# Biosynthesis of Vitamin K (Menaquinone) in Bacteria

RONALD BENTLEY* AND R. MEGANATHAN†

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

## INTRODUCTION

*Als der geistige Vater der Vitaminlehre ist wohl Gowland Hopkins zu betrachten . . . .*
F. Röhmann, 1916 (183)

*Research work on a disease (Johne's Disease) which affects any of the larger domesticated animals is necessarily very costly . . . . On this account our experiments, though covering a fairly wide field, have not been so numerous in some cases as we should have wished. In view of the importance of this disease to agriculturists, the question is one which should be investigated with public money. In our work on this disease, however, we have received no assistance from the Board of Agriculture or from the Development Fund Commissioners, even though appli-*

cations for a grant have been made after the essential part of our work—the cultivation of the bacillus—had been verified by the Danish Government bacteriologists.
F. W. Twort and G. L. Y. Ingram, 1913 (225)

In his Nobel Prize Lecture in 1929, Hopkins pointed out that "it is abundantly clear that before the last century closed there was already ample evidence available to show that the needs of nutrition could not be adequately defined in terms of calories, proteins and salts alone. . . . It is sure that until the period 1911–1912, the earlier suggestions in the literature pointing to the existence of vitamins lay buried" (107). At the time of Hopkins' Nobel Lecture, almost all of the work on vitamins had concerned animal and human nutrition. Fat- and water-soluble vitamins had been distinguished, but the first chemical structure, that of vitamin A, was not

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

determined until 1931. Hopkins was apparently unaware that, at the same time as his own work on vitamin requirements in animals, a growth factor requirement for "Johne's bacillus" (*Mycobacterium paratuberculosis*) was established by his fellow countrymen, Twort and Ingram (222, 224).

In June of 1910, these workers had considered a possible vaccine for Johne's disease—a chronic, specific enteritis principally affecting cattle. Whereas a specific bacillus (Johne's bacillus) was present in the intestinal mucous membrane and mesenteric glands of infected animals, all attempts to cultivate the organism on ordinary, artificial laboratory media had failed. They considered that these failures "must be due, either to some substance in the medium acting as a poison, or to the absence of some material or foodstuff necessary for its vitality and growth" (222, 224). Since related bacilli grew in ordinary laboratory media, the poison alternative seemed highly improbable; they were, therefore, forced "to conclude that the failure to grow the bacillus must be due to the absence of some necessary foodstuff." Further, the close relationships between the tubercle bacillus and Johne's bacillus (both live in the bodies of bovines, for instance) suggested that the former could grow in ordinary medium because it could elaborate a certain required substance, the "Essential Substance." When dead human tubercle bacilli were incorporated into an egg medium, Johne's bacillus was grown in culture for the first time. This result was announced by Twort in a preliminary fashion in 1910 (222) and more fully in 1911 (224). The timothy grass bacillus (*Mycobacterium phlei*) was also found to be an excellent source of Essential Substance. Extraction of *Mycobacterium phlei* cells with ethanol yielded a yellowish mass which supported growth; on further extraction with chloroform, the best stimulation was obtained with the material insoluble in chloroform. As Hanks has noted, this work provides the first discovery of a biological growth factor, a vitamin, for a microorganism (96). However, "der geistige Vater der Vitaminlehre" did not refer to Twort and Ingram's work in either his classical paper (106) or his Nobel Prize Lecture (107) and appears to have shared the feeling that bacterial nutrition and animal nutrition had no connection (69).

The nature of Essential Substance remained unexplored for 30 years. In the meantime, vitamin K had been characterized and shown to be a methyl naphthoquinone (see below). Woolley and McCarter (231) were aware of the report (6) that phthiocol, 2-methyl-3-hydroxy-1,4-naphthoquinone, isolated from *Mycobacterium tuberculosis* possessed vitamin K activity. Since Essential Substance was present in this orga-

nism and was to some extent soluble in fat solvents as well as in water, phthiocol, 2-methylnaphthoquinone, and a potent concentrate of vitamin K were tested as growth factors for Johne's bacillus. All three materials were shown to stimulate growth, but extracts of *Mycobacterium phlei* were somewhat more effective than any of the quinones. Whether, in fact, vitamin K can actually be considered to be required by strains of *Mycobacterium paratuberculosis* will be examined in a later section (Vitamin K-Requiring Bacteria). Writing in 1949, 1 year before his death and after the destruction of his institute in the London "blitz," Twort with obvious pride, but frustrated by the inability to obtain financial support, referred to his early work as follows: "The name Vitamin at that time had not been coined, although my 'Essential Substance' has since been named 'Vitamin K' " (223).

It would be of interest to speculate on the consequences of a serious and determined effort to characterize Twort's Essential Substance at an earlier date. However, the work "lay buried," and its significance was not appreciated until Knight called it to attention in 1936 (125). Remarkably, the Twort and Ingram paper of 1912 also contains a reference to the possibility of an ultramicroscopic virus working in symbiosis with Johne's bacillus. Although experiments to test this possibility were negative, later work led to another of Twort's major contributions to microbiology, the Twort-d'Herelle phenomenon of transmissible lysis (70).

Had the yellowish mass extracted from *Mycobacterium phlei* been examined at some time before say 1935, the identification of a naphthoquinone and vitamin K might have been achieved at an earlier date. In fact, possible connections between animal and bacterial metabolism remained unappreciated for many years after Twort and Ingram's work. It was not until 1934 that Fildes could write, "It is not impossible that substances shown by the bacterial chemist to be necessary for the proper growth of bacteria may subsequently be found to be necessary for the growth of animals" (69). Instead, vitamin K was discovered by the classical approach of animal nutrition. In 1929, Henrik Dam began nutritional studies with chickens, thus "lighting a candle which pushed back the darkness and revealed a new vitamin factor which is now recognized to be of vital importance to the health of mankind" (97). The history of the work of Almquist and Dam and their colleagues is, for the most part, well known (4, 59, 155), and some new and interesting reminiscences have appeared recently (5, 120, 172). Only those portions of the work of immediate interest to microbiologists will be noted here. It

is of unique interest that bacteria again came into the picture and, as will be seen, in a novel and unexpected manner.

To conclude this general introduction, it should be noted that available review articles in the general area of vitamin K have been listed by Suttie (207). A major function for vitamin K in mammalian metabolism has now been clarified: it is a required cofactor for the carboxylation (by $CO_2$) of protein-bound glutamate residues to form $\gamma$-carboxyglutamates (205). The functions of vitamin K in bacteria have been discussed in several review articles (88, 89, 118, 129, 208, 214). The vitamin has a major role as an electron carrier. The clinical uses of vitamin K are well known (148).

Many other 1,4-naphthoquinone derivatives are found in nature, particularly in plants and fungi, and have also been exploited by humans in many ways. For example, the plant metabolite lawsone (2-hydroxy-1,4-naphthoquinone) is the material responsible for the yellow-to-orange dyeing properties of henna; this material has been used by men and women for at least 2,000 years—reputedly, for instance, by Cleopatra and Mohammed (213). In more recent times, some naturally occurring naphthoquinones, or closely related materials, have been used or considered for use as antibiotics, e.g., the axenomycins (22), frenolicin (63), kalafungin (173), the nanaomycins (173), the naphthocyclinones (235), and the ansamycins (178).

## DISCOVERY OF VITAMIN K IN BACTERIA

The discovery of vitamin K biosynthesis in bacteria came about from detailed studies of the nutrition of chickens. In 1931 McFarlane et al. (156, 157) investigated the fat-soluble vitamin requirements of the chick. The basic rations included either fish meal (from white nonoily fish) or meat meal (from which the fat was partially extracted). When either meal was first extracted with ether, the animals suffered poor growth and, if injured, bled to death. The bleeding condition was most pronounced when ether-extracted fish meal (rather than meat) was used and was similar to that reported earlier by Dam. Dam's diets, however, were based on casein as the protein source (56, 57). Somewhat later, Holst and Halbrook described a "scurvy-like disease" in chicks, also using a fish meal ration (104). The feeding of 5 g of cabbage per bird during weeks 5 and 6 of deficiency gave a complete recovery.

At about this same time, Cook and Scott stated that the hemorrhagic condition in chickens "can be ascribed to the fish meals used in that they contained objectionable materials and/or lacked some accessory factor" (50). Reminiscent of the earlier work by McFarlane et al. (156,

157), these workers encountered no problems with a diet of "commercial meat scrap." The objectionable materials were said to be nitrogenous bases (51), and feeding a number of such compounds did produce hemorrhagic symptoms (e.g., mono-, di-, and trimethylamine, diethyl- and dipropylamine, ergot, nicotine). Further, methylamine was detected in fish meal. These workers unfortunately failed to appreciate the significance of one of their observations: when fish meal was washed with water and allowed to dry at 65°C, the syndrome was much reduced.

Since fish meals were used for animal feeding, the problem of "toxic fish meal" versus "non-toxic meat meal" became a cause célèbre. At that time, Almquist was working on problems of protein quality in animal protein concentrates for the feeding industry. He has recollected that "meat scraps were made mostly from the offal from meat packing, which would include the viscera and incidental manure, condemned livers, dead animals picked up from the hinterland, and what have you. The starting material often could be pretty 'ripe'. . . . It occurred to me that possibly the opportunity for bacterial action or other spoilage on the raw materials going into these animal protein concentrates might have something to do with the problem. So I moistened some good fish meal with water and let it stand in a warm cabinet. It stunk up the place" (H. J. Almquist, letter to R. Bentley, dated 26 March 1982 [a recollection of events that go back nearly half a century]). The water-moistened fish meal was examined by a graduate student, Halbrook (92), and found to prevent the hemorrhagic symptoms in chicks (Table 1). Water extraction followed by drying gave a similar result. If the water-extracted fish meal was treated with alcohol before drying, hemorrhagic symptoms were present (see Table 1). Halbrook concluded that the protective action of water-extracted fish meal "can only be explained by bacterial action, especially since mere moistening of the fish meal had a similar effect, whereas water extraction followed by moistening with ethyl alcohol to prevent bacterial action failed to prevent the symptoms from occurring."

Halbrook's thesis also records that untreated rice bran had little effect in preventing the occurrence of hemorrhagic symptoms, but did so when it was extracted with water and dried slowly under conditions conducive to bacterial action. With reference to either fish meal or rice bran, he concluded that "either a deficiency factor is synthesized by bacterial action or a toxic factor is destroyed." A fact arguing against the "toxicity theory" was his finding that the allegedly toxic trimethylamine hydrochloride (51) had no effect when added to water-extracted fish meal.

TABLE 1. Effect of various treatments of fish meal on occurrence of hemorrhages in chicks[a]

| Fish meal treatment | % of chicks with hemorrhagic symptoms[b] | No. of expt |
|---|---|---|
| Normal | 38.5–100 (77.2)[c] | 6 |
| Ether extracted | 83.0–86.0 (85.0) | 2 |
| Water extracted | 0.0–9.2 (2.3) | 4 |
| Water moistened | 0.0 | 1 |
| Water extracted, alcohol moistened | 83.3 | 1 |

[a] The fish meal was derived from Pacific Coast sardines, using equal parts of whole fish and heads and viscera, by a superheated steam-drying process. These data are abstracted from Table V of Halbrook's thesis (92).

[b] Each experiment involved 12 to 15 chicks. Different batches of fish meal were used in each experiment.

[c] Average is given in parentheses.

Some confusion had been caused in the early work since not all batches of fish meal allowed the development of the hemorrhagic syndrome. Again quoting Almquist's recollection, "Sometimes fish meals were made from offal from the canning operations, or from fish which had gone 'soft' and unfit for canning because of delay between catching and canning times. Sometimes they were almost entirely made from the cuttings from the canning operation." These meals were less likely to cause the bleeding problem, again supporting a role for bacterial action. Jukes has also suggested that the better microbiological state of some fish meal batches resulted from the cold temperatures in the sardine fishing grounds off the coast of California due to the Alaskan current. Sardine fishing was done at night, and under optimal conditions the fish were quickly transported, still cold, for immediate processing at the factory (120).

Almquist and Stokstad cited Halbrook's thesis work in a paper published in 1935 (9) and reported further experiments with fish meal which had been moistened and allowed to putrefy. By this time they were able to rationalize the nontoxic quality of meat meal by pointing out that it is "well known that many animal protein concentrates offered for poultry feeding are not protected from the action of microorganisms in the raw state and during manufacture." It was clear that "antihaemorrhagic power cannot be attributed specifically to any feed ingredient unless the possibility of action upon it by microorganisms has been guarded against" (10).

Unfortunately for Almquist, the "deficiency-toxicity" dispute had become heated at the University of California since commercial interests were at stake for the fish meal producers (no pun intended). A manuscript by Almquist and Stokstad was, for a time, actually embargoed by the administration of the University of California (4, 120); when submitted to *Science* it was rejected because of the previous claim that toxicity resulted from the presence of nitrogenous bases (51). After these delays, the paper was accepted by *Nature* (10), appearing in the 6 July issue of 1935 (no receipt date given). Almquist thereby lost priority to a paper in the same journal by Dam (58) which had been received on 19 March and appeared in the 27 April issue. In this paper, Dam suggested the name vitamin K for the antihemorrhagic factor and showed it to be present in hog liver fat, hemp seed, certain vegetables, and to a lesser extent in cereals. Vitamin K was derived from the spelling of coagulation in German and in the Scandinavian languages (59) and was also the first letter of the alphabet not then assigned to another vitamin. Dam was evidently of the opinion that Almquist should have shared the Nobel Prize for the discovery of vitamin K (4, 120). That he did not must presumably be attributed to the delay caused by the deficiency-toxicity dispute.

Strangely enough, a "replay" of toxicity versus deficiency occurred some years later when young rats, fed irradiated beef, were found to develop hemorrhages. As Matschiner has noted, several laboratories believed that a toxic principle was responsible rather than a nutritional deficiency of vitamin K (152). In fact, noncontaminated ground beef contains about 0.07 μg of vitamin K per g, and this amount is sufficient to protect rats against hemorrhage.

For some time, "putrefied fish meal" was a major source of what became known as vitamin $K_2$. In 1938, for example, Osterberg described the process in some detail (175). He wished to obtain material for a clinical trial of vitamin K in jaundice. Some 15 pounds (ca. 6.8 kg) of commercial fish meal (from tuna) was ether extracted (a staggering volume of ether must have been required) and, after drying, was moistened and allowed to putrefy for 1 week in a warm and moist atmosphere. After drying and extraction with petroleum ether, 15 ml of impure oil was obtained; the data suggest a vitamin K content of perhaps 15%.

Almquist and Stokstad considerably extended their observations. They showed, for example, that the vitamin could be biosynthesized, presumably by bacterial action, within the intestinal tract of chicks (11). This followed from the fact that droppings from chickens on a vitamin K-free diet could be extracted to yield material that

was adequate as a source of the antihemorrhagic vitamin. When droppings were collected in 1% phenol solution to inhibit further bacterial action, the potency of the extract was lower. Extreme care was necessary in these and other nutritional experiments. To prevent bacterial synthesis it was necessary, for instance, to remove feed which the chicks had carried to the water troughs.

The "fish meal organism," probably *Bacillus cereus*, was isolated and grown on substrates such as beef broth, fish meal broth, proteose-peptone broth, and nutrient agar; in each case it functioned as a rich source of vitamin K (8). Various pure bacteria were also grown on nutrient agar, and vitamin K was found, for example, in *Bacillus cereus*, *B. mycoides*, *B. subtilis*, *Chromobacterium prodigiosus* (= *Serratia marcescens*), *Escherichia coli*, *Mycobacterium tuberculosis*, *Sarcina lutea*, and *Staphylococcus aureus*. No activity was observed with extracts from yeast or *Pseudomonas aeruginosa* (now known to contain only ubiquinones). It was evident that the factor, vitamin K, was a product of the metabolism of many bacteria.

Bacteria continued to play an important role in the vitamin K story. Almquist became aware of the isolation of the naphthoquinone phthiocol (2-methyl-3-hydroxy-1,4-naphthoquinone) from *Mycobacterium tuberculosis* by Anderson and Newman (12). Since he had evidence that his vitamin K preparations were quinones and since *Mycobacterium tuberculosis* was a good source of vitamin K, he obtained a phthiocol sample. It was shown to have definite vitamin K activity and became the "first identified form of vitamin K" (6). With evidence accumulating for a phytyl group in vitamin $K_1$, Almquist and Klose condensed phytol and 2-methylnaphthoquinone to achieve a synthesis of vitamin $K_1$. This form of the vitamin, present in alfalfa and other green plants, is therefore 2-methyl-3-phytyl-1,4-naphthoquinone. Their paper (7), received by the *Journal of the American Chemical Society* on 21 July 1939, appeared at the same time as similar work from the laboratories of Fieser (67, received 12 August 1939) and Doisy (30, received 21 August 1939; a more detailed paper [144] carries the date 5 September 1939).

It was from bacterially putrefied fish meal that the first crystalline antihemorrhagic vitamin was prepared in Doisy's laboratory (excluding phthiocol from consideration). A page from R. W. McKee's notebook, dated 10 November 1938, has been published (160). The momentous crystallization of the purified vitamin occurred "a few days later." Since this crystalline bacterial material (161) was clearly different from the highly purified, but noncrystalline, material from alfalfa, the two were distinguished as vita-min $K_1$ (from green plants; now phylloquinone = leaf quinone) and vitamin $K_2$ (from bacteria; now menaquinone = *me*thyl *n*aphtho*quinone*).

It is perhaps striking that another 10 years were to elapse before the isolation of a menaquinone from a pure bacterial culture (as opposed to putrefied fish meal) was undertaken. In 1948 Tishler and Sampson isolated and crystallized a menaquinone from *B. brevis* (221). The saga of the antihemorrhagic vitamin present in bacteria has a final irony. Although Doisy and his colleagues (31, 161) characterized the material from the putrefied fish meal as 2-methyl-3-farnesylfarnesyl-1,4-naphthoquinone (i.e., MK-6 in present nomenclature), it was later shown that the major component is, in fact, MK-7; MK-6 is present but only as a minor component (114). It is now known that the natural menaquinones have all *trans* configurations for the appropriate side chain double bonds; the double bond of phylloquinone was also shown to be 2'-*trans* and the chiral centers at 7' and 11' were shown to be *R* (23). In the hydrogenated menaquinone from *Mycobacterium phlei* MK-9 (II-$H_2$), the configuration at the 7' position is also *R* (19).

It is now abundantly clear that bacteria contain both normal and modified menaquinone types; in addition, cyanobacteria contain phylloquinone rather than menaquinones. A comprehensive account of the various forms of vitamin K present in bacteria has been given by Collins and Jones (48). Common side chain variations in the menaquinones are hydrogenation of one or more of the isoprenoid double bonds and the introduction of oxygen atoms. Of particular importance for our present purposes is the occurrence of demethylmenaquinones (DMK). As will become apparent, the DMK are precursors to the menaquinones themselves.

In this review, we have made no attempt to be completely consistent with respect to the nomenclature of the various materials. The term vitamin K has been used in the initial introductory material and in those situations where physiological activities are discussed or where an inclusive term for materials with antihemorrhagic activity is needed. In other, more specific cases, the International Union of Pure and Applied Chemistry-International Union of Biochemistry nomenclature has been used (112).

## BIOSYNTHESIS OF MENAQUINONES

An introduction to the discovery of the menaquinone biosynthetic pathway will be given first along with a general description of the overall process. Subsequently, the work leading to the identification of intermediates will be reviewed

in detail, and the individual reactions of the pathway will be considered in terms of mechanism and enzymology, as far as is possible. Genetic considerations will then be covered separately.

