactivity in lawsone. With [U-^{14}C]alanine, there was a substantial labeling at C–2 and/or C–3 (73% of total lawsone activity) and with [U-^{14}C]aspartate at C–1 and/or C–4 (46%), as well as C–2 and C–3 (35%). In contrast, in the experiment with [2-^{14}C]glutamate, a very specific incorporation occurred with 99% of the lawsone activity being at C–1 and/or C–4. From this very significant observation, Campbell was able to conclude that the "missing three carbon unit" was derived from the three central carbon atoms of glutamate

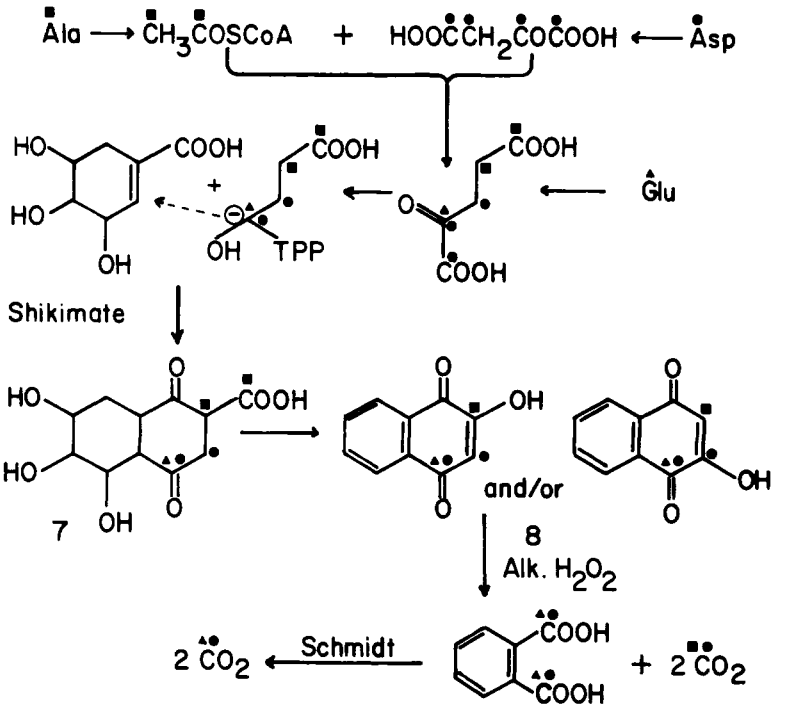


FIGURE 4. Amino acid utilization in lawsone biosynthesis. [U-^{14}C]Alanine is represented as ■ Ala, [U-^{14}C]aspartate as ● Asp, and [2-^{14}C]glutamate as ▲ Glu; TPP=thiamin pyrophosphate. In this figure, 2-ketoglutarate is assumed to be formed through the usual reactions of the tricarboxylic acid cycle (containing activity represented by ■ and ●) or by the deamination of glutamate (containing activity represented by ▲). The role of the thiamin pyrophosphate complex of succinic semialdehyde is more fully discussed under "Enzymes".

or, more likely, 2-ketoglutarate. The two carboxyl groups of 2-ketoglutarate were removed by decarboxylations at some stage.

A possible mechanism for utilization of 2-ketoglutarate by way of the thiamin pyrophosphate anion of succinic semialdehyde was suggested by Campbell (14) and is indicated in fig. 4. The detailed mechanism for the process, including evidence for the role of the proposed anion, is discussed under "Enzymes". The trihydroxydecalindione carboxylic acid, **7**, shown as a possible intermediate, is no longer considered to be involved.

A role for glutamate and/or 2-ketoglutarate was also demonstrated in bacterial systems (10,15). Glutamate was incorporated into MK by *E. coli*, *M. phlei*, *Corynebacterium diphtheriae* and *Streptomyces albus* with good I values (from 0.002 to 0.02%) and little dilution. In *E. coli*, which also contains ubiquinone, Q, the ratio of activity, MK:Q was high (20:1, 35:1) in two experiments; compare, for example, ratios of about 1.1:1 for acetate feeding. This was consistent with the known biosynthesis of ubiquinone *via* *p*-hydroxybenzoate, a process in which neither glutamate nor 2-ketoglutarate are involved. Chemical degradations showed that the use of [U-^{14}C]glutamate gave approximately equal labeling in C–1 (and/or C–4), C–2, and C–3. With [2-^{14}C]glutamate, the menaquinone samples from *E. coli* and *C. diphtheriae* were found to be labeled at C–1 and/or C–4, but there was no label in C–2 and C–3. Hence there was a very specific utilization of the C–2 atom of glutamate for one of the quinone carbonyl positions. In a feeding of [U-^{14}C]-2-ketoglutarate to *E. coli*, we found an incorporation (I=0.011%) similar to that for [U-^{14}C]glutamate (I=0.015%) (16). The ratio of activity MK:Q was 24:1, also comparable to that found in the glutamate experi-

ments. Chemical degradation established that the [U-^{14}C]-2-ketoglutarate contributed activity essentially equally to C–1 and/or C–4, C–2 and C–3 (16.3%, 17.0% and 14.6%, respectively).

To summarize, the basic precursors of the menaquinone nucleus can be considered to be shikimate, 2-ketoglutarate, mevalonate, and *S*-adenosyl-methionine, as shown in fig. 5.

