

Pathways of Biosynthesis of Aromatic Amino Acids and Vitamins and Their Control in Microorganisms

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INTRODUCTION

The aims of this review are to present an outline of the metabolic pathways leading to the aromatic amino acids and vitamins and to discuss how the flow of intermediates along these pathways is controlled. The general outlines of the pathways to the aromatic amino acids, phenylalanine, tyrosine, and tryptophan have been known for some time, and they were excellently reviewed by Umbarger and Davis (162). Since then, the situation regarding the "branch points" in aromatic biosynthesis has been clarified, and much information on the biochemical genetics

and control of the biosynthesis of aromatic amino acids has accumulated. In addition, the general outlines of the pathways leading to the metabolically important compounds found in small amounts, namely, 4-aminobenzoic acid, ubiquinone, vitamin K, and 2,3-dihydroxybenzoic acid, are partially understood. The latter compounds will be referred to as vitamins.

It is these more recent studies which we intend to emphasize with one important exception, the tryptophan operon. The biochemical genetics of this operon as a whole, and the enzyme tryptophan synthetase in particular, have been studied

intensively during recent years. The amount of information now available on these topics warrants a separate review; therefore, it is not our intention to deal with this work in detail. Various aspects of the work have been reviewed (171, 172); other recent general reviews on aromatic biosynthesis generally, or on specific topics, are also available (12, 48). There has been, of necessity, some selection in the papers cited, but further references may readily be found through these.

A general outline of the pathways to be discussed consists of a "common pathway" leading through shikimate to chorismate, after which there is branching to the individual pathways (Fig. 1).

INTERMEDIATES IN AROMATIC BIOSYNTHESIS

Common Pathway

The common pathway involves the condensation of two products of carbohydrate metabolism, phosphoenolpyruvate and erythrose 4-phosphate, to give a straight chain seven-carbon compound which is then cyclized through shikimate to chorismate (Fig. 2).

In recent work, the main advance has been the clarification of the region of the branch point (Fig. 1) where, from chorismate, a series of individual pathways diverge. After the establishment of 3-enolpyruvylshikimate 5-phosphate as an intermediate on the common pathway (101,

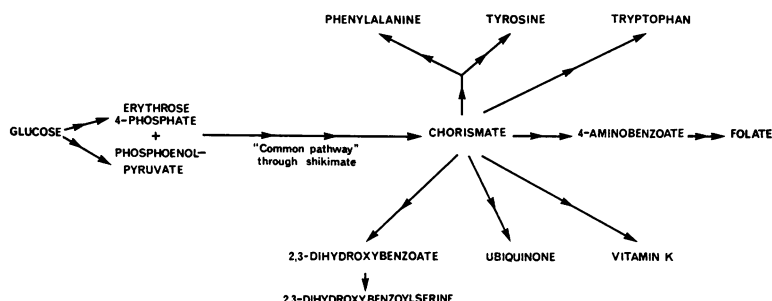


FIG. 1. General outline of pathways for the formation of aromatic amino acids and vitamins in *E. coli*.

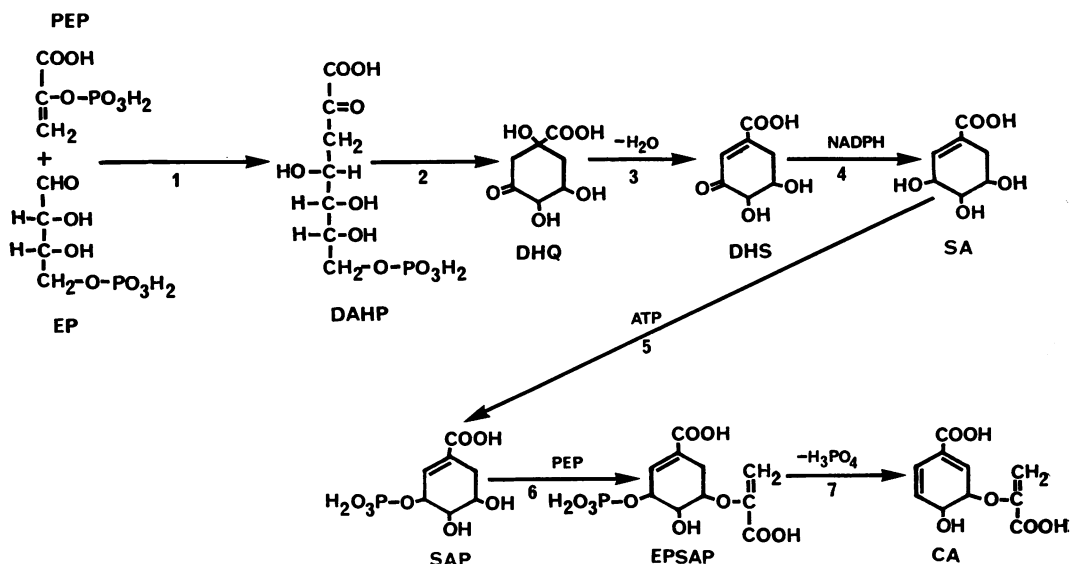


FIG. 2. Intermediates in the common pathway of aromatic biosynthesis. Abbreviations: PEP, phosphoenolpyruvate; EP, erythrose 4-phosphate; DAHP, 3-deoxy-D-arabino-heptulosonic acid 7-phosphate; DHQ, 5-dehydroquinic acid; DHS, 5-dehydroshikimic acid; SA, shikimic acid; SAP, shikimic acid 5-phosphate; EPSAP, 3-enolpyruvylshikimic acid 5-phosphate; CA, chorismic acid. Trivial names of enzymes and some references to purification and cofactors:— (1), 3-deoxy-D-arabino-heptulosonate 7-phosphate synthetase (DAHP synthetase; 48, 154, 164); (2), 5-dehydroquininate synthetase (153); (3), dehydroshikimate reductase; (4), shikimate kinase; (5), 3-enolpyruvylshikimate 5-phosphate synthetase (102); (6), chorismate synthetase (119).

102), two groups studying the conversion of shikimate to anthranilate showed that 3-enolpyruvylshikimate 5-phosphate was a precursor of anthranilate, as well as of phenylpyruvate and 4-hydroxyphenylpyruvate (73, 138). It was suggested that a specific branch point compound was involved, and this compound was sought by examining a mutant in which the pathways to tryptophan, tyrosine, and phenylalanine were blocked (71, 72).

Using ultraviolet irradiation followed by penicillin selection, a strain requiring both tryptophan and tyrosine was isolated from a tryptophan auxotroph which accumulated anthranilate. The double mutant was then treated to obtain the triple mutant (*Aerobacter aerogenes* 62-1), in which tryptophan and tyrosine were essential for growth, whereas phenylalanine stimulated growth. Cell-free extracts were prepared from this strain grown with excess tryptophan to repress the enzyme system forming anthranilate. These cell extracts formed a new compound from a mixture of shikimate, ribose-5-phosphate, adenosine triphosphate (ATP), and Mg^{2+} . This compound could be converted to anthranilate in the presence of glutamine by cell extracts of a multiple aromatic auxotroph with a metabolic block immediately after 3-enolpyruvylshikimate 5-phosphate. The new compound was readily isolated on paper chromatograms and could be shown to be enzymically converted not only to anthranilate but also to prephenate (and thence to phenylpyruvate and 4-hydroxyphenylpyruvate), 4-hydroxybenzoate (at that time, a bacterial vitamin of unknown function), and 4-aminobenzoate (68, 71, 72).

The new intermediate was named chorismic acid (chorismic meaning separating) and found to be excreted by whole cells of *A. aerogenes* 62-1. It was isolated first as the barium salt and later as the free acid, and its chemical structure was determined (56, 66, 67, 69). Chorismic acid and its salts are unstable, and they decompose under physiological conditions to give a mixture of 4-hydroxybenzoate and prephenate, the latter compound giving phenylpyruvate in acid solution (66, 72).

Chorismate, presumably because of a permeability barrier, does not act as a growth factor that will replace the amino acid or vitamin requirements of multiple aromatic auxotrophs. The instability of chorismate at 37 C (66) necessitates the detection of any growth response during a short period after its addition. Figure 3 shows the results of an experiment in which the ability of chorismate to substitute for the 4-aminobenzoate requirement of a multiple aromatic auxotroph (a mutant unable to carry out a

reaction of the common pathway) of *Escherichia coli* was tested. The concentration of 4-aminobenzoate required for half-maximal growth of such an auxotroph is about 10^{-8} M, but the addition of a large excess of chorismate (5×10^{-4} M) did not support growth. The addition of dimethylsulfoxide (5%), which has been shown to increase cellular permeability (61), did not affect the results.

The instability of chorismate and its inability to promote growth probably were factors in the branch point compound not being discovered earlier. Metzenberg and Mitchell (115) examined a mutant of *Neurospora crassa*, which probably accumulated chorismate, in an attempt to find a branch point compound, but they found prephenate among other compounds.

Chorismate has also been isolated from culture fluids of *E. coli* (107), *N. crassa* (41), and *Saccharomyces cerevisiae* (107), and it is also metabolized by cell extracts from *Lactobacillus arabinosus* (103), *N. crassa* (41), *Claviceps paspali* (106), yeast (50, 104), and plants (27), indicating the general role of the compound in aromatic biosynthesis.

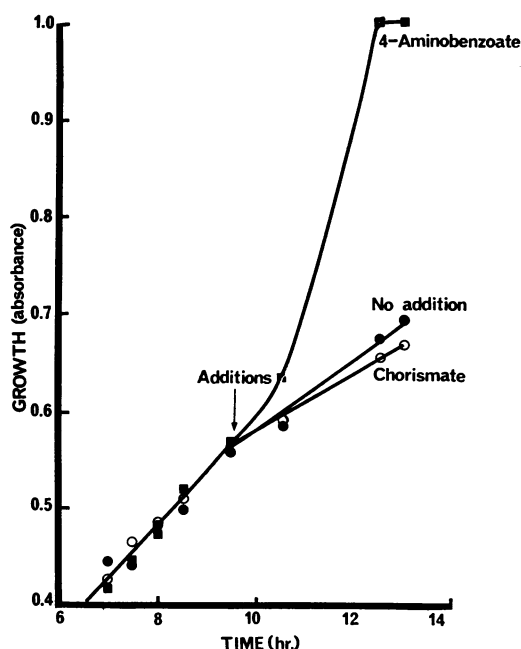


FIG. 3. Inability of chorismate to replace the 4-aminobenzoate requirement of a multiple aromatic auxotroph (*E. coli* AB2839). Growth tests were carried out as described previously (174). Medium contained multiple aromatic supplement with 4-aminobenzoate limiting growth at an absorbance of about 0.6. 4-Aminobenzoate (10^{-8} M) or chorismate (5×10^{-4} M) was added at time indicated.

Early experiments on the incorporation of ^{14}C -glucose into aromatic amino acids in *A. aerogenes* were not consistent with the scheme as outlined, and they suggested that the shikimate ring was not used, as such, as a precursor of the aromatic amino acids (for references, see 149). However, similar experiments have now been carried out with *E. coli* (149), giving results which are consistent with the pathway of aromatic biosynthesis as it is now understood.

Tryptophan Pathway

No new intermediates in the tryptophan pathway have been found recently, and the pathway is as set out in Fig. 4.

The postulated intermediate, *N*-(5'-phosphoribosyl)-anthranilate, has not been isolated and chemically characterized. Its existence has been recognized by the enzymic formation of a compound with a lower intensity of fluorescence than that of anthranilate by cell extracts of *E. coli*, *A. aerogenes*, *Salmonella typhimurium*, *Saccharomyces cerevisiae*, and *Pseudomonas aeruginosa* (45, 50, 51, 57). The compound is very labile (see 44), particularly under acid conditions, breaking down to regenerate anthranilate. Further evidence that the labile compound is an intermediate in tryptophan biosynthesis is provided by the observation that it is formed by cell extracts from some tryptophan auxotrophs, whereas extracts from other tryptophan auxotrophs will convert it to more stable compounds further along the tryptophan pathway (51).