## General Information

The work described in the preceding section clearly showed that bacteria have a high ability for menaquinone biosynthesis. However, before 1964, the biosynthetic question had not received any experimental attention. Fieser et al. (68) had suggested that phylloquinone might be derived by condensation of phytol with 2-methyl-1,4-naphthoquinone, and they had also attempted to make a connection between the two farnesyl residues (two $C_{15}$) found in the menaquinone from putrefied fish meal (as noted earlier, only a minor component) and the known presence of squalene, $C_{30}H_{50}$, in fish oils. In 1964, Martius and Leuzinger (150) found that phylloquinone and 2-methyl-1,4-naphthoquinone could be converted to menaquinone by the action of the vitamin K-requiring *Bacteroides melaninogenicus* (then termed *Fusiformis nigrescens*). Furthermore, it was shown that 1,4-naphthoquinone itself was converted to menaquinone by *Bacteroides melaninogenicus* and that the required methyl group was contributed by methionine (206). The role of methionine as the methyl group donor has been confirmed for the biosynthesis of MK-9 (II-H$_2$) by *Mycobacterium phlei* and *Mycobacterium smegmatis* (81, 117) and for MK-8 in *E. coli* (53, 64, 115). Schiefer and Martius also observed the conversion of 2-methyl-1,4-naphthoquinone to menaquinone, using animal mitochondrial preparations (194). The isoprenoid side chain was contributed by pyrophosphate esters of polyisoprenoid alcohols. This work pointed to a mevalonoid origin for the second side chain of vitamin K. In 1967, Threlfall et al. were able to show the utilization of mevalonate for the biosynthesis of phylloquinone (219), and in 1969 Hammond and White extended these observations to a bacterial system (93). It is now generally agreed that the primary precursors of the two side chains of menaquinones are methionine and mevalonate.

Studies of the origin of the naphthoquinone nucleus proceeded more slowly than those of the other isoprenoid quinones and the related cyclized forms such as vitamin E. In part, this slow development stemmed from the low levels of menaquinones and biosynthetic enzymes present in bacteria, and in part it was from the fact that the biosynthetic pathway has turned out to be unique and to involve unprecedented reactions. Most of the presently available information concerning vitamin K biosynthesis in general has, however, been obtained from ex-

periments with bacteria. The work reviewed here will be concerned almost exclusively with menaquinone biosynthesis in a rather small number of bacteria (generally *E. coli*, *Mycobacterium phlei*, and *Bacillus subtilis*). The only other possible experimental organisms available for a study of vitamin K biosynthesis are green plants and cyanobacteria. Although phylloquinone biosynthesis seems to be generally the same as that of menaquinones, the exact pathway in plants remains unclear at the present time, and virtually no work with enzyme systems has been carried out.

In 1964, Cox and Gibson observed the conversion of [$G$-$^{14}$C]shikimate into both ubiquinone and menaquinone by *E. coli*, thus providing the first evidence for a role for the shikimate pathway (52). The incorporation ($I$) value was not given; the dilution ($D$) can be calculated to be 9.4 for menaquinone and 7.9 for ubiquinone; $I$ and $D$ have the usual meanings (41). Chemical degradation of two labeled samples of *E. coli* menaquinone (MK-8) showed that essentially all of the radioactivity was retained in the phthalic anhydride. Hence it was concluded that "the benzene ring of the naphthaquinone (*sic*) portion of vitamin $K_2$ (MK-8) arises from shikimate in *E. coli*." These authors also suggested that shikimate was first converted to chorismate. Soon afterwards, more complete chemical degradations of menaquinone derived from radioactive shikimate established that all seven carbon atoms of this precursor were incorporated into the menaquinone molecule (43). The remaining three atoms of the naphthoquinone nucleus were subsequently found to be derived from 2-ketoglutarate; both carboxyl groups of this precursor were removed at some stage (40, 180, 181).

The work just summarized established that the immediate precursors of the menaquinones were as follows: shikimate (chorismate) plus noncarboxyl carbon atoms of 2-ketoglutarate forming the naphthoquinone nucleus, with the methyl and isoprenoid side chains obtained, respectively, from *S*-adenosylmethionine and an isoprenoid alcohol pyrophosphate ester. Two important aromatic intermediates were subsequently characterized. They are the benzenoid derivative *o*-succinylbenzoate (OSB; 60) and the naphthalenoid compound 1,4-dihydroxy-2-naphthoate (DHNA; 182). The broad outlines of the biosynthetic pathway to menaquinones are summarized in Fig. 1. Evidence has also been obtained for the participation of at least two other intermediates; each possibility will be discussed in detail below.

The branch of the shikimate pathway through OSB is also responsible for the biosynthesis of phylloquinone (215), some simpler plant naphthoquinones such as lawsone and juglone (60),
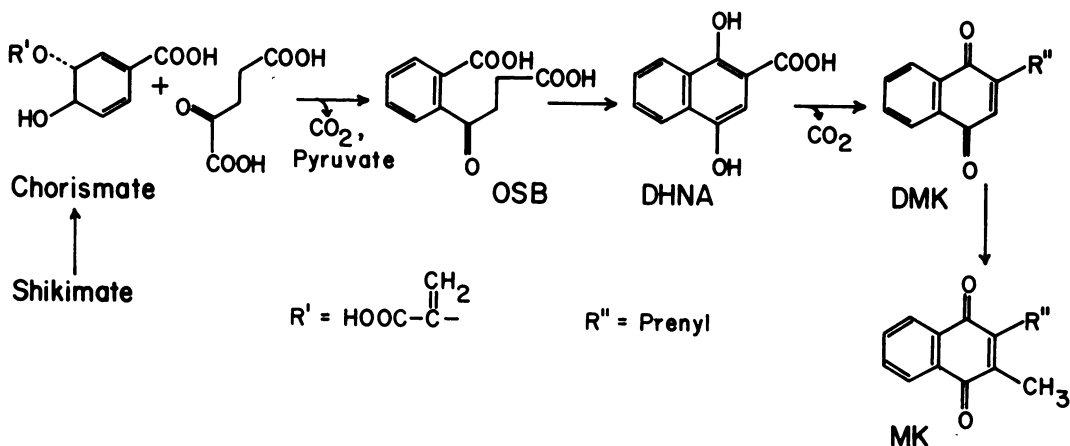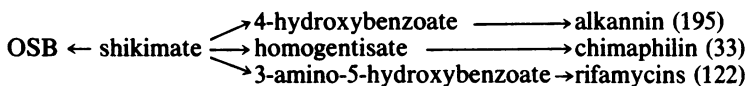
FIG. 1. Major intermediates in menaquinone biosynthesis. The ketoglutarate unit is not identified due to space limitations. The abbreviations used here and elsewhere are as follows: OSB = o-succinylbenzoate (4-[2'-carboxyphenyl]-4-oxobutyrate); DHNA = 1,4-dihydroxy-2-naphthoate; DMK = demethylmenaquinone; MK = menaquinone.

and (with the addition of further carbon atoms derived from mevalonate) of some plant anthraquinones such as those of *Rubia peregrina* (60). OSB is also a precursor to the orchid alkaloid shihunine (134) in *Dendrobium pierardii* and *D. lohohense*. Shikimate pathway branches lead to other plant naphthoquinones by way of different intermediates, as indicated:

reduced naphthalene material geosmin, produced by various streptomycetes, is apparently a degraded sesquiterpene derived from mevalonate (26).

### Role of Shikimate

After their early observation (52) that shikimate was a menaquinone precursor, Cox and

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                        ┌→4-hydroxybenzoate ──────→alkannin (195)
OSB ← shikimate ═══→homogentisate ──────────→chimaphilin (33)
                        └→3-amino-5-hydroxybenzoate →rifamycins (122)
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Furthermore, it has always appeared likely that the naphthoquinone (and related) ring systems of rifamycins and similar antibiotics were derived from the shikimate pathway (24). This expectation has been upheld by the discovery of 3-amino-5-hydroxybenzoate as a rifamycin precursor (122).

Most of the 1,4-naphthoquinones found in nature, however, are produced by plants and fungi, and the majority of these are derived by "polyketide" pathways (24); some bacterial naphthoquinones are also produced in this way (e.g., 5,8-dihydroxy-2,7-dimethoxy-1,4-naphthoquinone produced by a *Streptomyces* strain [158]). One final pathway for naphthoquinone biosynthesis must be noted. In a few cases, plant naphthoquinones are derived entirely from mevalonate. In bacteria, however, this precursor is used sparingly; few, if any, bacteria produce sterols. However, as noted, the isoprenoid side chain of menaquinones derives from this material as do the long-chain alcohols such as bactoprenol. Although not a naphthoquinone, the

Gibson in 1966 (53) converted the labeled menaquinone to 1,4-diacetoxy-2-methylnaphthalene-3-acetic acid by the procedure used earlier for ubiquinone (27). This material was more vigorously oxidized with $KMnO_4$ to form phthalic anhydride (Fig. 2); in two experiments this material contained 92 or 95% of the activity present in MK-8.

It had become apparent at about this time that purification of radioactive menaquinone samples by the usual chromatographic procedures was unreliable: contamination of such samples by other lipids was observed. Esters of fatty and aromatic acids were particularly troublesome (17, 42, 82). In addition, Cox and Gibson had used materials of relatively low specific activity (the maximum activity in their phthalic anhydride sample was ca. 8 cpm/mg), and their chemical degradation did not reveal whether all of the shikimate carbons were incorporated into the naphthoquinone nucleus. Campbell et al. (43, 44), therefore, carefully purified menaquinone samples by use of the (lipophilic) Sephadex
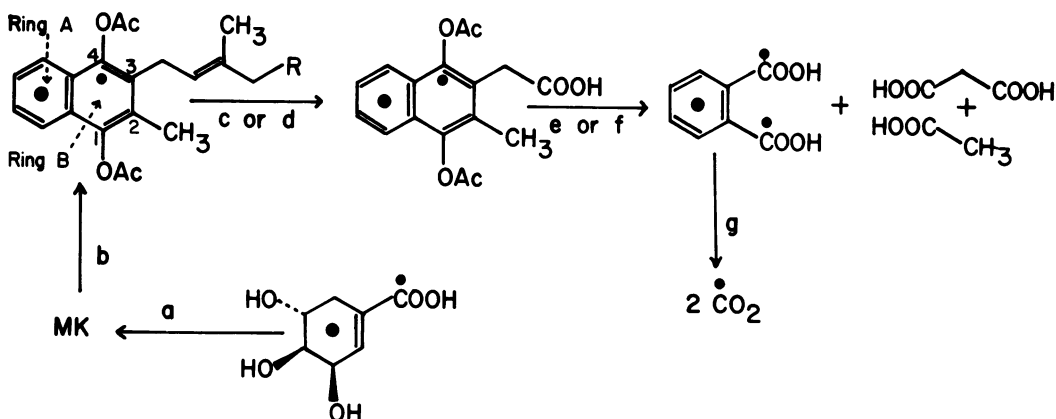
FIG. 2. Utilization of all seven carbon atoms of shikimate for menaquinone biosynthesis. Radioactivity is indicated as ●, and the same symbol within a six-membered ring implies that all carbon atoms are labeled. The sequence is as follows; a, bacterial biosynthesis; b, reductive acetylation of the menaquinone; c, $O_3$/KMnO$_4$ (53); d, OsO$_4$/HIO$_4$, then KMnO$_4$ (43); e, KMnO$_4$ in acetone, only phthalate being isolated (53); f, H$_2$O$_2$ (43); g, Schmidt degradation.

LH-20 and Sephadex LH-50 (170). The samples were converted to the diacetate of the menaquinol for verification of radiochemical purity.

In this work, the incorporation from shikimate to menaquinone ranged from 0.1 to 1.6% with *E. coli*, and the dilution values were 290 to 5.3. With *Mycobacterium phlei* and *Streptomyces albus* the incorporations were lower (0.02 and 0.007%, respectively) and the dilutions were higher (3,300 and 16,000, respectively). A chemical degradation was devised so that all carbon atoms of the naphthoquinone nucleus could be recovered. By treatment with OsO$_4$-HIO$_4$ and then KMnO$_4$, the atoms of the nucleus (along with two carbons from the polyisoprenoid side chain) were obtained as 1,4-diacetoxy-2-methyl-3-naphthalene acetic acid. Further degradation of the latter with H$_2$O$_2$ yielded phthalic acid, acetic acid, and malonic acid (see Fig. 2). Schmidt degradation of the phthalate yielded the carboxyl carbons as CO$_2$. When [$G$-$^{14}$C]shikimate was used as precursor, the quinone carbon atoms, C-1 plus C-4, contained 12 to 16% of the total radioactivity of the menaquinone (experiments using *E. coli*, *Streptomyces albus*, and *Mycobacterium phlei*). The "generally labeled" shikimate ([$G$-$^{14}$C]shikimate) used in these experiments was a commercial preparation obtained by exposing *Ginkgo biloba* seedlings to $^{14}$CO$_2$. Chemical degradations established that, on average, the COOH group of the shikimate contained 15.6% of the total radioactivity. Hence, it was clear that, in these organisms, all seven carbon atoms of shikimate were incorporated into the menaquinone molecule and the carboxyl carbon of shikimate provided one (or both) of the carbonyl functions of the menaquinone (43, 44).

At the same time, Leistner et al. (135) also examined the conversion of shikimate into menaquinone in the following organisms: *Bacillus megaterium*, *Bacillus subtilis*, *E. coli*, "*Micrococcus lysodeickticus*," *Proteus vulgaris*, and *Sarcina lutea*. The highest incorporations (1.1 and 2.7%) were obtained with *Bacillus megaterium*. Radioactive menaquinone samples from the latter organism were directly oxidized with KMnO$_4$ to phthalic acid; this acid was then decarboxylated. It was again observed that the phthalate had all of the menaquinone radioactivity. The two carboxyl groups of phthalate contained a total of 13.9% of the menaquinone activity, in agreement with the work just cited (43, 44). Thus, it was clear that the ring of shikimate was incorporated intact into ring A of a variety of bacterial menaquinones; the shikimate carboxyl was also utilized, becoming either one (or possibly both) of the quinone carbonyl groups. The correct situation is shown in Fig. 2, and evidence in support of it will be discussed.

In ingenious experiments, Leduc et al. investigated which of the shikimate atoms provide the two atoms at the A/B ring junction (133). Attempts to resolve this question by the use of [1,6-$^{14}$C$_2$]shikimate were frustrated by difficulties in purifying degradation products; however, the use of [3-$^3$H]shikimate and degradation to a mixture of 3- and 4-nitrophthalates provided a solution. The question is complicated by possible symmetry in a biosynthetic intermediate (as noted below, this is not the case) and by actual symmetry in a degradation product (phthalate). The experiments can be best understood with reference to Fig. 3. Following isolation of labeled MK-9 (II-H$_2$) after administration of [3-
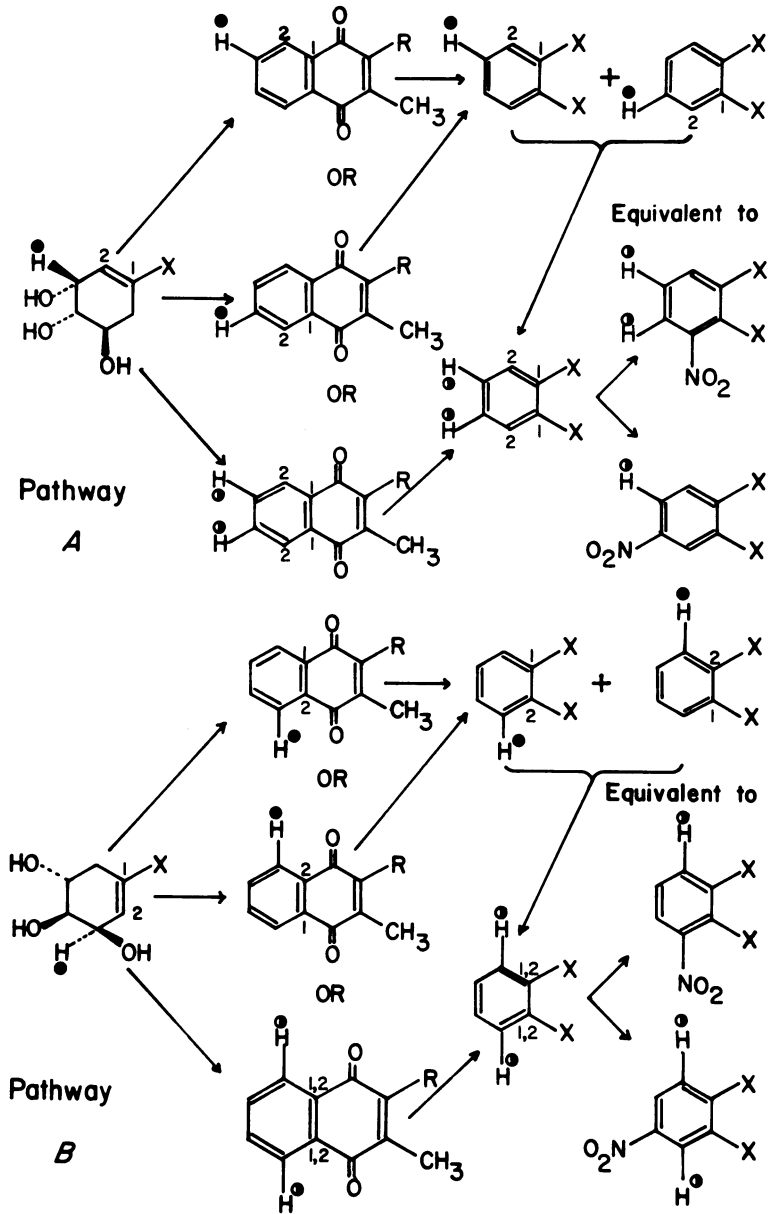
FIG. 3. Origin of the atoms at the menaquinone ring junction. R = prenyl; X = COOH. The numbers identify atoms from shikimate at all times; $^3$H radioactivity is indicated as ●, and if a 1:1 dilution has arisen, it is indicated as ◐. Two possible pathways, A and B, are considered. For each of the pathways, there are two possible incorporation modes without a symmetrical biosynthetic intermediate and a third in which randomization could have taken place with a symmetrical intermediate. After conversion of the labeled menaquinones to phthalates, nitration gave a mixture of the 3-nitro and 4-nitro derivatives; the "top" nitrophthalate in the figure is the 3-nitro derivative; the "bottom" nitrophthalate is the 4-nitro derivative. The nitrophthalates were separated before determination of radioactivity.