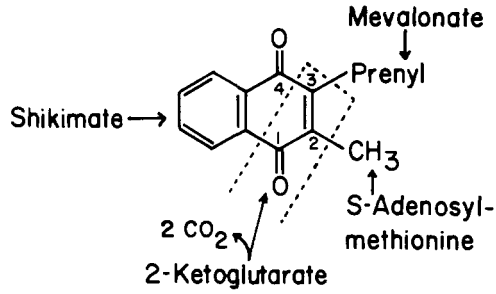


FIGURE 5. The basic precursors for menaquinone biosynthesis.

INTERMEDIATES

Several lines of evidence had indicated that any intermediates in menaquinone biosynthesis had to be non-symmetrical (5), and two such aromatic compounds have been identified. In 1970, Dansette and Azerad (17) postulated a role for the eleven carbon benzenoid compound, *o*-succinylbenzoate, OSB (**10**). They synthesized a ^{14}C labeled material and found that it was, indeed, an excellent precursor for bacterial menaquinones and for some other plant naphthoquinones and anthraquinones. We confirmed their results for bacterial menaquinone biosynthesis and were also able to show that when OSB was labeled in the side chain carboxyl group (*i.e.*, [1-^{14}C]-4-(2'-carboxyphenyl)-4-oxobutyrate) no activity was incorporated into menaquinones (10). Hence this carboxyl group, which is derived from the number 5 carboxyl group of ketoglutarate, is lost during menaquinone biosynthesis (see fig. 6).

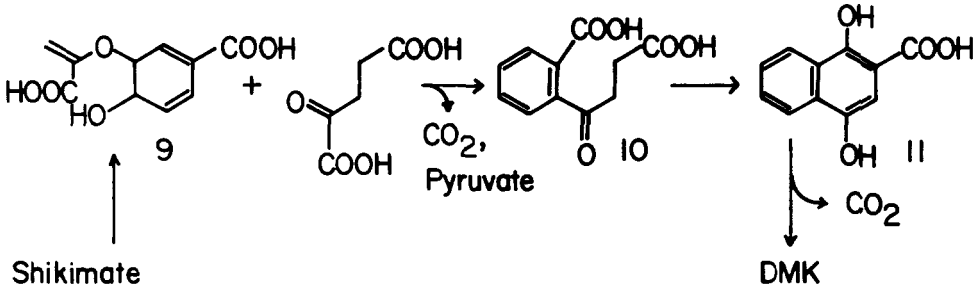


FIGURE 6. Intermediates in menaquinone biosynthesis. The conversion of shikimate to chorismate presumably involves the usual enzymes of the "shikimate pathway".

Robins *et al.* (15) suggested a second aromatic intermediate, 1,4-dihydroxy-2-naphthoate, DHNA (**11**). The first evidence that it had a role in menaquinone biosynthesis was the demonstration that it could substitute for the usual vitamin K requirement of the bacterium *Bacteroides melaninogenicus* (18). Furthermore, an excellent conversion of labeled DHNA to menaquinone was obtained in work with anaerobic cultures of *E. coli* (19); anaerobic conditions were used to minimize the oxidation of DHNA.

There is now extensive evidence confirming the roles of OSB and DHNA in menaquinone biosynthesis (5), and these two aromatic materials are accepted as major intermediates. Their position in the biosynthetic pathway is shown in Fig. 6; this figure also indicates a role for chorismate, **9**, which will be discussed more fully under "Enzymes". More recently, at least one other intermediate, a derivative of OSB, has been characterized; there may be others involved in the steps leading to the formation of OSB. These possibilities are described in connection with the enzymology of the individual reactions.

While there has been considerable discussion of possible roles for materials such as naphthoquinone, 2-methylnaphthoquinone, and naphthol, these materials are not now considered to be intermediates. One of the difficulties encountered in all of the biosynthetic work was the purification of menaquinone samples. Despite conclusive evidence that menaquinone samples, isolated and purified by chromatographic techniques, are frequently contaminated with fatty acid esters and aromatic acid esters, completely rigorous purification of labeled menaquinone samples has frequently been neglected. Some of the claims that materials such as naphthol are intermediates must be attributed to these contamination problems. In our own work, purification on columns of (lipophilic) Sephadex LH–20 and LH–50 was found to be particularly suitable to avoid the presence of contaminants.

A further difficulty in all of this work was the very small amounts of MK which could be isolated from cultures of a reasonable size. It was also a discouraging fact that the more interesting naphthoquinone nucleus represents only about 20% of the isolated menaquinone.

ENZYMES

Our most recent work has been concerned with the enzymes carrying out individual reactions in the biosynthetic sequence. It is convenient to note at the beginning that other investigators have studied a methylase system (20–22), responsible for transferring a methyl group from *S*-adenosylmethionine; a prenyltransferase system (23,24), responsible for transferring the polyisoprenoid side chain from a pyrophosphate precursor; and a reductase (20), responsible for saturating one or more double bonds in the polyisoprenoid side chain.

Formation of OSB

Cox and Gibson had originally suggested that conversion of shikimate to chorismate (**9**, fig. 6) was required for menaquinone biosynthesis. That chorismate indeed provides the "branch point" was confirmed by the use of bacterial mutants blocked after shikimate; such mutants did not form menaquinones. More detailed information has now come from our studies of an enzyme system which actually produces OSB from chorismate and 2-ketoglutarate (25). To provide a sensitive assay for OSB formation, radio glc has been used. Following incubation of chorismate and [U-^{14}C]2-ketoglutarate with a cell-free extract, the reaction was terminated with acid and a little "cold" OSB added as carrier. After extraction of OSB from the incubation mixture with ethyl acetate and removal of the solvent, the residue was subjected to thin layer chromatography on Anasil OF plates (Chloroform:methanol:acetic acid, 70:30:5). The OSB was located by a blue color produced on exposure to 254 nm uv light. The OSB was eluted from the plates, converted to a dimethyl derivative by exposure to diazomethane, and then examined by radio glc.