Because of the complexity of the reaction catalyzed by anthranilate synthetase, intermediates between chorismate and anthranilate have been proposed (102, 108, 136, 151). The enzymic evidence suggests that a protein com-

plex metabolizes chorismate through anthranilate to *N*-(5'-phosphoribosyl)-anthranilate. Evidence that it is possible to trap an intermediate has been obtained by Somerville and Elford (147), who found that partially purified anthranilate synthetase from *E. coli* would catalyze the formation of a hydroxamate when incubated with chorismate, glutamine, and hydroxylamine. Although the structure of the hydroxamate is not yet known, the evidence obtained suggests that the overall conversion of chorismate to anthranilate may be separable into two steps, the first utilizing glutamine and the second being dependent on Mg^{2+} . Several possible intermediates have been tested, but they do not serve as precursors of anthranilate (108, 151). Any mechanistic scheme for the amination of chorismate must take into account the finding of Srinivasan (151) that the amide nitrogen of glutamine is transferred to the carbon 2 of chorismate (Fig. 4). (The numbering of the carbons in compounds of the common pathway is conventionally taken from the numbering of shikimic acid, which is itself incorrect because the order of numbering should be through the double bond and not away from it.) This information was gained by growing a tryptophan auxotroph in a medium containing (3,4- ^{14}C)-glucose and determining the distribution of the ^{14}C -labeled atoms in the excreted anthranilate. A comparison of the distribution of the ^{14}C -label in shikimate isolated from cultures of the appropriate mutant during earlier experiments with the distribution of label in the ^{14}C -anthranilate showed the position of insertion of the nitrogen atom.

The source of the nitrogen atom for anthranilate formation has been the subject of a number of studies. In the earlier experiments (150, 152),

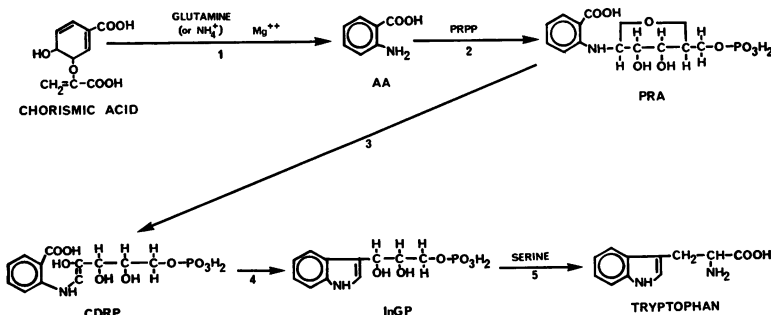


FIG. 4. Intermediates in the tryptophan pathway. Abbreviations:- AA, anthranilic acid; PRA, *N*-(5'-phosphoribosyl)-anthranilic acid; CDRP, *1*-(*o*-carboxyphenylamino)-*1*-deoxyribulose 5-phosphate; InGP, indoleglycerol phosphate. Trivial names of enzymes and some recent references to purification and cofactors:- (1), anthranilate synthetase (123); (2), anthranilate-5'-phosphoribosyl-*1*-pyrophosphate phosphoribosyl transferase (PR transferase); (3), *N*-(5'-phosphoribosyl)-anthranilate isomerase (35); (4), indoleglycerol phosphate synthetase (35); (5), tryptophan synthetase (34, 81, 169).

it seemed that L-glutamine was the likely source of the nitrogen atom and that the amide nitrogen of glutamine was utilized (55, 152). A reexamination of this problem (55) showed that, if buffered at high pH, ammonium ions in relatively high concentrations would serve as an effective nitrogen source for anthranilate formation by crude cell extracts of *A. aerogenes*. Furthermore, it has been possible to obtain evidence that ammonium ions may be used for anthranilate formation directly in vivo rather than via glutamine as an obligate intermediate (70). Thus, cell suspensions of a double auxotroph that required tryptophan and were unable to form glutamine excreted anthranilate when incubated in a glucose-NH₄⁺ salts-buffer mixture.

Pathways to Phenylalanine and Tyrosine

The intermediates between chorismate, phenylalanine, and tyrosine, namely, prephenate, phenylpyruvate and 4-hydroxyphenylpyruvate (Fig. 5), have been known for a number of years (162). These intermediates are concerned in the phenylalanine and tyrosine pathways in *E. coli*, *A. aerogenes*, *Saccharomyces cerevisiae*, and *N. crassa* (8, 105, 162).

Although no new intermediates have been discovered, it has been found that the details of the pathway in different organisms vary in the way in which the known intermediates are metabolized.

Pathway to 4-Aminobenzoic Acid

The molecule of folic acid (Fig. 6) contains a benzene moiety which is inserted as 4-aminobenzoic acid (16); the biosynthesis of the latter compound is being studied at present. Some multiple aromatic auxotrophs were shown to require 4-aminobenzoate for growth (38), and it seems that whether a requirement is shown by

such an auxotroph depends on the completeness of the metabolic block in the common pathway. "Leaky" mutants will allow sufficient flow of intermediates along the common pathway to satisfy the requirement for aromatic vitamins. Weiss and Srinivasan (166) showed that 4-aminobenzoate could be formed from shikimate 5-phosphate plus glutamine by cell-free extracts of bakers' yeast. It was then shown (155) that the amide nitrogen of glutamine was the precursor of the amino group in the aromatic amine by using ¹⁵N-labeled glutamine and studying the effect of glutamine analogues on the conversion.

The fact that mutants blocked between 3-enol-pyruvylshikimate 5-phosphate and chorismate required 4-aminobenzoate for growth in addition to the amino acids (40) indicated that chorismate might well be a precursor of the bacterial vitamin. Cell-free extracts of the strain which accumulated chorismate (*A. aerogenes* 62-1) were found to convert chorismate to 4-aminobenzoate in the presence of L-glutamine (68). Two different approaches to the problem of 4-aminobenzoate synthesis are currently being used. Mutants of both *N. crassa* (52) and *E. coli* (83) requiring 4-aminobenzoate for growth have been divided into two classes by genetic mapping. Hendler and Srinivasan (80) reported "cross-feeding" between the mutant strains of *N. crassa*, but no "cross-feeding" was found by Huang and Pittard (83) with the *E. coli* auxotrophs. The existence of different classes of mutants suggested that at least two reactions were involved in the specific pathway for the synthesis of the vitamin, that is, between chorismate and 4-aminobenzoate. More direct biochemical evidence now supports this concept.

Cell extracts of yeast have been fractionated by ammonium sulfate treatment yielding two

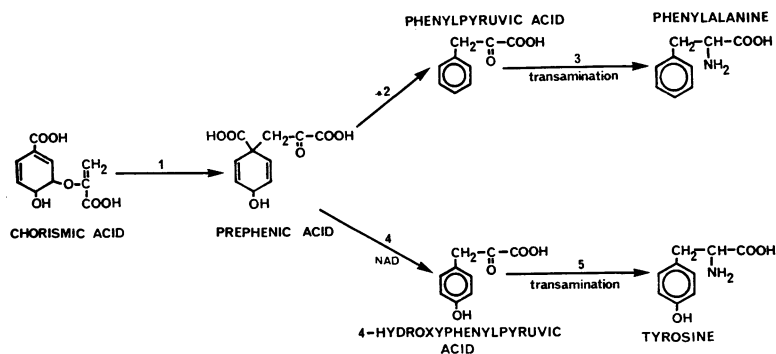


FIG. 5. Intermediates in the biosynthesis of phenylalanine and tyrosine. Trivial names of enzymes:— (1), chorismate mutase; (2), prephenate dehydratase; (3), phenylalanine transaminase; (4), prephenate dehydrogenase; (5), tyrosine transaminase.

fractions, neither of which alone forms 4-aminobenzoate from chorismate plus glutamine, but the two fractions do so when mixed (80). Evidence for an intermediate in *E. coli* has been obtained by the use of cell extracts from suitable mutants (M. Huang, unpublished data). The two mutations affecting 4-aminobenzoate synthesis have been transferred into a strain of *E. coli* K-12 unable to convert chorismate along the pathways to the amino acids. Cell extracts from the resulting strains have been tested for their ability to convert chorismate into 4-aminobenzoate. Neither extract alone will carry out the conversion, which is, however, carried out by a mixture of the two extracts. The little that is known about 4-aminobenzoate synthesis in *E. coli* is shown in Fig. 7.

Intermediates in Ubiquinone Biosynthesis

Ubiquinone occurs in a wide variety of microorganisms and other cells (32). The structure of ubiquinone is shown in Fig. 8. The number of isoprenoid units in the side chain varies with the species (32), although ubiquinones with varying numbers of isoprenoid units may be isolated

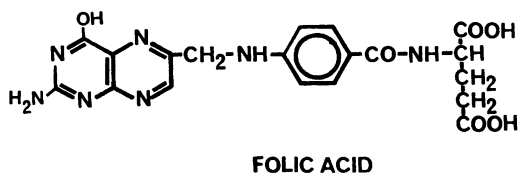


FIG. 6. Structure of folic acid.

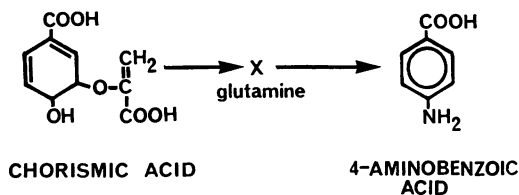


FIG. 7. Biosynthesis of 4-aminobenzoic acid.

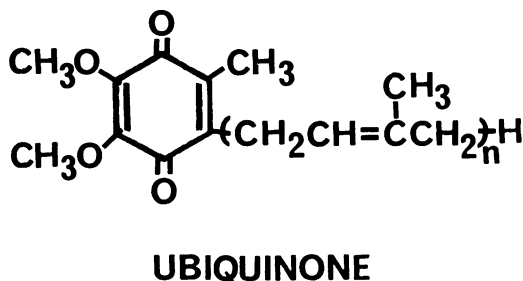


FIG. 8. Structure of ubiquinone.

from the one organism (64, 92). In *E. coli* and *A. aerogenes*, the ubiquinone with a forty-carbon atom side chain is the predominant form. We are concerned here with the quinone nucleus of ubiquinone, which is derived from an aromatic precursor.

The observation of Rudney and Parson (139) that ^{14}C -4-hydroxybenzaldehyde was incorporated into the benzoquinone ring of ubiquinone in *Rhodospirillum rubrum* provided the first definite evidence for an intermediate in ubiquinone biosynthesis. ^{14}C -4-Hydroxybenzoic acid, as well as the aldehyde, was then shown to be incorporated into ubiquinone in *Azotobacter vinelandii*, bakers' yeast and rat kidney (129) and *R. rubrum* (130). The relationship of ubiquinone to the shikimic acid pathway was shown by the demonstration that ^{14}C -shikimic acid was incorporated into ubiquinone in *E. coli* (28). In the latter experiments, it was shown that an excess of unlabeled 4-hydroxybenzoate in the medium together with the ^{14}C -shikimate "swamped" the labeling of ubiquinone, providing further evidence that 4-hydroxybenzoate lay on the pathway.

4-Hydroxybenzoate had been shown to have vitamin-like activity for multiple aromatic auxotrophs of *E. coli* many years before (37), although the requirement was not an absolute one. Multiple aromatic auxotrophs of *E. coli* growing on a glucose-mineral-salts medium supplemented with the aromatic amino acids and 4-aminobenzoate formed ubiquinone only when 4-hydroxybenzoic acid was added (29). However, in similar experiments with multiple aromatic auxotrophs of *A. aerogenes*, ubiquinone was formed, suggesting that an alternative pathway to ubiquinone may exist in these cells. Experiments with cell-free extracts of *A. aerogenes* (29; F. Gibson and R. Bayly, unpublished data) indicate 4-hydroxybenzoate is formed from tyrosine (Fig. 9), although the conversion of 4-hydroxyphenylpyruvate to 4-hydroxybenzaldehyde occurs spontaneously at physiological pH; it is not known whether there is an enzyme carrying out this step (130). Under conditions where extracts of *A. aerogenes* readily convert 4-hydroxybenzaldehyde to 4-hydroxybenzoate, extracts of *E. coli* K-12 are unable to do so, probably accounting for the inability of the multiple aromatic auxotrophs of the latter strain to form ubiquinone, although there is tyrosine in the medium.