$^3$H]shikimate to *Mycobacterium phlei*, the sample was oxidized with KMnO$_4$ to phthalate. The latter was nitrated (HNO$_3$-H$_2$SO$_4$) to a mixture of 3- and 4-nitrophthalates; the acids were separated by thin-layer chromatography on cellu-

lose. If the phthalate carries $^3$H at positions 4 and 5, the 3-nitro derivative carries $^3$H in two positions (4 and 5) and the 4-nitro carries it only in one (position 5). Thus, for pathway A the ratio of radioactivity, 3-nitro/4-nitro = 2. For path-
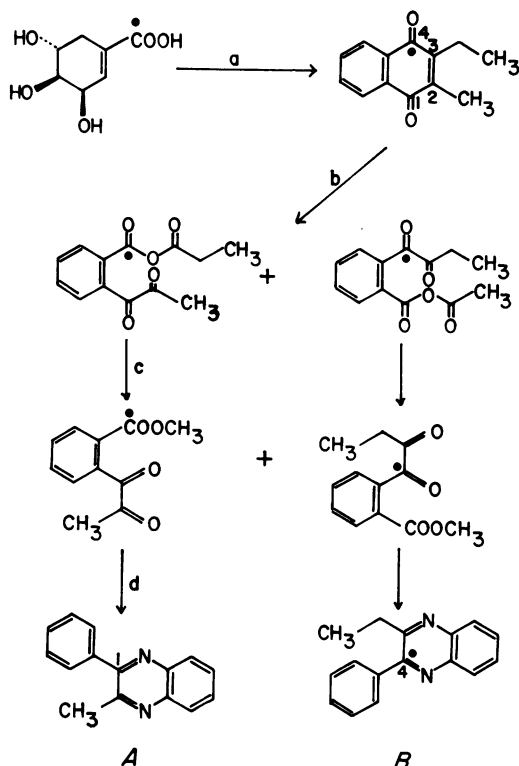
FIG. 4. Utilization of shikimate carboxyl group in menaquinone biosynthesis. The reactions are as follows: a, biosynthetic conversion of [7-$^{14}$C]shikimate to MK-9 (II-H$_2$) by *Mycobacterium phlei*, followed by chemical conversion to 2-methyl-3-ethyl-1,4-naphthoquinone; b, reaction with O$_3$; c, hydrolysis and esterification of the anhydride mixture followed by separation of the two diketoesters by gas chromatography; d, formation of quinoxalines, followed by ester hydrolysis and decarboxylation of the resulting acids. Compound *A* formed by this sequence contained only 3.6% of the original radioactivity of the menaquinone, and compound *B* contained 101%.

way *B* the situation is reversed. The 3-nitro derivative contains $^3$H at position 6; the 4-nitro contains it at positions 3 and 6. Hence, the radioactivity ratio in this case is 3-nitro/4-nitro = 0.5. Since the experimentally determined value was 0.5, pathway *B* operates. In other words, the atoms of the A/B ring junction are provided by C-1 and C-2 of shikimate. Not answered by this work, however, was the question, Does the shikimate carboxyl give rise to C-1, to C-4, or to both C-1 and C-4 of the naphthoquinone nucleus?

That the carboxyl group of shikimate provided C-4 was shown by Baldwin et al. (21). In this work, shikimate was labeled in the carboxyl group. A chemical degradation yielding C-1 and C-4 of the naphthoquinone ring, as separate

chemical entities, was devised as shown in Fig. 4. The end products of the degradation were two quinoxaline derivatives, one containing C-1 but not C-4 (compound *A*, Fig. 4) and the other containing C-4 but not C-1 (compound *B*, Fig. 4). It was found that only compound *B* contained $^{14}$C (101% of the MK-9 [II-H$_2$] activity). Hence, the origin of the C-4 carbonyl in the shikimate carboxyl was demonstrated. This work also demonstrated that there are no symmetrical intermediates in the menaquinone biosynthetic pathway.

It has also been shown (193) that in menaquinone biosynthesis from shikimate the *pro-R* hydrogen at position 6 is eliminated (Fig. 5). The required precursors, (6S)-[7-$^{14}$C, 6-$^3$H]- and (6R)-[7-$^{14}$C, 6-$^3$H]shikimate, were obtained enzymatically from (E)- and (Z)-[3-$^3$H]phosphoenolpyruvate (71, 174). The organism used was *Bacillus megaterium* 248, a shikimic acid auxotroph. Very high incorporations were obtained with this mutant (15 to 17%). The incorporations were determined for both $^3$H and $^{14}$C, and the ratios of activity are shown in Table 2. Hence, $^3$H retention from the 6S material was 84.0%, and that from the 6R material was only 18.6%. These experiments are consistent with a role for chorismate (see below) since the same *pro-6R* hydrogen of shikimate is eliminated during chorismate biosynthesis.

As a result of their initial work, Cox and Gibson had suggested chorismate as the "branch point" for menaquinone formation. This suggestion was, in part, based on the observation that addition of 3,4-dihydroxybenzaldehyde or adrenaline to *E. coli* cultures diminished the incorporation of radioactivity from shikimate into menaquinone (the following materials were without effect on the incorporation: catechol, phenylpyruvate, 4-hydroxyphenylpyruvate, 2,3-dihydroxybenzoate, 4-hydroxybenzoate, 3,4-dihydroxybenzoate, menadione). 3,4-Dihydroxybenzaldehyde (or some related pyrocatechol derivative) was known to be required for growth of certain aromatic auxotrophs of *E. coli* and was described as the "sixth factor" (61, 62). Cox and Gibson also found that
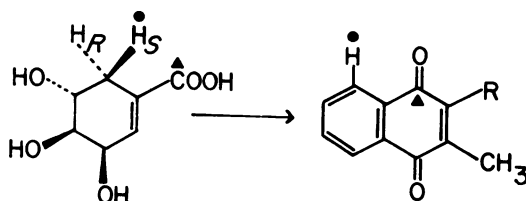


FIG. 5. Stereospecificity of the conversion of shikimate to menaquinone. R = Prenyl. The precursor shown is (6S)-[7-$^{14}$C, 6-$^3$H]shikimate.

an auxotrophic strain of "*Aerobacter aerogenes*" (strain 170-44) did not form detectable quantities of menaquinone when grown on medium containing phenylalanine, tyrosine, tryptophan, and 4-aminobenzoate. Similar results with this strain and with *E. coli* 159-4, which is blocked after shikimate, were obtained by Dansette and Azerad (60) and Leduc et al. (133). When 3,4-dihydroxybenzaldehyde was present at a final concentration of 10 μM, menaquinone was produced (ubiquinone was produced under all growth conditions). This "*A. aerogenes*" strain is unable to convert 5-enolpyruvoyl-shikimate 3-phosphate into chorismate. These results strongly implicated chorismate as the common branch point. The authors stated, furthermore, that there was little reason "to suppose that 3,4-dihydroxybenzaldehyde itself is on the direct route of biosynthesis of vitamin K." A clearly defined role for chorismate as a substrate for the enzymatic synthesis of OSB has now been demonstrated (162) and is discussed below.

The possible role of 3,4-dihydroxybenzaldehyde has remained enigmatic. Radioactive samples of 3,4-dihydroxybenzaldehyde and the corresponding acid were not incorporated into menaquinones by cultures of *E. coli*, *Mycobacterium phlei*, *Bacillus megaterium*, *Proteus vulgaris*, and "*A. aerogenes*" (43, 44, 83, 135).

Guérin et al. (83) were unable to stimulate "*A. aerogenes*" 170-44 to produce menaquinone on addition of 3,4-dihydroxybenzaldehyde (contrary to Cox and Gibson); furthermore, Leistner et al. (135) reported only a 40% suppression of the incorporation of activity from labeled shikimate into menaquinone with *E. coli* in the presence of 3,4-dihydroxybenzaldehyde, whereas Cox and Gibson found a larger value (although no numerical value was determined by these authors, the figure reproduced in their paper suggests a suppression of at least 85%). Other workers found a slight increase in shikimate incorporation when 3,4-dihydroxybenzaldehyde was added. It is now the general consensus that neither 3,4-dihydroxybenzaldehyde nor the corresponding acid plays any role in menaquinone biosynthesis.

### A Possible Role for 1-Naphthol?

In 1967 Sandermann and Simatupang had isolated 2,2-dimethylnaphthochromane from teak wood and had suggested on comparative phytochemical grounds that 1-naphthol would be an early precursor of the chromane, plant naphthoquinones, and materials such as tectol (188). This possibility was tested for menaquinone biosynthesis in the same year by Leistner et al. (135). Using *Bacillus megaterium*, 1-[1-$^{14}$C]-naphthol was reported to be incorporated to the extent of 1.5% (purification by thin-layer and

TABLE 2. Stereospecificity of the shikimate → menaquinone conversion

| Compound | $^3$H/$^{14}$C ratio | |
| --- | --- | --- |
| | (6S)-[7-$^{14}$C,6-$^3$H] shikimate expt | (6R)-[7-$^{14}$C,6-$^3$H] shikimate expt |
| Precursor shikimate | 8.70 | 6.92 |
| Menaquinone | 7.30 | 1.29 |

reversed-phase thin-layer chromatography to constant specific activity). On oxidation to phthalate all of the menaquinone activity was retained (102%), and all of the radioactivity was located in the carboxyl groups. As a result, the following partial pathway was suggested: 1-naphthol → 1,4-naphthoquinol → 1,4-naphthoquinone → menadione → MK. Similar results with 1-[1-$^{14}$C]-naphthol administration ($I$ = 0.3%) to *Staphylococcus aureus* were reported by Hammond and White (93, 94). In their work, the labeled samples of menaquinone were also degraded with permanganate to phthalate and phthalic anhydride. The phthalate derivatives contained about 87% of the menaquinone $^{14}$C.

However, despite these chemical degradations, it now appears most probable that the menaquinone samples in this work were contaminated with impurities that were difficult to remove. Using more rigorous purifications, other workers have failed to show conversion of 1-naphthol to menaquinone in *Mycobacterium phlei*, "*Micrococcus lysodeikticus*," *Bacillus megaterium* (several strains, including that used by Zenk and his colleagues), *Proteus vulgaris*, and *Proteus mirabilis* (21, 35, 44, 64, 83).

A problem with 1-naphthol is its surprising instability in growth medium. On standing 1-[1-$^{14}$C]-naphthol in sterile growth medium in the absence of bacteria at 37°C for 44 h, only 0.06% of original radioactivity was reisolated as 1-naphthol (21; H. Rapoport, personal communication). The medium used was as follows: glucose, 3%; Casamino Acids, 1.3%; potassium fumarate, 0.1%; Tween 80, 0.2%; $K_2HPO_4$, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.003%; $FeSO_4 \cdot 7H_2O$, 0.002%; pH adjusted to 7.0 with KOH. In distilled water under the same conditions, 96% of radioactivity was recovered as 1-naphthol. Not only does this instability present a problem in biosynthetic experiments with 1-naphthol, but there is, in addition, the possible degradation of 1-naphthol by bacterial action. For example, a soil *Pseudomonas* grown on 1-naphthol as sole carbon source produced 3,4-dihydro-3,4-dihydroxy-1(2H)-naphthalenone as an early intermediate (227). Other workers found 4-hydroxy-3,4-dihydro-1(2H)-naphthalenone as a bacterial degradation product of 1-naphthol (34).

When the origin of the oxygen atoms in mena-

quinone was studied, further evidence eliminating 1-naphthol was obtained (203). For this work, *Mycobacterium phlei* was grown either in ordinary water with an $^{18}O_2$ atmosphere or in $H_2{}^{18}O$ and ordinary oxygen. Special isolation techniques were used to prevent (nonenzymatic) exchange of the quinone oxygens with water. Menaquinone, MK-9 (II-$H_2$), obtained by growth in the presence of $^{18}O_2$ contained no excess of $^{18}O$ above the natural abundance. On the other hand, in a medium containing $H_2{}^{18}O$ the quinone oxygen atoms were shown to contain $^{18}O$ (in all cases samples were converted by pyrolysis to carbon monoxide for mass spectrometric analyses). If 1-naphthol were a biosynthetic intermediate to menaquinone, introduction of a second oxygen function would be necessary. Presumably this would require an "aromatic hydroxylase" enzyme. Since most such enzymes utilize molecular oxygen, incorporation of $^{18}O$ (from $^{18}O_2$) into menaquinone would be expected. No such incorporation was observed, so these experiments also cast considerable doubt on the proposed role for 1-naphthol.

### Origin of the "Three Carbon" Unit

Since all seven carbon atoms of shikimate were incorporated into the naphthoquinone nucleus, a search was undertaken for the source of the remaining three carbons (44, 121). Likely candidates, such as pyruvate, glycerol, and diethyl malonate, were not well converted to MK-8 by *E. coli* or to MK-9 (II-$H_2$) by *Mycobacterium phlei*. (*I* values, 0.0001 to 0.003%). Hammond and White also reported a low incorporation (*I* = 0.035%) of activity from [2-$^{14}C$]glycerol into the menaquinones of *Staphylococcus aureus* (93). [1-$^{14}C$]- and D-[$U$-$^{14}C$]ribose were also not utilized effectively (*I* values, 0.003 to 0.017%). When the three-carbon amino acids alanine and serine were examined, the *I* values were only slightly higher (0.005 to 0.01%) but, surprisingly, very low dilution values were obtained (1.1 to 1.4), suggesting a rather direct utilization.

Radioactive acetates were reasonably well utilized by *Mycobacterium phlei* (up to *I* = 0.22% for [2-$^{14}C$]acetate); as expected, most of the incorporated $^{14}C$ was associated with the isoprenyl side chain (about 80%). Surprisingly, chemical degradation showed significant activity in ring A. Since the aromatic portion of tyrosine was also found to be radioactive, it was clear that this incorporation occurred by leakage of acetate radioactivity into the shikimate pool (44). The carboxyl carbon of acetate labeled C-1 or C-4 (or both) of ring B to some extent, whereas the methyl carbon tended to label C-2

or C-3 (or both) of ring B. Acetate utilization by *E. coli* was much lower than with *Mycobacterium phlei* (0.002% with [1-$^{14}C$]acetate). When D-[1-$^{14}C$]glucose was used for growth of *E. coli*, higher incorporations were obtained (0.006 to 0.04%). Most of the incorporated $^{14}C$ (about 80%) was associated with the isoprenyl side chain; there was again a tendency to find some $^{14}C$ at C-2 or C-3 (or both) of ring B, presumably as a result of the formation of [2-$^{14}C$]acetate (or acetyl coenzyme A [CoA]).

These experiments with acetate, coupled with the observations that three carbon amino acids were incorporated (albeit poorly) but with low dilution suggested further testing of intermediates of the citric acid cycle. The first test of the hypothesis used a system for which the chemical degradations were simpler than those for menaquinones. Various plants produce lawsone (2-hydroxy-1,4-naphthoquinone), and it was known that shikimate utilization was involved (236); furthermore, in juglone (5-hydroxy-1,4-naphthoquinone) biosynthesis in *Juglans regia*, C-2 or C-3 (or both) of ring B were formed from the methylene carbon of malonate or the methyl carbon of acetate (136; compare the bacterial results just described). Campbell, therefore, administered [1-$^{14}C$]- and [$U$-$^{14}C$]alanine, [$U$-$^{14}C$]aspartate, and [2-$^{14}C$]glutamate to excised shoots of *Impatiens balsamina* plants, assuming they would be converted, respectively, to pyruvate, oxaloacetate, and 2-ketoglutarate (40). Of these precursors, the first three showed *I* values of 0.03 to 0.33%; the incorporation with [2-$^{14}C$]glutamate was substantially higher, namely, 1.36%. The chemical degradation used was oxidation to phthalate and $CO_2$, with further decarboxylation of phthalate. It was found that [1-$^{14}C$]-alanine gave a rather random distribution of radioactivity. With [$U$-$^{14}C$]-alanine, there was substantial labeling at C-2 or C-3 or both (73% of total lawsone activity); and with [$U$-$^{14}C$]aspartate, at C-1 or C-4 or both (46%), as well as C-2 and C-3 (35%). In the experiment with [2-$^{14}C$]glutamate, a very specific incorporation occurred: 99% of the lawsone activity was at C-1 or C-4 or both.

Campbell concluded from this very significant observation that the three central carbon atoms of ketoglutarate constituted the "missing three carbon unit" (Fig. 6). He suggested that the unit actually attacking shikimate was the thiamin pyrophosphate (TPP) adduct of succinic semialdehyde. This anion could be formed by the first (decarboxylase) enzyme of the ketoglutarate dehydrogenase complex, and the addition was mechanistically a Michael addition; the required anion could also have been formed by a separate decarboxylase. It was clear that in the formation of the naphthoquinone system, both carboxyls
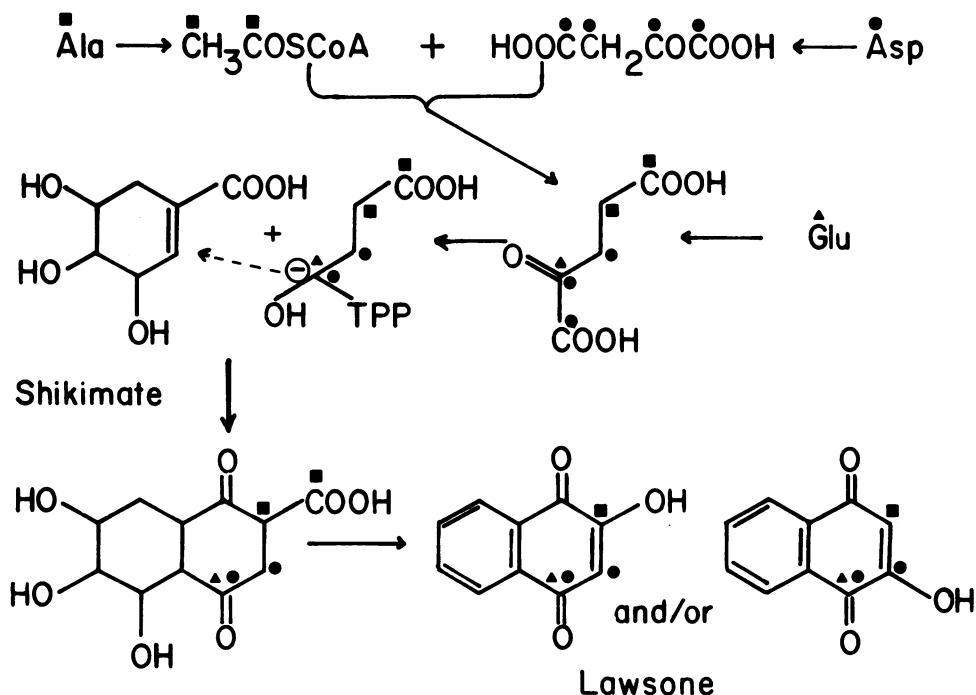
FIG. 6. Amino acid utilization in naphthoquinone biosynthesis. The amino acids were administered to *Impatiens balsamina* plants and lawsone was isolated. The labeling in the amino acids was uniform for Ala ($^{14}$C = ■), uniform for Asp ($^{14}$C = ●), and at the 2 position for Glu ($^{14}$C = ▲). It was assumed that 2-ketoglutarate was formed by transamination from Glu or by tricarboxylic acid cycle reactions from acetyl CoA and oxaloacetate as shown. This drawing is based on the original (40) and shows a trihydroxydecalindione carboxylic acid as a possible precursor; this precursor is no longer considered to be involved.

of the glutamate-ketoglutarate precursor had to be removed.

A role for glutamate-ketoglutarate was quickly confirmed in bacterial systems. Glutamate was incorporated into MK by *E. coli*, *Mycobacterium phlei*, *Corynebacterium diphtheriae*, and *Streptomyces albus* with good *I* values (0.002 to 0.02%) and little dilution (44, 181). In *E. coli*, which also contains ubiquinone, the ratio of activity MK/Q was high (20:1, 35:1) in two experiments (cf., for example, ratios of about 1.1:1 for acetate feeding). This was in line with the known biosynthesis of ubiquinone via *p*-hydroxybenzoate, a route giving no place to glutamate or ketoglutarate. In chemical degradations, the use of [*U*-$^{14}$C]glutamate gave approximately equal labeling in C-1 (or C-4), C-2, and C-3. With [2-$^{14}$C]glutamate, the menaquinone samples from *E. coli* and *Corynebacterium diphtheriae* were shown to be labeled at C-1 or C-4 or both, but there was no label in C-2 and C-3. Hence, there was a specific utilization of glutamate C-2 for one of the quinone carbonyl positions. In a feeding of 2-[*U*-$^{14}$C]ketoglutarate to *E. coli*, Robins and Bentley found an incorporation (*I* = 0.011%) comparable to that for [*U*-

$^{14}$C]glutamate (*I* = 0.015%) (180). The ratio of activity MK/Q was 24:1, a value in line with that found in the glutamate experiments. Chemical degradation established that the 2-[*U*-$^{14}$C]ketoglutarate contributed activity essentially equally to C-1 or C-4 or both, C-2, and C-3 (16.3, 17.0, and 14.6%, respectively).