In the early experiments, *E. coli* strain AN 154 was used as the source of enzyme extracts (obtained by the use of a French pressure cell). This mutant was chosen since it is blocked in all of the aromatic biosynthetic pathways except that for menaquinone. It was hoped that the enzyme would be more readily obtained since the only possible way in which chorismate could be utilized was for mena-

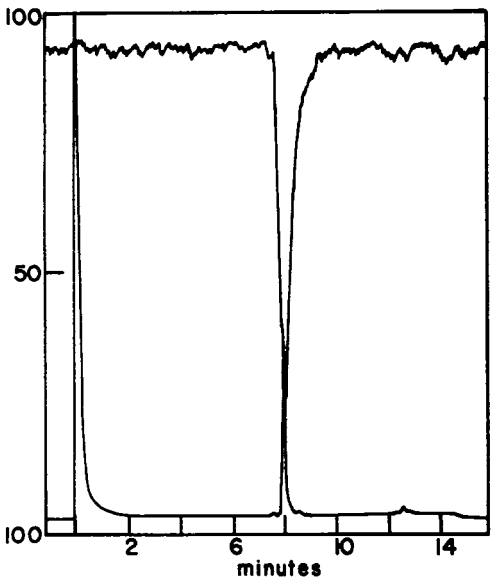


FIGURE 7. Enzymatic synthesis of OSB in cell-free extracts of *E. coli* PL2024. The incubation mixture contained the following components (amounts are given as μmoles); 5 μCi of [U-^{14}C]-2-ketoglutarate, 0.24; chorismate, 1.4; and TPP, 0.11. The amount of extract was 0.5 ml and the total volume was brought to 3.0 ml with 0.1 M glycylglycine buffer, pH 8.5. Following incubation at 30° for 30 min, the reaction was terminated with acid and the mixture was extracted with ethyl acetate as previously described (25). The OSB was purified by thin layer chromatography on Anasil OF plates; the solvent was chloroform:methanol:acetic acid, 70:30:5. The recovered OSB was converted to the dimethyl derivative (diazomethane) and was then examined by radio glc. The two tracings shown were recorded simultaneously; the upper trace is from the proportional counter component (full scale deflection = 1000 cpm) and the lower trace is from the flame ionization detector (sensitivity setting, 8 x 10^{-11} amps).

quinone biosynthesis. Following the demonstration that *E. coli* AN 154 extracts did produce OSB (25), it has now proved possible to show the same process in wild type *E. coli* extracts. A typical result is shown in fig. 7. The extract from *E. coli* PL 2024 was incubated with chorismate and [U-^{14}C]2-ketoglutarate; a mass peak with the same retention time as that of standard dimethyl OSB coincides exactly with a peak of radioactivity as recorded by the proportional counter. In control experiments, it was shown that the radioactivity peak was not produced in the absence of chorismate. An important observation was that no radioactive dimethyl OSB was produced when [1-^{14}C]2-ketoglutarate was used as a substrate. This observation is, of course, consistent with the observed loss of this carboxyl group during *in vivo* studies of OSB biosynthesis (26). In other experiments it has been shown that [U-^{14}C]shikimate is also converted to OSB due to the presence of the necessary enzymes for the synthesis of chorismate from shikimate.

Campbell had originally proposed that shikimate reacted with the thiamin pyrophosphate—succinic semialdehyde anion in a Michael addition process (14); an analogous reaction can be postulated for chorismate. The TPP-succinic semialdehyde complex could be derived from 2-ketoglutarate and TPP by the action of the first (decarboxylase) enzyme of the 2-ketoglutarate dehydrogenase complex, or by a separate but similar enzyme. It was gratifying to find that the enzymatic synthesis of OSB by our extracts was stimulated by the addition of TPP to the incubation mixtures. By chance, *E. coli* AN 154 is a thiamin auxotroph; in our work, growth was possible on a trypticase soy broth medium without further addition of thiamin; the trypticase soy broth presumably contained sufficient thiamin (in fact, we have obtained evidence that different batches of this medium vary in thiamin content). Routine trypticase soy broth growth

TABLE 1. Stimulation of OSB synthesis in *E. coli* extracts by the presence of thiamin pyrophosphate (TPP).

Organism and growth condition	OSB Formation[a]	
	With TPP	Without TPP
	pmoles/30 min/mg protein	
AN154, grown without thiamin	62.4	0[b]
AN154, grown with thiamin	66.7	23.6
Wild type, PL2024[c]	25.4	6.1

[a]OSB synthesis was assayed by radio glc with [U-^{14}C]-2-ketoglutarate as the substrate (25).

[b]In this particular case, zero formation of OSB occurred. However, in other experiments, variable results were obtained, and in some cases OSB formation was observed; the amount produced was never more than 10% of that of the complete system with added TPP.