The pathway from 4-hydroxybenzoate to ubiquinone is not well established, but the complete sequence shown in Fig. 10 has been proposed (63). A number of isoprenoid compounds have been isolated from cells of *R. rubrum* and *P. ovalis* (63, 65, 128, 129), and the plausible scheme of Fig. 10 has been advanced. However, it should

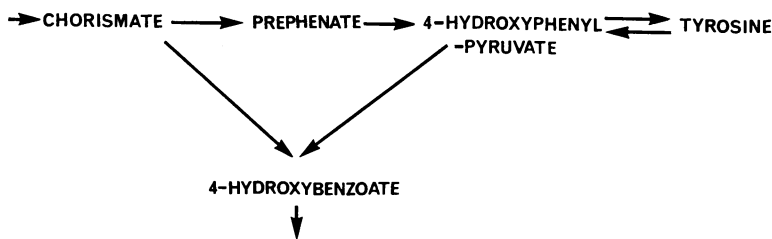


FIG. 9. Alternative pathways of 4-hydroxybenzoate formation.

be emphasized that not all of the compounds set out in Fig. 10 have been isolated; furthermore, other related compounds not in the scheme have been isolated (65). The only reaction which has been studied with cell-free extracts is the conversion of chorismate into 4-hydroxybenzoate (71). Recently, this reaction was studied in greater detail and the enzyme was partially purified (I. G. Young, *unpublished data*). The results of investigation of other reactions of the proposed pathway, with cell-free enzymes, are awaited with interest.

Another approach to the problem of the biosynthesis of ubiquinone is to isolate mutants unable to carry out specific reactions in the pathway and to look for accumulated precursors. This approach has been used with *E. coli* K-12, but ubiquinone does not give a growth response and is not required for growth in a glucose-mineral-salts medium. Hence, the usual methods of mutant selection cannot be used. However, an indirect method of selection has been developed (31) which is based on the assumption that ubiquinone is essential for electron transport and that, therefore, a ubiquinoneless strain of *E. coli* would grow fermentatively on a glucose medium but be unable to grow on a reduced substrate such as malate or succinate as sole source of carbon. By testing strains of the desired phenotype for their ability to form ubiquinone, a number of ubiquinoneless strains were isolated (31; G. B. Cox, *unpublished data*). One of the strains contains two mutations affecting ubiquinone biosynthesis (31). These mutations have been separated, by conjugation, into different strains. Examination of these strains for accumulated intermediates has shown that one of them (*ubiA*⁻) accumulates 4-hydroxybenzoate and the other (*ubiB*⁻) accumulates octaprenylphenol (G. B. Cox, *unpublished data*). The continuation of this approach should yield further information about the biosynthesis of ubiquinone.

The methyl group of methionine has been shown to serve as the source of the methoxy-methyl groups and the ring-methyl group of ubiquinone in *Mycobacterium phlei* and *E. coli* (90, 91).

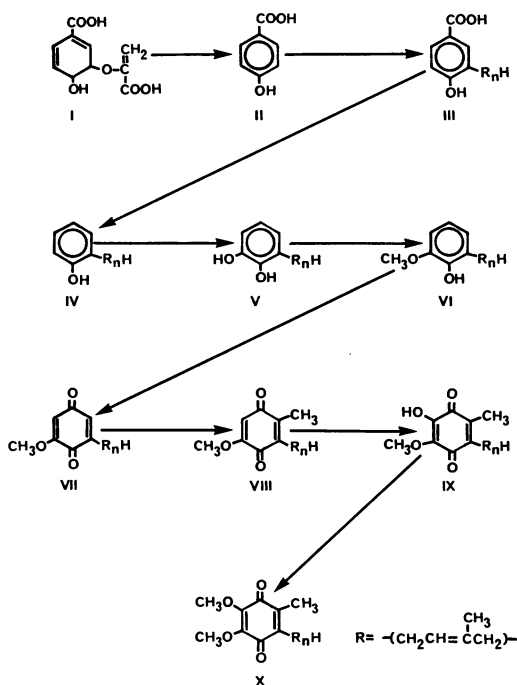
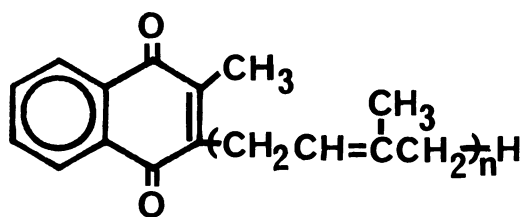


FIG. 10. Proposed pathway of ubiquinone biosynthesis.

The pathway outlined above may be of general significance since ¹⁴C-4-hydroxybenzoic acid is incorporated into ubiquinones in animal tissues (129), a plant (167), and a protozoan (118). In the last two cases, ¹⁴C-shikimate was also incorporated into ubiquinone.

Intermediates in Vitamin K Biosynthesis

As in the case of the ubiquinones, a number of forms of vitamin K occur in microbial cells. They have a common naphthoquinone nucleus and a side chain which varies in the number of isoprenoid units and the degree of saturation and stereochemistry of the side chain (6, 32, 53, 86, 143). The basic structure of vitamin K is shown in Fig. 11. One exception to this general structure is the 2-desmethyl nucleus in the vitamin K isolated from *Haemophilus influenzae* (100).



VITAMIN K₂

FIG. 11. Structure of vitamin K₂.

Virtually nothing was known about the biosynthesis of the naphthoquinone nucleus of vitamin K until 1964, when it was observed that *E. coli* growing in the presence of ¹⁴C-shikimate incorporated the label into the naphthoquinone nucleus (28). It was later shown that the shikimate was incorporated into the benzene ring of the naphthoquinone (29). More recently, evidence has been obtained that the carboxyl group of shikimate is incorporated into vitamin K (19) and also into a plant naphthoquinone (177).

It is likely that the branch point from the common pathway for vitamin K biosynthesis is at chorismate, although the fact that chorismate does not act as a growth factor means that incorporation of ¹⁴C-chorismate by the use of whole cells cannot be tested. However, a multiple aromatic auxotroph of *A. aerogenes* blocked between 3-enolpyruvylshikimate 5-phosphate and chorismate did not form vitamin K (29), and an excess of unlabeled phenylpyruvate or 4-hydroxyphenylpyruvate did not "swamp" the labeling of vitamin K formed from ¹⁴C-shikimate. The possibility still remains that the branch point is at prephenate.

The pathway from the branch point to vitamin K is at present unknown. Two compounds, 3,4-dihydroxybenzaldehyde and α -naphthol, have been suggested as possible intermediates. In early experiments, it was found that a compound with some growth factor activity for multiple aromatic auxotrophs, 3,4-dihydroxybenzaldehyde (39), would "swamp" the incorporation of ¹⁴C-shikimate into vitamin K, although the effect was not as marked as the effect of 4-hydroxybenzoate on the incorporation of label into ubiquinone (28). Recently, it has been shown that ³H-3,4-dihydroxybenzaldehyde is not incorporated into vitamin K in *E. coli*, *Bacillus subtilis*, or *M. phlei* (19, 98), although the results of the swamping experiment in *E. coli* were confirmed (98). α -Naphthol has been suggested as a precursor of vitamin K (98), following the observation that

¹⁴C- α -naphthol is incorporated into vitamin K. Further evidence is needed, preferably with cell-free systems, to establish that α -naphthol is directly on the pathway of vitamin K biosynthesis and to clarify the effects observed with 3,4-dihydroxybenzaldehyde. No satisfactory system for the formation of the naphthoquinone nucleus of vitamin K by cell-free extracts has been devised, despite one promising report (4, 5).

It has been established for a number of organisms that the 2-methyl group of the quinone nucleus of vitamin K, like that of ubiquinone, is derived from methionine (4, 90, 91, 97).

As in studies of ubiquinone biosynthesis, the isolation of suitable mutants would assist in the search for possible intermediates. One mutant of *E. coli* K-12 unable to form vitamin K was isolated during a search for ubiquinoneless mutants (31) and is being examined for possible accumulation products, but a rational procedure for the isolation of mutants blocked in the specific pathway of biosynthesis of vitamin K has yet to be devised.

Pathways Involving 2,3-Dihydroxybenzoate

The importance of 2,3-dihydroxybenzoate in bacterial metabolism was emphasized recently with the observation that it is an essential growth factor for some multiple aromatic auxotrophs of *E. coli* (30, 174). However, it has been known for some time that 2,3-dihydroxybenzoate and certain compounds containing the phenolic acid are formed by microbial cells. Ito and Neilands (88) isolated 2,3-dihydroxybenzoylglycine from the culture media of *B. subtilis* growing in an iron-deficient medium. Since then, 2,3-dihydroxybenzoate and related compounds have been identified as metabolic products formed by other organisms including *A. aerogenes* (132), *C. paspali* (3), *Aspergillus niger* (160), *Streptomyces griseus* (54), *S. rimosus* (21) and *E. coli* (15, 174). In *C. paspali* (161) and *Aspergillus niger* (156), it appears that 2,3-dihydroxybenzoate may be formed from tryptophan, but in *A. aerogenes* and *E. coli*, it is formed more directly from chorismate. Crude cell extracts of *A. aerogenes* 62-1 (174) or of a similar mutant of *E. coli* K-12 (R. K. J. Luke, unpublished data) form 2,3-dihydroxybenzoate when incubated with chorismate, Mg²⁺ and nicotinamide mononucleotide (NAD). The pathway in *A. aerogenes* has been examined in detail (Fig. 12). Evidence for at least two steps in the conversion of chorismate to 2,3-dihydroxybenzoate was provided by the observation that crude extracts of *A. aerogenes* continued to form 2,3-dihydroxybenzoate after all the chorismate had been removed. When NAD was not added

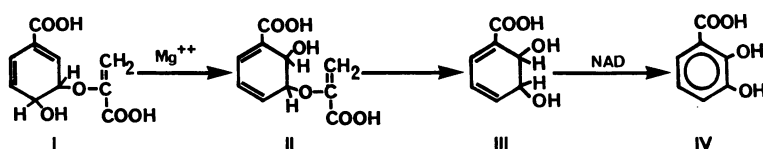


FIG. 12. Conversion of chorismate (I) to 2,3-dihydroxybenzoate (IV) through isochorismate (II) and 2,3-dihydro-2,3-dihydroxybenzoate (III).

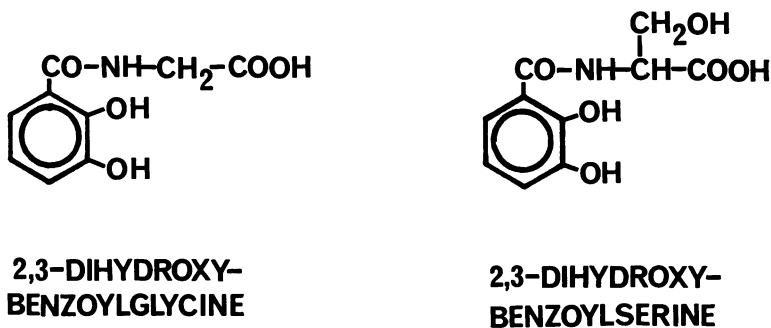


FIG. 13. Structures of 2,3-dihydroxybenzoylglycine and 2,3-dihydroxybenzoylserine.

to the reaction mixture, chorismate was removed at about the same rate as when NAD was added. The compound formed by metabolism of chorismate in the absence of NAD was isolated, examined by nuclear magnetic resonance and mass spectrometry (175; I. G. Young, *unpublished data*), and identified as 2,3-dihydro-2,3-dihydroxybenzoic acid (Fig. 12).

Fractionation of crude extracts of *A. aerogenes* 62-1 by chromatography on diethylaminoethyl (DEAE) cellulose gave a fraction which formed an intermediate capable of being converted to 2,3-dihydro-2,3-dihydroxybenzoate and to 2,3-dihydroxybenzoate (I. G. Young, *unpublished data*). The new intermediate, for which the trivial name isochorismic acid is suggested, is very unstable but has been isolated and identified (I. G. Young, T. J. Batterham, and F. Gibson, *unpublished data*) as the compound II of Fig. 12.