As a result of this work, the origin of all of the carbon atoms of menaquinones became known (Fig. 7).
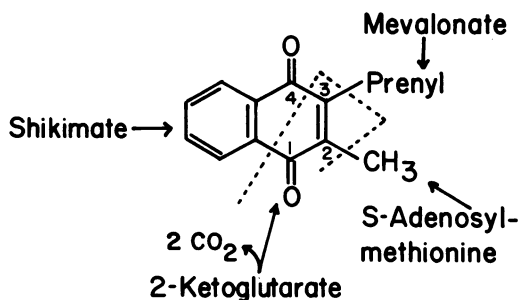


FIG. 7. Primary biosynthetic precursors of menaquinones.

## First Aromatic Intermediate, o-Succinylbenzoate

The discovery of a new pathway from chorismate was foreseen by Young et al. in 1969 (233). In isotope competition experiments, neither 3-(1'-carboxyvinyloxy)benzoate (a chemical precursor to 3-hydroxybenzoate) nor 3,4-dihydro-3,4-dihydroxybenzoate (known to be produced enzymatically from chorismate) reduced $^{14}C$ incorporation from shikimate into MK-8 of *E. coli*. They concluded that if "none of the known compounds derived from chorismic acid are concerned in vitamin K biosynthesis, it is probable that one more metabolic conversion of chorismic acid is yet to be found" (233).

Important evidence for a new intermediate, the benzenoid compound OSB, was quickly obtained by Dansette and Azerad (60). (Interestingly, this important compound had been first prepared in 1884 [184]). Earlier, however, they had suggested a possible role for carboxyphenylpyruvate (133). These earlier speculations were influenced by the finding that C-2 of malonate or acetate was incorporated into C-2 or C-3 or both of the naphthoquinone ring of juglone in the plant *Juglans regia* (136). It was suggested that the carboxyphenylpyruvate was derived from isochorismate; hence, all nine carbon atoms of chorismate would have been retained in the naphthoquinone nucleus, the remaining one being the central methylene of malonate. However, o-[2-$^{14}C$]carboxyphenylacetate, a potential further intermediate from carboxyphenylpyruvate, either as the free acid or ester, was not incorporated into menaquinone by *Mycobacterium phlei* or "*A. aerogenes*" 170-44 (133).

With the knowledge that three carbons of the naphthoquinone were obtained from ketoglutarate, and reasoning that a condensation with chorismate might give an aromatic compound directly, they then synthesized OSB (60). The compound supported growth and allowed menaquinone formation in *E. coli* 159-4 (a mutant blocked after shikimate) and with "*A. aerogenes*" 170-44 (blocked after 5-enolpyruvylshikimate 3-phosphate). Furthermore, [$^{14}C$]OSB was incorporated into MK-9 (II-H$_2$) in *Mycobacterium phlei* with a low dilution ($I = 70\%$; $D = 1.85$). Much smaller $I$ values were obtained in "*A. aerogenes*" 62-1 and *E. coli* K-12 (0.18 and 2.25%, respectively). In these organisms, $D$ values were measured for both MK-8 and DMK-8. With "*A. aerogenes*," the two $D$ values were 0.14 and 0.38, and in *E. coli* they were 8 and 18.5. Thus, despite the relatively low incorporation in these bacteria compared with *Mycobacterium phlei*, the utilization of OSB proceeded with good specificity (Fig. 8). Also of importance, in the last two organisms, was the lack of $^{14}C$ in ubiquinone.

The MK-9 (II-H$_2$) from *Mycobacterium phlei* was degraded to phthalate with KMnO$_4$; decarboxylation of the phthalate showed that all of the label of the menaquinone was localized in C-1 or C-4 or both (see Fig. 8). [2',4-$^{14}C_2$]OSB was also found to be an excellent precursor of MK in *Bacteroides melaninogenicus* ($I = 0.9\%$; $D = 1.8$) (182). A somewhat higher incorporation of [2,3-$^{14}C_2$]OSB into menaquinone of *E. coli* K-12 was reported later; in this work $I = 1.8\%$ and $D = 6.3$ (44). Campbell et al. also showed that [1-$^{14}C$]OSB was not incorporated under the same conditions. Hence, the "aliphatic" carboxyl of OSB is clearly lost during MK biosynthesis (see Fig. 8).

It was not until 1981 that a direct study of OSB biosynthesis was carried out (164). For this work, glutamate samples specifically labeled with $^{14}C$ at C-5 and C-1, or uniformly labeled, were administered to *E. coli* AN209, a mutant shown to accumulate OSB (see below). The isolated OSB was converted to a dimethyl derivative for examination by radiogas chromatography. When the precursor glutamate contained $^{14}C$ at position 5, or was uniformly labeled, the dimethyl OSB was radioactive. From [1-$^{14}C$]glutamate, however, the dimethyl OSB was without $^{14}C$. Hence, in OSB biosynthesis, C-1 of glutamate is lost and C-2 to C-5 are retained.

## 1,4-Dihydroxy-2-Naphthoate, a Naphthalenoid Intermediate

In 1969, Campbell suggested a Claisen-type reaction as the mechanism for formation of the naphthalenoid nucleus. The reaction involved the carboxyl group introduced by shikimate (chorismate) and the C-4 methylene of ketoglutarate (40). A trihydroxy-carboxy-decalindione was written as a possible intermediate. Later, Campbell et al. and Robins et al. suggested a role for 1,4-dihydroxy-2-naphthoate possibly formed from the decalin derivative (44, 181). Dansette and Azerad (60) realized that the dihydroxynaphthoate could be directly formed by a Claisen-type process on OSB (Fig. 9). They administered [$^{14}C$]OSB and unlabeled DHNA to the plant *Impatiens balsamina*, but found no change in incorporation compared with controls.

The first evidence implicating DHNA as a menaquinone intermediate was obtained in 1973 (182). The growth of a vitamin K-requiring strain of *Bacteroides melaninogenicus* was strongly stimulated by DHNA; this material was effective at $10^{-5}$ M and was comparable to phylloquinone or MK-9. Among the nonquinonoid naphthalene compounds, it is the most effective growth stimulator for this organism. Furthermore, [2,3-$^{14}C_2$]DHNA was administered to *E. coli*, using anaerobic growth conditions to minimize oxida-
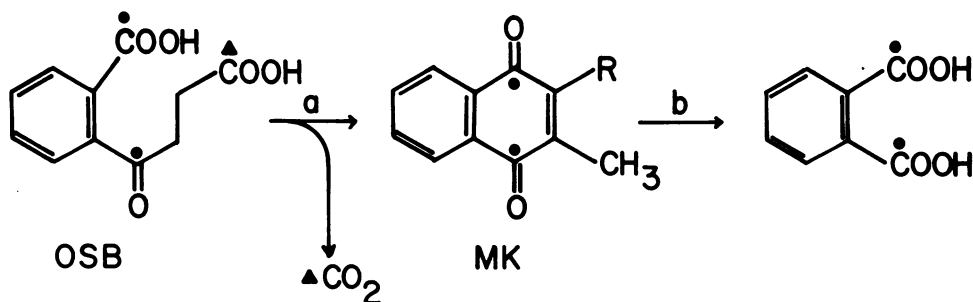
FIG. 8. Utilization of OSB for menaquinone biosynthesis. R = Prenyl. The results of two separate experiments have been combined. The reactions are: a, biosynthetic utilization; b, degradation to phthalate either directly with KMnO$_4$ or as shown in Fig. 2.

tive degradation (25). A high incorporation into MK-8 was obtained ($I$ = 3.3%) coupled with a low $D$ value (1.4). (By contrast, in a control experiment under the same conditions, 1,4-[1,4-$^{14}C_2$]naphthoquinone was less efficiently incorporated and with higher dilutions: $I$ = 0.3%; $D$ = 14.5.) The MK-8 derived from the [2,3-$^{14}C_2$]DHNA was degraded to phthalate and malonate. The phthalate was without radioactivity, and the malonate contained 50% of the activity of the naphthylacetate (see Fig. 2 for reactions). Hence, the conversion apparently was a direct one, without degradation. The carboxyl group is, of course, removed at some further stage (see Fig. 1).

It is of interest that DHNA is degraded by cell-free extracts of a nonfluorescent pseudomonad (grown on *m*-cresol) to pyruvate and phthalate (108). This reaction was postulated to involve a keto derivative of OSB. In phenanthrene-grown *Aeromonas* sp. S45P1, the related

1-hydroxy-2-naphthoate is degraded to 2-carboxybenzaldehyde and hence to phthalate (123).

### Role of Naphthalene Compounds Other than 1-Naphthol

In addition to the somewhat contradictory results obtained in the incorporation experiments with radioactive 1-naphthol, equally contradictory results have been obtained with some other naphthalene compounds. Some of the observations are summarized in Table 3. In four organisms, there is apparently a utilization of labeled menadione for MK biosynthesis; these observations indicate that the final stage in menaquinone biosynthesis is prenylation rather than methylation. In the case of *Staphylococcus aureus*, this possibility gains some credence from the fact that menadione (i.e., MK-0) can actually be shown to be present along with other, more usual isoprenylogs (93).
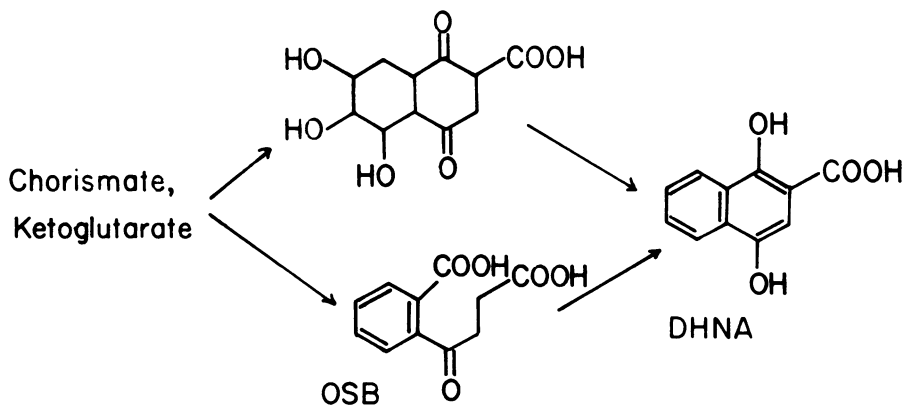
To conclude that menadione is, in some cases,



FIG. 9. Formation of 1,4-dihydroxy-2-naphthoate. The (no longer accepted) trihydroxydecalindione carboxylic acid was proposed by Campbell et al. (44) and Robins et al. (181), and OSB was proposed by Dansette and Azerad (60).

TABLE 3. Incorporation of naphthalenoid materials into bacterial menaquinones

| Precursor | Organism | Incorporation | Reference |
|---|---|---|---|
| [methyl-$^{14}$C]menadione | "Aerobacter aerogenes" 170-44 | + | 83 |
| [methyl-$^{14}$C]menadione | Bacillus megaterium | + | 217 |
| [methyl-$^{14}$C]menadione | Bacteroides melaninogenicus[a] | + | 150 |
| [methyl-$^{14}$C]menadione | Staphylococcus aureus | + | 93, 217 |
| [methyl-$^{14}$C]menadione | Escherichia coli | − | 44, 217 |
| [methyl-$^{14}$C]menadione | Micrococcus luteus | − | 220 |
| [methyl-$^{14}$C]menadione | Mycobacterium phlei | − | 44, 217 |
| 1,4-[1,4-$^{14}$C]naphthoquinone | Mycobacterium phlei | − | 44 |
| 1,4-[1-$^{14}$C]naphthoquinone | "Aerobacter aerogenes" 170-44 | + | 83 |
| 1,4-[1,4,5,8-$^{14}$C]naphthoquinone | "Aerobacter aerogenes" 170-44 | + | 83 |
| 1,4-[5,8(?)-$^{3}$H]naphthoquinone | Bacteroides melaninogenicus | + | 150 |
| [methyl-$^{3}$H,1',2'-$^{14}$C]phylloquinone | Bacteroides melaninogenicus | + | 150 |

[a] [methyl-$^{3}$H]menadione was also examined in this organism.

a precursor to menaquinones, it would be necessary to carry out further experiments. These would involve rigorous purification of samples derived from tracer experiments with labeled menadione. The problem is illustrated by work on the administration of [methyl-$^{14}$C]menadione to E. coli and Mycobacterium phlei. Although the initial extracts containing the MK fractions were strongly radioactive, on gel filtration chromatography followed by derivatization, the radioactivity was lost (44). Unfortunately, rigorous purification procedures such as these have generally not been used, so the precise significance of a positive incorporation result is difficult to assess.

The incorporations observed in some cases with labeled samples of naphthoquinone are also difficult to evaluate. Since the evidence strongly indicates that a symmetrical intermediate is not involved in menaquinone biosynthesis, it appears that some kind of aberrant pathway must be involved, if indeed the results are not due to the presence of radioactive contaminants. Two organisms, "A. aerogenes" 170–44 and Bacteroides melaninogenicus, seem to be particularly active in converting naphthalenoid compounds to menaquinones. The Bacteroides melaninogenicus strain examined by Martius and Leuzinger (150) was actually the Lev strain and required a supplement of vitamin K for growth. A particularly striking result was the conversion of phylloquinone, labeled in the methyl group with $^{3}$H and in the phytyl side chain with $^{14}$C to a menaquinone containing only $^{3}$H. This observation implies that the phytyl side chain was removed and replaced with the typical menaquinoid side chain. This result may again be suspect. The purification used was a Craig countercurrent distribution, and on a single separation the MK fractions appeared to be labeled with both isotopes. On repetition of the counter-

current distribution, it appeared that the $^{3}$H activity showed two maxima; the second was very small and contained a reduced level of $^{14}$C. However, the experimental evidence (150 [Fig. 3b]) is less than convincing, although it was claimed that this small peak was MK-9. A much larger peak containing large amounts of both $^{14}$C and $^{3}$H was also present. Martius and his colleagues also observed that radioactive phylloquinone was converted to MK-4 in animals when administered orally, but not when administered by injection (29). This again suggested that the phylloquinone side chain was removed by intestinal bacteria. The methodology relied on the Craig countercurrent distribution technique and is not above suspicion.

Whatever the merits of these incorporation experiments, the genetic and enzymological studies in organisms such as Bacillus subtilis and E. coli, and the enzymological work in Micrococcus luteus and Mycobacterium phlei, provide no indication for a role for menadione or naphthalenoid derivatives other than DHNA in menaquinone biosynthesis.

A further complication in work on the possible significance of naphthalenoid compounds is the known toxicity of many of these materials for bacteria (14). In particular, substituted naphthoquinones are quite inhibitory to the growth of bacteria (131). For 50% inhibition of growth of E. coli and Staphylococcus aureus, the concentrations ranged from $4.76 \times 10^{-5}$ to $36.0 \times 10^{-5}$ M and $0.09 \times 10^{-5}$ to $9.0 \times 10^{-5}$ M, respectively (61 compounds were examined in this work). In the more sensitive Staphylococcus aureus, menadione had a 50% growth inhibition concentration of $0.55 \times 10^{-5}$ M, and phylloquinone had one of $11.1 \times 10^{-5}$ M. Even in a bacterium with a specific growth requirement, toxicity may occur beyond a critical concentration (139). In Bacillus cereus, the antibacterial action of mena-

dione results from a specific inhibition of RNA synthesis in growing cells (124).

## INDIVIDUAL REACTIONS IN MENAQUINONE BIOSYNTHESIS

### Formation of *o*-Succinylbenzoate

The formation of OSB from chorismate and 2-ketoglutarate (glutamate) represents an unusual synthesis of an aromatic compound from a cyclohexadiene structure. These reactions provide the largest unknown area of the entire biosynthetic pathway, and it is only recently that a cell-free synthesis of OSB has been demonstrated by Meganathan (162). Improved techniques for the isolation, purification, and identification of small quantities of OSB were devised (164); a major component was the use of radiogas chromatography to examine OSB as its dimethyl ester. A cell-free extract of *E. coli*, prepared by use of a French press, was incubated with 2-[$U$-$^{14}$C]ketoglutarate and chorismate in the presence of thiamine pyrophosphate (TPP). In the absence of chorismate, no OSB was produced, and in the absence of TPP there was a decreased synthesis of OSB. The organism originally used was an *E. coli* mutant, AN154, blocked in all of the aromatic pathways with the exception of that for menaquinone (see Genetics of Menaquinone Biosynthesis). The occurrence of "OSB synthase" activity has, however, now been demonstrated in wild-type strains (R. Meganathan and R. Bentley, unpublished data).

The chemistry of this reaction is clearly complex; the fundamental addition of the succinyl side chain has for long been considered to require the succinic semialdehyde anion complex of TPP (40). If this is indeed the case, as many as five separate stages might be needed for the overall process: (i) formation of the succinic semialdehyde-TPP complex from 2-ketoglutarate; (ii) addition of the succinic semialdehyde-TPP complex to chorismate; (iii) regeneration of TPP; (iv) removal of the pyruvoyl group originally associated with chorismate; and (v) removal of the hydroxyl group originally associated with chorismate.

### Formation of succinic semialdehyde-thiamine pyrophosphate complex. 

A succinic semialdehyde-TPP anion is presumably formed during the action of the 2-ketoglutarate dehydrogenase complex by the first (decarboxylase) enzyme (Fig. 10). Although this first enzyme of the ketoglutarate dehydrogenase complex remains as a prime candidate, an alternate and separate decarboxylase cannot be ruled out at this time. After dialysis, the *E. coli* OSB synthase preparations were stimulated by the addition of TPP, and these same extracts showed stimulation by

TPP when 2-ketoglutarate decarboxylase activity was assayed by ferricyanide reduction (91; modified to read absorbance at 420 nm). When extracts were prepared from a 2-ketoglutarate decarboxylase-negative mutant of *E. coli*, there was a decreased incorporation of $^{14}$C from 2-[$U$-$^{14}$C]ketoglutarate into OSB. This observation is consistent with the known leakiness of this group of mutants and their ability to grow anaerobically on lactatefumarate media.

**Subsequent reactions.** Figure 11A shows a possible reaction sequence in which the ordering of the stages is as follows: anion addition, removal of TPP, removal of the pyruvoyl group, and removal of the hydroxyl. An analogy for the proposed biosynthetic process has been provided recently by a stereoselective synthesis of decalin derivatives (113; Fig. 12).

It is not easy to predict how many enzymes are needed for the various stages. The maximum is probably four, in addition to the decarboxylase. This number could be reduced if more than one stage was catalyzed by a single enzyme or if some of the reactions were concerted. For example, the anion addition (step 1, Fig. 11A) and removal of TPP (step 2, Fig. 11A) could perhaps require only one enzyme. An attractive possibility for anion addition concerted with removal of the chorismate OH group is shown as step 1 in Fig. 11B. If the enzyme also catalyzed TPP removal (step 2, Fig. 11B), only two enzymes, in addition to the decarboxylase, would be required. A concerted mechanism for the addition of glutamate to chorismate during the action of anthranilate synthetase was proposed earlier (204, 212); in this case, addition takes place at position 6 of chorismate, and direct evidence for a postulated intermediate has, apparently, not been obtained.

