The Shikimate Pathway — A Metabolic Tree with Many Branches

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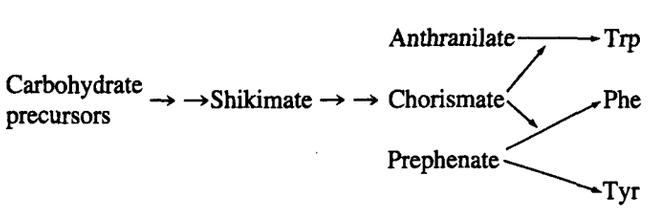
Dedication

This review is dedicated to the memory of Marian L. Bentley (née Blanchard). As was always the case, she provided me with much encouragement to undertake the task. Unhappily, a terminal cancer did not allow her to rejoice in its conclusion.

"And time remember'd is grief forgotten"
(Chorus from "Atalanta" by Algernon Charles Swinburne)

I. INTRODUCTION

The aromatic amino acids, phenylalanine, tyrosine, and tryptophan, are formed by a reaction sequence leading from the two carbohydrate precursors, D-erythrose 4-phosphate and phospho*enol*pyruvate, via shikimate, to further pre-aromatic and aromatic compounds. A branching of this "shikimate pathway" occurs after the formation of chorismate; indeed, the name of this compound (suggested by an ecclesiastical authority) is derived from the Greek, meaning fork. One branch leads to anthranilate and hence to tryptophan. The other branch leads to prephenate, which by a further branching, forms phenylalanine and tyrosine. Thus, the "classical" shikimate pathway may be represented as follows:



Ever since the recognition of chorismate as an intermediate in the pathway, there has been a tendency to regard it as the only branch point intermediate in the biosynthesis of aromatic compounds. Thus, an authoritative text (1988) states that "The branchpoint compound for all these diverse products (that is, the aromatic amino acids, vitamins E and K, folic acid, ubiquinone, and plastoquinone and certain metal chelators, such as enterochelin) is *chorismate* . . .".[1] Since two of the mentioned compounds (vitamin K and enterochelin) actually branch from the pathway at isochorismate, this statement is misleading; moreover, it neglects the important pathway from tyrosine to ubiquinone in animals.

It is now obvious that the shikimate pathway contains not one but multiple branch points. As explicated herein, there are more initial branches from isochorismate than from chorismate, and almost certainly all of the pathway intermediates function as branch points. In addition, an alternate branch route to phenylalanine and tyrosine via arogenate has been discovered. Yet more branching takes place when the "end-product" aromatic amino acids act as precursors to countless further metabolites. In fact, the biosynthesis of many aromatic components in various organisms is accomplished not only by a very complex assembly of alternate routes (i.e., branches), but by organization of enzyme activities as mono- or polyfunctional polypeptides, by utilization of specific cofactors in dehydrogenase reactions, and by sophisticated regulatory mechanisms, including compartmentalization.

In 1979, Floss suggested that in view of the key role of chorismate (as then perceived), the pathway would more appropriately be named the "chorismate pathway".[2] Also, due to the subsequent discovery of the major role of isochorismate and of other branch points, adoption of this name would have been counterproductive. Another possible name, "the aromatic pathway", is inappropriate since many aromatic compounds originate in polyketide and mevalonate pathways; a further complication for this name is formation of some (nonaromatic) cyclohexane compounds by the pathway. For want of a better name, it seems best to continue to refer to "the shikimate pathway". The emphasis on shikimate is in harmony with the historical fact that it was first isolated more than a century ago from *Illicium religiosum*. Although the term "secondary metabolite" had not then been coined,[3] shikimate was for many years treated as a typical secondary metabolite lacking any biological function. When its important role was recognized, shikimate became one of the very few compounds to make the change from obscure secondary metabolite to vitally important primary metabolite.

Since humans (and many other species) lack the capacity for aromatic biosynthesis, other than the aromatization of the steroid ring A, and since the operation of the shikimate pathway in plants and microorganisms produces nutritionally essential amino acids, vitamins, and cofactors, this pathway assumes

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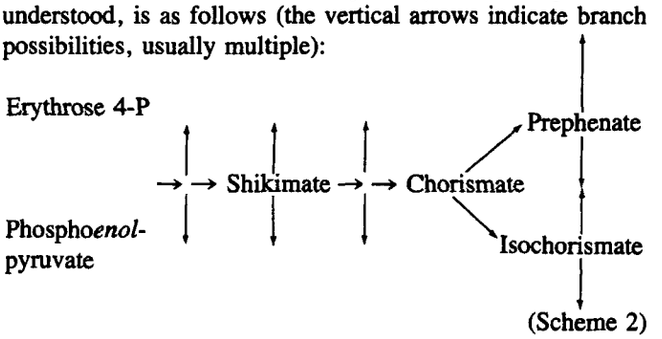
prime importance. Despite this significance for human nutrition the shikimate pathway usually receives short shrift in texts of biochemistry, microbiology, and molecular biology.

Furthermore, there have been relatively few biochemically oriented reviews of the overall shikimate pathway in recent years. Haslam's classic text, *The Shikimate Pathway*, first published in 1974, remains a most useful source book.[4] Another book, *The Biosynthesis of Aromatic Compounds*, was published in 1980, but covers the literature only to about 1973.[5] It includes much of the same material as Haslam's text, and also describes the other pathways for aromatic biosynthesis. Papers presented at a 1985 symposium of The Phytochemical Society of North America have been published,[6] and a particularly interesting chapter by Weiss recalls early work on the shikimate pathway. A chemically oriented journal, *Natural Product Reports*, reviews the biosynthesis of shikimate metabolites; the most recent review covers material published to the end of 1987.[7]

In light of the multiple branches from isochorismate, the components of the basic "trunk" of the pathway tree is expanded here to include this compound. Thus, an abbreviated form of the common shikimate pathway trunk, as presently understood, is as follows (the vertical arrows indicate branch possibilities, usually multiple):



(Scheme 2)

The trunk of the tree is firmly rooted in carbohydrate catabolism, and there are two main branches, one to prephenate and the other to isochorismate (see also Figure 1). Equal significance is given to isochorismate and prephenate since, on the one hand, prephenate gives rise to two essential amino acids, and on the other, isochorismate gives rise to essential factors such as vitamin K and many important iron-chelating "siderophores".