[c]This strain, unlike AN154, is not a thiamin auxotroph.

medium was used; the formation of OSB was considerably stimulated on the addition of thiamin pyrophosphate to the incubations (see table 1). Addition of thiamin to the growth medium gave a small stimulation of enzymatic activity, about 2.5 fold. The "OSB synthase" activity has also been obtained in extracts of wild-type *E. coli* (not auxotrophic for thiamin). Addition of TPP during incubation of extracts from the wild type cells gave a 4-fold stimulation of activity. Thus, there is good evidence that TPP is, indeed, involved in the biosynthesis of OSB. The greatest stimulation was observed when TPP was added to extracts of the thiamin auxotrophic strain, which had been grown in trypticase soy broth with no further additions, *i.e.*, under conditions where the cell presumably had only minimal amounts of thiamin available.

In addition to this evidence for a role for TPP in OSB synthesis, we have obtained preliminary evidence that the 2-ketoglutarate decarboxylase activity of *E. coli* is also stimulated by TPP as measured by use of the ferricyanide reduction assay. Furthermore, extracts of a 2-ketoglutarate decarboxylase negative mutant of *E. coli*, *suc* A72 (27) tend to show a reduced incorporation of activity from [U-^{14}C]2-ketoglutarate into OSB. That some OSB synthesis does occur in these extracts is to be attributed to the known leakiness of this mutant.

The chemistry of the synthesis of OSB from chorismate and 2-ketoglutarate is clearly complex. Several enzyme activities and possibly one or more intermediates are presumably involved. This question will be discussed in more detail in connection with the use of *men* mutants of *E. coli*.

Conversion of OSB to DHNA

The first enzyme system to be obtained (in 1975) which was concerned in the biosynthesis of the aromatic nucleus was one converting OSB to DHNA (22); this conversion was demonstrated by the use of radioactive OSB. Active enzyme extracts were prepared from *E. coli* by use of the French press (22) or from *M. phlei* by sonication (28). In the presence of a suitable side chain donor, these extracts also converted OSB to menaquinones, thus implying the presence of a methyl and prenyl transferase. An important observation was that the conversion of OSB to DHNA was a direct one; *i.e.*, there was no breakdown of OSB to smaller molecules such as acetate, followed by resynthesis in some way. For proof of the direct conversion, [1-^{13}C]OSB was prepared and was converted to DHNA by the enzyme system of *Mycobacterium phlei* (fig. 8). An intact conversion process would yield DHNA containing a single ^{13}C atom in the carboxyl group. A detailed

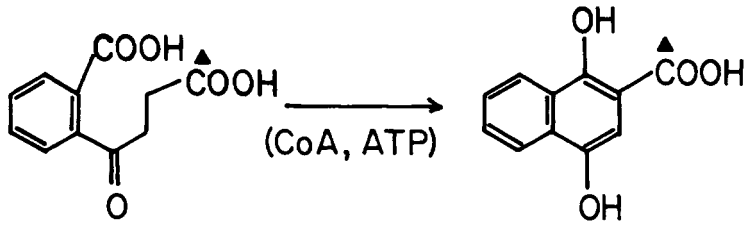
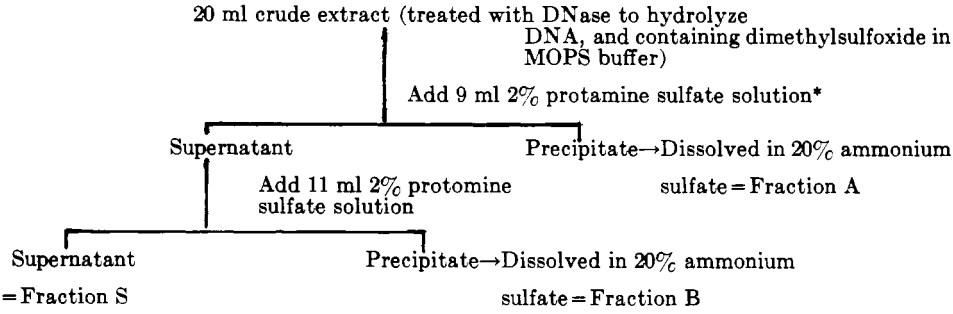


FIGURE 8. The direct conversion of OSB, **10**, to DHNA, **11**. Isotope from [1-^{13}C] OSB is shown as ▲. This labeled substrate was incubated with an enzyme extract from *M. phlei*.

analysis of the mass spectrum of the product DHNA, derivatized to methyl-4-methoxy-1-hydroxy-2-naphthoate, showed that this was, indeed, the case. In particular, there was a metastable ion at m/z 147.9 (200→172, loss of CO) in the mass spectrum of methyl-4-methoxy-1-hydroxy-2-naphthoate of the normal material and at m/z 147.18 (201→172, loss of ^{13}CO) in the ^{13}C enriched material.

It was of considerable interest that these systems producing DHNA from OSB were found to be dependent on the presence of coenzyme A (CoA) and ATP (22). This suggested the possible existence of an OSB–CoA compound as an intermediate with the involvement of two separate enzymes—one to form the intermediate, and the other to complete the cyclization to the naphthalenoid compound. In 1979, it was shown that two enzyme activities could, in fact, be separated from *M. phlei* extracts by the use of protamine sulfate (29). In these experiments, 20% dimethylsulfoxide had originally been included in the hope of increasing stability. However, it developed that the presence of this material was essential to obtain the fractionation. The protocol finally adopted is shown below.



*The exact amount of protamine sulfate solution required varies from batch to batch; with a new batch of protamine sulfate, small scale experiments were conducted first to determine the optimum amount to be used.