Some evidence for two enzymes comes from the fact that there are two groups of *E. coli* mutants, *menC* and *menD*, which require OSB for growth and are, therefore, blocked in OSB biosynthesis (55, 84, 85). This suggests the possible occurrence of a definite intermediate in the formation of OSB from chorismate and 2-ketoglutarate. To investigate this possibility, cell-free extracts were prepared from *menC* and *menD* mutants. After incubation of these extracts separately with 2-[$U$-$^{14}$C]ketoglutarate and chorismate followed by protein denaturation, ethyl acetate extraction was performed in the hope of isolating any such intermediate. The two materials obtained after removal of ethyl acetate were then further incubated with an extract from the other mutant. Evidence was obtained that the *menC* mutant did, in fact, form an intermediate and that this intermediate was converted to OSB by the extract from the *menD*
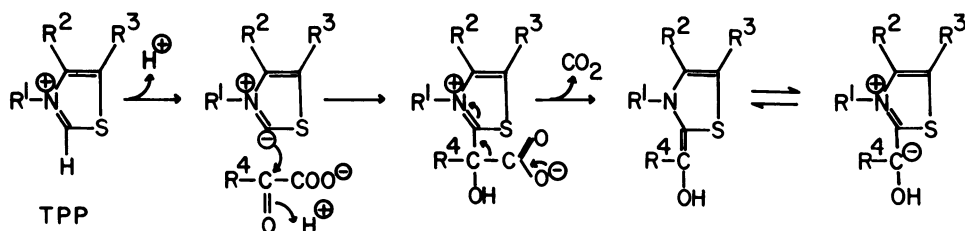
FIG. 10. Formation of the succinic semialdehyde-TPP anion. $R^1$ = Pyrimidine component of TPP; $R^2$ = $CH_3$; $R^3$ = $CH_2CH_2OP_2O_6^{3-}$; $R^4$ = $CH_2CH_2COOH$. The suggested anion is shown as the final structure; it is one possible resonance structure.

mutant (R. Meganathan and R. Bentley, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, K138, p. 159). It appears from these preliminary experiments that the *menD* gene codes for an enzyme which is responsible for the formation of an intermediate, "X," and that the enzyme coded for by the *menC* gene converts this intermediate to OSB:

$$\text{Chorismate + succinic semialdehyde-TPP} \xrightarrow{menD} \text{X} \xrightarrow{menC} \text{OSB}$$

$$2\text{-ketoglutarate + TPP} \xrightarrow{\phantom{menD}} CO_2$$

The chemical nature of X remains to be determined; assuming that it does not contain TPP,

three possible structures for the intermediate are indicated in Fig. 11.

### Formation of 1,4-Dihydroxy-2-Naphthoate

The enzymatic conversion OSB → DHNA was first demonstrated in *E. coli* (37) and *Mycobacterium phlei* (159) extracts. It was of considerable interest that the conversion showed an absolute dependence on the presence of CoA and ATP. However, initial attempts to purify the enzyme(s) were not successful. It was subse-
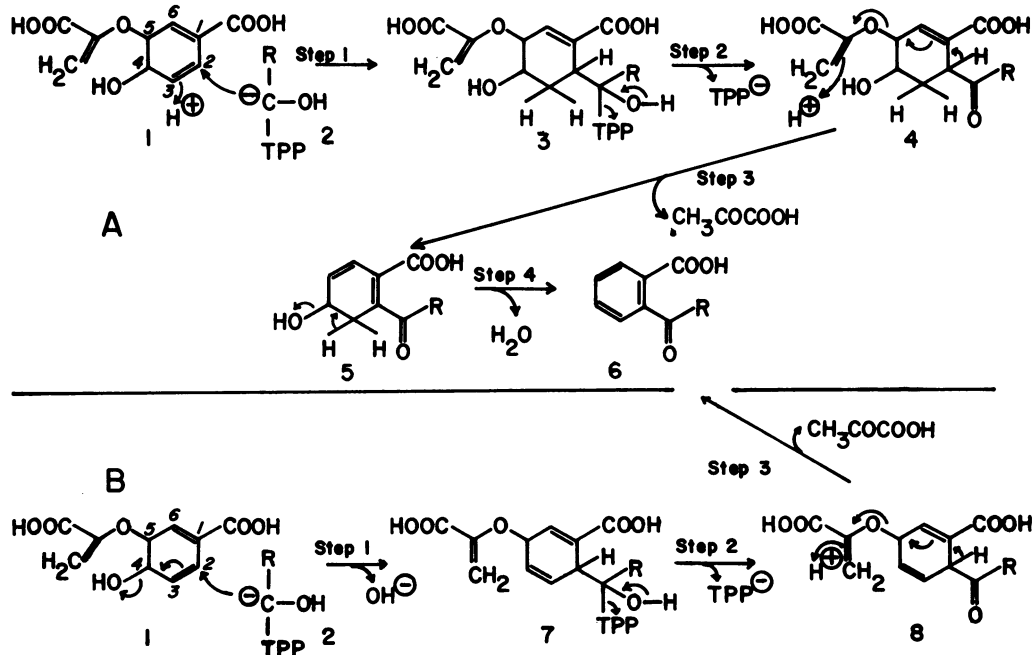


FIG. 11. Reaction mechanisms for OSB biosynthesis. R = $CH_2CH_2COOH$. Compounds are identified as follows: 1, chorismate; 2, succinic semialdehyde-TPP anion; 6, OSB. The postulated intermediate, X, could have structure 4, 5, or 8, assuming it does not contain TPP.
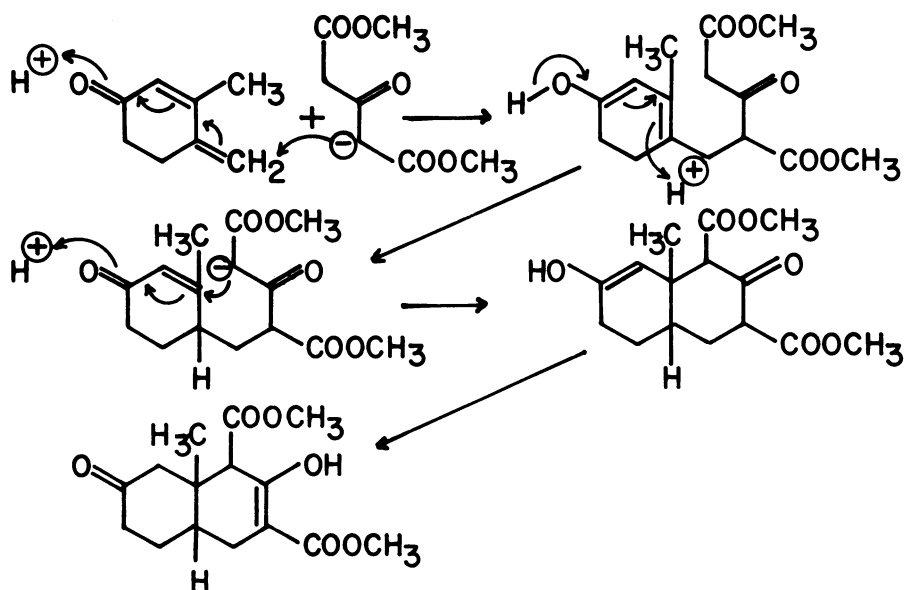
FIG. 12. Chemical analogy for the biosynthetic reaction. The chemical process was carried out with KF in dimethyl sulfoxide.

quently found that a cell-free enzyme extract from *Mycobacterium phlei* could be treated with protamine sulfate in the presence of 20% dimethyl sulfoxide so that one protein was precipitated and a second remained in solution; neither protein alone formed DHNA, but a combination of the two did so (163). These facts, combined with the following, provided strong evidence that a CoA derivative of OSB was involved as an intermediate in the overall conversion. (i) By the use of [$^{14}$C]ATP, it could be shown that AMP and pyrophosphate were formed during the reaction, which is typical of those ligases forming CoA esters. (ii) When the protein remaining in solution (on treatment with protamine sulfate as just described) was incubated with [2-$^{14}$C]OSB, ATP, and CoA, the spirodilactone derivative of OSB was produced (but no DHNA). This result could be explained by formation of the (unstable) OSB-CoA derivative, followed by a spontaneous, nonenzymatic lactonization. These processes are summarized in Fig. 13.

The two enzymes have been termed OSB-
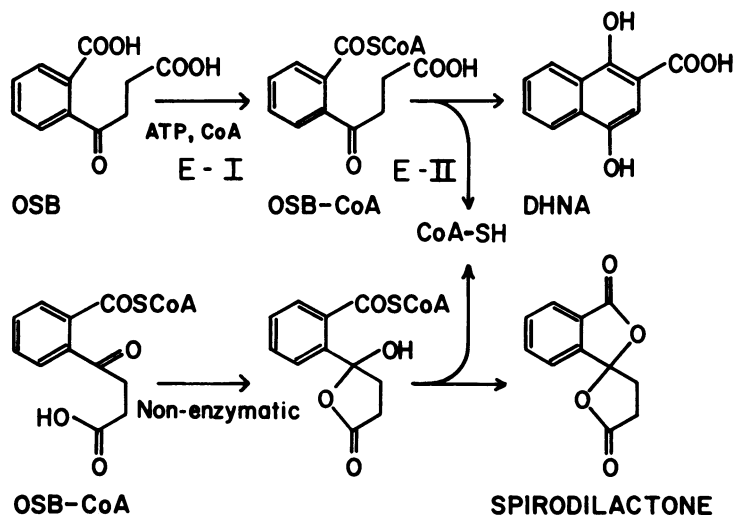


FIG. 13. Formation of OSB-CoA and its conversion to DHNA by an enzyme and to a spirodilactone nonenzymatically. The enzymes involved are: E-I = OSB-CoA synthetase; E-II = DHNA synthase.

CoA synthetase (E-I) and DHNA synthase (E-II); the separability of the *Mycobacterium phlei* enzymes has received an independent confirmation (98). Further evidence for their existence came from enzymological studies on mutants of *Bacillus subtilis* and *E. coli* (165; D. J. Shaw, J. R. Guest, R. Meganathan, and R. Bentley, unpublished data).

Extracts from a (wild-type) *men*$^+$ *Bacillus subtilis* catalyzed DHNA formation at a somewhat higher pH, 7.5 to 8.5, than did *Mycobacterium phlei* extracts (pH 6.9). Extracts of mutant strains RB413 (*men-325*) and RB415 (*men-329*) produced DHNA in combination with extracts from either RB388 (*men-310*) or RB397 (*men-312*); no DHNA was formed by any extract separately. Complementation analysis with preparations of OSB-CoA synthetase and DHNA synthase from *Mycobacterium phlei* showed that RB388 (*men-310*) and RB397 (*men-312*) lacked OSB-CoA synthetase but did possess DHNA synthase; the reverse situation held with RB413 (*men-325*) and RB415 (*men-329*). Mutants defective in the structural gene for OSB-CoA synthetase are now termed *menE*, and those defective in the structural gene for DHNA synthase are termed *menB*. *E. coli* mutants deficient in one or the other enzyme have similarly been identified by the same methods.

In this work, fresh cells were used and were lysed with lysozyme. In addition to unchanged OSB and DHNA, the thin-layer chromatograms showed a third spot which was identified as the spirodilactone of OSB. We believed that formation of spirodilactone resulted from a low level of DHNA synthase in the extracts; when a DHNA synthase preparation from *Mycobacterium phlei* was added to the incubation mixtures, spirodilactone formation was suppressed and DHNA production increased. No difficulty was experienced in showing DHNA formation from OSB with extracts prepared from 5-year-old spray-dried cells of *Micrococcus luteus*. There is, therefore, no ready explanation for the failure of Hutson and Threlfall to show DHNA formation in extracts from *Micrococcus luteus*.

In earlier work with *E. coli*, we had sometimes encountered some formation of the spirodilactone (37); it appears that bacterial extracts contain different levels of DHNA synthase and with those that we have examined the situation is as follows: *Mycobacterium phlei* extracts never show formation of spirodilactone; *E. coli* extracts sometimes show formation of spirodilactone; *Micrococcus luteus* and *Bacillus subtilis* extracts always show formation of spirodilactone. The OSB-CoA derivative is rather unstable and, unless a high level of DHNA synthase

$$\text{OSB} \xrightarrow[\text{OSB-CoA synthetase}]{menE} \text{OSB-CoA} \xrightarrow[\text{DHNA synthase}]{menB} \text{DHNA}$$

It was also observed that when extracts from *menB* mutants of *Bacillus subtilis* and *E. coli* were incubated with [$^{14}$C]OSB, ATP, and CoA, radioactive spirodilactone was the only product formed. Further, the spirodilactone formation could be suppressed by adding DHNA synthase from *Mycobacterium phlei*. This result again supported the formation of an unstable OSB-CoA compound which decomposed to spirodilactone by elimination of CoA-SH (see Fig. 13).

Other workers were unable to demonstrate the conversion of OSB to DHNA by using cell-free extracts prepared from spray-dried cells of *Micrococcus luteus* (111); similar negative results were also obtained in a limited number of experiments with *E. coli* extracts. With the *Micrococcus luteus* extracts, they routinely observed the formation of OSB spirodilactone on incubation of OSB with CoA and ATP. *Micrococcus luteus* contains high levels of menaquinone (about five times those in *E. coli*), and these surprising results raised the possibility of an alternate pathway for menaquinone biosynthesis. In our hands, incubation of cell-free extracts of *Micrococcus luteus* under the same conditions did lead to the formation of DHNA (166).

activity is present, spirodilactone formation occurs with elimination of CoA-SH (see Fig. 13).

It is not known at present whether these differing responses result from different levels of DHNA synthase relative to OSB-CoA synthetase in different organisms or whether it reflects possible loss of DHNA synthase activity on extraction from cells. So far, most attention has been given to the two enzymes present in *Mycobacterium phlei*, *Micrococcus luteus*, and *Bacillus subtilis* (163, 165, 166). In general, DHNA synthase appears to be less stable than the OSB-CoA synthetase. For example, in *Mycobacterium phlei* preparations, the OSB-CoA synthetase shows a definite resistance to low pH and enzymatic activity can be recovered after exposure of the preparations to 0.1 N HCl for 5 min. Under these conditions, the *Mycobacterium phlei* DHNA synthase is completely inactivated (163).

The OSB-CoA synthetase from *Mycobacterium phlei* has been purified approximately 1,200-fold (Table 4); on acrylamide gel electrophoresis, however, there is present one major and two or three minor bands (R. Meganathan, C. Dippold, and R. Bentley, unpublished data).

TABLE 4. Purification of OSB-CoA synthetase

| Procedure | Fraction no. | Vol (ml) | U (ml)[a] | Total U | Protein (mg/ml) | Activity (U/mg of protein) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|---|---|---|
| Extract | | 37.1 | 24.5 | 909.0 | 30.00 | 1.23 | 100 | |
| Protamine sulfate precipitation | S[b] | 59.4 | 12.7 | 754.4 | 2.90 | 4.30 | 83 | 3.6 |
| Acid treatment and dialysis | | 49.8 | 11.8 | 587.6 | 0.69 | 17.10 | 65 | 13.9 |
| $(NH_4)_2SO_4$ precipitation and dialysis | | 1.5 | 367.6 | 551.4 | 3.80 | 96.70 | 61 | 78.6 |
| Affi-Gel Blue Column | 12–14 | 3.0 | 156.9 | 470.7 | 0.15 | 1,046.00 | 52 | 850.0 |
| Matrex Gel Green A | 12–14 | 3.0 | 125.1 | 375.3 | 0.08 | 1,564.00 | 41 | 1,271.0 |

[a] Unit = nanomoles of DHNA produced per 30 min.
[b] S, Supernatant.

In this purification the use of relatively nonspecific affinity columns was particularly useful; possibly construction of OSB-containing affinity columns might lead to a complete purification.

In *E. coli* these two enzymes do not behave as just described. Attempts to use the dimethyl sulfoxide-protamine sulfate method for the separation of the *E. coli* enzymes have led to loss of all DHNA synthase activity, although the OSB-CoA synthetase activity was retained. A similar result was obtained with ion-exchange chromatography. Furthermore, the *E. coli* OSB-CoA synthetase is inactivated by acidic conditions under which the *Mycobacterium phlei*, *Bacillus subtilis*, and *Micrococcus luteus* enzymes retain activity.

### Structure of o-Succinylbenzyl–Coenzyme A Intermediate

In 1981, Heide and Leistner achieved the isolation of the putative OSB-CoA derivative (98). A preparation of OSB-CoA synthetase from *Mycobacterium phlei* was prepared by the protamine sulfate precipitation methods just described. After incubation of [4'-$^{14}$C]OSB, ATP, CoA, and $Mg^{2+}$ with the enzyme preparation, separation was attained by paper chromatography (Whatman 3 MM paper; butanol-acetic acid-water, 5:2:3). The CoA derivative ($R_f = 0.48$) was eluted with 3 M formic acid and was further purified on a Hg-Sepharose column which retained residual CoA-SH. Formation of the $^{14}$C-labeled CoA derivative was only observed in the presence of enzyme and ATP. Use of $^3$H-labeled CoA-SH also gave a radioactive product; with both labels, it was possible to show that the $^3$H/$^{14}$C ratio was that expected from a mono-CoA derivative rather than a di-CoA ester. The

OSB-CoA derivative was active as a substrate with DHNA synthase, as expected.

The OSB-CoA ester was relatively unstable, as had been concluded before its isolation. It was most stable at acid pH and was converted to OSB spirodilactone plus CoA-SH under neutral conditions and to OSB plus CoA-SH under alkaline conditions.

Subsequently, Leistner and his colleagues have provided evidence that the CoA moiety is located on the aromatic carboxyl group of OSB (128). In this work, paper chromatography was replaced by thin-layer chromatography on cellulose for the isolation of the ester. The nonesterified carboxyl was reacted with diazomethane, and the resulting diester (CoA, CH$_3$) was hydrolyzed under mild conditions to cleave the thioester bond. The $^{14}$C-labeled product was compared with reference samples of "aliphatic" and "aromatic" ester by thin-layer chromatography on silica gel. (Reference samples were obtained by partial hydrolysis of dimethyl OSB and were identified on the basis of $R_f$ values and $^1$H-nuclear magnetic resonance and mass spectra. In particular, the base peak at $m/z$ 149 was obtained by the fragmentation shown in Fig. 14.) This work provided convincing evidence that the aromatic carboxyl carries the CoA unit, as had been originally suggested (37, 159, 163). The correct location of the CoA unit is, in fact, shown in Fig. 13 and 14.

Similar results have been obtained in our laboratory (R. Meganathan, G. Emmons, L. A. Ernst, I. M. Campbell, and R. Bentley, unpublished data). [$^{14}$C]OSB was incubated with purified preparations of OSB-CoA synthetase obtained from *Mycobacterium phlei*; products were separated by thin-layer chromatography on cellulose plates (*n*-butanol–acetic acid–water,

5:2:3). The slowest-moving peak ($R_f$ = 0.51) was removed, dissolved in methanol, and treated with diazomethane. The labile thioester bond was subsequently hydrolyzed at pH 8.0 to yield a monomethyl OSB. This product was subjected to the action of diazoethane, and the mixed methyl ethyl ester of OSB was examined by radiogas chromatography and mass spectrometry. For identification purposes, dimethyl and diethyl derivatives of OSB were prepared by alkylation, respectively, with diazomethane and diazoethane; mixed esters were obtained by exchange reactions. Study of the mass spectra of these known compounds indicated the characteristic fragment ion shown in Fig. 14. The mixed methyl ethyl ester obtained from enzymatically synthesized OSB-CoA was shown to have the ethyl group on the aromatic carboxyl, indicating location of the CoA at that position.