Where possible, this review gives a detailed description of the enzymes and reaction mechanisms involved in the shikimate pathway trunk, and emphasizes papers published after 1980. A further section reviews the branch products derived from the trunk components. Whereas for the main trunk metabolites the current emphasis is on gene structure, nucleotide and protein sequences, overexpression and purification of enzymes, and reaction mechanisms, the branch reactions are poorly characterized except in a few cases (e.g., the aromatic amino acids). While products derived from the "classical end products" (phenylalanine, tyrosine, tryptophan) are logically regarded as shikimate metabolites, a description of all of these materials would make this review excessively long. For instance, it is estimated that there are now about 1000 naturally occurring coumarins, which derive, at least in part, from the shikimate pathway. Moreover, the various systems for transporting aromatic amino acids into cells of microorganisms are not considered; this subject has been reviewed.[9]

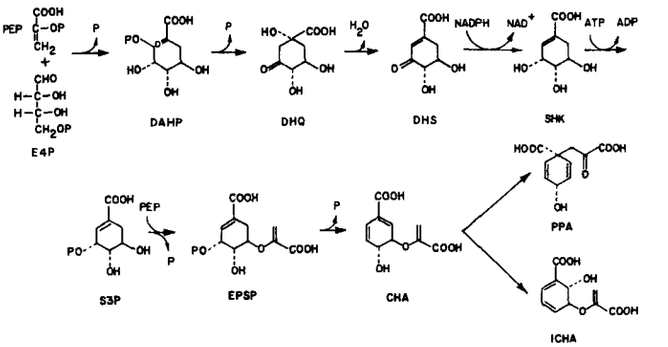


FIGURE 1. The main trunk of the SHK pathway. For convenience in visualizing relationships between carbon atoms, DAHP is drawn here as an open chain ketone, even though it reacts as a pyranose arrangement. The phrase, "the common shikimate trunk", was used earlier.[8]

"Chemical" numbering is used for shikimate and its derivatives, thus locating the cyclohexene double bond between carbon atoms 1 and 2. In the earlier biochemical literature, this bond was numbered as between carbons 1 and 6. Since chemical numbering is used by the Nomenclature Committee of the International Union of Biochemistry in naming the pathway enzymes, it is unfortunate that the older system is still used.[10] Amino acids will be referred to by the standard three-letter abbreviations; unless otherwise indicated, the L configuration is intended. The following abbreviations are used for the main trunk of the shikimate pathway: phospho*enol*pyruvate, PEP; D-erythrose 4-phosphate, E4P; 3-deoxy-D-*arabino*-heptulosonate 7-phosphate, DAHP; 3-dehydroquinate, DHQ; 3-dehydroshikimate, DHS; shikimate, SHK; shikimate 3-phosphate, S3P; 5-*enol*pyruvyl shikimate 3-phosphate, EPSP; chorismate CHA; isochorismate, ICHA; and prephenate, PPA. This usage is admittedly inconsistent in that shikimate is SHK, whereas in derivatives it is S; it avoids, however, the length of EPSHKP for 5-*enol*pyruvyl shikimate 3-phosphate. Other generally used abbreviations are arogenate, AGN; phenylpyruvate, PPY; 4-hydroxyphenylpyruvate, HPP; quinate, QA (again inconsistent since Q is used in DHQ); and PP, pyrophosphate. Other abbreviations used on a limited basis are defined in appropriate sections.

Since the distinction between synthetase and synthase has been abandoned, all such enzymes are referred to as synthases. The following common organisms are always referred to in abbreviated forms: *Aspergillus nidulans, A. nidulans; Bacillus subtilis, B. subtilis; Escherichia coli, E. coli; Klebsiella pneu-*

moniae, K. pneumoniae (in earlier literature, *Aerobacter aerogenes); Neurospora crassa, N. crassa; Pseudomonas aeruginosa, P. aeruginosa; Saccharomyces cerevisiae, S. cerevisiae;* and *Salmonella typhimurium, S. typhimurium.*

Several chemical syntheses of compounds of interest have been described recently. In addition to those in a comprehensive list for SHK and CHA,[11] these may be noted: methyl SHK;[12-14] methyl SHK with ^2H label at 6R or 6S;[15] methyl 4-epi-SHK;[16] methyl 5-epi-SHK;[17] ICHA;[18] methyl 6S-fluoro-SHK;[19] (Z)-9-methyl-CHA.[20] In the figures, stereochemistry at chiral centers is indicated by "dashed" bonds (below the paper plane) and a plain bond rather than a wedge (above the paper plane). Methyl groups are generally represented as a line without the addition of CH_3, and similarly, CH_2 is omitted in representation of *enol*pyruvoyl derivatives.

II. THE MAIN TRUNK OF THE SHIKIMATE PATHWAY

Although the formation of PPA and ICHA always involves the same chemical intermediates (see Figure 1), there is considerable variation from organism to organism in the organization of the participating enzymes. In enteric bacteria such as *E. coli,* the first seven reactions are catalyzed by separate enzymes which can be purified independently; the reaction, CHA → PPA, is catalyzed by a CHA mutase associated as a bifunctional enzyme with either PPA dehydratase (leading to phe) or PPA dehydrogenase (leading to tyr). However, *E. coli* mutants are known without the PPA activities. The conversion, CHA → ICHA, is catalyzed by a separate enzyme that is part of a polycistronic operon for enterobactin biosynthesis. *E. coli* also shows another commonly found feature, the occurrence of some enzymes in isozymic forms (e.g., DAHP synthase). Twelve genes (see Table 1), widely scattered on the *E. coli* chromosome, direct the synthesis of the *E. coli* enzymes.

A very different situation is found in some fungi *(A. nidulans, N. crassa),* in *S. cerevisiae* and other yeasts, and in *Euglena.* In these organisms, the second through sixth reactions are catalyzed by a pentafunctional polypeptide, encoded by a single structural gene. Thus, in *N. crassa,* the "arom complex" consists of two identical polypeptide chains with M_r of 165,000. In addition to the bifunctional CHA mutase-PPA dehydratase and CHA mutase-PPA dehydrogenase, bifunctional DAHP synthase-CHA mutase and DHQ dehydratase-SHK dehydrogenase enzymes are known in some organisms.