The functional form of 2,3-dihydroxybenzoate is not known. As mentioned above, 2,3-dihydroxybenzoylglycine (Fig. 13) is formed by *B. subtilis*. A similar compound formed by *E. coli* was tentatively identified as 2,3-dihydroxybenzoylserine (15). The structure of the serine conjugate (Fig. 13) excreted by *E. coli* and *A. aerogenes* has recently been established by synthesis and by comparison with the natural product (I. G. O'Brien, *unpublished data*).

It has been observed that these compounds are formed in large quantities when cells are grown in iron-deficient media (14, 88; B. R. Byers and C. E. Lankford, *Bacteriol. Proc.*, p. 43, 1967). The enzymes forming 2,3-dihydroxybenzoate in *A.*

aerogenes (174) and the enzyme system converting 2,3-dihydroxybenzoate to 2,3-dihydroxybenzoylserine in *E. coli* (14) are strongly repressed by iron or cobalt ions. Iron (or in one case, manganese ions) will replace the 2,3-dihydroxybenzoate requirement of multiple aromatic auxotrophs (174). These observations support the suggestion of Ito and Neilands that the glycine conjugate might play an important role in iron transport (88). However, the effects of other metals may mean that these phenolic compounds are also important in the metabolism of metals other than iron.

Mutants of *E. coli* K-12 requiring 2,3-dihydroxybenzoylserine have been isolated to aid in the study of the biosynthetic pathway and for studies on function (R. K. J. Luke, *unpublished data*).

Other Phenolic Growth Factors

Other phenols have been found to act as growth factors. Tyrosine or lower concentrations of phenols such as protocatechuic acid or catechol acted as growth factors for a species of *Sarcina* (77) or *Micrococcus lysodeikticus* (140). The pathways to these compounds are not known in the above organisms; however, in *N. crassa*, protocatechuic acid is formed from the common pathway intermediate, 5-dehydroshikimic acid (78). This compound has also been shown as the source of both protocatechuic acid and catechol in *A. aerogenes* by experiments with whole cells (133) and cell extracts (A. F. Egan, *unpublished data*).

A species of *Pseudomonas* can convert anthranilate into catechol (157).

ISOENZYMES AND PROTEIN AGGREGATES
CONCERNED IN AROMATIC
BIOSYNTHESIS

Although not all of the reactions concerned in aromatic biosynthesis have been studied in detail, a number of interesting features have been revealed. These include the occurrence of isoenzymes, protein aggregates carrying out more than one reaction, and the catalysis of two reactions by one polypeptide. A brief survey of the occurrence of these features in the various pathways follows, and some aspects will be dealt with in more detail when discussing metabolic regulation of the various pathways of biosynthesis.

Common Pathway

Isoenzymes have been found for three of the reactions of the common pathway. 3-Deoxy-D-arabino-heptulosonate 7-phosphate synthetase (DAHP synthetase), the first enzyme, has an important function in the regulation of aromatic biosynthesis and will be discussed in detail. Inhibitor studies, ammonium sulfate fractionation, and column chromatography have provided evidence for the presence of isoenzymes of DAHP synthetase (often three) in a wide variety of microorganisms (17, 48, 49, 62, 93, 94, 145, 163, 164), of which the best studied are *E. coli* and *N. crassa*. In *B. subtilis*, however, only a single DAHP synthetase is present (96), as judged by the results of enzyme purification, inhibitors, and the isolation of auxotrophic mutants lacking DAHP synthetase activity, which are the result of single-step revertible mutations.

Although multiple aromatic auxotrophs of *N. crassa* that lack shikimate kinase activity may be isolated (74), no similar mutants have been reported from the widely studied species

of *E. coli*, *A. aerogenes*, *S. typhimurium*, and *B. subtilis*. The presence of two distinct shikimate kinases in *S. typhimurium* (120), and possibly in *B. subtilis* (125), probably accounts for the lack of shikimate kinase mutants in this species, and a similar explanation may apply to the other species. In *N. crassa*, however, no auxotrophs lacking dehydroquinase were isolated, and again it was possible to demonstrate the presence of two enzymes (75); one of the enzymes was constitutive and the other was inducible.

In two organisms, *B. subtilis* and *N. crassa*, protein aggregates carrying out more than one reaction on the common pathway have been described. A single revertible mutation in one strain of *B. subtilis*, resulting in loss of chorismate mutase activity, gave a strain which simultaneously lost DAHP synthetase activity. Gel filtration of cell extracts suggested that one of the shikimate kinases might also be complexed with the two enzymes mentioned (125). All the enzymes of the common pathway, with the exception of the first (DAHP synthetase) and the last (chorismate synthetase), are associated as a multienzyme complex in *N. crassa* (74).

Tryptophan Pathway

One of the best examples studied of a protein aggregate is the terminal enzyme in tryptophan biosynthesis in *E. coli*, tryptophan synthetase, which can be separated into two proteins, A and B (34, 171). Tryptophan synthetase from *S. typhimurium* is also dissociable, but that from *N. crassa* is not dissociable, although it shows many similarities to the *E. coli* enzyme (13). Although there are five recognizable reactions in the specific pathway of tryptophan biosynthesis, it seems that in *E. coli* there are only three enzymic steps, since anthranilate synthetase and 5'-phosphoribosyl-1-pyrophosphate phosphoribosyl (PR) transferase activities are associated

TABLE 1. Combination of PR transferase from *A. aerogenes* with anthranilate synthetase from *E. coli*

Strain ^a	Anthranilate synthetase		PR transferase	
	Approx molecular weight	Inhibition by tryptophan	Approx molecular weight	Inhibition by tryptophan
<i>A. aerogenes</i> (wild type)	170,000	+	170,000	+
<i>A. aerogenes</i> NC3	Activity not detectable		90,000	—
<i>E. coli</i> K-12 (wild type)	170,000	+	170,000	+
<i>A. aerogenes</i> NC3, and <i>E. coli</i> D 9778 ^b	170,000	+	170,000	+

^a Anthranilate synthetase activity was not detectable in *A. aerogenes* NC3. No activity was detectable in *E. coli* D 9778.

^b *E. coli* D 9778 was obtained from C. Yanofsky.

in a protein complex (89), *N*-(5'-phosphoribosyl)-anthranilate isomerase and indoleglycerol phosphate synthetase activities are carried by the same polypeptide (35), and tryptophan synthetase carries out the final step.

Anthranilate synthetase is evidently associated with PR transferase in *E. coli* (89) and *S. typhimurium* (10, 11), since the two activities travel together during ultracentrifugation in sucrose gradients. Also, it was observed (89) that a nonsense mutation affecting the *D* gene coding for PR transferase activity in *E. coli* affected anthranilate synthetase activity, although the latter could be detected on mixing extracts of such cells with extract from cells in which the anthranilate synthetase was affected by a mutation affecting the *E* gene. Purification of the protein complex from *E. coli* results in loss of PR transferase activity because of instability (C. Yanofsky, *personal communication*). However, purification of anthranilate synthetase from *A. aerogenes* simultaneously purifies the second activity (57; A. F. Egan, *unpublished data*). Furthermore, it is possible to obtain evidence that anthranilate synthetase from *E. coli* K-12, which is inactive alone, will combine with the PR transferase from *A. aerogenes* to form an active complex (89; A. F. Egan, *unpublished data*). This evidence is summarized in Table 1. Thus, anthranilate synthetase and PR transferase in wild-type cells of these strains form a protein aggregate with a molecular weight of about 170,000, and both reactions are inhibited by tryptophan. The mutant of *A. aerogenes* (NC3) lacking anthranilate synthetase activity contains PR transferase activity which has a lower molecular weight, as judged by sucrose gradient centrifugation, than that in extracts from wild type, and it has lost its sensitivity to tryptophan. The mutant strain of *E. coli* used (D9778) has lost PR transferase activity and anthranilate synthetase activity owing to a mutation in the gene coding for PR transferase activity (89). When crude cell extracts from the mutants are incubated together and examined by centrifugation in a sucrose gradient, there is found a peak of anthranilate synthetase—PR

transferase activity with the molecular weight and sensitivity to tryptophan of the aggregate from wild type *A. aerogenes* or *E. coli*.

It has recently been found that PR transferase activity is not necessary for anthranilate synthetase activity in *P. putida* and that anthranilate synthetase itself in this organism is separable into two components (S. W. Queener and I. C. Gunsalus, *Bacteriol. Proc.*, p. 136, 1968).

It can be seen that in *E. coli* (89) and *S. typhimurium* (10) anthranilate synthetase activity is the result of aggregation of two polypeptides. Similar combinations of polypeptides have been demonstrated for enzymes of the tryptophan pathway in a variety of organisms, but the relationships between the genes and the enzymes vary with the species. This point is well illustrated by reference to an extensive study by Hütter and DeMoss (84) of the biochemistry and genetics of a variety of microorganisms, particularly fungi.

Phenylalanine and Tyrosine Pathways

Examination of chorismate mutase activity in *A. aerogenes* by chromatography of cell extracts showed that there were two enzymes (24). One of these activities (chorismate mutase P) was associated with the next enzymic activity on the phenylalanine pathway, namely, prephenate dehydratase (*see* Fig. 5), and both activities were absent in a phenylalanine auxotroph. The protein carrying out these activities was named the P protein. The second chorismate mutase (T) is associated with prephenate dehydrogenase. This protein aggregate from *A. aerogenes* has been highly purified (R. G. H. Cotton, *unpublished data*) and is dissociable into two subunits, neither of which alone has either chorismate mutase or prephenate dehydrogenase activity (25, 26). Both activities of the T protein may be lost as the result of a single reversible mutation (24). The pathways in *A. aerogenes* 62-1 may be represented as in Fig. 14. The pathways in *E. coli* appear to be the same as in Fig. 14, but a mutant has been found in *E. coli* that has lost prephenate de

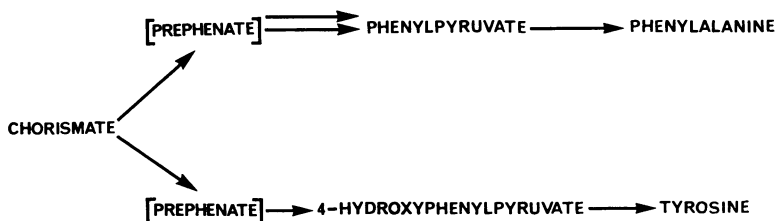


FIG. 14. The pathways to phenylalanine and tyrosine in *E. coli* K-12 and *A. aerogenes* 62-1. There is a second prephenate dehydratase in *A. aerogenes* 62-1.

hydratase activity but still has two chorismate mutases (134).

In *A. aerogenes* 62-1, the strain which accumulates chorismate, there is a second prephenate dehydratase (A) which is still present in phenylalanine auxotrophs (28; Fig. 14). No specific function can be ascribed to this enzyme and it is not present in the only other strain of *A. aerogenes* examined (R. G. H. Cotton, unpublished data). *A. aerogenes* 62-1 has lost both T and P protein activities, but a reversion to tyrosine independence also removes the phenylalanine requirement. The simplest explanation is that the prephenate formed by the T protein is then converted by the remaining prephenate dehydratase A into phenylpyruvic acid, thus bypassing the metabolic block.

Other organisms that have been examined differ from *E. coli* and *A. aerogenes*. In *N. crassa* (8) and the bean (*Pisum sativum*) (27), there appears to be one chorismate mutase activity with branching of the pathways at prephenate (as in Fig. 5). In extracts of one strain of *B. subtilis*, three distinct species (CM₁, CM₂, and CM₃) of chorismate mutase could be separated by chromatography on DEAE-cellulose, whereas extracts of another strain contained only the CM₃ species (109). Unlike the *E. coli* and *A. aerogenes* system, there was no association of chorismate mutase with either of the subsequent activities.