## Prenylation Reaction

In the presence of farnesyl pyrophosphate and $Mg^{2+}$, cell-free extracts of *E. coli* were shown to convert DHNA to MK-3 or DMK-3 or both (25). In more detailed investigations, Shineberg and Young obtained a membrane-bound enzyme, 1,4-dihydroxy-2-naphthoate octaprenyltransferase, from *E. coli*; this enzyme was active with either synthetic solanesyl pyrophosphate or (natural) octaprenyl pyrophosphate, but solanesyl monophosphate was not a substrate (201). The enzyme showed a lipid and $Mg^{2+}$ requirement. The overall conversion, DHNA → DMK, actually requires three stages: removal of the DHNA carboxyl as $CO_2$, the attachment of the isoprenoid residue, and a quinol → quinone oxidation. The demethylmenaquinol is a likely intermediate, and possibly its conversion to DMK is a spontaneous process (Fig. 15). The question of whether more than one enzyme is involved has not been answered unequivocally; the available evidence, however, suggests one enzyme, possibly with a concerted mechanism. Evidence for a single enzyme is that the decarboxylation product, 1,4-naphthoquinol, cannot exist as even a transient intermediate since this would lead to a symmetrical situation. Furthermore, as will be described below, *menA* mutants of *E. coli* accumulate DHNA and not 1,4-naphthoquinol or 1,4-naphthoquinone (234).

The 1,4-dihydroxy-2-naphthoate octaprenyltransferase has some features in common with 4-hydroxybenzoate octaprenyltransferase involved in ubiquinone biosynthesis (both are membrane bound and require $Mg^{2+}$). In addition, both of these enzymes appear to use a common pool of membrane-bound octaprenyl pyrophosphate as the prenyl donor (127, 234). Genetic evidence, however, indicates that they are quite distinct. The side chain lengths of the menaquinones are probably determined by the availability of the isoprenyl pyrophosphate substrate(s) within the membranes. In addition to the situation just described for *E. coli*, there is an association between the polyprenyl pyrophosphate synthetase isolated from *Bacillus subtilis*, which produces all *trans*-heptaprenyl pyrophosphate (211), and the fact that this organism produces exclusively MK-7. It is of interest in this connection that an enzyme reacting DHNA with phytyl pyrophosphate has been detected in spinach chloroplasts (197); this enzyme is involved in the biosynthesis of phylloquinone. A similar enzyme is presumably present in the cyanobacteria, which also biosynthesize phylloquinone.

Saito and Ogura have also investigated a prenyl transferase enzyme in the membrane fraction of *Micrococcus luteus* (185). This enzyme was found to be relatively nonspecific with respect to the prenyl unit; both all *trans*-farnesyl pyrophosphate and all *trans*-geranylgeranyl pyrophosphate were effectively converted to DMK. The all *trans* forms of octaprenyl pyrophosphate and farnesyl and geranyl phosphate were less effective. Inactive compounds were dimethylallyl pyrophosphate, *trans*-farnesol, and *trans*-octaprenol. It appears from these results that a *trans* configuration in the 2 double bond is important. With regard to the prenyl acceptor, a greater specificity was observed. As expected, DHNA was very active, and the fol-
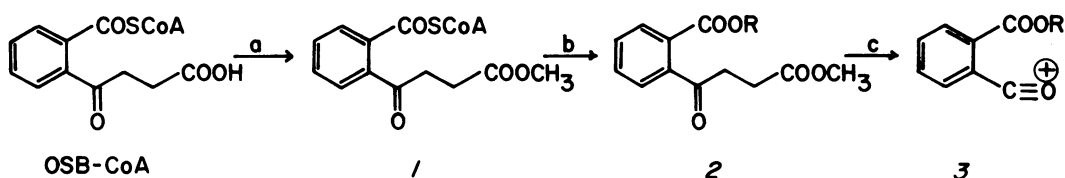


FIG. 14. Structure of OSB-CoA. Two sets of experiments are covered. In the work of Leistner and colleagues (128) the OSB-CoA derivative was converted with $CH_2N_2$ (a) to the methyl ester, 1; the acid, 2, R = H, was obtained on mild alkaline hydrolysis (b). On mass spectrometry (c), this material provided the ion, 3, R = H, $m/z$ = 149. In unpublished work from our laboratory, the same methyl ester, 1, was hydrolyzed (b) at pH 8; the product was realkylated with diazoethane to 2, R = $C_2H_5$. On mass spectrometry (c), the ion, 3, R = $C_2H_5$, $m/z$ = 177, was obtained.
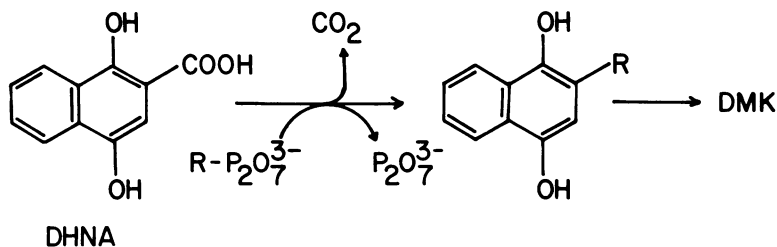
FIG. 15. Prenylation of 1,4-dihydroxy-2-naphthoate. R = Prenyl.

lowing were much less effective as acceptors: 2-carboxy-4-hydroxy-α-tetralone, 1,4-dihydroxy-3-methyl-2-naphthoate, and 1-hydroxy-2-naphthoate. A number of substituted benzoic and naphthoic acids and naphthalenes were inactive.

## Methylation of Demethylmenaquinone

A cell-free extract of *Mycobacterium phlei* was shown to incorporate radioactivity from L-[$^{14}$CH$_3$]methionine into menaquinones; ATP and MgCl$_2$ were required, probably to facilitate the formation of *S*-adenosylmethionine (18, 45). Similarly, the conversion of DMK-3 to MK-3 was demonstrated in *E. coli* extracts by using *S*-[$^{14}$CH$_3$]adenosyl-L-methionine (37). The structural requirements of the methylase system were investigated by Samuel and Azerad (187). DMK-1 was not a substrate, and a side chain containing two or more prenyl units was required. DMK-3 and DMK-4 showed maximal activity, which decreased progressively up to about DMK-9. Saturation of one or more of the isoprene units, other than the first, had little effect on the methylation rate; both DMK-3 (II-H$_2$) and demethylphylloquinone were good substrates. Saturation of the first double bond (adjacent to the nucleus) resulted in loss of activity. The *trans* configuration was necessary in the first double bond. Structural changes in the naphthoquinone ring, by partial saturation (5,8-dihydro-2-phytyl-1,4-naphthoquinone) or by replacement (2,3-dimethylbenzoquinone), resulted in loss of activity.

The methylase enzyme (*S*-adenosylmethionine:2-demethylmenaquinone methyltransferase) is localized in the particulate membrane fraction of *Mycobacterium phlei*. Lipids could be removed from the preparations by treatment with acetone to yield a powder, stable for some months. However, attempts at further solubilization and purification were not satisfactory, and reproducible results could not be obtained. A change in substrate specificity occurred in the presence of a phospholipid fraction; under these conditions, DMK-2 was the preferred substrate. Investigations with [*methyl*-$^2$H$_3$]methionine

have shown that in formation of MK-9 (II-H$_2$) by *Mycobacterium smegmatis* all three $^2$H atoms of the methyl group were transferred ("mécanisme-CD$_3$") (117). A similar result has been obtained for MK-8 produced by *E. coli* strain 518 (115) and for the formation of a hydroxylated phylloquinone by the alga *Euglena gracilis* (218). In contrast, only two hydrogens are transferred, for example, in the biosynthesis of the "extra" methyl group of C$_{28}$ sterols and in the formation of tuberculostearic acid by *Mycobacterium smegmatis* (132).

## Formation of Reduced Isoprenyl Units

Although the formation of menaquinones with one or more isoprene units in the reduced condition is common (48), little is known of the biosynthesis of these materials. The reduction of MK-9 to MK-9 (II-H$_2$) has been demonstrated in cell-free extracts of *Mycobacterium phlei* (18). The reduction required NADH or NADPH as electron donor, but nothing further is known concerning this enzyme.

## GENETICS OF MENAQUINONE BIOSYNTHESIS

### *men* Mutants of *Escherichia coli*

The most complete genetic information relating to menaquinone biosynthesis has been obtained with mutant strains of *E. coli*. Five separate genes have been identified, and for three of these the association with a particular biosynthetic enzyme has been established. The other two genes are concerned in the first committed step of menaquinone biosynthesis where the prearomatic compound chorismate is converted to the fully aromatic OSB by a complex sequence of reactions. The precise role of these two genes is not yet clear. The enzymological consequences of the mutations were discussed above (Individual Reactions in Menaquinone Biosynthesis); hence, no further descriptions of enzymology will be given here. The present understanding of the relationship of the *men* genes to the biosynthetic pathway is summarized below for convenience.

$$\text{Chorismate} \xrightarrow{menD} \text{``X''(?)} \xrightarrow{menC} \text{OSB} \longrightarrow$$

OSB synthase

$$\xrightarrow{menE} \text{OSB-CoA} \xrightarrow{menB} \text{DHNA} \xrightarrow{menA} \text{DMK}$$

OSB-CoA          DHNA synthase          DHNA octa-
synthetase                              prenyl transferase
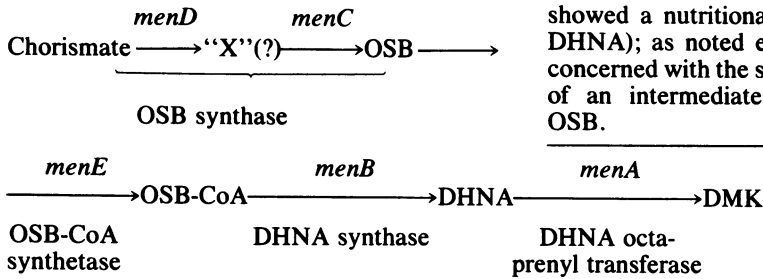
showed a nutritional requirement for OSB (or DHNA); as noted earlier, these genes may be concerned with the synthesis and transformation of an intermediate between chorismate and OSB.

The first *E. coli* mutant, deficient in menaquinone biosynthesis, was isolated during a search for ubiquinone-deficient mutants; strains were selected which were unable to grow on malate as the source of carbon and energy after mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (54). Subsequently, this mutation was shown to be cotransducible with *metB* and *argE* at frequencies of 30 and 50%, respectively; hence the gene, designated *menA*, was tentatively located at min 78 on the *E. coli* chromosome (169). It should be noted that *E. coli* mutants deficient in genes for both ubiquinone and menaquinone (i.e., *ubiA menA*) have been constructed by transduction techniques (228). In growth studies on glucose media the generation time increased in *ubi* strains and was very prolonged in the double quinone mutant. The following generation times were determined: AN387 (*ubi*+ *men*+) and AN386 (*ubi*+ *men*), both 60 min; AN385 (*ubi men*+), 180 min; AN384 (*ubi men*), 420 min. Clearly, menaquinone has a role in aerobic metabolism when ubiquinone is absent. In addition to these strains, another double quinone mutant, AN187 (*ubiD menA*), was also constructed by transduction techniques (169).

In 1975, Young isolated menaquinone-deficient *E. coli* mutants by treatment of strain AB3311 with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (201, 232). The mutants selected were those unable to grow on succinate as a sole carbon source, but able to grow on glucose; the selection procedure was a fortuitous one. The organisms of one group were *menA* mutants and were found to be unable to attach the isoprenyl side chain; hence, such mutants accumulated DHNA. A second group of mutants was originally termed *menB* and was found to accumulate OSB (201, 232). These mutants, blocked in the conversion of OSB to DHNA, were later shown to consist of two groups, now termed *menE* and *menB* (D. J. Shaw, personal communication).

Guest identified two further groups of mutants, *menC* and *menD*, which were selected for their inability to use fumarate as a terminal electron acceptor (84, 85). These mutants

The *menB*, *-C*, and *-D* genes form a cluster at 48.5 min on the *E. coli* linkage map; the *menA* gene (originally placed at 78 min) is actually located at 88 min according to the recalibrated linkage map (20). A transducing phage carrying some of the *men* genes has been isolated from a pool of lambda phages that were constructed from R · HindIII digests of *E. coli* DNA and the corresponding insertion vector (86). This phage, λG68 [λ*menCB(D)*], was used in attempts to complement lesions in *menB*, *-C*, and *-D*; complementation did occur with *menB* and *menC* mutants but the *menD* mutants were transduced at low frequencies or not at all. Thus, the transducing phage contains functional *menC* and *menB* genes, but only part of the *menD* gene. Restriction analyses established the presence of a bacterial DNA fragment (11.5 kilobases) linked by an R · HindIII target to the right arm of the λ genome but fused to the left arm of the vector (86). Studies with this phage have led to a reinterpretation of the mapping data; the gene order is now believed to be as follows: *nalA-....menC....menB....menD....purF*. The exact location of the *menE* gene has not been determined. The single *menE* mutant that is available, AN213, has been found to be leaky, allowing sufficient menaquinone to be formed for growth to occur anaerobically on lactate-fumarate media (D. J. Shaw, personal communication).

### *men* Mutants of *Bacillus subtilis*

Mutants of *B. subtilis*, an organism that does not contain ubiquinone, were obtained by nitrosoguanidine treatment and were selected by simultaneous resistance to two aminoglycoside antibiotics by Taber and his colleagues (209). The use of aminoglycoside antibiotic resistance for the selection of *men* mutants derives from the role of quinones in transport of these antibiotics into the bacterial cell (36, 210). The extent of aminoglycoside entry is related to the presence and efficiency of electron transport and the generation of an electrochemical proton gradient. Hence, those organisms resistant to aminoglycoside antibiotic action are likely to be *men*

mutants. Taber has noted that the MK concentration must be very low before aminoglycoside resistance is expressed (208).

The *B. subtilis* mutants, obtained as just described, were divided into two groups: group I, whose nutritional requirement was satisfied either by OSB or DHNA; and group II, comprising those capable of growing only in the presence of DHNA. The group I mutants could not be separated either biochemically or genetically into *menC* and *menD*. However, group II mutants could be divided into two groups on the basis of syntrophy experiments, fine-structure mapping, and in vitro complementation by cell-free extracts (209). The term *menE* for mutants lacking OSB-CoA synthetase was first applied to *B. subtilis* strains, and *menB* was used for those deficient in DHNA synthase (165). The *menC*, *menD*, *menE*, and *menB* genes were localized between *bioB* and *ald* on the *B. subtilis* genome by genetic mapping with bacteriophage PBS1 (209). By three-point transductional crosses with *bioB* and by cotransductional frequencies with *ksgB*, the *men* genes were found to be grouped in the probable order, *bioB....menE....menC*, *-D....menB....ksgB*. It will be noted that this ordering is different from that proposed for the *E. coli* genes.

### men Mutants of Staphylococcus aureus

Menadione-requiring, small-colony mutants of *S. aureus* (another organism without ubiquinone) were obtained in 1968 by selection with neomycin (192). In the absence of menadione, growth was poor and there were reduced biochemical activity and respiration, as well as deficient pigment formation. Menadione concentrations of 0.1 to 1.0 μg per ml of medium gave rise to fully restored growth and respiration; higher concentrations were inhibitory. Some of the mutants were also stimulated by shikimate and formation of menaquinones was demonstrated. Hence, these strains (then termed *meq*) were truly vitamin K-deficient mutants. It was later shown that some of the mutants were deficient at the level of the common aromatic pathway, others prior to naphthoquinone ring formation, and others at the level of synthesis of the isoprenoid side chain (190, 191). Despite the potential usefulness of these mutants, little further work with them has been reported. It has been shown that menaquinone deficiency is accompanied in every case by an impaired nitrate respiration. Reinitiation of MK biosynthesis (e.g., by addition of shikimate) restores electron transport. These results clearly implicate menaquinone in nitrate respiration (189).

A mutant of *S. aureus*, auxotrophic for menadione and glycerol, was obtained from a glycerol auxotroph by treatment with kanamycin and selection on nutrient agar with and without menadione (80). This organism makes the normal array and distribution of isoprenologs from the menadione provided. The enzymatic lesion, prior to menadione, has not been determined and it is not clear whether menadione is a normal biosynthetic intermediate in this organism (see also Individual Reactions in Menaquinone Biosynthesis).

Menadione-requiring strains of *S. aureus*, similar to those of Sasarman et al., have been isolated clinically (1). In one study of eight strains isolated from patients, four required thiamine and four required menadione. One of the menadione-requiring strains was isolated from a patient receiving warfarin ("anti-vitamin K") after surgery. The menadione-requiring strains were resistant to aminoglycoside antibiotics.

## FACTORS INFLUENCING MENAQUINONE BIOSYNTHESIS

### Aerobic Versus Anaerobic Growth

Many bacteria biosynthesize both ubiquinones and menaquinones (48). In these organisms, the interrelationships between the amounts of the various quinones are complex and are influenced by aerobic or anaerobic growth. For instance, among those bacteria able to use fumarate as a terminal electron acceptor, the major quinone patterns appear to be MK alone, DMK alone, MK plus Q, or DMK plus Q (129). A complete account of these effects, which would of necessity have to consider the functions of the quinone components, is beyond the scope of this review. Only those aspects relating directly to biosynthesis will be considered here.

Despite some early conflicting reports (32, 137, 177), it is now generally recognized that menaquinone biosynthesis in facultative anaerobes is increased by anaerobiosis. At the same time, ubiquinone biosynthesis is diminished, and there are also changes in the various cytochromes. In *E. coli* there is considerable variation in the observed effects, depending to some extent on the cultural conditions and on the particular strain which is examined. An extreme case was encountered with *E. coli* B/r, where ubiquinone biosynthesis was reduced to a very low level indeed under anaerobic conditions (Table 5). A smaller change in ubiquinone content was noted in *E. coli* AN98 (*argE metE*). In this strain the aerobic cultures contained 252 nmol of Q-8 per g (wet weight), and the anaerobic cultures contained 140 nmol of Q-8 per g (wet weight) (169): at the same time, the menaquinone content increased from 104 to 246 nmol of MK-8 per g (wet weight).

TABLE 5. Levels of ubiquinone (Q) and
menaquinone (MK) in *E. coli* B/r under aerobic and
anaerobic conditions (177)

| Conditions | nmol/g, dry wt | |
|---|---|---|
| | Q | MK |
| Stationary culture, aerobic[a] | 190 | 150 |
| Vigorous aeration, log phase[b] | 570 | 26 |
| Vigorous aeration, resting phase[c] | 330 | 24 |
| Anaerobic[d] | 3 | 250 |

[a] Average from two experiments.
[b] Average from four experiments.
[c] Average from three experiments.
[d] Single experiment.

Since some organisms contain all three quinone types, Q, DMK, and MK, complete information requires analysis for all three. It should be noted that there are significant differences in the standard redox potentials of the two naphthoquinones (101, 102). For MK, $\Delta E'_0 = -74$ mV; for DMK, $\Delta E'_0 = +36$ mV. Thus, in contrast to Q ($\Delta E'_0 = +113$ mV) and MK, DMK can act equally well in succinate respiration and in fumarate reduction; MK can function in fumarate reduction but not succinate respiration (228).

Work with *Haemophilus influenzae* RAMC 18 Bensted will illustrate these points (102). This organism normally biosynthesizes DMK only. The quinone content of disrupted bacteria was depleted by pentane extraction, and reincorporation of Q, MK, or DMK was studied. For electron transport from NADH to fumarate, MK and DMK were very effective and more active than Q by a factor of 4 to 5. For electron transport from succinate to $O_2$, Q and DMK were effective, and MK had only a slight effect. It appears that electron transport depends not only on the redox potential but also on the chemical structure of the quinone.