A particular consideration in plant biochemistry is whether any given enzyme is located in the chloroplast or in the cytosol. The SHK pathway for biosynthesis of aromatic amino acids is usually assigned to the chloroplast compartment. In a recent detailed study with young pea seedling shoots, density gradient analysis and high-performance anion-exchange chromatography established that DHQ dehydratase, SHK dehydrogenase, and EPSP synthase were predominantly chloroplastic. Analysis of lysed-chloroplast preparations showed that the first six enzymes of the SHK pathway were present. It was concluded that chloroplasts "are a major site for the biosynthesis of the common precursors of the aromatic amino acids".[21]

Nevertheless, it has become clear that several of the pathway enzymes can be detected as isozymic forms in the cytosol in many and perhaps all plants. The existence of cytosolic DAHP synthase (DAHP synthase-Co^{2+}) and cytosolic CHA mutase suggested that the intervening succession of enzymes was also present in the cytosol.[22] It is likely that an intact dual pathway exists in the cytosol, and is concerned with secondary metabolite biosynthesis but not with aromatic amino acid formation.[23] The evidence is strongest for DAHP synthase and CHA mutase, and two forms of anthranilate synthase are known. Details are given in connection with individual enzymes.

Many of the main trunk enzymes are present in low concentrations in organisms such as *E. coli* so that purification is difficult. However, recombinant DNA technology for gene cloning, (particularly in *E. coli*), has changed the situation dramatically. Several of the enzymes are now available readily in mg, and even 100 mg, amounts. Moreover, at least one (EPSP synthase from *E. coli*) has been crystallized. It is hoped that three-dimensional studies of these important proteins will soon be possible. Two enzymes for trp biosynthesis have also been crystallized and three-dimensional structures determined.

The organization followed here is to review the separable enzyme activities first, followed by a discussion of bifunctional complexes. A separate section considers the pentafunctional "arom" complexes. Volume 142 of *Methods in Enzymology*[24] contains much useful information concerning purification of several of the enzymes, assay methods, and substrate preparation. Not all enzymes of interest to readers of this present review are covered, and there have, of course, been further developments since the journal volume was published in 1987.

The regulation of the SHK pathway has been extensively studied. Since there are several reviews of this topic, [8,9,25,26] the regulatory mechanisms are discussed here only in broad, general terms. Some recent papers cover general regulatory aspects for these microorganisms: *Acholeplasma laidlawii,*[27] *Neisseria gonorrhoeae,*[28] *P. aeruginosa,*[29] *Anabaena variabilis,*[30] *Pichia guilliermondii,*[31] *Nocardia meditteranei,*[32] *Norcardia sp.* 239.[33] In plants, recent papers concern tobacco callus under shoot-forming conditions[34] and *Cinchona succirubra.*[35]

A. 3-Deoxy-D-*Arabino*-Heptulosonate 7-Phosphate Synthase

The recommended name of the enzyme, EC 4.1.2.15, which forms 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) from E4P and PEP, is phospho-2-dehydro-3-deoxyheptonate aldolase. Because of the lengthy chemical name it is referred to as DAHP synthase. The regulatory forms of the enzyme, found in different organisms, are described as follows:

Table 1
The Enzymes and Genes for Prephenate and Isochorismate Synthesis in *E. coli*

Reaction	EC no.	Enzyme name	Gene	Map position (min)
1	4.1.2.15	DAHP synthase		
		DAHP synthase-tyr	*aroF*	57
		DAHP synthase-phe	*aroG*	17
		DAHP synthase-trp	*aroH*	37
2	4.6.1.3	3-Dehydroquinate synthase	*aroB*	75
3	4.2.1.10	3-Dehydroquinate dehydratase	*aroD*	37
4	1.1.1.25	Shikimate dehydrogenase	*aroE*	72
5	2.7.1.71	Shikimate kinase	*aroL*	9
6	2.5.1.19	5-*Enol*pyruvylshikimate 3-phosphate synthase	*aroA*	20
7	4.6.1.4	Chorismate synthase	*aroC*	51
8	5.4.99.5	Chorismate mutase		
		Chorismate mutase-prephenate dehydratase	*pheA*	57
		Chorismate mutase-prephenate dehydrogenase	*tyrA*	57
9	5.4.99.6	Isochorismate synthase	*entC*	13

DAHP synthase-O	Allosterically insensitive
DAHP synthase-X	Inhibited by X
DAHP synthase-X(Y)	Inhibited by both X and Y, with X being the more powerful
DAHP synthase-M^{2+}	Stimulated by divalent metal, M^{2+}

Thus, DAHP synthase-CHA(Trp) refers to an enzyme subject to feedback inhibition by both CHA and trp, with CHA being the stronger inhibitor.

The reaction proceeds with C-O cleavage rather than P-O cleavage (see Figure 2), and H_Z of PEP becomes H_S at C-3 of DAHP.[36,37] As recently as 1988, this cleavage pattern was still described as surprising and not understood.[38] A reaction mechanism suggested by Ganem[39] postulates addition of an enzyme —SH group (E-SH) to PEP. A key step for which chemical analogies are available is a migration of the E-S- group, with a concomitant elimination of phosphate. The effect of this step is to provide a modified substrate with the phosphate group of PEP replaced by E-S- (Figure 2).

The Z-fluoro analog of PEP was an alternate substrate for *E. coli* DAHP synthase-Phe; the product was a 3-fluoro-DAHP, with assumed S configuration.[40] Phosphonate analogs of E4P (4-deoxy-D-*erythro*-tetrose 4-phosphonate and 4,5-dideoxy-D-*erythro*-pentose 5-phosphonate) were converted to the corresponding phosphonate or homophosphonate analogs of DAHP by *E. coli* DAHP synthase-Tyr.[41] The DAHP synthase of *E. coli* and other microorganisms was inhibited by the phosphonate compound, glyphosate, a widely used herbicide.[42,43] In yeasts, this inhibitory action was specific for DAHP synthase-

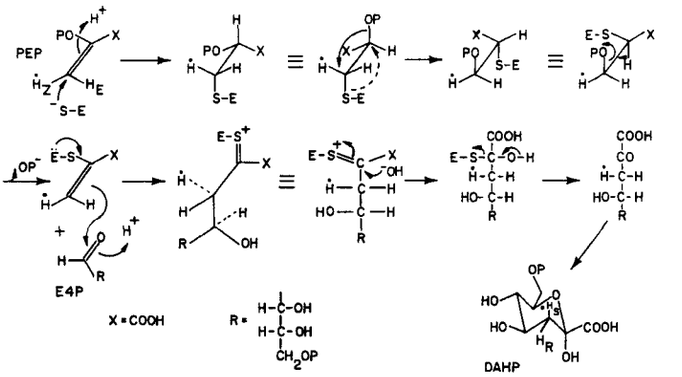


FIGURE 2. Possible reaction mechanism for DAHP synthase. X = —COOH. Conformational rearrangements are shown by the equivalence symbol, ≡. Note that the elimination of the phosphate ion provides for the observed C-O cleavage. E-S^- = sulfhydryl group on the enzyme. The final DAHP structure is shown in the usual $^4C_1$ conformation.