REGULATION OF THE COMMON PATHWAY

Enzymes of the common pathway provide an intermediate, chorismic acid, which is a common precursor molecule of tyrosine, phenylalanine, tryptophan, folic acid, ubiquinone, vitamin K, and 2,3-dihydroxybenzoylserine (or 2,3-dihydroxybenzoylglycine). Of these end-products only the three amino acids appear to play an important role in controlling the rate of synthesis of chorismic acid. In different microorganisms, this control is affected either by feedback inhibition alone or by a combination of feedback inhibition and repression.

In feedback inhibition, it would be possible for the various end products to effectively control the common pathway by one of at least four different mechanisms. (i) In "cumulative inhibition", as reported by Woolfolk and Stadtman for glutamine synthetase (170), each inhibitor adds its effect to the total inhibition of the enzyme, in which, however, the combined effect of any two inhibitors is less than the sum of their single inhibitions. (ii) In "concerted or multivalent inhibition" (36), two end products are required together before any significant inhibition occurs. (iii) "Sequential feedback inhibition" is carried out by a single molecule whose accumulation

TABLE 2. Percentage of inhibition of DAHP synthetase isoenzymes of *Escherichia coli* K-12 by the aromatic amino acids^a

DAHP synthetase	Tyrosine		Phenylalanine		Tryptophan	
	10 ⁻³ M	10 ⁻⁵ M	10 ⁻³ M	10 ⁻⁵ M	10 ⁻³ M	10 ⁻⁵ M
(Tyr)	95	50	5	0	0	0
(Phe)	40	0	95	60	0	0
(Trp) ^b	0	0	0	0	60	20

^a Data taken from unpublished results of B. J. Wallace and J. Pittard. Single isoenzymes were assayed in crude cell-free extracts obtained from mutant strains possessing only one functional DAHP synthetase isoenzyme. The reaction mixture contained erythrose-4-phosphate (0.5 μmole), phosphoenolpyruvate (0.5 μmole), sodium phosphate buffer (pH 6.4; 25 μmoles), a rate-limiting amount of enzyme and inhibitors at the final concentrations shown above.

^b Co²⁺ 10⁻³ M was added to the reaction mixture.

is in turn controlled by several end products (124). (iv) In "feedback inhibition of isoenzymes," the reaction is carried out by more than a single enzyme, and a balanced control is possible because an inhibitable isoenzyme exists for each major end product. In this case, it is expected that the inhibition caused by two end products will equal the sum of the inhibitions caused by each one separately. If extensive cross-inhibitions occur, this result will not be obtained and the final distinction between (i) and (iv) may depend on the physical separation of different isoenzymes.

Theoretically, similar possibilities exist for the repression of the formation of the enzymes of the common pathway. If there is no duplication of enzymes, a system of multivalent repression could ensure that only in the presence of all the end products of the terminal pathways would the enzymes of the common pathway be repressed. Alternatively, if multiple enzymes are formed for any particular reaction, repression of the formation of individual enzymes by individual end products offers a reasonably efficient system of control.

Studies on the regulation of the common pathway of aromatic biosynthesis in a number of different microorganisms suggest that the control of the first reaction of the pathway, the conversion of erythrose-4-phosphate and phosphoenolpyruvate to DAHP, by inhibition or repression, or both, is an important factor in the control of the common pathway. Therefore, this reaction will be considered separately from the other reactions of the common pathway. The control

of this first reaction has also recently been reviewed by Doy (48).

Feedback Inhibition of DAHP Synthetase

Smith et al. (145) demonstrated the existence of two DAHP synthetase isoenzymes in *E. coli*, DAHP synthetase (tyr) and DAHP synthetase (phe); DAHP synthetase (tyr) was inhibited by tyrosine and DAHP synthetase (phe) was inhibited by phenylalanine. Brown and Doy (17), also working with *E. coli* W, demonstrated the existence of a third isoenzyme, DAHP synthetase (trp), the activity of which was apparently not inhibited by either phenylalanine, tyrosine, or tryptophan, but the formation of which was repressed by tryptophan. The presence of three isoenzymes in *E. coli* K-12 has been confirmed by the isolation of mutants which have lost one or more of their isoenzymes, and by the identification of these three activities using chromatography on DEAE-cellulose (163, 164; K. D. Brown and W. K. Maas, Federation Proc., p. 338, 1966).

With *E. coli* W, DAHP synthetase (phe) and DAHP synthetase (tyr) activities have been separated by ammonium sulfate fractionations of crude cell-free extracts (49). With *E. coli* K-12, recombinant strains have been isolated which contain only one of the three isoenzymes (164). In these strains, the activity of a single isoenzyme can be assayed in crude cell-free extracts. In both systems, the sensitivities of DAHP synthetase (tyr) and DAHP synthetase (phe) to inhibition by phenylalanine, tyrosine, and tryptophan have been determined (49). Similar results have been obtained with both organisms, and the results of studies of inhibition with *E. coli* K-12 are summarized in Table 2.

There have been many reports to the effect that DAHP synthetase (trp) from either *E. coli* W or *E. coli* K-12 is not inhibited by tryptophan (49, 94, 107, 164). However, Doy has recently reported a 32% inhibition of this enzyme from *E. coli* W (46). Because of the early difficulties in establishing *in vitro* inhibition of DAHP synthetase (trp), experiments have recently been carried out to demonstrate inhibition of this enzyme in whole cells (*unpublished data*). When the *trypR*⁻ gene is present in a strain of *E. coli* K-12 which possesses only DAHP synthetase (trp), this enzyme and the enzymes of the tryptophan operon are synthesized constitutively; i.e., their rate of synthesis is no longer affected by the presence or absence of tryptophan. These strains rely entirely on DAHP synthetase (trp) to carry out the first reaction of the pathway, and they have a mean generation time in minimal medium

of 3 hr, compared with 80 min for the wild-type cells. When tryptophan is added to the medium, even though it has no effect on the formation of DAHP synthetase (trp), the mean generation time of these strains is increased to 12 hr. When a further mutation which prevents the conversion of DAHP to dehydroquininate is introduced into these strains, added tryptophan (5×10^{-4} M) reduces the rate of accumulation of DAHP by 80%. The cells in both instances, however, contain the same levels of the enzyme DAHP synthetase (trp). Further studies with extracts have shown that in the presence of Co^{2+} (10^{-3} M) DAHP synthetase (trp) is inhibited 60% by 10^{-3} M L-tryptophan (*unpublished data*).

Using strains that each possess only one of the isoenzymes, it has been possible to isolate feedback-resistant mutants. The DAHP synthetase (trp) of one mutant isolated shows an inhibition of 20% at 10^{-3} M L-tryptophan, compared with 60% inhibition of the wild-type enzyme. The DAHP synthetase (phe) of another feedback resistant mutant shows an inhibition of 30% at 10^{-4} M L-phenylalanine, compared with 92% for the wild-type enzyme (J. Pittard, *unpublished data*). Ezekiel has also reported the isolation of mutant strains from *E. coli* K-12, in which DAHP synthetase (phe) is no longer inhibited by phenylalanine (59). Although there have been no reports yet of mutant strains in which DAHP synthetase (tyr) is feedback-resistant, there is no reason to believe that these should be difficult to isolate.

In *S. typhimurium*, the situation would appear to be very similar to that existing in the case of *E. coli* (76). In crude cell-free extracts, inhibitions by phenylalanine and tyrosine are found to be additive when both amino acids are added together, suggesting the presence of two separate isoenzymes. Furthermore, DAHP synthetase (tyr) and DAHP synthetase (phe), inhibitable by tyrosine and phenylalanine, respectively, can be separated by ammonium sulfate fractionation.

Evidence for the existence of a third isoenzyme, DAHP synthetase (trp), comes from the isolation of mutant strains unable to grow in minimal medium supplemented with phenylalanine and tyrosine but able to grow in minimal medium. Mutant strains lacking DAHP synthetase (tyr) or DAHP synthetase (phe) have also been isolated in this organism (76). No fraction of the DAHP synthetase activity of *S. typhimurium* has yet been found to be inhibited by tryptophan (76, 93). In view of the difficulty experienced in *E. coli*, however, this failure to demonstrate inhibition *in vitro* may not reflect the true *in vivo* situation.

A survey of the inhibition of DAHP synthetase

from a variety of gram-negative and gram-positive organisms by phenylalanine, tyrosine, and tryptophan has recently been carried out (94). In some cases (e.g., *Hydrogenomonas* sp.) phenylalanine and tyrosine exerted a cumulative feedback inhibition on DAHP synthetase. In other organisms, the effects of the individual amino acids were additive. Therefore, it was concluded that each amino acid was inhibiting a different isoenzyme. Many cases in which tryptophan could inhibit part of the DAHP synthetase activity were described, and there were some strains which appeared to possess only a single enzyme inhibited by a single amino acid. As the authors point out, the dilemma posed by these last mentioned strains, which are able to grow in the presence of the amino acid which totally inhibits DAHP synthetase *in vitro*, may well be resolved when they are studied in more detail.

In *Saccharomyces cerevisiae*, results suggest (47, 105) that there are only two DAHP synthetase isoenzymes, one inhibited by tyrosine [DAHP synthetase (tyr)] and one inhibited by phenylalanine [DAHP synthetase (phe)]. Mutants lacking either enzyme have also been isolated (117). The growth of these strains is inhibited by phenylalanine or tyrosine, respectively, confirming the existence of only two isoenzymes. Furthermore, a recombinant strain which has neither isoenzyme is unable to grow on minimal medium and possesses no detectable DAHP synthetase activity (116).

In *N. crassa*, there are three isoenzymes, one inhibited by tyrosine, one by phenylalanine, and one by tryptophan (46, 48, 93). One fraction, which can be isolated by chromatography on Sephadex G-100, has been shown to be inhibited 100% by tryptophan (93). Studies of the regulation of aromatic amino acid biosynthesis in *C. gaspali* reveal the presence of three isoenzymes (106). One of these is inhibited by phenylalanine, one by tyrosine, and one by tryptophan. In this case the tryptophan-inhibitable isoenzyme constitutes approximately 60% of the total activity.

B. subtilis and a number of other strains of *Bacillus* have a different means of inhibiting the first reaction of the pathway (95). In these strains, there appears to be only a single DAHP synthetase enzyme which is inhibited by either chorismic acid or prephenic acid. The accumulation of chorismate and prephenate is, in turn, controlled by the amino acids phenylalanine, tyrosine, and tryptophan, acting on the reactions of the terminal pathways which utilize chorismate (124). These reactions will be dealt with in a later section. In addition to the various strains of *Bacillus*, strains of *Staphylococcus*, *Gaffkya*, *Flavobacterium*,

Achromobacter, and *Alcaligenes* show sequential feedback inhibition of DAHP synthetase by either prephenate or chorismate. The DAHP synthetase from *Xanthomonas*, on the other hand, is inhibited approximately 86% by chorismate, but it shows 10% or less inhibition by prephenate (94).

Repression of DAHP Synthetase

The early work from two laboratories (17, 145) established that in *E. coli* W there were three DAHP synthetase isoenzymes and that the formation of each was repressed by a single amino acid, e.g., DAHP synthetase (tyr) by tyrosine, DAHP synthetase (phe) by phenylalanine, and DAHP synthetase (trp) by tryptophan. In addition, cross repression of DAHP synthetase (tyr) by phenylalanine and tryptophan and DAHP synthetase (phe) by tryptophan was also reported (18). Recently, the derepression of the DAHP synthetase isoenzymes has been studied by growing an aromatic auxotroph of *E. coli* K 12 in a chemostat under conditions in which single aromatic amino acids, in turn, limit the growth rate (K. D. Brown, 1968. Genetics, *in press*). When either tryptophan or phenylalanine limits growth, DAHP synthetase (phe) is derepressed, and it is inferred from these results that phenylalanine and tryptophan together are required for the repression of DAHP synthetase (phe). DAHP synthetase (tyr) is only derepressed when tyrosine is the limiting amino acid and when phenylalanine and tryptophan are present at 10^{-4} M. If these last two amino acids are present in higher concentrations (10^{-3} M), derepression of DAHP synthetase (tyr) is greatly reduced. DAHP synthetase (trp), measured as DAHP synthetase activity not inhibited by either phenylalanine or tyrosine, is derepressed when the growth rate is limited by tryptophan (K. D. Brown, 1968. Genetics, *in press*). Studies carried out on the repression of DAHP synthetase (tyr) in strains containing only this isoenzyme confirm the finding that DAHP synthetase (tyr) is repressed in the presence of high concentrations of phenylalanine and tryptophan (B. J. Wallace, unpublished data).