In an early approach to the study of all quinone components, three representative organisms were examined. Anaerobiosis was found to influence the content of MK and DMK differently. As anticipated, anaerobic growth diminished ubiquinone formation from 1.5- to 3-fold in *Escherichia freundii*, *Proteus mirabilis*, and *Aeromonas punctata* (230). The combined amounts of MK plus DMK increased anaerobically by 1.45-fold in *E. freundii* and 1.58-fold in *Proteus mirabilis*; there was no change, however, with *Aeromonas punctata*. For the individual naphthoquinone components, the amounts of MK increased under anaerobic conditions, whereas the amounts of DMK decreased and, in

the case of *Proteus mirabilis*, DMK was not found at all. The changes in the ratio DMK/MK for aerobic versus anaerobic growth were, respectively, 2.0 to 0.4 (*E. freundii*), 0.8 to 0.0 (*Proteus mirabilis*), and 13 to 3.3 (*Aeromonas punctata*).

A series of *E. coli* mutants carrying two possible combinations of genes for ubiquinone and menaquinone biosynthesis (*ubi*$^+$, *ubi*, *men*$^+$, *men*) has also been examined with respect to changes in the levels of Q, MK, and DMK for the change from aerobic to anaerobic conditions (228). The change in Q concentration for anaerobiosis was to about 20% or less of the aerobic values; the combined MK plus DMK increased 2.7-fold (*ubi*$^+$ *men*$^+$) or 1.85-fold (*ubi* *men*$^+$) under anaerobic growth conditions. In terms of the individual naphthoquinone components, DMK concentrations increased somewhat, and the MK concentrations increased considerably (Table 6). In these mutants, the changes in DMK/MK ratio were from 7.6 to 1.0 (aerobic → anaerobic, *ubi*$^+$) and from 3.5 to 1.7 (aerobic → anaerobic, *ubi*). These changes are different from that observed in *E. freundii*.

The following organisms also reduce their Q content in favor of increased DMK content when cultivated anaerobically in the presence of fumarate: *Haemophilus parainfluenzae* HIM 412-6 and NCTC 4101, *H. haemoglobinophilus*, *H. parasuis*, and *H. paragallinarum* (103). These are examples of organisms which do not contain MK components.

Some strains of *E. coli* K-12 actually synthesize relatively high levels of Q under anaerobic conditions, in the presence of fumarate. These levels are in the range of 50 to 70% of those obtained under aerobic conditions (2). The levels are higher than those for cells grown anaerobically with nitrate as electron acceptor (228). It is worth mentioning that under anaerobic conditions the hydroxylations required in Q biosynthesis are carried out by three "alternative" hydroxylation reactions, not involving molecular oxygen (rather than by the aerobic monooxygenases). The aerobic monooxygenases are probably flavin enzymes rather than cytochrome P-450 type enzymes (127). The oxygen-dependent synthesis of Q-8 in *E. coli* appears to have a standby position in the anaerobic cell and can be activated quickly if oxygen becomes available (126). It is of interest to recall that in menaquinone biosynthesis the quinone oxygen atoms are not derived from molecular oxygen (203); this is, of course, consistent with the known biosynthetic pathway for MK. These oxygen atoms derive from the carboxyl group originally associated with shikimate and the carbonyl group originally present in 2-ketoglutarate. Despite the general structural similarities, the quinone functions in

the naphthoquinones are biosynthetically very different from those in the benzoquinones.

The increased biosynthesis of MK in *E. coli* under anaerobic conditions is apparently related to its role as an obligatory hydrogen carrier for the oxidation of dihydroorotate coupled to fumarate reduction (169). It has, apparently, not been determined whether DMK can function in this system. In any event, this system is probably inhibited by molecular oxygen. It has also been suggested that the low levels of Q with anaerobic growth are an expression of a regulatory mechanism (169) rather than an interference with a biosynthetic reaction (229).

In line with the general effects of anaerobiosis which have just been summarized is an effect of KCN. Aerobic growth of *E. coli* with an oxidizable substrate (e.g., succinate) in the presence of KCN leads to an approximately ninefold increase in menaquinone content; the ubiquinone content is essentially unchanged (16). At the same time, the cytochromes synthesized are typically the same as those in anaerobically grown cells.

An increased formation of menaquinones under aerobic conditions occurred when *Staphylococcus aureus* cultures were shifted from anaerobic to aerobic growth (74). This organism is, of course, one which does not contain ubiquinone and so menaquinone is used for electron transport to oxygen. The increase was about 1.6-fold. The various isoprenylogs were affected differently; during the shift, the amount of MK-9 increased and that of MK-7 decreased. Expressed as percentage of the total MK content, the changes for anaerobic to aerobic were as follows: MK-7, 26.0 to 18.9; MK-8, 66.0 to 62.5; MK-9, 8.0 to 18.6. The reason for these changes is not clear.

## Other Factors Influencing Menaquinone Biosynthesis

Little attention has been paid to the influence of general cultural conditions on the biosynthesis of menaquinones. Conclusions from one

study (28) with a strain of *Serratia marcescens* are as follows. (i) The greatest amount of Q-8 and MK-8 was present during stationary-phase development (2 to 3 days) on an optimal medium. (ii) The amount of MK-8 was little influenced by changes in glucose concentration from 10 to 80 g/liter. (iii) The best growth and yield of MK-8 were obtained in the presence of 20 to 50 g of glutamate per liter. (iv) There was little change in the ratio of MK-8/Q-8 as a function of medium composition and growth phase.

During exponential growth of *Staphylococcus aureus*, the proportions of the various menaquinone isoprenologs changed, although the total amount remained essentially constant ($2.0 \pm 0.1$ μmol/g, dry weight). MK-8 increased from 36 to 70% of the total during seven to eight doublings, and in the same period the proportions of MK-0, MK-1, MK-5, MK-6, and MK-7 decreased (93).

When *Staphylococcus aureus* is grown at 25°C, the cells contain more total menaquinone than at 20°C (28% more) or 37°C (16% more). There are also differences in the amounts of the various menaquinone isoprenologs as growth temperature changes (119). During a shift down from 37 to 25°C, total menaquinones increased by about 20% and the proportions of very short-chain components decreased, whereas MK-8 increased. No explanation was offered for these changes.

Since diphenylamine was known to suppress the formation of carotenoids and ubiquinone in various bacteria, Salton and Schmitt examined the effect of this material on the menaquinone content of membranes isolated from "*Micrococcus lysodeikticus*," *Sarcina lutea*, and *Bacillus megaterium* (186). At diphenylamine concentrations of 50 μg/ml of medium, the menaquinone content was reduced to 77% of the normal value with "*Micrococcus lysodeikticus*" and to 84% with *Sarcina lutea*. These levels of diphenylamine reduced the carotenoid content to 10% or less of the normal. With the nonpigmented *Bacillus megaterium*, diphenylamine concentra-

TABLE 6. Levels of demethylmenaquinone (DMK) and menaquinone (MK) in *E. freundii* and *E. coli* strains under aerobic and anaerobic conditions

| Strain | nmol/g, wet wt[a] | | | | | |
| | Aerobic | | | Anaerobic | | |
| | DMK | MK | DMK/MK | DMK | MK | DMK/MK |
|---|---|---|---|---|---|---|
| *E. freundii* | 160 | 80 | 2.0 | 90 | 260 | 0.4 |
| *E. coli* AN387 (*ubi⁺ men⁺*)[b] | 38 | 5 | 7.6 | 58 | 59 | 1.0 |
| *E. coli* AN385 (*ubi men⁺*) | 120 | 34 | 3.5 | 179 | 106 | 1.7 |
| *E. coli* AN386 (*ubi⁺ menA*) | <2 | <2 | | <2 | <2 | |
| *E. coli* AN384 (*ubi menA*) | <2 | <2 | | <2 | <2 | |

[a] The amounts are expressed as nanomoles per gram, dry weight, for *E. freundii* (230).
[b] Data for all *E. coli* mutants are from reference 228. These results are for anaerobic growth in the presence of nitrate.

tions of 12.5 and 25 μg/ml of medium reduced the content of MK-7 to 90% of the normal, a much smaller effect. Clearly, the biosynthesis of menaquinones was less sensitive to the effect of diphenylamine than was that of the carotenoids. In similar work with *Staphylococcus aureus* under both aerobic and anaerobic conditions, diphenylamine inhibited menaquinone biosynthesis by about 50% (95). The diphenylamine concentrations in this work did not influence the growth rate; cyclic carotenoid biosynthesis was inhibited by either 25 to 35% (anaerobically) or 60 to 90% (aerobically). The mechanism by which diphenylamine exerts these effects is apparently not known.

Some observations have been made on the effect of 5-aminolevulinate, a precursor to heme, on quinone levels in *E. coli* strains (90). A 5-aminolevulinate-deficient mutant of *E. coli* was obtained and was grown both aerobically and anaerobically in the presence or absence of 5-aminolevulinate. Under aerobic conditions the formation of menaquinone was decreased by the presence of 5-aminolevulinate, but there was no such action under anaerobic conditions (Table 7). The precise significance of this effect is not clear.

The lack of action of 5-aminolevulinate under anaerobic conditions has been confirmed in the AN359 strain of *E. coli*, which carries a mutation in the *hemA* gene (13). Under anaerobic conditions, in the absence of 5-aminolevulinate, growth was about one-third of the rate with excess 5-aminolevulinate present. Cytochromes could not be detected in the membranes of those cells grown without 5-aminolevulinate, but the levels of naphthoquinones were normal. It may be noted that other workers have implicated a menaquinone requirement in the anaerobic biosynthesis of heme, specifically for the oxidation of protoporphyrinogen (116).

## VITAMIN K-REQUIRING BACTERIA

In 1945, a comprehensive review of the growth requirements of bacteria for vitamins contained the statement ". . . most of the fat-soluble vitamins are not known to have any potency for bacteria" (176). In a listing of 130 bacteria, the only reference to vitamin K was veiled: *Mycobacterium paratuberculosis* was listed as requiring "anti-hemorrhagic compounds." Clearly, the ability to synthesize demethylmenaquinones and menaquinones was widespread among bacteria. Since that time, however, several instances of other vitamin K-requiring bacteria have been discovered. The best documented cases are discussed here. Other examples of a requirement for menaquinone, or for a biosynthetic precursor thereof, are provided by the *men* mutants of *E. coli*, *Bacillus*

TABLE 7. Influence of 5-aminolevulinate on *E. coli* mutants deficient in heme biosynthesis

| Strain | 5-Amino-levulinate | nmol/g, wet wt | | |
|---|---|---|---|---|
| | | Q | MK | DMK |
| 1, aerobic | − | 86 | 30 | —[b] |
| 1, aerobic | + | 84 | 3 | — |
| 1, anaerobic | − | 36 | 34 | — |
| 1, anaerobic | + | 38 | 27 | — |
| AN359, anaerobic | − | 73 | 33 | 50 |
| AN359, anaerobic | + | 78 | 31 | 52 |

[a] Strain 1 is a 5-aminolevulinate synthetase-deficient mutant (90); AN359 has a mutation in the *hemA* gene.
[b] —, Not determined.

*subtilis*, and *Staphylococcus aureus* (see Genetics of Menaquinone Biosynthesis). On the practical level, it is worth noting that vitamin K, as menadione, is added to the brain heart infusion broth (supplemented) medium used for culturing many anaerobes. This addition is recommended, for instance, for isolation of anaerobes from various clinical specimens (99).

### *Mycobacterium paratuberculosis*

In their early work on the cultivation of *M. paratuberculosis* (see Introduction), Twort and Ingram recognized clearly that not all bacteria and not all strains of the same bacterium were sources of Essential Substance (224, 225). Production of the growth factor by *M. phlei* was found to be dependent on culture media components; thus, glycerol-containing media were good substrates for Essential Substance production. Since bioassay required about 2 months for the growth of *M. paratuberculosis*, progress was necessarily slow.

When Woolley and McCarter reported in 1940 that *M. paratuberculosis* could be grown on media containing phthiocol, 2-methylnaphthoquinone, or a vitamin K concentrate, it appeared that this organism did have a vitamin K requirement. However, they also realized that none of these materials was as effective as an extract of *M. phlei* (231). In an examination of the same question, Glavind and Dam showed that a concentrated bovine tuberculin preparation, which was known to have a definite vitamin K activity, stimulated growth of a *M. paratuberculosis* strain about threefold (78). These results (Table 8) were obtained before 1941, using a strain isolated in Denmark. A strain obtained by Dam in 1946 from W. A. Hagan, Ithaca, N.Y. (on the suggestion of D. W. Woolley and J. R. McCarter) showed less stimulation. This strain was clearly different from that used earlier, since it gave about 10 times as much growth under

similar conditions. In the early experiments, 2-methylnaphthoquinone, or the corresponding hydroquinone disulfate, gave at most only very slight growth stimulation and amounts of 250 μg per ml of medium were actually inhibitory. With phthiocol, a somewhat more consistent stimulation (average yield, 97 versus 81 mg for controls) was seen. In all of these experiments, there was a considerable variation (Table 8). These authors concluded that the tuberculin preparation contained a growth-stimulating factor different from vitamin K. They also stated that "the strains used in the two laboratories (i.e., Woolley and McCarter vs. Glavind and Dam) might have been different with respect to their vitamin K requirements."

A new growth factor for *M. paratuberculosis* was investigated by Francis et al. beginning in 1949 (72, 73, 202). These authors used strain 129 of "*Myco. johnei*" (sic) obtained from R. E. Glover. This strain could not be easily adapted to growth in the absence of growth factor. From *M. phlei* they isolated a crystalline aluminum complex of a material termed mycobactin (now, mycobactin P). Optimal growth of *M. paratuberculosis* required 40 to 80 μg of mycobactin per ml of medium. This was the first of several mycobactins, now recognized as iron-chelating growth factors for mycobacteria (202). Francis et al. observed no effect of phthiocol, 2-methylnaphthoquinone, and vitamin K in their assay with *M. paratuberculosis*. They concluded that most, if not all, of the activity of simple extracts of *M. phlei* in promoting growth of *M. paratuberculosis* could be accounted for by mycobactin P (it is present to the extent of about 1% of dry weight of *M. phlei*).

The work on the chemistry and microbiology of the mycobactins is certainly elegant and voluminous. The various members of the group are generally recognized as growth factors for mycobacteria, including *M. paratuberculosis*. An argument favoring the identity of Twort's Essential Substance with mycobactin concerns the reported solubility characteristics. The best stimulation of the growth of *M. paratuberculosis* was reported by Twort and Ingram (224, 225) to be obtained with material insoluble in chloroform; there was evidence, however, that it was to some extent soluble in lipid solvents. These characteristics do not agree well with the excellent lipid solubility of vitamin K and the general difficulty of obtaining it in a water-soluble form; for instance, a form of vitamin $K_1$ used clinically is actually an aqueous colloidal preparation (AquaMEPHYTON).

As a result of the discovery of the mycobactins, any possible role of vitamin K has, since 1949, been minimized. For example, an extensive discussion of *M. paratuberculosis* and other

TABLE 8. Stimulation of growth of *Mycobacterium paratuberculosis* by tuberculin

| Tuber-culin addition (ml) | Expt no. | mg of sediment/200 ml of growth medium | | | |
|---|---|---|---|---|---|
| | | Danish strain, 1941 | | U.S. strain, 1946 | |
| | | Individual flasks | Avg | Individual flasks | Avg |
| 0 | 1 | 59, 65, 75, 112 | 78 | 843, 856 | 850 |
| 0 | 2 | 68, 73, 90, 91 | 81 | | |
| 0.02 | 1 | 194, 235, 251 | 226 | 1,586, 1,627 | 1,607 |
| 0.02 | 2 | 199, 206 | 203 | | |

host-dependent microbes does not contain any reference to vitamin K (96). Nevertheless, for the specific case of *M. paratuberculosis*, it is perhaps not heretical to suggest that the picture is not totally clear. For example, in 1970, an investigator well aware of the role of mycobactin showed that "water soluble vitamin K" (the sodium dibenzoylsulfonate of 2-methylnaphthohydroquinone) could be substituted for *M. phlei* extract in the isolation and repeated subculture of *M. paratuberculosis* (47). Coletsos noted that the culturing of *M. paratuberculosis*, despite mycobactin, is burdened with a considerable number of failures, so that stocks of this organism are extremely rare. In his work, three strains (one isolated from a cow; two isolated from sheep) were maintained for 15 years on media containing the menadione derivative.

The problem of the actual growth requirement(s) is made more complex by the "circumvention of the mycobactin requirement of *Mycobacterium paratuberculosis*" (167). For instance, during the first transfer of strain 68 from the mycobactin-containing Trypticase (BBL Microbiology Systems)-glycerol medium onto Watson-Reid medium (pH 5.5) a mycobactin requirement was observed. Subsequently, 18 transfers were made without mycobactin. Furthermore, autoclaved Watson-Reid medium contained an unidentified growth factor, not present in filtered medium. (This work did not consider a possible vitamin K requirement.)

Although an evaluation of all of the nutritional and other factors for growth of *M. paratuberculosis* is beyond the scope of this review, it appears to us that the existence of vitamin K-requiring strains is still possible. It would not be beyond the bounds of probability to suggest that there may even be strains requiring both vitamin K and mycobactin for optimal growth. (Compare the double requirement for vitamin K and heme of *Bacteroides melaninogenicus*, discussed below.) Ironically, the crude extracts of *M. phlei*, used for a long time for the growth of *M. paratuberculosis*, probably contained significant amounts of both materials. In other bacteria, both vitamin K-requiring and vitamin K-

independent strains are well known (see below). There is clearly much variation in *M. paratuberculosis*, and it is known that phage-induced changes in mycobacteria may be severe. For instance, lysogenization of *M. phlei* F89 with mycobacteriophage B2hF89 gave a strain with acquired properties characteristic for *M. smegmatis* (75).

It is known that within the *M. avium* group of mycobacteria there are various degrees of mycobactin dependence, as is the case with *M. paratuberculosis* (151). In particular, with *M. avium*, mycobactin dependence occurs when media are inoculated with small numbers of viable units; large inocula "presumably contain sufficient mycobactin associated with the organisms to enable growth to occur."

Possibly the discovery of mycobactin has led to the selection of mycobactin-requiring strains. It would be of considerable interest to isolate *M. paratuberculosis* from an animal with Johne's disease and to examine in detail the effects of vitamin K and mycobactin, separately and in combination. With the exception of the work of Coletsos in 1970, any possible effect of vitamin K on freshly isolated strains of *M. paratuberculosis* has apparently not been investigated. In any event, such fresh isolations are now uncommon. Between the years 1973 to 1979 the Central Laboratory of Veterinary Research (Alfort, France) isolated some 332 cultures of mycobacteria from 590 pathological specimens of different origin. Of these, only two strains were identified as *M. paratuberculosis* (216).

Since vitamin K is present to a large extent in the diet of bovines and is also biosynthesized by other intestinal bacteria, it may well have been used as a growth factor by *M. paratuberculosis*. It may be more than coincidence that it is the intestinal mucous membrane which is uniquely the site of the lesions produced by this organism (47). It is also a remarkable coincidence that both vitamin K and some of the mycobactins are derived from the shikimate pathway (110, 149). Those mycobactins containing 6-methylsalicylic acid rather than salicylic acid, however, derive the aromatic acid by way of polyketide pathways (109).

Thus, at least this one strain of *M. paratuberculosis* is able to biosynthesize menaquinones. However, mycobactin biosynthesis by any strain of this organism has, apparently, not been demonstrated.