Tyr,[44] and in *E. coli* the glyphosate inhibition was overcome by Co^{2+}.[44] The action of this inhibitor on plant DAHP synthases is discussed later; for work on its major target, See Section II.F.7.

1. DAHP Isozymes

Before discussing the properties of specific DAHP synthases, it is helpful to consider the multiple forms of this enzyme. As information concerning the SHK pathway was obtained, it became apparent that as the first of the "main trunk" enzymes,

DAHP synthase, was a likely target for regulation of the metabolic flow. By 1968, it was known that a variety of types of feedback inhibition were present in various microorganisms.[45] Thus, *E. coli* and *S. cerevisiae* contained isozymic forms of this enzyme that were inhibited by the aromatic amino acids. On the other hand, in *B. subtilis* there was a single enzyme with feedback inhibition by CHA and PPA. Moreover, in *B. subtilis,* DAHP synthase and CHA mutase formed a multifunctional protein (Section II.K).

The work just briefly summarized is merely the tip of the iceberg. This enzyme is now known to possess the largest number of allosteric regulatory patterns thus far described for any one protein. These patterns have utility in taxonomy since they appear to delineate taxa at about the generic level,[8] and in some cases can further differentiate genera.[46,47] The observed inhibition patterns include the presence of isozymes and sequential, concerted, cumulative, and unimetabolite patterns of feedback inhibition.

2. DAHP Synthases of E. coli

The three DAHP synthase isozymes of *E. coli* are encoded by the genes *aroF* (DAHP synthase-Tyr), *aroG* (DAHP synthase-Phe), and *aroH* (DAHP synthase-Trp). More detail than can be provided here is available.[9] The activities and rates of synthesis of these isozymes are differentially affected by the aromatic amino acids; hence, the cell can change synthetic rates rapidly in response to the exogenous availability of the aromatic amino acids. ^{13}C NMR spectroscopy, used as a noninvasive probe to study whole cells of *E. coli* K 12 derivatives, indicated that feedback inhibition of the DAHP synthase isozymes is the major quantitative mechanism for controlling carbon flow in the SHK pathway.[48] *aroF* exists as an operon with a regulatory component, *tyrR*; this operon has been discussed in detail elsewhere.[9,49]

The properties of the three *E. coli* isozymes are summarized in Table 2. The DAHP synthase-Trp is only a minor component. Both of the two major isozymes are unstable in the absence of PEP, and both are Fe^{2+}-containing proteins. All of the isozymes have been purified to homogeneity and the gene DNA sequences have been determined (Table 2). The sequences show considerable areas of homology. The amino acid homologies are (indicated as percentages) *aroH, aroF*,48 *aroG, aroF*,53 and *aroH, aroG*,57. When all three are compared,[142] (41%) of the residues are identical. The high degree of sequence similarity for the three isozymes suggests a common evolutionary origin. The DAHP synthase-Phe has evolved most recently since it is absent from all other members of the Gram-negative cluster-containing enteric bacteria. All genera of the contemporary Enterobacteriaceae family possess this recently evolved DAHP synthase-Phe in addition to the two other isozymes.[58]

3. DAHP Synthases of Other Microorganisms

Some investigations of DAHP synthase from other microorganisms are summarized in Table 3. The following organisms not listed there show a pattern of three isozymes: *K. pneumoniae, Aeromonas hydrophila,* and *Alteromonas putrefaciens*.[58] In the mycoplasma, *Acholeplasma laidlawii,* a DAHP-Tyr was present, but the inhibition did not increase with increased concentration of tyr (43% inhibition at 0.1 and 0.5 m*M* tyr); the residual activity may have been due to the presence of DAHP synthase-O. The overall DAHP synthase activty was stimulated in a concentration-dependent manner by trp.[27] Two DAHP synthase isozymes (tyr and phe) present in *Anabaena variabilis* also showed the unusual feature of trp stimulation.[30] This feature was also observed with plant enzymes (see Section II.A.4).

DAHP synthase plays an important role in a classification of organisms depending on whether their metabolism is geared to the efficient utilization of materials available in the external environment, or whether their metabolism is more inwardly directed with end products (e.g., the aromatic amino acids) made entirely by the cell. Organisms such as the cyanobacteria gear the regulation of the SHK pathway to the endogenous

Table 2
The DAHP Synthase Isozymes of *E. coli*[a]

Enzyme	Strain	Gene	ORF bp	Amino acid residues	Subunit M_r calc.	Subunit M_r obs.	Native enzyme	Ref.
DAHP synthase-Phe[b]	K12, HE401	*aroG*	1077	350	37,997	35,000	Tetramer	50–52
DAHP synthase-Tyr[b]	K12	*aroF*	1068	356	38,804	39,000	Dimer	51, 53–55
DAHP synthase-Trp	Plasmids, pAROH924, and pAHH1	*aroH*	1041	347	—	39,000	Dimer	56, 57

[a] For all of the enzymes, the complete nucleotide sequences of the genes have been determined and homogeneous proteins have been obtained.
[b] Unstable in absence of PEP; known to contain Fe^{2+}.