It has been known for some time that a mutation in a gene, *trpR*, can cause derepression of the enzymes of the tryptophan operon (23). Studies of *trpR*⁻ strains of *E. coli* K-12 which either possess all three DAHP synthetase isoenzymes or possess only the single isoenzyme, DAHP synthetase (trp), have demonstrated that DAHP synthetase (trp) is also produced constitutively in *trpR*⁻ strains (K. D. Brown, 1968. Genetics, *in press*).

A second class of mutants has been isolated (*unpublished data*) in which the control of DAHP synthetase (*trp*) has been altered. These mutants also make this enzyme constitutively but still possess a normally repressible tryptophan operon. Since the mutations conferring this change are closely linked to the structural gene for DAHP synthetase (*trp*), these mutants may turn out to be operator constitutive mutants. In these mutants, DAHP synthetase (*trp*) is not only produced constitutively, but is much less sensitive to inhibition by tryptophan. This pattern resembles that of certain 5-methyl tryptophan-resistant mutants of *E. coli* possessing mutations in the anthranilate synthetase gene, as reported by Somerville and Yanofsky (148). Mutations in a third gene, the *trpS* gene, have an indirect effect on the levels of DAHP synthetase (*trp*) and the enzymes of the tryptophan operon; this will be discussed later.

The tyrosine analogues 4-aminophenylalanine and 3-thianaphthenealanine have been found to repress the formation of DAHP synthetase (*tyr*), although they are not activated by tyrosyl-tRNA synthetase. Since these compounds can per se repress the formation of DAHP synthetase (*tyr*), it has been proposed that tyrosine uncomplexed to any transfer RNA molecule should also function as corepressor (137).

4-Aminophenylalanine at relatively low concentrations (10^{-4} M) completely inhibits the growth of a recombinant strain of *E. coli* K-12 possessing only DAHP synthetase (*tyr*). Since 4-aminophenylalanine does not inhibit the activity of this enzyme (146), the inhibition of growth is presumably caused by the repression of its formation. Using this system, it is a simple matter to isolate mutant strains in which DAHP synthetase (*tyr*) is no longer repressed by tyrosine. Several of these strains have been isolated, and one group in particular has been studied in detail (B. J. Wallace and J. Pittard, *J. Bacteriol.*, *in press*). In this case, a mutation in a gene designated as *tyrR*, which is situated in the general region of the tryptophan operon (*see* Fig. 15), causes derepression of DAHP synthetase (*tyr*), chorismate mutase T and its associated prephenate dehydrogenase, and transaminase A. It also has an effect on the repression of the shikimate kinase enzyme. In other words, those enzymes normally repressible by tyrosine are made constitutively by these mutants. The sensitivity of DAHP synthetase (*tyr*) to feedback inhibition by tyrosine is, however, unchanged, as would be expected from the fact that the *tyrR* gene and the *aroF* gene, the structural gene for DAHP synthetase (*tyr*), are widely separated on the chromosome.

TABLE 3. *Repressed and derepressed levels of the DAHP synthetase isoenzymes in mutant strains of E. coli K-12 possessing only a single DAHP synthetase isoenzyme^a*

DAHP synthetase	Specific activities in extracts prepared from ^b	
	Fully repressed cells	Fully derepressed cells
(Tyr)	0	50
(Phe)	11	18
(Trp)	0.4	5-10

^a Conditions under which the cells were grown are described in the text. Values obtained in the case of strains possessing *trpR*⁻ or *tyrR*⁻ mutations are: DAHP synthetase (*tyr*) in a strain possessing *tyrR*⁻, 150; DAHP synthetase (*trp*) in a strain possessing *trpR*⁻, 4.

^b Specific activity = 0.1 μ mole of DAHP formed per mg of protein per 20 min at 37 C.

In strains possessing the *tyrR*⁻ mutation, high concentrations of phenylalanine and tryptophan no longer repress DAHP synthetase (*tyr*), indicating that the gene product of the *tyrR* gene is involved in this apparent cross-repression (B. J. Wallace, *unpublished data*). No strains have yet been reported that are derepressed for the DAHP synthetase (*phe*) isoenzyme.

One interesting feature of the production of each of the isoenzymes in *E. coli* K-12 and in *E. coli* W (K. D. Brown, *Genetics*, *in press*; 18) is to be found in the differences between fully repressed and fully derepressed values for each one. In studies carried out in our laboratories, repressed values have been determined by growing mutants containing single isoenzymes in the presence of the three aromatic amino acids plus shikimic acid (10^{-6} M), to satisfy the requirement for aromatic vitamins, and harvesting cells in late exponential phase. Derepressed values have been obtained using strains containing only a single isoenzyme, which were also unable to convert DAHP to 5-dehydroquinic acid (DHQ) because of a mutation in the *aroB* gene. These were grown, either in limiting shikimic acid or in a mixture of the three amino acids in which the relevant amino acid was present in growth limiting concentrations. In the latter case, shikimic acid (10^{-6} M) was also added.

The results of this study are indicated in Table 3. Two interesting points emerge from this table. The first is that under derepressed conditions DAHP synthetase (*tyr*) activity is much higher than that of either DAHP synthetase (*phe*) or DAHP synthetase (*trp*). The second point of interest is that the variation in levels of DAHP

synthetase (phe) from repressed to derepressed state is very small by comparison with DAHP synthetase (tyr). Furthermore, although DAHP synthetase (tyr) and DAHP synthetase (trp) are repressed to very low values, DAHP synthetase (phe) exhibits much higher values for maximally repressed conditions.

Inhibition appears to play a much more important role in the regulation of DAHP synthetase (phe) activity than does repression. Recombinant strains possessing only DAHP synthetase (phe) are unable to grow in minimal medium supplemented with phenylalanine or with phenylalanine plus tryptophan. Mutant strains, however, in which the DAHP synthetase (phe) is feedback-resistant suffer no reduction in growth rate when these amino acids are added to the medium, even though the enzyme is as repressible in these strains as in the parent (J. Pittard, unpublished data). Therefore, repression by itself exerts little control on the *in vivo* activity of DAHP synthetase (phe). By contrast, however, 4-aminophenylalanine, which acts only as a corepressor, can completely inhibit growth of a strain possessing only DAHP synthetase [(tyr) B. J. Wallace and J. Pittard, *J. Bacteriol.*, in press], and a strain possessing only a feedback-resistant DAHP synthetase (trp) isoenzyme has its growth rate halved by the addition of tryptophan (J. Pittard and J. Camakaris, in preparation).

In *S. typhimurium*, at least a 10-fold derepression of the total DAHP synthetase activity occurs when cells are transferred from medium containing excess phenylalanine and tyrosine to one in which these amino acids are present in very low (1 $\mu\text{g/ml}$) concentration (76). However, whereas DAHP synthetase (tyr) is derepressed about 10-fold in a "leaky" multiple aromatic auxotroph derived from *S. typhimurium* strain LT2, DAHP synthetase (phe) is not derepressed (189). In contrast, in a mutant strain of LT2 resistant to β -2-thienylalanine a 12-fold derepression of DAHP synthetase (phe) was observed (176). Other studies involving different strains of *S. typhimurium* indicate that when cells are grown in minimal medium, DAHP synthetase (tyr) is the predominant isoenzyme, but both DAHP synthetase (tyr) and DAHP synthetase (phe) can be derepressed about 10-fold by making suitable changes in the growth conditions (93).

In *Saccharomyces cerevisiae*, it has been reported that the formation of neither of the two DAHP synthetases is repressed by phenylalanine, tyrosine, or tryptophan (47, 105).

In *N. crassa*, there appear to be three distinct DAHP synthetase isoenzymes. Although their formation is not repressed by either tyrosine,

phenylalanine, or tryptophan to levels lower than those found in wild-type strains growing in minimal medium, derepression can be demonstrated by the use of auxotrophic strains, thus demonstrating that some form of specific control does exist (48).

In *B. subtilis*, the formation of the single DAHP synthetase enzyme is repressed by the aromatic amino acids (125), but no detailed studies of this repression have yet been reported.

Inhibition of Other Enzymes of the Common Pathway

Studies of the control of a number of biosynthetic pathways have shown that when feedback inhibition occurs, it almost always affects the enzyme carrying out the first reaction in a particular biosynthetic sequence. Since the net result of feedback inhibition is to stop the wasteful flow of intermediates along a pathway, it is not surprising that the enzyme of the first reaction normally functions as the major control point.

There is currently only one reported case of feedback inhibition of an enzyme of the common pathway other than DAHP synthetase, and it is interesting to note that in this case the affected enzyme is found in close association with DAHP synthetase. In *B. subtilis*, three enzymes, DAHP synthetase, chorismate mutase, and shikimate kinase, form a protein aggregate (125). The activity of both DAHP synthetase and shikimate kinase is feedback-inhibited by both chorismate and prephenate.

Repression of Other Enzymes of the Common Pathway

Relatively little work has been carried out on the repressibility of the enzymes of the common pathway other than DAHP synthetase. Studies involving the growth of an aromatic auxotroph of *E. coli* K-12 in a chemostat under various conditions failed to show any significant repression or derepression of either dehydroquinase synthetase or dehydroquinase (K. D. Brown, 1968. *Genetics*, in press). Fewster (60) failed to find any variation in the level of shikimate kinase activity in many strains of *E. coli* when grown in the presence or absence of the aromatic amino acids. Similarly, in *S. typhimurium* it has recently been reported that the addition of excess aromatic amino acids to wild-type cells growing in minimal medium failed to repress any of the enzymes involved in converting DAHP to chorismate (76).

In contrast to these results, it has recently been shown (J. Pittard et al., in preparation) that under certain conditions the shikimate kinase activity of *E. coli* K-12 can be considerably

derepressed. When a wild-type strain is grown in the presence of the aromatic amino acids, only a twofold repression of this activity occurs, in comparison with extracts from cells grown in minimal medium. When, however, strains possessing only DAHP synthetase (*trp*), which are either *trpR*⁺ or *trpR*⁻, are grown in minimal medium, the shikimate kinase activity is derepressed seven- to eightfold by comparison with fully repressed wild-type values. A similar result is obtained when a strain which possesses the *tyrR*⁻ mutation and has only DAHP synthetase (*tyr*) is grown in minimal medium. The addition of the aromatic amino acids to the minimal medium represses the formation of kinase activity in every case, although in strains containing either the *trpR*⁻ or the *tyrR*⁻ mutations, the fully repressed values are approximately double those obtained in the corresponding *trpR*⁺ and *tyrR*⁺ strains. These results are summarized in Table 4. It can also be seen from Table 4 that when either shikimic acid, tyrosine, or tryptophan limits the growth of an aromatic auxotroph, in contrast to when these aromatic amino acids are present in excess, a three- to fourfold derepression of the kinase activity occurs. When, however, phenylalanine limits growth, there is no derepression of kinase activity. Although these results do not indicate any simple system of control, they do clearly demonstrate that the levels of this particular enzymic activity can be subject to considerable variations. Before these studies can be interpreted in terms of any specific model, it is necessary to establish whether the activity that is measured in crude cell-free extracts represents one or more than one shikimate kinase enzyme. It has recently been demonstrated that in *S. typhimurium* there are two shikimate kinase enzymes which can be separated from each other by chromatography on DEAE-cellulose (120).

REGULATION OF THE TRYPTOPHAN PATHWAY

Feedback Inhibition

In *E. coli* (89), anthranilate synthetase and PR transferase have been shown to form an enzyme aggregate such that, although the PR transferase activity can function by itself, only the aggregate has appreciable anthranilate synthetase activity.

Both anthranilate synthetase and PR transferase activity are inhibited by tryptophan. Feedback-resistant mutants have been obtained by the use of tryptophan analogues such as 5-methyl tryptophan. In a number of cases, these strains are found to be both feedback-resistant and to produce the enzymes of the tryptophan pathway constitutively (121, 148; C. Cordaro and E. Balbinder, *Bacteriol. Proc.*, p. 51, 1967).