Preliminary experiments showed that DHNA production from OSB occurred with a mixture of fractions B and S, but not with combinations of A and S, or A and B; none of the fractions alone gave any DHNA. Experiments were, therefore, devised to investigate the possible formation of an OSB-CoA intermediate by one of the fractions, B or S. The procedure was as follows:

a. Incubate one fraction (B or S) with OSB, CoA, and ATP for 30 min
b. Add 3N HCl to a final concentration of 0.1N HCl to denature the enzyme(s) of the first incubation
c. Return to neutral pH, add the second fraction (S or B) and incubate for 30 min
d. Analyze for DHNA production.

When fraction B was used as the source of the enzyme for the first incubation, no DHNA was obtained with any conditions (see table 2, experiments 1 and 2).

TABLE 2. DHNA synthesis by two enzyme fractions from *M. phlei*.

Experiment number	First Incubation			Second Incubation[a]		
	Fraction used	Amount mg	Reaction time min	Fraction used	Amount mg	DHNA yield nmol/tube
1	B	4.1	0[b]	S	1.23	<0.1
2	B	4.1	30[b]	S	1.23	<0.2
3	S	1.23	0[b]	B	4.1	6.4
4	S	1.23	30[b]	B	4.1	8.1
5	S	1.23	0[c]	B	4.1	0.5
6	S	1.23	30[c]	B	4.1	1.8

[a]The reaction time for the second incubation was always 30 minutes. DHNA assay was carried out by the spectrophotofluorometric assay (28).
[b]The final HCl concentration was 0.1 N.
[c]The final HCl concentration was 0.5 N.

With fraction S in the first incubation a good synthesis of DHNA was obtained (table 2, experiment 4). Surprisingly, there was also a substantial DHNA synthesis in the control, treated with acid at zero time. This indicated that fraction S was apparently somewhat stable in 0.1N HCl. When the acid concentration used in the "denaturation" was increased to 0.5N HCl (table 2, experiment 5) DHNA synthesis could be repressed almost completely. However, this denaturation after the first 30 minute incubation also appeared to decompose any possible intermediate since the yield of DHNA was relatively low (table 2, experiment 6). Despite these difficulties, the amount of DHNA formed when the first incubation was treated with acid at 30 min was always higher than that produced with acid treatment at zero time (table 2, compare experiment 4 with 3, and experiment 6 with 5); the differences were not large but were consistently observed. These experiments provided tentative evidence for the formation of an intermediate (**12**) by an enzyme present in fraction S and its conversion to DHNA by fraction B (see fig. 9). In addition, they suggested that the presumed intermediate had a limited stability.

Other experiments served to reinforce the conclusions. As is generally the

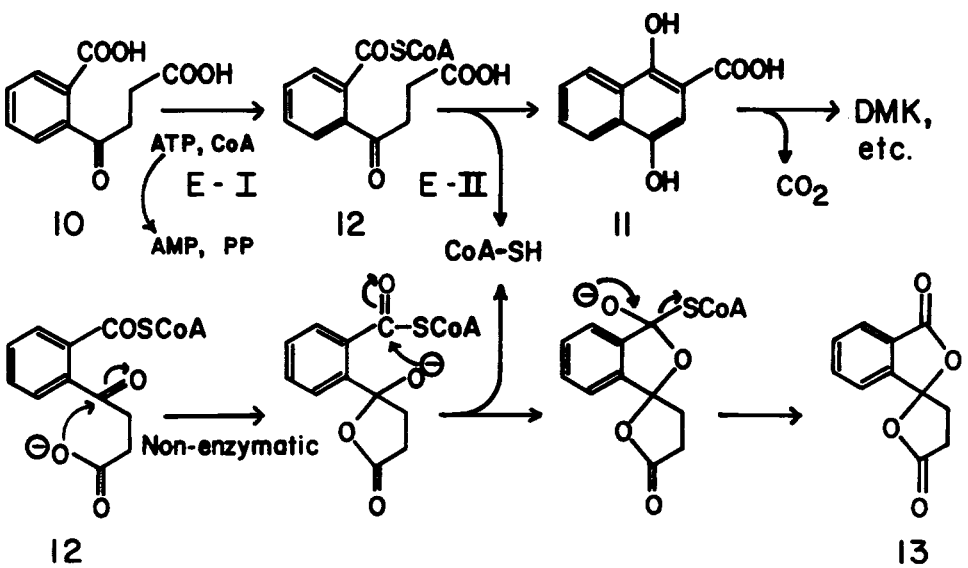


FIGURE 9. The role of OSB-CoA in the biosynthesis of DHNA and the menaquinones. E-I = OSB-CoA synthetase, E-II = DHNA synthase. On the bottom line of this figure, OSB-CoA, **12**, has been redrawn (and with loss of a proton) to illustrate the non-enzymatic conversion of this intermediate to the spirodilactone form of OSB, **13**.

case for the formation of acyl CoA derivatives, fraction S (but not fraction B) reacted with OSB, CoA and ATP, with the formation of the characteristic product, AMP. This suggested the reaction was as follows: OSB+CoA-SH+ATP→ OSB-S-CoA+AMP+PP. Furthermore, when fraction S was incubated with a radioactive OSB, CoA, and ATP, the formation of a cyclized dilactone form of OSB (**13**) was demonstrated by scanning of thin layer chromatograms. This "spirodilactone" formation could readily be accounted for by assuming it to be a non-enzymatic decomposition product of the OSB-CoA intermediate (*i.e.*, OSB-S-CoA→OSB-spirodilactone+CoA-SH). In the presence of fraction B, the formation of spirodilactone was repressed.