Table 3
DAHP Synthases In Microorganisms

Organism[a]	Major isozyme	Minor isozyme(s)	Ref.
Bacteria			
Acinetobacter calcoaceticus	DS-Tyr (>95%), Tyr NC wrt E4P and PEP, M_r 43,000	DS-O, M_r 44,700	59
Anabena variabilis	DS-Tyr	DS-Phe	30
Anacystis nidulans (*Synechococcus* sp. strain PCC 6301)[b]	DS-Tyr		60
Bacillus polymyxa	DS inhibited to same extent by any single aromatic amino acid and each may occupy the same allosteric site; possibility of three isozymes apparently not considered		61
Bacillus subtilis	DS-PPA (CHA)		62
Lysobacter enzymogenes	DS-CHA (Trp)		63
Neisseria gonorrhoeae	DS-Phe		28
Oceanospirillum minutulum	DS-Tyr, Tyr NC wrt E4P, PEP	DS-O	63
Oceanospirillum sp[c]	DS-Tyr, Tyr NC wrt E4P, UC wrt PEP	DS-Trp, Trp NC wrt E4P, UC wrt PEP	63
Pseudomonads, group I[d]			
Pseudomonas aeruginosa	DS-Tyr (PPY)[e], Co^{2+} activates, no end product repression M_r 137,000	DS-Trp, Co^{2+} activates, M_r 175,000	64
Pseudomonads, group II			
Pseudomonas cepacia	DS-Tyr (Phe), may represent two isozymes		64
Pseudomonads, group III			
Pseudomonas acidovorans	DS-Phe (proportions of these isozymes not determined)	DS-Tyr	65
	Complex inhibition by DTT, M_r 67,000	Requires DTT for stabilization, stimulated by Co^{2+}, M_r 251,000	
Pseudomonads, group IV			
Pseudomonas diminuta	DS-Trp (CHA)		46, 47
Pseudomonads, group V			
Xanthomonas campestris	DS-CHA (Trp), no activation by divalent cations		66
Serpens flexibilis	DS-Tyr, Tyr NC wrt E4P, PEP	DS-Trp (CHA), Trp NC wrt E4P, UC wrt PEP	67
Yeasts, fungi			
Candida maltosa	DS-Phe (55%), inhibited by PPY and to lesser extent by AGN, PPA; no inhibition by glyphosate	DS-Tyr (45%), glyphosate C wrt E4P, NC wrt PEP, strong inhibition by HPP	68, 69
Neurospora crassa[f]	DS-Tyr (54%), partially purified, unstable	DS-Phe (32%), partially purified, unstable; DS-Trp (14%), homogeneous, tetramer, subunit M_r 52,000	70, 71
Pichia guilliermondii	DS-Tyr	DS-Phe	31
Rhodotorula glutinis	DS-Phe, unstable	DS-Tyr, relatively stable, partially purified, stimulated by Mn^{2+}, Co^{2+}; DS-Trp, relatively stable, partially purified, stimulated by Mn^{2+}	72
Sporobolomyces salmonicolor	Possesses all three isozymes		44
Hansenula henricii	Possesses DS-Phe and DS-Tyr		73

[a] Abbreviations: DS, DAHP synthase; C, competitive inhibition; NC, noncompetitive inhibition; UC, uncompetitive inhibition; wrt, with respect to; DTT, dithiothreitol.

[b] This autotrophic cyanobacterium is an example of an "endo-oriented" organism.

[c] This cluster includes *O. beijerinckii*, *O. maris*, *O. linum*, and *O. japonicum*.

[d] For the classification of Pseudomonads on the basis of biosynthetic mechanisms for phe and tyr, see References 46, 47, and 64.

[e] Some regulatory mutants have a "DS-Tyr which is completely insensitive to feedback inhibition by tyr, but which is still inhibited by PPY.

[f] The *N. crassa* genes are *arom6*, *arom7*, and *arom8*.

formation of initial pathway intermediates. Such "endo-oriented" organisms, *inter alia*, use an "asymmetric" pattern for the end-product control of DAHP synthase; thus, the cyanobacteria usually have DAHP synthases of the single effector type. In an "exo-oriented" organism the machinery must respond efficiently so as to utilize those aromatic amino acids that are available exogenously. Hence, in *E. coli*, the three DAHP isozymes show a "symmetrical" inhibition pattern in which each isozyme is feedback inhibited by and also repressed by one of the aromatic amino acids.[74] *P. aeruginosa* possesses both types of regulation (early- and late-pathway). The route of phe biosynthesis via PPY ressembles that of *E. coli* in enzyme makeup and regulation, whereas the unregulated AGN route ressembles that of late-pathway regulation in cyanobacteria.[75]

The evolutionary history of DAHP synthase isozymes in a phylogenetic cluster of prokaryotes ("superfamily B") that includes *E. coli* indicates that the most ancient organisms probably possessed DAHP synthase-O and DAHP synthase-Tyr.[63] The "dinosaurs" of this superfamily, retaining this ancestral isozyme feature, are the *Oceanospirillum minutulum* and *Acinetobacter* species. The likely major evolutionary events were

1. Evolution of the unregulated DAHP synthase-O to an isozyme with sensitivity to trp as well as weak sensitivity to CHA, i.e., DAHP synthase-O → DAHP synthase-Trp(CHA) (group I *Pseudomonads*)
2. Conversion of DAHP synthase-Trp(CHA) to DAHP synthase-Trp
3. The new appearance of a third isozyme, DAHP synthase-Phe
4. In addition to these three major events, a small cluster of organisms (group V *Pseudomonads* and *Lysobacter*) was formed by loss of DAHP synthase-Tyr and evolution of DAHP synthase-Trp(CHA) to one with a reversed sensitivity pattern, DAHP synthase-CHA(Trp)

A phylogenetic distribution of the DAHP synthase isozymes in 32 bacteria and 4 bacterial groups has been described.[76] For more details of the evolutionary relationships for the SHK pathway, other papers by Jensen and his colleagues[27,47,59,64-66,77] should be consulted.