TABLE 4. A comparison of the specific activities of shikimate kinase in different strains of *E. coli* K-12 grown under different conditions^a

Phenotype	Specific activities in extracts prepared from cells grown in	
	Minimal medium	Minimal medium plus phenylalanine, tyrosine, and tryptophan (each, 10 ⁻³ M)
Wild type	3.4	1.6
Multiple aromatic auxotroph	— ^b	1.2 ^c
Prototroph possessing only DAHP synthetase (<i>trp</i>)	12.0	2.5
As above, but possessing the <i>trpR</i> ⁻ mutation	12.1	3.9
Prototroph possessing only DAHP synthetase (<i>tyr</i>)	4.9	1.7 ^c
As above, but possessing the <i>tyrR</i> ⁻ mutation	10.7	3.0

^a Specific activity = 0.1 μmole of substrate utilized per 20 min per mg of protein at 37 C.

^b When the aromatic auxotroph was grown in limiting shikimate, limiting tryptophan, limiting tyrosine, and limiting phenylalanine, respectively, specific activities were 3.5, 4.1, 5.4, and 0.5.

^c 4-Aminobenzoic acid and 4-hydroxybenzoic acid (10⁻⁶ M) were also added to the medium.

These examples occur in *E. coli* K-12, *E. coli* W, and *S. typhimurium*. Only in the *E. coli* K-12 mutants, however, has it been established that both changes were the result of a single mutation (148).

In *Chromobacterium violaceum* (J. Wegman and I. P. Crawford, *Bacteriol. Proc.*, p. 115, 1967), *P. putida* (33), *B. subtilis* (124), *Saccharomyces cerevisiae* (50, 105), and *N. crassa* (42), tryptophan acts as a feedback inhibitor of anthranilate synthetase, although in *C. paspali* (106), tryptophan does not inhibit the activity of anthranilate synthetase.

Repression

In *E. coli*, the structural genes for the enzymes of the tryptophan pathway are organized into the now well-characterized tryptophan operon. Studies of various operator-constitutive mutants have shown that the expression of all these genes is controlled by a single operator locus (112, 113, 148). Furthermore, it has been shown (87) that all the enzymes of the tryptophan pathway are repressed or derepressed in a coordinate fashion. The regulation of these enzymes is controlled by

the *trpR* gene, which was originally described by Cohen and Jacob (23).

In the biosynthesis of at least two other amino acids, histidine and valine (58, 122, 142), it has been demonstrated that histidyl-transfer ribonucleic acid (tRNA)_(his) and valyl-tRNA_(val) are the active corepressors and not the amino acids themselves. Consequently, mutations affecting tRNA molecules or amino acid activating enzymes can also cause derepression of enzymes in a biosynthetic pathway. Furthermore, in diploids these mutations would be expected to be recessive in the same way in which a mutation which caused the formation of a nonfunctional aporepressor is expected to be recessive. Hirst and DeMoss (Bacteriol. Proc., p. 114, 1967) have studied the relationship between the size of the free tryptophan pool and the repression of tryptophan synthetase in *E. coli*. They find that changes in this pool do not affect repression of tryptophan synthetase, and they conclude that either there is more than a single intracellular pool of tryptophan or that tryptophan itself is not the corepressor.

A class of mutant strains which require tryptophan for growth has recently been described (43, 82, 111) in which the mutations causing tryptophan dependence map in the *trpS* gene, which is located between the *aroB* and the *pabA* genes and far away from the tryptophan operon. In spite of the inability of these strains to grow without added tryptophan, all of the enzymes of the tryptophan operon can be detected in their cell-free extracts. The levels of these enzymes in extracts prepared from derepressed *trpS*⁻ cells are, however, only one-third or less of the levels obtained in extracts from derepressed *trpS*⁺ cells (82). The level of DAHP synthetase (*trp*) is similarly lowered in *trpS*⁻ strains (J Pittard and J. Camakaris, *in preparation*). Because of these observations, it seemed possible that the *trpS*⁻ strains may be producing a super-repressor analogous to the *i*^s mutants of the *lac* operon (82, 111). Doolittle and Yanofsky (43), however, have recently demonstrated that the *trpS* gene codes for the tryptophanyl-tRNA synthetase enzyme, and that *trpS*⁻ mutants have a greatly reduced ability to charge tryptophan-specific tRNA. By contrast with the histidyl-tRNA synthetase mutants, in which the poor charging of histidyl-tRNA causes derepression of the histidine enzymes, the enzymes of the tryptophan pathway are not derepressed in the tryptophanyl-tRNA synthetase mutants. Therefore, it has been suggested that tryptophan, and not tryptophanyl-tRNA, is the active corepressor for the tryptophan operon (43). The low values which have been observed for enzymes of the

tryptophan operon and for DAHP synthetase (*trp*) in *trpS*⁻ strains is probably, therefore, a direct consequence of an internal accumulation of tryptophan by these strains.

In *S. typhimurium*, a single tryptophan operon exists, although it has been suggested that in this organism the tryptophan operon contains two separate promoter genes instead of one as in *E. coli* (10). Mutant strains resistant to 5-methyl tryptophan and derepressed for enzymes of the tryptophan pathway have been isolated, but the mutations have not yet been mapped (176). Preliminary studies in both *Saccharomyces cerevisiae* and *N. crassa* (50) indicate that repression plays an important role in these organisms. No repression of the tryptophan enzymes has been found in *C. paspali* (106), and it has been reported that tryptophan synthetase formation is specifically induced in *P. putida* by indoleglycerol-phosphate (33).

REGULATION OF THE TYROSINE PATHWAY

Feedback Inhibition

In *A. aerogenes* and *E. coli*, the first two reactions of the tyrosine pathway are carried out by a single enzyme (24). Tyrosine is a feedback inhibitor of the second of these activities, prephenate dehydrogenase (90% inhibition at 10⁻³ M), but it does not affect the first, chorismate mutase T (24; B. J. Wallace, *unpublished data*). In strains of *B. subtilis*, there are one or more chorismate mutase enzymes and a separate prephenate dehydrogenase enzyme. Chorismate mutase is not inhibited by tyrosine, but this amino acid does inhibit the prephenate dehydrogenase enzyme [90% at 10⁻³ M (124)].

In *S. cerevisiae*, prephenate dehydrogenase activity is activated by phenylalanine (105). In *C. paspali*, prephenate dehydrogenase is inhibited by tyrosine (106).

Repression

In *A. aerogenes* and *E. coli*, the formation of chorismate mutase T and its associated prephenate dehydrogenase activity are strongly repressed by tyrosine (24). In *E. coli*, tyrosine has also been shown to repress the formation of transaminase A, an enzyme which converts 4-hydroxyphenylpyruvate to tyrosine (144). Mutations in a gene (*tyrR*) which is located at some distance on the chromosome from *tyrA* (the structural gene for chorismate mutase T) and its associated prephenate dehydrogenase cause constitutive synthesis of chorismate mutase T and prephenate dehydrogenase, transaminase A, and DAHP synthetase [(*tyr*) B. J. Wallace and J. Pittard, *in preparation*].

AroF, the structural gene for DAHP synthetase (*tyr*), and *tyrA* are closely linked in the *E. coli* chromosome and may be part of an operon. The isolation of a mutant strain which has lost DAHP synthetase (*tyr*) activity and which produces greatly reduced levels of chorismate mutase T and prephenate dehydrogenase (B. J. Wallace, unpublished data) lends support to this possibility. No operator constitutive mutants have yet been found. In *S. cerevisiae* (105), tyrosine does not repress prephenate dehydrogenase or chorismate mutase. In this strain, however, prephenate dehydrogenase is induced by phenylalanine. The genetic units involved in this process of induction have not yet been studied.

REGULATION OF THE PHENYLALANINE PATHWAY

Feedback Inhibition

In *A. aerogenes* and *E. coli*, phenylalanine feedback inhibits prephenate dehydratase activity (90 to 100% inhibition at 10^{-3} M). Although phenylalanine has no effect on the associated chorismate mutase P in *E. coli* (J. Pittard, unpublished data), it causes 65% inhibition of the chorismate mutase P of *A. aerogenes* (24). In *B. subtilis*, phenylalanine inhibits prephenate dehydratase (124). Mutant strains of *B. subtilis* have been isolated that are resistant to β -2-thienylalanine. In some of these mutants, phenylalanine activates prephenate dehydratase instead of inhibiting it (22). In *N. crassa* and *C. paspali*, there appears to be a single chorismate mutase enzyme. The activity of this enzyme is inhibited by phenylalanine and by tyrosine, but the inhibition is reversed and the enzyme is activated by L-tryptophan (7, 106). In *C. paspali*, phenylalanine also inhibits prephenate dehydratase activity (106).

Repression

In *A. aerogenes* and *E. coli*, phenylalanine represses the formation of chorismate mutase P and its associated prephenate dehydratase. The variation in activity between maximally repressed and derepressed levels is much less in the case of chorismate mutase P than in that of chorismate mutase T (24; B. J. Wallace and J. Pittard, unpublished data), and a transaminase enzyme which is involved in the formation of phenylalanine is found not to be repressed by phenylalanine (144). DAHP synthetase (*phe*) shows similar small variations in activity between maximally repressed and derepressed conditions. No regulator genes associated with the control of this pathway have yet been identified.

REGULATION OF THE PATHWAYS OF VITAMIN BIOSYNTHESIS

Although there is no doubt that the relative amounts of any aromatic vitamin formed by bacterial cells can vary as a result of mutations or changes in growth conditions, little information is available concerning the mechanisms that normally control their synthesis. In part, this lack of information is due to the fact that the details of the pathways leading to the biosynthesis of the aromatic vitamins are still being worked out.

Mutant strains of *Staphylococcus aureus* which overproduce and excrete 4-aminobenzoic acid have been reported (168). The formation of 2,3-dihydroxybenzoate and related compounds has been shown to be markedly influenced by the medium in which the cells are grown. Thus the amount of 2,3-dihydroxybenzoate and 2,3-dihydroxybenzoylglycine produced in cultures of *B. subtilis* is inversely proportional to the iron content of the growth medium (88). The formation of enzymes concerned in the biosynthesis of 2,3-dihydroxybenzoate by *A. aerogenes* (174) and 2,3-dihydroxybenzoylserine by *E. coli* (14) is repressed by the presence of iron or cobalt in the growth medium.

The presence of the aromatic amino acids also inhibits the production of 2,3-dihydroxybenzoate and 3,4-dihydroxybenzoate by washed cell suspensions of *A. aerogenes* (133). These effects in *A. aerogenes* can be explained by feedback inhibition of the DAHP synthetase system (A. F. Egan, unpublished data).

Vitamin K and ubiquinone levels are affected by conditions of aeration (135), and in mutants which are unable to form one of the quinones, there is a several-fold increase in the level of the remaining quinone (31).

There is no indication that the aromatic vitamins play any effective role in the control of the common pathway. On the other hand, since a strain of *E. coli* K-12 which has mutations in the structural genes for DAHP synthetase (*tyr*), DAHP synthetase (*phe*), and DAHP synthetase (*trp*) possesses no detectable DAHP synthetase activity, there does not appear to be a fourth DAHP synthetase isoenzyme which is concerned with vitamin biosynthesis. The results of these in vitro tests are confirmed by the observation that this same strain grows slowly with a mean generation time of 280 min in a medium containing phenylalanine, tyrosine, and tryptophan. When, however, either shikimic acid (10^{-6} M) or 4-aminobenzoic acid, 4-hydroxybenzoic acid, and 2,3-dihydroxybenzoic acid (each at 10^{-6} M) are also added to the medium, the growth rate is

returned to normal with a mean generation time of 80 min (B. J. Wallace and J. Pittard, *in preparation*).