All of these observations were consistent with the hypothesis that fraction S contained an OSB-CoA synthetase activity, while fraction B contained a cyclizing enzyme, DHNA synthase (see below and fig. 9).

$$\text{OSB+CoA-SH} \xrightarrow[\text{ATP} \swarrow \searrow \text{AMP+PP}]{\text{OSB-CoA synthetase (Fraction S)}} \text{OSB-S-CoA} \xrightarrow{\text{DHNA synthase (Fraction B)}} \text{DHNA+CoA-SH}$$

More recently, Leistner and his colleagues have confirmed the separability of the two enzymes from *M. phlei*, have isolated the OSB-CoA derivative, and determined that the two components were present in a 1:1 ratio (30,31). To determine the precise location of the CoA moiety, the OSB-CoA derivative was methylated with diazomethane, forming the methyl ester, **14**. This ester was subjected to a mild hydrolysis to cleave the thioester bond and yield the mono ester, **15**, R=H. The product of these treatments could have contained a methyl group on either the "aromatic" or "aliphatic" carboxy group. Mass spectrometric data indicated that it was located on the "aliphatic" position; hence, in OSB-CoA, the CoA moiety is located on the aromatic carboxyl (**12**) as had originally been suggested (see fig. 10). In similar experiments, we have converted the same monomethyl ester (**14**) to a mixed methyl ethyl ester (**15**, R=C_2H_5) by further reaction with diazoethane. This material was identified as having the structure, **15**, R=C_2H_5, by combined glc-mass spectrometry. This result agrees with that of Leistner and his colleagues.

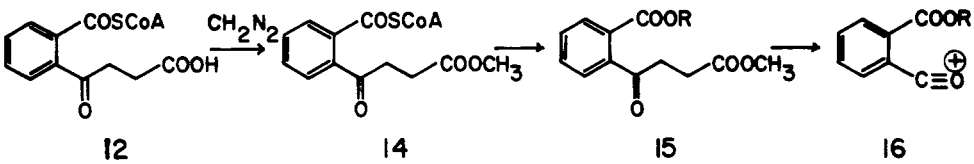


FIGURE 10. Location of the CoA residue in OSB-CoA. The methyl derivative of OSB-CoA, **14**, was subjected to a mild hydrolysis to cleave the thioester bond. In the work of Leistner and his colleagues (32), the resulting product, **15**, R=H, was examined directly. In our own work, the hydrolysis product was realkylated with diazoethane to **15**, R=C_2H_5 for gc/ms. On mass spectrometry, **15**, R=H, yields an ion, **16**, R=H, m/z=149; for **15**, R=C_2H_5, the ion, **16**, R=C_2H_5, has m/z=177.

We have now demonstrated the presence of the two enzymes for the conversion, OSB→DHNA, in *B. subtilis*, *E. coli*, and *Micrococcus luteus*. There are, however, significant differences in the extracts prepared from these various microorganisms. With *M. phlei*, incubation of crude, unfractionated enzyme extracts with [2,3-$^{14}C_2$] OSB results in the production of only DHNA but never the OSB-spirodilactone (this material can be shown to be formed using Fraction S by itself). With *E. coli* extracts, spirodilactone formation was sometimes seen, and, with *Micrococcus luteus* and *B. subtilis*, the spirodilactone was always produced. These differing results suggest one of two explanations. The ratio of activities, OSB-CoA synthetase:DHNA synthase, is not optimal, so that the amount of DHNA synthase is inadequate to cope with all of the OSB-CoA which is produced. Alternatively,

the DHNA synthases in different bacteria may have different stabilities and, in some cases, activity is lost during extraction and manipulation. Evidence supporting the latter possibility is the fact that the two enzymes of *E. coli* cannot be separated by the protamine sulfate treatment. From such experiments, active OSB-CoA synthetase can be obtained, but DHNA synthase activity is lost.

The *E. coli* system shows another difference—the OSB-CoA synthetase in this organism does not resist the mild acid treatment (e.g., 0.1N HCl, 5 min) under which the same enzyme from *Micrococcus luteus*, *M. phlei*, and *B. subtilis* retains activity.

GENETICS

Several bacterial mutants have been described which are defective in the menaquinone biosynthetic pathway (5). The menaquinone genes are referred to as *men* and some of the mutants, particularly in *B. subtilis* and *E. coli*, have been analyzed by genetic methods (32,33). In addition to these mutants, a few bacteria have been found to have a growth requirement for vitamin K, even though these are "wild type" strains and not selected mutants. The classical example of a bacterium deficient in menaquinone biosynthesis is the strict anaerobe, *Bacteroides melaninogenicus*; however, not all natural isolates of this organism show a vitamin K requirement.

For the conversion of chorismate to demethylmenaquinone, five separate genes are involved; one of these, termed *menA*, is concerned in the prenylation reaction, DHNA→DMK (23,34). These mutants actually accumulate DHNA during growth. They were obtained by treatment of *E. coli* with mutagenic agents, and those mutants unable to grow on succinate as sole carbon source were selected (actually a fortuitous selection procedure). Another group of mutants obtained in this work was originally termed *menB*; they were found to be blocked in the conversion of OSB to DHNA. More recently, these mutants have been shown to consist of two groups, now termed *menE* and *menB*. Guest identified two further groups of *E. coli* mutants which had been selected for their inability to use fumarate as a terminal electron acceptor; these mutants, termed *menC* and *menD*, showed a nutritional requirement for OSB (or DHNA) (35,36). In addition to the *E. coli* mutants, Taber and his colleagues have obtained *B. subtilis men* mutants by treatment with nitrosoguanidine and selection for simultaneous resistance to two aminoglycoside antibiotics (32). This selection method derives from the fact that quinones play a role in transporting the antibiotics into the bacterial cell. Mutants obtained were characterized as *menB* and *menE*, but in this organism, *menC* and *menD* mutations could not be separated genetically.