### Bacteroides melaninogenicus

A vitamin K requirement for some strains of *B. melaninogenicus* has been discovered. The chain of circumstances leading to this is strikingly similar to that which provided evidence for a growth factor requirement for *Mycobacterium paratuberculosis*. In 1928 Burdon (38) investigated "the nonspore-bearing, black pigment producing, anaerobic microorganism, "*Bacterium melaninogenicum*" (sic), which had been described and named in a brief note a few years earlier (171). Burdon believed it was probable that the earlier workers had not obtained pure cultures. Growth of such pure cultures was found to be "uncertain, slow, and usually meager." However, the organism exhibited "to a marked degree the habit of growing in very intimate mixture with other bacteria . . . ." In mixed cultures, under anaerobic conditions, growth on blood agar was found to be rapid and luxuriant. A "melanin-like" pigment was formed, and the red color of the hemoglobin containing agar eventually was completely lost. The bacterium was widely distributed on normal mucous membranes and skin in humans, particularly in the mouth and on the external genitalia.

Although Burdon had noted the fact of good growth in mixed cultures, he was apparently not aware of the work of Twort and Ingram, nor did he postulate the formation of any special substance(s) by the contaminants. He spoke of a symbiosis between it and other organisms such as *Streptococcus viridans* (39).

It required another 15 years before the pigment was recognized as a heme compound, not melanin (198). The name, *Fusiformis nigrescens*, was then proposed for the organism, and subsequent revisions now have led to the presently used *B. melaninogenicus*.

In 1954, a gram-negative, nonmotile anaerobe was isolated (along with other organisms) from

$$\text{Shikimate} \rightarrow \text{chorismate} \begin{array}{c} \nearrow \text{OSB} \rightarrow \text{MK} \\ \searrow \textit{iso}\text{-chorismate} \rightarrow \text{salicylate} \rightarrow \text{mycobactin} \end{array}$$

One strain of *M. paratuberculosis* has been shown to contain menaquinone; MK-9 (II-H$_2$) was the major component with smaller amounts of MK-9 and MK-8 (49). The distribution pattern was similar to that in other mycobacteria. (It was not stated whether the particular strain examined was mycobactin dependent, and apparently mycobactin was not used for growth.)

the mouth of a patient with a clinical diagnosis of hypertrophic gingivitis (146). The organism, K110, produced a black pigment and was assigned to the "ill-defined species *Bacteroides nigrescens*." This organism normally grew in association with a streptococcus, JS9, from which it was separated with difficulty. However, "growth occurred in Difco thioglycollate broth

only when 20% of a sterile Seitz filtrate of 5-day broth culture of JS9 was added.'' It was later found that filtrate of a hemolytic *Micrococcus aureus* was more effective than filtrate of JS9. A little later, Macdonald et al. (147) noted that "cross-streaking strain K110 against all of the strains disclosed that in the presence of strains JR3, JR4, JR5 or JS9, strain K110 grew well. It was found to grow poorly in the presence of strain JB3B and not at all with any of the other strains." (Strains JR4, JR5, and JS9 were apparently streptococci, JR3 was an aerobic gram-positive, nonmotile rod, and JB3B was also a gram-positive nonmotile rod.) It was clear from this work that K110 required a growth factor, which could be obtained in a water-soluble form from various bacteria.

In 1958, Lev also isolated an organism, apparently "*Fusiformis nigrescens*," from the rumen contents of cows (138). It was first isolated associated with an anaerogenic strain of *Proteus*. On attempting to grow it in pure culture, the organism died out after giving rise to atypical forms. Mindful of the work with *Mycobacterium paratuberculosis* and *Mycobacterium phlei* and the work of Woolley and McCarter with vitamin K, Lev added a suspension of menadione in water to a blood agar plate. Good growth of "*F. nigrescens*" was obtained and serial subculture was possible. Menadione was also able to revive atypically growing cultures.

Similarly, Gibbons and Macdonald found that human strains of *B. melaninogenicus*, which grew as satellites adjacent to *Staphylococcus aureus*, presumably by deriving a growth factor, could also be grown and maintained by addition of menadione and, indeed, a number of other naphthoquinones and naphthalene derivatives (77). Benzenoid and anthraquinonoid materials were ineffective. In this work, 12 of 14 strains of *B. melaninogenicus* isolated from the human mouth were found to require hemin for growth. Half of the isolates, in addition, required the growth factor from *Staphylococcus aureus* or a vitamin K replacement. Clearly, there are three groups of *B. melaninogenicus* strains. (i) those growing on Trypticase soy broth plus 0.05% sodium thioglycolate (basal); (ii) those requiring basal medium plus heme; (iii) those requiring basal medium plus heme plus vitamin K.

A further nutritional complication is that for a rumen strain of *B. melaninogenicus* succinate can replace the heme requirement in the presence of vitamin K, and, in addition, succinate can to some extent replace the vitamin K requirement in the presence of heme (141). Also, succinate increased the growth rate when added to a blood- and vitamin K-supplemented culture. The precise role of succinate is not entirely clear. Labeled succinate is, however, incorpo-rated into ceramide phosphorylethanolamine, ceramide phosphoglycerol, and other phospholipids. In vitamin K-depleted cultures, addition of vitamin K increases this incorporation. It is of interest that in another *Bacteroides*, *B. ruminicola*, an unusual reductive carboxylation of succinate to 2-ketoglutarate has been reported (3). In growing cultures of this organism, addition of $[1,4-^{14}C_2]$succinate leads to $^{14}C$ in amino acids as well (particularly glutamate, arginine, proline, aspartate, threonine, and alanine). A possible connection between succinate, 2-ketoglutarate, and menaquinone biosynthesis cannot be ruled out.

In a study of a large number of oral isolates (human, canine) of *B. melaninogenicus* and *B. asaccharolyticus*, the strains were found to fall into two classes: asaccharolytic organisms produced butyric acid whereas saccharolytic (*B. melaninogenicus*) organisms actually produced succinic acid (154). Of 177 strains of *B. melaninogenicus* isolated from humans, about 11% required vitamin K; on the other hand, of 160 strains isolated from dogs, 68% required vitamin K. There was no correlation between vitamin K dependency and the source of the human isolates. *B. asaccharolyticus*, which is found in the oral cavity, also shows a vitamin K requirement with some strains. Of 23 human isolates, about 21% were vitamin K dependent. Of 30 canine isolates, 90% were vitamin K dependent (154).

Before 1973, a number of synthetic compounds had been examined as replacements for the growth factor or vitamin K requirement of *B. melaninogenicus* (77, 139, 145, 206). Although several naphthalene and naphthoquinone compounds had this ability, benzene derivatives which were examined did not (benzoquinone, hydroquinone, pyrogallic acid, phthalic acid, and γ-phenylbutyric acid) (145). However, in 1973 Robins et al. were able to show that three monocyclic compounds replaced the vitamin K requirement of Lev's strain of *B. melaninogenicus* (182). These three compounds, shikimate, chorismate, and OSB, were all materials identified as menaquinone precursors; another precursor, DHNA, behaved similarly. Benzoic acid, phthalic acid, and ubiquinone could not replace vitamin K. (Phthalic acid had been examined earlier by Macdonald. We regret that we omitted to reference that work [145] when our paper appeared.)

In general, the genus *Bacteroides* appears to be well endowed with a biosynthetic capacity for menaquinones: 36 of 37 strains have been shown to contain no ubiquinones but a variety of menaquinones from MK-5 to MK-14 (199). "*Bacteroides melaninogenicus* subsp. *levii*" (140) was stated to contain neither menaquinones nor ubiquinones; contrary results had, however, been

reported earlier (179). Although identified as subspecies *levii*, the strain used by Shah and Collins (140) was not obtained from Lev and was isolated from a cattle horn abscess rather than from bovine rumen contents (as was Lev's strain). It was not stated whether this strain showed a vitamin K requirement, although menadione was apparently added to the growth medium. Robins et al. (182) also found MK-9 and MK-10 in Lev's strain in a ratio of 2:1. It appears that in this strain the defect arises before shikimate; aromatic amino acids are presumably available from the blood generally present in the medium.

### *Lactobacillus bifidus* var. *pennsylvanicus*

A batch of commercial lactulose (Bifiterlose; 4-*O*-β-D-galactopyranosyl-D-fructofuranose) was found to enhance the growth of *L. bifidus* var. *pennsylvanicus* in the presence of human milk (or materials such as *N*-acetyl-D-glucosamine and $NH_3$). By extraction of 1.8 kg of lactulose with methanol, followed by further extraction from water with ether, a brown syrup was obtained (2.97 g). By chromatography on a cellulose column and vacuum sublimation, a pale yellow sublimate (25.4 mg) was obtained. After further crystallization, this material was identified as menadione. Synthetic menadione was also shown to function in the same way; it was described as a "supplementary factor," required in addition to that for *N*-acetyl-D-glucosamine-containing saccharides (79). Why the particular batch of carbohydrate contained menadione is not known; lactulose is obtained by epimerizing lactose, and contamination of the latter also seems unlikely. A few other naphthoquinones, including phylloquinone, were also shown to function in the same way as menadione; benzoquinone and hydroquinone were inactive.

### Other Microorganisms

An oxygen-dependent and menadione-requiring variant of *Haemophilus parainfluenzae* was obtained from cultures grown on heated blood agar (143). Of two colony types present, only one showed the requirement. The response occurred only over a limited range (0.5 to 2.5 µg/ml) and was limited to menadione, menadione bisulfite, 2-methyl-4-amino-1-naphthol, and 2,3-dimethyl-1,4-naphthoquinone. The response occurred on blood-Lemco broth but not on blood or heated blood agar plates. Strains of *Haemophilus parainfluenzae* generally contain either ubiquinone and demethylmenaquinone or only demethylmenaquinone (102, 103).

An investigation of the nutritional requirements of a number of anaerobic coryneforms led to the identification of four strains of *Propionibacterium acnes* type II with nutritional require-

ments for heme and vitamin K (cf. *Bacteroides melaninogenicus*). Ten other water-soluble vitamins were also present in the defined medium. It was suggested that the heme and vitamin K were required for synthesis of cytochromes (65).

A brief note from the 15th Joint Leprosy Research Conference indicated that vitamin $K_3$ (0.005 µg/ml) and vitamin $B_{12}$ (0.16 µg/ml) significantly enhanced growth of *Mycobacterium lepraemurium* and suggests that these materials may have given possible multiplication of *M. leprae* (168).

In 1942, an apparent influence of vitamin K on the growth of intestinal bacteria, particularly *E. coli*, was reported (196). It was found that in young chicks *E. coli* grew rather slowly. If chicks were maintained for 26 days on a diet producing vitamin K deficiency, the intestinal *E. coli* largely disappeared. Vitamin K administration restored the coli count by up to 70% after 8 to 18 days. There were morphological differences when the isolated intestinal bacteria were grown on media with and without vitamin K. It would be of interest to see this work confirmed; it has received little attention since most workers do not regard *E. coli* as requiring vitamin K for growth.

In *Aspergillus niger*, menadione was said to exert a slight growth stimulation at low concentration but rapidly inhibited growth as the concentration was increased (226). The result is surprising since fungi do not contain menaquinones.

### Are the Vitamin K-Like Growth Factors Secreted by Bacteria Actually Menaquinones?

It is common to ascribe response of a "vitamin K-dependent" organism, such as *Bacteroides melaninogenicus*, to another organism by assuming the excretion of naphthoquinones. To quote one example, it has been stated that "naphthoquinones were detected in *Bacteroides fragilis* using a naphthoquinone-dependent strain of *Bacteroides melaninogenicum*" (36). Indeed, the stimulation of growth of vitamin K-dependent bacteria by the presence of other bacteria has been used or suggested for use as an assay for vitamin K. It appears that rarely, if ever, in such work has a distinction been made among several possibilities. The test organism could: (i) secrete into the agar a menaquinone (or menaquinol) in the typically lipophilic form; (ii) secrete into the agar a menaquinone (or menaquinol) in a modified, watersoluble form; (iii) secrete into the agar a biosynthetic precursor of menaquinone, likely as a water-soluble material.

(i) Since menaquinone is typically localized in the cytoplasmic membrane (130), it seems unlikely that it would be easily excreted by bacteri-

al colonies into agar in its lipophilic form. Although bacteriolytic enzymes are present, for example, in *Staphylococcus aureus* (15), the possible role of such enzymes in the release of menaquinone is not known.

(ii) Lev and Milford have provided evidence for the existence of water-soluble factors with vitamin K activity in pig liver and in *Bacteroides melaninogenicus* (142). The pig liver extracts appeared to contain a protein with vitamin K bound to it in some manner; microbiological activity was associated entirely with this form (and no ether-extractable vitamin K was present). Similar, water-soluble forms, presumably protein bound, were obtained from *Bacteroides melaninogenicus*. When this organism was grown in the presence of vitamin $K_1$, a lipid soluble form was also present. Such protein-bound forms of vitamin K might be excreted by bacterial colonies into agar.

(iii) Whereas it could be argued that the most likely material to be excreted into agar would be a water-soluble menaquinone precursor (e.g., OSB, DHNA) this possibility has generally been overlooked in work dealing with vitamin K assay. However, the "cross feeding" of specific *men* mutants by other mutants or by wild-type *E. coli* and *Bacillus subtilis* is well known (85, 209).

It is not impossible that Twort and Ingram's Essential Substance could have contained a vitamin K precursor, and some of the growth factors for *Bacteroides melaninogenicus* have properties more closely resembling an aromatic acid than a menaquinone. For example, the properties of the growth factor produced by *Staphylococcus aureus* were summarized as follows (145): (i) withstood 15 min at 100°C, but 15 min at 121°C gave 95% loss of activity; (ii) not inactivated by vacuum drying, ethylene oxide, or UV light; (iii) ether extractable at pH 1.5 with retention of activity; (iv) absorbed by charcoal and both anion- and cation-exchange resins; (v) gummy residue obtained on evaporation was soluble in NaOH or ethanol, and redissolved residue was active; (vi) extracts (ether?) of alkaline preparation at pH 11.5 were inactive. These properties, in particular, the stability to UV radiation, do not agree with those of a menaquinone. In this case, therefore, a good argument can be made that the growth factor is actually a water-soluble precursor of menaquinone.

### Are Growth Factors with Vitamin K Activity Converted to Menaquinones?

In many of the studies of the vitamin K requirements of bacteria, the growth supplement used has been the readily available menadione or phylloquinone rather than the menaquinone

which would be expected as the normal bacterial component. There has been little study of the mechanisms by which materials with vitamin K activity are actually taken up by growing bacterial cells and are then subjected or not to further transformations. Unfortunately, the results that have been obtained are somewhat contradictory. In the early work of Martius with *Bacteroides melaninogenicus* (obtained from Lev) (see Biosynthesis of Menaquinones) conversion of labeled menadione and phylloquinone to menaquinone was said to have been achieved (150). Whether or not the purifications obtained in this work were adequate is now difficult to decide. When the same organism was grown by others with phylloquinone as the supplement, a substantial amount of phylloquinone itself was actually recovered from the cells. Only a trace of other quinones was present; this material was shown by mass spectrometric analysis to be a mixture of MK-9 and MK-10 (D. J. Robins and R. Bentley, unpublished data). Other growth factors for this organism (shikimic acid, OSB, DHNA, 1,4-naphthoquinone) gave mixtures of MK-9 and MK-10, and an abnormal mass spectrometric pattern was obtained in one experiment with 6-methyl-1,4-naphthoquinone as the growth factor, as well as a much lower yield of "quinone." As noted earlier, other workers have stated that "neither menaquinones or ubiquinones were detected in '*Bacteroides melaninogenicus* subsp. *levii* (JP2)' " (140).

In studies of a *men* mutant of *Staphylococcus aureus*, menadione was used as the growth factor (191). Isolation of quinonoid material from the cells "revealed a spectrum (UV) differing from that of vitamin $K_2$ (30)," i.e., MK-6. It was concluded, therefore, that such cells did not contain a normal menaquinone component. The UV spectrum was derived from material recovered from thin-layer chromatography, and it is possible that the abnormal spectrum resulted from the presence of contaminants; a rigorously purified compound was probably not examined. In any event, there has been no further characterization of this material. In work with a *Staphylococcus aureus* strain which produced menaquinones, Hammond and White observed that radioactive menaquinones, from MK-2 to MK-9, were not taken up by exponentially growing cultures (93). The samples were added to culture media in dimethyl sulfoxide solution, and essentially 100% of added activity was recovered in the culture media. With MK-0 (menadione), about 0.5% of the added material was incorporated by the bacterial cells.

### VITAMIN K BIOSYNTHESIS BY INTESTINAL BACTERIA

Beginning with the pioneering work of Almquist and Stokstad (9), the biosynthesis of vita-

min K by intestinal bacteria has come to be recognized as an important component in animal and human nutrition. With the exception of the chick, it is, in fact, not easy to induce vitamin K deficiency in experimental animals. With the development of powerful antibiotics, vitamin K-responsive hypoprothrombinemia became a significant clinical problem (210), and the antibiotic-associated defect in vitamin K biosynthesis can actually prove to be lethal (105). To cite only one recent example, gastrointestinal bleeding was encountered in patients being treated with cefamandole (105).

Some work on the role of intestinal bacteria has been carried out with gnotobiotic rats (46). When these animals are fed a vitamin K-free diet, they rapidly develop a hemorrhagic condition which can be reversed by associating them with bacteria isolated from conventional animals (87). A variety of bacteria isolated from the oral cavity or feces of rats was tested singly and in combination. Bacterial suspensions were sprayed into the containers for the gnotobiotes (control, vitamin K-deficient, germfree animals showed no response to uninoculated media). The following bacteria gave no significant reversal: rat oral strains of *Lactobacillus acidophilus* and a diphtheroid organism; rat enteric strains of a sporeformer and two *Bacteroides* strains. However, an *E. coli* strain from rat feces and an unclassified sarcina-like micrococcus did reverse the vitamin K deficiency symptoms. This was the first demonstration that an experimentally induced vitamin deficiency could be reversed by colonization of the host animal by a single strain of bacteria.

Evidence for a role for bacterial biosynthesis in bovines comes from a study of the menaquinone composition in liver. Bovine liver contains MK-10, -11 and -12; these three menaquinones and MK-13 as well have also been isolated from bovine rumen contents (153). It appears likely that the liver menaquinones are bacterial in origin and are deposited after intestinal absorption. It has been postulated that the vitamin K content of liver is determined, in fact, by nutritional sources and is not dependent on metabolic events. In support of this statement is the fact that the liver of the horse, a herbivore which is not a ruminant, contains only phylloquinone (152).

The lower part of the intestinal tract, where the bacterial density is highest, seems the likely site for vitamin K absorption. In rats, it has been shown that the large bowel can absorb bacterially synthesized menaquinone (although it has no role in the absorption of lipids). The absorption rate appears to be more than adequate to provide the animal with the daily requirement (100). A more complete discussion of this question is given by Suttie (207).

Knowledge of the physiology of vitamin K in humans is far from complete: it was noted recently that "the fundamental question of the relative importance of the diet or intestinal micro-flora in providing man's requirements for vitamin K still remains to be answered" (200).

Since *Bacteroides* species are among the most numerous of the bacteria inhabiting the human intestinal tract and since strains such as *Bacteroides fragilis* do synthesize vitamin K, it was suggested that *Bacteroides fragilis* types were more significant in providing the human vitamin K requirement than were *E. coli* strains (76).

Other workers have investigated human bacterial strains and have shown that vitamin K is "produced by some strains of *Bacteroides fragilis*, bifidobacteria, clostridia and *Streptococcus faecalis*." Although this conclusion is reasonable, it should be noted that the method used was "a plate test, with *Bacteroides melaninogenicus* as the indicator organism" (66). Possible problems in this regard have been discussed earlier. It seems clear that the major bacterial population contributing vitamin K to human nutrition remains to be identified with certainty.

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