There are indications that not all of the DAHP synthetase isoenzymes play an equal role in vitamin biosynthesis. When a mutant strain of

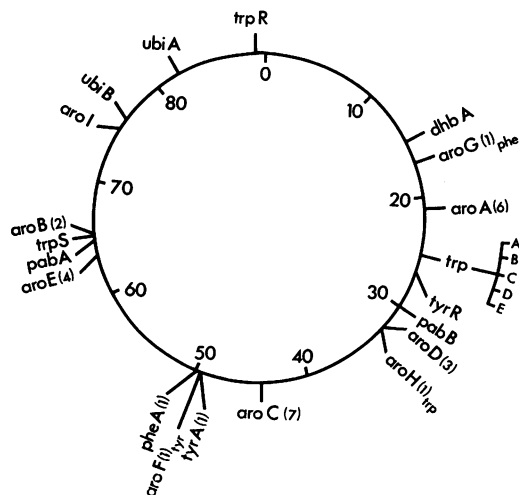


FIG. 15. A map of the *E. coli* chromosome showing the relative positions of genes concerned with aromatic biosynthesis. The chromosomal locations of the genes *ubiA*, *ubiB*, *aroI*, and *tyrR* are based solely on the data of interrupted mating experiments. The exact order of the genes *pheA*, *aroF*, *tyrA* and *aroH*, *aroD* has not been determined. Genes coding for enzymes of the common pathway have the prefix *aro*. Genes coding for enzymes of the tryptophan, phenylalanine, or tyrosine pathways have the prefixes *trp*, *phe*, and *tyr*, respectively. Genes concerned with the biosynthesis of ubiquinone have the prefix *ubi*. Genes concerned with the biosynthesis of *p*-aminobenzoic acid have the prefix *pab*, and those concerned with the biosynthesis of 2,3-dihydroxybenzoic acid, *dhb*. Two genes concerned with regulation which affect both the common pathway and one of the terminal pathways have been given the prefix relevant to the terminal pathway, e.g., *trpR*, *tyrR*. The uppercase letters given to the genes have no significance with regard to the relative positions of the enzymes in the biosynthetic sequences. For example, *aroA* does not code for the first enzyme of the common pathway. The numbers in parenthesis describe the particular reaction with which the gene is concerned. For example, *tyrA* (1) codes for the first reaction in the terminal pathway of tyrosine biosynthesis and *aroF* (1)_{tyr} codes for one of the three isoenzymes involved in the first reaction of the common pathway. The subscript *tyr* denotes that it codes for DAHP synthetase (*tyr*); *aroG* (1)_{phe} codes for DAHP synthetase (*phe*) and *aroH* (1)_{trp} codes for DAHP synthetase (*trp*). The function of *aroI* is yet to be determined. The gene *trpS* is the structural gene for tryptophanyl-tRNA synthetase. The formal representation of the chromosome is in accordance with the recommendations of Taylor and Trotter (159).

E. coli containing only DAHP synthetase (*tyr*) is inoculated into minimal medium supplemented with phenylalanine, tyrosine, and tryptophan, in the presence and absence of 10^{-6} M shikimate, its growth rate in the absence of shikimate is very slow (mean generation time of 9 hr). In the presence of 10^{-6} M shikimate, however, it grows with a mean generation time of 120 min. In a strain containing only DAHP synthetase (*trp*), the addition of shikimate to medium containing phenylalanine, tyrosine, and tryptophan causes only a slight stimulation of growth rate, whereas in the strain containing DAHP synthetase (*phe*), this addition makes no difference to the growth rate. In medium not containing shikimate but containing phenylalanine, tyrosine, and tryptophan, the mean generation times of strains containing only DAHP synthetase (*trp*) and DAHP synthetase (*phe*) are 114 and 130 min, respectively (B. J. Wallace and J. Pittard, *in preparation*). It would appear, therefore, that either DAHP synthetase (*phe*) or DAHP synthetase (*trp*) can provide enough DAHP synthetase activity for vitamin synthesis even in the presence of phenylalanine, tyrosine, and tryptophan.

A mutant strain of *N. crassa* lacking DAHP synthetase (*tyr*), however, exhibits a growth requirement for 4-aminobenzoic acid in the presence of phenylalanine, tyrosine, and tryptophan. Since wild-type *N. crassa* does not exhibit this requirement in the presence of these amino acids, it would appear that in this organism it is probably DAHP synthetase (*tyr*) which provides enough DAHP synthetase activity for vitamin synthesis under repressed conditions (48). Since the enzymes of the common pathway other than the DAHP synthetases do not appear to be repressed to such levels as would interfere with vitamin biosynthesis, no special controls are required to ensure the supply of chorismate for vitamin syntheses. However, systems of control of each of the terminal pathways to the vitamins must exist to ensure the production of less of the vitamins than of the aromatic amino acids.

CHROMOSOMAL DISTRIBUTION OF GENES CONCERNED WITH AROMATIC BIOSYNTHESIS

In *E. coli* K-12, genetic analyses of mutant strains that have been carried out over the last few years permit many of the genes concerned with aromatic biosynthesis to be given specific map locations (23, 31, 43, 82, 83, 111, 134, 158, 163, 173; R. Luke, *unpublished data*). The position of these genes on the *E. coli* chromosome is shown in Fig. 15.

Although the function of the *aroI* gene has not yet been determined, a mutant strain has been isolated which requires phenylalanine, tyrosine, and tryptophan for growth at 42 C. A genetic analysis of this strain shows that the mutation maps in the position designated by the *aroI* gene (J. Pittard and E. M. Walker, unpublished data). The gene product of the *aroI* gene has not yet been identified.

The normal functions of the regulator genes, *trpR* and *tyrR*, have not yet been determined, and structural genes for shikimate kinase(s) and one or more transaminases have yet to be identified.

For the genes coding for the tryptophan pathway, organization into a single operon is well established (87, 112, 113). As can be seen from Fig. 15, the only other genes which, as a result of position and function, show a possibility of such an arrangement are the *aroF* and *tyrA* genes and possibly the *arcH* and *aroD* genes. In neither pair, however, has contiguity been established, and in the latter pair it can already be calculated that under conditions in which *aroH* is repressed *aroD* is not affected.

On the other hand, it is possible that *aroF* and *tyrA* may constitute an operon. In general, however, the gene distribution is such that any system of control affecting several genes will probably resemble that already described for arginine biosynthesis (110) for which the term regulon has been proposed.

Genetic studies of *S. typhimurium* have so far concentrated on genes coding for enzymes of the common pathway and of the terminal pathways of phenylalanine, tyrosine, and tryptophan biosynthesis. The general distribution of these genes resembles that found for *E. coli* (76, 126, 141; E. Gollub et al., Federation Proc., p. 337, 1956), although minor discrepancies between the two maps still exist (134). Although mutants that are derepressed for enzymes of the tryptophan pathway have been isolated in *Salmonella*, the mutations causing this change have not yet been studied (176).

In *B. subtilis*, all the genes concerned with the tryptophan pathway are closely linked to each other on the chromosome (2, 20, 123, 125). Moreover, genes for dehydroquinase synthetase, prephenate dehydrogenase, 3-enolpyruvyl-shikimate 5-phosphate synthetase, and one form of the chorismate mutase enzyme are located on the same transforming molecule of deoxyribonucleic acid (DNA) as the tryptophan cluster. There is, however, no evidence at the moment to suggest that these genes form an operon like structure. A gene for DAHP synthetase, *aroA*, and one for a second chorismate mutase, *aroG*, are closely

linked on another molecule of transforming DNA (123). In the latter case, there is also a close association of the two gene products, DAHP synthetase and chorismate mutase, in an enzyme aggregate. Genes for dehydroshikimate reductase, dehydroquinase, chorismate synthetase, and prephenate dehydratase have also been mapped but are not linked to each other or to the tryptophan cluster.

In *N. crassa*, the structural genes for enzymes of the tryptophan pathway are unlinked to each other (1), although the gene products of two of these genes combine to form a molecular aggregate (42). Genes coding for enzymes for each of the reactions of the common pathway, with the exception of the third step for which there are two dehydroquinase enzymes (74), have all been mapped. Genes coding for dehydroshikimate reductase, shikimate kinase, dehydroquinase synthetase, and 3-enolpyruvyl-shikimate 5-phosphate synthetase have been shown to be closely linked on linkage group II (74, 79). A study of a class of polarity mutants suggests that these four genes and a gene for dehydroquinase activity form a single unit of transcription (74). It was originally postulated that this gene cluster may in fact constitute an operon, but neither operator nor regulator mutants have been found to substantiate this proposal (74). Mutant strains lacking each of the three DAHP synthetase isoenzymes have also been isolated and the mutations have been mapped. The gene for DAHP synthetase (*tyr*) is located on linkage group VI, the gene for DAHP synthetase (*phe*) on linkage group I, and the gene for DAHP synthetase (*trp*) either on linkage group I or II (48).

CONCLUSION

In recent years, knowledge of the pathways of biosynthesis of the aromatic amino acids has been extended, and it is probable that most, if not all, of the intermediates concerned are now known. The emphasis has now changed from the determination of the pathways themselves to the study of the biochemical genetics related to the pathways and to a detailed examination of certain key reactions. The branched pathways leading to the aromatic amino acids have provided a valuable system for experiments on enzyme repression and feedback inhibition. The isolation of mutant strains possessing only a single DAHP synthetase, DAHP synthetase (*tyr*), DAHP synthetase (*phe*), or DAHP synthetase (*trp*) has greatly simplified the study of the role of these enzymes in the regulation of metabolism. Only some of the genes concerned with the regulation of aromatic biosynthesis have so far been identified, and the

normal function of these genes has yet to be determined. Even after all the genes and their products have been identified, the understanding of the interplay between these various systems in wild-type cells *in vivo* will require a great deal more work involving new and sensitive experimental approaches.

In all of the organisms in which the regulation of aromatic biosynthesis has been studied, the rate of synthesis of chorismic acid (the end product of the common pathway) appears to be affected primarily by control of the first reaction of the common pathway. At least three different systems have evolved to allow a balanced control of the common pathway by the major end products, tyrosine, phenylalanine, and tryptophan. (i) In the gram-negative enteric microorganisms, and in *N. crassa*, *S. cerevisiae*, and *C. paspali*, the first reaction of the common pathway is carried out by isoenzymes (usually three). Each of these DAHP synthetases appears to be end product inhibited and repressed, primarily by one of the aromatic amino acids, although the effects of the amino acids are not completely specific. (ii) In many strains of *Bacillus*, *Staphylococcus*, *Flavobacterium*, *Achromobacter*, and *Alcaligenes*, a single enzyme carries out the first reaction of the common pathway. This DAHP synthetase activity is sensitive to inhibition by chorismate or prephenate. Control of the common pathway in these organisms is apparently exerted by "sequential feedback inhibition," in which the amino acids inhibit reactions on their particular pathways causing the accumulation of chorismate or prephenate, or both; these, in turn, inhibit DAHP synthetase activity. (iii) *Hydrogenomonas* sp. appear to possess a single DAHP synthetase enzyme which is subject to "cumulative end product inhibition" by phenylalanine and tyrosine.

The control of the terminal pathways differs in detail from organism to organism, but it may involve feedback inhibition or repression, or both. Feedback inhibition is exerted on anthranilate synthetase (by tryptophan), prephenate dehydrogenase (by tyrosine), and prephenate dehydratase (by phenylalanine). Chorismate mutase is inhibited by phenylalanine or tyrosine, or by both, in some organisms, and in *N. crassa* and *C. paspali* it is stimulated by tryptophan which also reverses inhibition by the end products.

At present, there is no evidence, apart from the repression (by iron) of the enzymes concerned in 2,3-dihydroxybenzoate synthesis to suggest that the biosynthesis of the aromatic vitamins is controlled by repression or end

product inhibition. More work will have to be done on the regulation of formation of the aromatic vitamins before any generalization can be made.

The pathways of biosynthesis of the aromatic vitamins are being clarified, and the isolation of mutants unable to form ubiquinone, vitamin K, or the 2,3-dihydroxybenzoate group of compounds will assist in studying the biosynthetic pathways leading to, and the function of, these compounds.

The comparative biochemistry of the pathways and control mechanisms will no doubt continue to be studied actively. Experiments with bacteria have thus far played a major role in the elucidation of the pathways, but work with other microorganisms and higher plants has indicated that, although the intermediates are likely to be the same, there may be differences in details of organization and control of the pathways within different cells.

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