In *S. cerevisiae*, DAHP synthase-Phe is encoded by the *ARO3* gene, and DAHP synthase-Tyr by *aro4*. Strains with a single *aro3* and a double *aro3aro4* mutation have been obtained. The *ARO3* gene was cloned in a 1.9 kb fragment; the DAHP synthase levels of strains with such plasmids were about 50-fold higher than those of wild-type strains.[78] The *ARO3* gene was sequenced and encoded a protein with 370 amino acids and M_r = 42,137.[79]

Both of these yeast genes responded equally well to the "general control" of amino acid biosynthesis.[80] The general control activator protein, GCN4, is essential for a basal level of *aro3* gene expression in *S. cerevisiae* grown in presence of amino acids. Under amino acid starvation the *ARO3* gene is derepressed to a higher transcription rate by GCN4.[81]

For various *Streptomyces*, the DAHP synthase activity was either partially inhibited by trp or suffered no inhibition from any of the aromatic amino acids. These organisms apparently contain a single enzyme species.[82] The enzyme from *Nocardia mediterranei* was inhibited by E4P; low levels of inhibition were observed with trp.[82,83] In *Nocardia* sp. 239, DAHP synthase was inhibited in a cumulative fashion by the aromatic amino acids. No evidence was obtained for the existence of isozymes. A virtually homogenous enzyme preparation was obtained and found to be a tetramer of subunits with approximate M_r = 41,000.[33]

4. DAHP Synthase in Plants

Although less than 1 decade ago it appeared that plant enzymes did not show inhibition patterns by the aromatic amino acids or materials such as CHA and PPA,[25] the situation has changed markedly. Not only are inhibition patterns now recognized, but there are cases of activation. Moreover, as for bacteria, the DAHP synthase picture is complex and there are several features which are not found with the bacterial enzymes. One generalization is that the plant DAHP synthases appear to be less stable than those of bacteria; however, a few of them have been purified to homogeneity.

Much of the work with plant DAHP synthases carried out prior to about 1985 is probably in need of experimental reexamination. As demonstrated in this article, isozymes, DAHP synthase-Co^{2+} and DAHP synthase-Mn^{2+}, have now been clearly characterized. Not only do these isozymes differ in cellular location and in showing different responses to light during growth, but the assay conditions for one isozyme are also completely inappropriate for the other (pH optima, dithiothreitol presence). It is thus possible that in early work where only one enzyme type was described, a second type may have been overlooked.

Carrot roots contain three forms of DAHP synthase activity, named as enzymes I, II, and III. While I and II remain relatively uncharacterized, enzyme III was purified 338-fold to electrophoretic homogeneity. The native enzyme (M_r = 103,000) was a dimer of subunits (M_r = 53,000). This enzyme showed hysteretic behavior and was activated by physiological concentrations of trp and to a lesser extent by tyr. The enzyme may undergo a conformational change or a change in subunit aggregation faciliated by trp that is slow in relation to catalysis. The observed feedback activation by trp was interpreted as an early regulatory signal for polyphenol biosynthesis. In addition, Mn^{2+} activated enzyme III.[84] DAHP synthase, also subject to activation by trp and tyr, was observed in extracts from carrot cells grown in suspension cultures.[85]

These activation effects with carrot enzymes contrast with results reported for a highly purified (1000-fold) DAHP syn-

thase from cauliflower florets. This enzyme contained an essential —SH group, required Mn^{2+}, and was not influenced either by aromatic amino acids or CHA and PPA.[86]

In other plant tissues, inhibitory effects of aromatic amino acids have been noted. The enzyme from *Zea mays* shoots was inhibited by trp, but not phe or tyr; thiol compounds were necessary for activity, Mn^{2+} was an activator, and isozymic forms were not demonstrated.[87] DAHP synthase preparations from pea leaves were inhibited by tyr; the inhibition was reversed by phe and trp. A partially purified preparation was slightly stimulated (5%) by phe or trp. An end-product insensitive enzyme was perhaps present in the preparations.

The DAHP synthase from *Solanum tuberosum* tubers purified to electrophoretic homogeneity, was obtained as two distinguishable forms with isoelectric points of 7.8 and 8.4. It was a dimer with M_r = 110,000 and showed hysteric behavior. The enzyme was stabilized by dithiothreitol, and stimulated by trp and Mn^{2+}. Other divalent metals such as Fe, Zn, Cu, were strongly inhibitory.[88] In contrast to this work, the presence of two isozymes, DAHP synthase-Co^{2+} and DAHP synthase-Mn^{2+}, has been clearly established in recent work with potato tubers.[22]

Several of the plant DAHP synthases just described were stimulated by Mn^{2+}. However, in mung bean *(Vigna radiata)*[89] and tobacco *(Nicotiana silvestris)*,[90] two isozymes, one responding to Mn^{2+} and the other to Co^{2+} were present. In mung beans, the DAHP synthase-Co^{2+} had an absolute requirement for a divalent cation, with Co^{2+} being the most effective. The second isozyme, DAHP synthase-Mn^{2+}, did not require Mn^{2+}, but was stimulated by this metal. The more stable DAHP synthase-Co^{2+} was partially purified and separated from accompanying phosphatase and CHA mutase activities. The Co^{2+} requirement was partially satisfied by Mg^{2+} and Mn^{2+}. This isozyme was not influenced by SHK pathway intermediates or by the aromatic amino acids. However, the secondary metabolite, caffeic acid, was an effective inhibitor.

The DAHP synthase-Mn^{2+}, which was still contaminated with considerable phosphatase activity, was active in the absence of Mn^{2+}, but 0.4 m*M* Mn^{2+} gave a 2.6-fold increase in activity. Unlike the cobalt-sensitive enzyme, there were four allosteric effectors for the Mn enzyme. Inhibitory effects were observed with trp, PPA, and AGN; CHA was an activator. This complex pattern of allosteric regulation was interpreted as a control mechanism of sequential feedback inhibition governing overall pathway flux.[89]

Detailed investigations of the DAHP synthase isozymes were carried out in cultured cells of *N. silvestris*. This plant has a number of technical advantages, including rapid growth in suspension cultures.[91] The isozymes were separated by DEAE-cellulose chromatography and had distinctively different properties in addition to the requirement for divalent metal.[90] The Mn^{2+}-activated enzyme had a pH optimum of 8.0 and required dithreothreitol; activation by this material was hysteretic. The Co^{2+}-activated enzyme had a pH optimum of 8.6 and was inhibited by dithiothreitol. Moreover, the two isozymes were distinguished by their cellular locations; the DAHP synthase-Mn^{2+} was located in chloroplasts, while the DAHP synthase-Co^{2+} was located in the cytosol. The partially purified (37-fold) DAHP synthase-Co^{2+} from *N. silvestris* had M_r = 440,000. Prior to the recognition of the two isozymes, "DAHP synthase" of *Pisum sativum* was reported to occur in intact density gradient-purified chloroplasts.[21]

It is not immediately clear whether the Co^{2+}-stimulated enzyme is present in most plant tissues. This is because the best conditions for the assay of this enzyme are not optimal for the DAHP synthase-Mn^{2+} (different pH optima and dithiothreitol requirements — see the earlier section). Hence, the Co^{2+}-stimulated enzyme may have been overlooked in some of the earlier experiments reported here. Preliminary observations suggested that the presence of the two isozymes is general in plants; they were noted in spinach, cauliflower and broccoli florets, and seedlings of soybean, alfalfa, squash, wheat, and rye[90] in addition to the plants already described.