In collaboration with Drs. Guest, Shaw and Taber, we have investigated the enzyme defects in some of these mutant strains of *E. coli* and *B. subtilis*, particularly with reference to the formation of the aromatic nucleus (37,38).

Chorismate to OSB

As noted earlier, the chemistry of this conversion is obviously complex; some possible reaction schemes are shown in fig. 11. Five separate operations can be distinguished for the overall conversion of chorismate+2-ketoglutarate to OSB+pyruvate. They are as follows.

i. Formation from 2-ketoglutarate of a succinic semialdehyde-thiamin pyrophosphate complex. This process is, of course, accompanied by a decarboxylation.

ii. Addition of the succinic semialdehyde-thiamin pyrophosphate complex to chorismate

iii. Regeneration of thiamin pyrophosphate

iv. Removal of the pyruvoyl group originally associated with chorismate

v. Removal of the hydroxyl group originally associated with chorismate

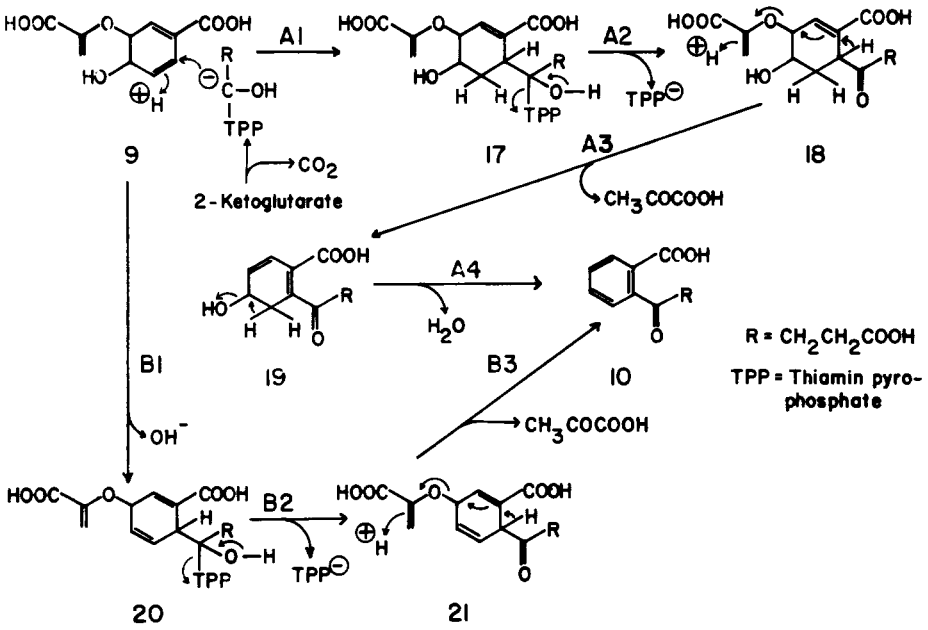
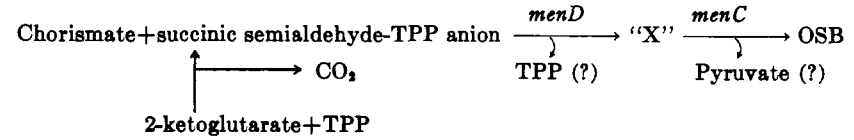


FIGURE 11. Possible mechanisms for OSB synthesis. Two separate possibilities, A and B, are represented by steps A1 to A4, and B1 to B3. In mechanism A, the initial anion addition requires acquisition of a proton. In mechanism B, the anion addition is concerted with the elimination of the hydroxy group of chorismate. Although the reactions suggested here involve regeneration of TPP before the removal of the pyruvate component, a further possibility is that TPP is retained until a much later stage.

As for the formation of the succinic semialdehyde-TPP complex, we have already noted the requirement for thiamin pyrophosphate in the enzymatic synthesis of OSB in *E. coli* extracts. The required anion can presumably be obtained by the 2-ketoglutarate decarboxylase component of the 2-ketoglutarate dehydrogenase complex or by the action of a similar, but separate, enzyme. A maximum of four further enzymes might then be required to complete the synthesis of OSB (steps A1 to A4, fig. 11). However, the fact that only two genes have been identified for this part of the menaquinone biosynthetic pathway, *menC* and *menD*, suggests that only two separate enzyme activities are involved. An attractive possibility is that the anion addition is concerted with removal of the OH group of chorismate (step B1, fig. 11). If this enzyme also catalyzed TPP removal (step B2, fig. 11), only one further enzyme would be required (step B3, fig. 11).