There are further complexities for plant DAHP synthases. A preliminary report shows that the DAHP synthase-Mn^{2+} responded to light during plant growth. Thus, when dark- and light-treated plants were compared, the activity of the Mn^{2+} sensitive enzyme increased 11-fold. On the other hand, the DAHP synthase-Co^{2+} was unchanged under these conditions.[91] Other workers found that wounding potato or tomato tissue induced DAHP synthase, with an actual increase in the amount of the enzyme.[92] Moreover, the specific activity of plastidic DAHP synthase-Mn^{2+} in cultured parsley cells was increased 2- to 3-fold by a cell wall fraction of the fungus *Phytophthora megasperma;* however, the cytosolic DAHP synthase-Co^{2+} was not affected. Transcriptional regulation was indicated since actinomycin D or cycloheximide prevented the increase in DAHP synthase-Mn^{2+} activity.[93]

5. Action of Glyphosate on Plant DAHP Synthases

In 1982, the mung bean DAHP synthase-Co^{2+} was shown to be inhibited by glyphosate.[94] An important finding was that the two *N silvestris* isozymes behaved differently with this inhibitor.[95] The DAHP synthase-Mn^{2+}, (located in chloroplasts) was resistant to glyphosate inhibition, while the DAHP synthase-Co^{2+} (located in the cytosol) was inhibited. Spectrophotometric observations provided evidence for formation of a cobalt(II):glyphosate complex with electronic properties consistent with octahedral coordination.

With *Solanum tuberosum* cells grown in suspension culture, the DAHP synthase levels varied during the growth cycle.[96] The specific activity increased up to day 15 (midway through the linear growth phase) and thereafter declined. If glyphosate was added to the culture medium of cells grown for 9 d, there

was an increase in the specific activity of DAHP synthase. Although glyphosate caused this *in vivo* induction of DAHP synthase activity, there was no effect on the enzyme in *in vitro* experiments. The increase was due to an increase in enzyme amount, probably as a result of induced *de novo* protein synthesis. The possible presence of Co^{2+}- and Mn^{2+}-dependent isozymes was not studied in this work. A similar increase in DAHP synthase levels was reported when a glyphosate-tolerant cell line of *Nicotiana tabacum* was grown in the absence of glyphosate.[97]

The availability in the chloroplast of the two substates required by DAHP synthase is of concern. As an intermediate of the Calvin-Bassham cycle, E4P can be obtained directly in the chloroplast by photosynthesis. However, $^{14}CO_2$ was only poorly incorporated into aromatic amino acids when highly purified intact spinach chloroplasts were examined.[98] Addition of phosphoglycerate mutase and enolase to the incubation medium gave a 10-fold increase of CO_2 fixation into phe and tyr, suggesting the absence of these enzymes from the chloroplast. Hence, PEP, the other substrate for DAHP synthase, was likely to be synthesized in the cytoplasm and then imported into the chloroplast. More recently, intact spinach chloroplasts were shown to possess the following effective, but low capacity, pathway for synthesis of PEP and pyruvate following CO_2 fixation:

$$CO_2 \rightarrow \text{3-phosphoglycerate} \rightarrow \text{2-phosphoglycerate} \rightarrow \text{PEP} \rightarrow \text{pyruvate}.$$

The rate limiting step was likely the chloroplast phosphoglycerate mutase.[99]

B. 3-Dehydroquinate Synthase

This enzyme (EC 4.6.1.3) forming DHQ, formally named 7-phospho-3-deoxy-D-*arabino*-heptulosonate phosphate lyase, is abbreviated as DHQ synthase. The enzyme has generally been assumed to require Co^{2+} and the homogeneous enzyme from an overexpression strain of *E. coli* did contain 1 mol of tightly bound Co^{2+}. On incubation with EDTA enzyme activity was lost rapidly and a stable but inactive protein was formed. The presence of substrate DAHP prevented this inactivation. Full activity was restored by Co^{2+} and partial activity (about 50%) by Zn^{2+}. On the basis of its greater bioavailability, it was proposed that Zn^{2+} was the actual functional divalent metal *in vivo*. Zn^{2+} was also bound at a second, lower affinity, inhibitory site.[100] Interestingly, the *arom* protein from *N. crassa* also has a Zn^{2+} requirement (see Section II.L).

The conversion of DAHP to DHQ requires considerable chemistry, specifically oxidation, β-elimination, reduction, and finally an intramolecular aldol condensation, Indeed, "The mechanistic details of the transformation reflect both clever functional group manipulation and stereochemical dexterity on the part of the enzyme."[101] One aspect of the clever manipulation is that the —CHOH group at C-5 of DAHP is temporarily oxidized to —C=O to facilitate proton removal and then phosphate elimination. After phosphate elimination the ketone is reduced, producing the —CHOH group of DHQ with the same configuration as was originally present. Since the C-7 phosphate group must be removed, intervention of an enol is likely. Thus, the reaction can be represented as a first approximation by the sequence of Figure 3. This sequence indicates that the reaction inverts configuration at C-7 of DAHP.

NAD is the cofactor for the oxidation-reduction processes and the purified enzyme binds 1 mol of NAD^+. Dissociation of NAD from the holoenzyme is a complex process. Even in the presence of DAHP, NAD analogs can be bound with high affinity. Unless high-purity NAD is used, catalytic activity may be lost as a result of the presence of unidentified impurities which function as inhibitors.[101]

Although DAHP was shown in Figure 3 in a straight chain conformation, the involvement of a pyranose form is likely. Reaction of DAHP with 4-hydrazinobenzoate proceeded with a small burst (corresponding to only 0.3% of the final absorbance) and then continued slowly with first order kinetics, suggesting that over 99% of the DAHP was pyranoid.[102] Moreover, synthetic 2-deoxy DAHP (Figure 4A, R = H) was a substrate for DHQ synthase (the enzyme used was a homogeneous material from an overproducing *E. coli* strain—refer to the later section).[103] Without the —OH group at C-2, this analog cannot undergo the pyranose → straight chain ketose isomerization. Phosphate was eliminated from this substrate, the product being an *enol* ether (Figure 4B, R = H). Hence, with this synthetic substrate analog, the enzyme apparently catalyzed the first three steps of the normal reaction: oxidation, elimination, reduction.