Our work to date has not provided a complete picture; however, preliminary investigations suggest that an intermediate is involved and that it is sufficiently stable to be isolated (39). In this work, cell-free extracts were prepared from *menC* and *menD* mutants of *E. coli*. Each extract was then subjected separately to the following sequence of operations: Incubate with chorismate and [U-^{14}C] ketoglutarate; denature protein with acid; extract the denatured incubation mixture with ethyl acetate; remove solvent. The residue obtained after solvent removal was then further incubated with an extract from the other mutant, that is, if a *menC*$^-$ mutant was used for the first incubation, the residue was further incubated with an extract from a *menD*$^-$ mutant, and *vice versa*. It was found that the *menC*$^-$ mutant yielded an extract which formed a ^{14}C labeled sample of OSB on incubation with a *menD*$^-$ extract. This was not the case when the order of incubations was reversed. It appears, therefore, that the *menD* gene codes for an enzyme which is responsible for the production of an unidentified inter-

mediate, "X". The enzyme which is coded for by the *menC* gene then is responsible for converting "X" to OSB. This situation is represented below.



The chemical nature of "X" is presently unknown, although some possible structures are **17–21** of fig. 11. It is also unknown at what stage TPP and pyruvate are removed; the fact that "X" can be extracted with ethyl acetate possibly suggests, however, that it does not contain TPP. In this case, possible "X" structures are **18**, **19**, and **21**.

OSB→DHNA

Considerably more information is available concerning this portion of the menaquinone biosynthetic pathway; it has been possible to link the *menB* and *menE* genes with the two enzymes described earlier, OSB-CoA synthetase and DHNA synthase. This was achieved first with mutant strains of *B. subtilis* (37) and more recently with *E. coli* mutants (38). *B. subtilis* strains RB413, RB415, RB338 and RB397, did not form DHNA on separate incubation with OSB; when extracts from RB413 were combined with either RB388 or RB397 extracts, DHNA formation took place. Similarly, RB415 extracts formed DHNA in combination with extracts from RB388 or RB397. It was then possible to pinpoint the specific defect in each mutant by the use of purified samples of OSB–CoA synthetase and DHNA synthase from *M. phlei*. Results of such experiments for both *B. subtilis* and *E. coli* mutants are given in table 3. It can be seen that *B. subtilis* RB413

TABLE 3. Enzymological characterization of *menB* and *menE* mutants of *B. subtilis* and *E. coli*.

A. Production of DHNA by *menE* mutants on complementation with OSB–CoA synthetase

Organism used for extract preparation	DHNA production[a] nmole/30 min/tube
B. subtilis, RB388	5.2
B. subtilis, RB397	6.6
E. coli, AN213	4.7

[a]In the assays with the *B. subtilis* extracts, there was added 18 U of *M. phlei* OSB–CoA synthetase; and with the *E. coli* extracts, there was added 13 U of OSB–CoA synthetase. Assay conditions have been described previously (37). The amounts of protein present in the extracts were as follows: RB388, 8.7 mg; RB397, 8.0 mg; AN213, 14.2 mg.

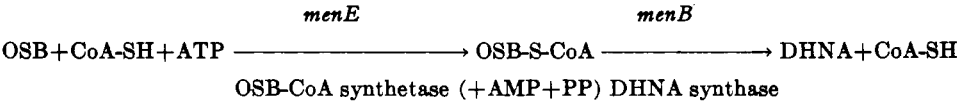
B. Production of DHNA by *menB* mutants on complementation with DHNA synthase

Organism used for extract preparation	DHNA production[b] nmole/30 min/tube
B. subtilis, RB413	18.4
B. subtilis, RB415	15.7
E. coli, JRG962	8.3
E. coli, JRG1205	8.2
E. coli, AN209	9.3

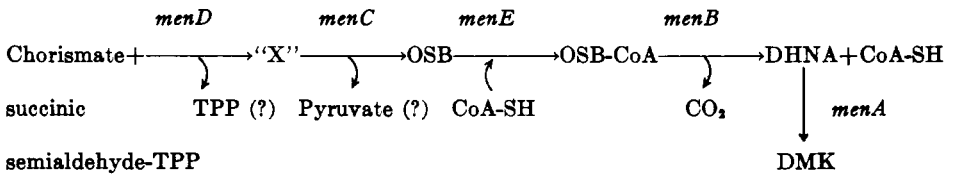
[b]The amount of DHNA synthase (from *M. phlei*) used with the *B. subtilis* extracts was 28 U, and with the *E. coli* extracts, 13 U. The amounts of protein in the extracts were as follows: RB413, 9.0 mg; RB415, 8.7 mg; JRG962, 15.1 mg; JRG 1205, 12.6 mg; AN209, 14.8 mg.

and RB415 possess DHNA synthase but not OSB-CoA synthetase, and the reverse situation is true for RB388 and RB397. Mutants defective in the structural gene for OSB-CoA synthetase are now termed *menE* mutants, those defective for DHNA synthase are *menB* mutants. As shown in table 3, *menB* and *menE*

mutations have also been identified in *E. coli* by the same methods. For this part of the pathway, the picture that has emerged is as follows:



To summarize, the overall genetic identifications for the pathway from chorismate to DMK are as follows. The only evidence supporting the proposed removal of TPP in the formation of "X" is the fact that "X" can be easily extracted into ethyl acetate.



ACKNOWLEDGMENTS

Work from the authors' laboratory was supported in part by a grant from the U.S. National Institutes of Health (GM 20053). We are grateful to Drs. J. R. Guest, H. Taber, and I. G. Young for providing the mutant bacterial strains, to Drs. I. M. Campbell and L. Ernst, and Mr. G. Emmons for assistance in determining the structure of OSB-CoA, and to Christine Dippold for excellent technical assistance.

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