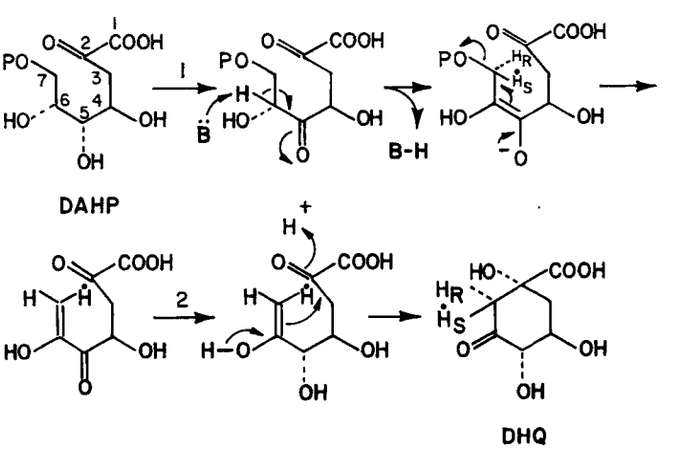


FIGURE 3. Simplified reaction mechanism for DHQ synthase. For step 1, NAD^+ is converted to NADH; the reverse process occurs in step 2.

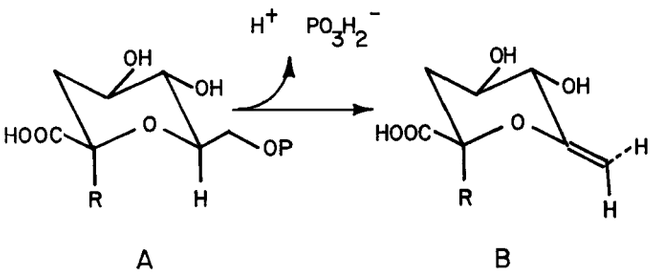


FIGURE 4. Action of DHQ synthase on 2-deoxy-DAHP (A, R = H).

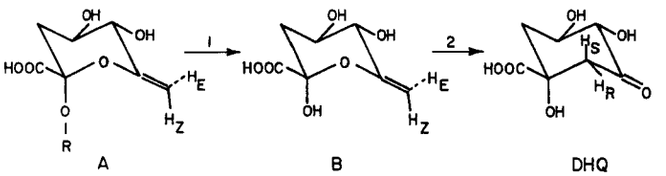


FIGURE 5. Attempted synthesis of *enol*pyranose intermediate, B, for DHQ synthase. In A, R = —CH_2—C_6H_4—NO_2. Step 1, photolysis at 0°C; step 2, spontaneous reaction.

By analogy, it was reasonable to postulate an *enol* pyranose (Figure 4B, R = OH) as the actual intermediate formed from DAHP itself. An attempted synthesis of the *enol* pyranose (Figure 4B, R = OH, and Figure 5B) by photochemical irradiation (at 0°C) of the corresponding *o*-nitrobenzylketal (Figure 5A) gave only DHQ.[101] While some intermediates were observed by reaction at lower temperatures, none of them predominated prior to DHQ formation. Hence, conversion of the *enol* pyranose (Figure 5B) to DHQ was at least as rapid as the steps involved in removal of the *o*-nitrobenzyl group. Since the spontaneous conversion of the *enol* pyranose to DHQ was so rapid, the actual catalysis by DHQ synthase probably concluded with this intermediate; *in this case, the aldol cyclization was nonenzymatic.*

Photolysis of the *o*-nitrobenzyl ketal, labeled with ^2H in the Z position at C-7, gave rise to (2R)-[2-^2H]-DHQ (Figure 5). This was consistent with the known conversion of the 7-H_R proton of DAHP to the 2-H_R proton of DHQ (inversion of configuration), assuming that the phosphate elimination was syn. Evidence for syn stereochemistry of phosphate elimination was obtained with 2-deoxy-DAHP labeled with ^2H at C-7 (S configuration) (see Figure 6A). After conversion of the *enol* product (Figure 6B) to the bicyclic ketone (Figure 6C), NMR analysis indicated that the ^2H was in the E position.[103]

More information concerning the possible transition state for the aldol condensation was derived by considering the overall stereochemistry for the syn elimination. As already noted, during conversion of DAHP to DHQ, the C-7 configuration of DAHP undergoes inversion. To satisfy both of these observations (syn elimination, overall inversion) the most likely possibility was a chairlike transition state.[104] It can be obtained by a 180° rotation about the bond between C-5 and C-6. This also seems likely since the preferred conformation for DHQ is a chair structure. The C-6 to C-7 double bond adds to the re face of the C=O bond at C-2 from above. The overall picture for the complex reactions catalyzed by DHQ synthase is shown in Figure 7. Enzymatic steps lead to intermediate C (Figure 7), which proceeds further, probably by nonenzyme catalyzed steps, to DHQ itself.

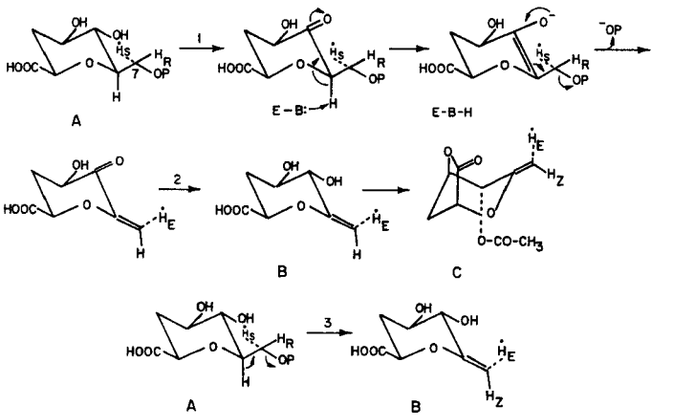


FIGURE 6. Evidence for syn stereochemistry in the DHQ synthase reaction. E-B = enzyme with basic group, B. The substrate, A, is 2-deoxy-DAHP with (S-^2H) at C-7. The overall syn stereochemistry is more easily visualized by the simplified reaction, A → B, shown at the bottom of the figure.

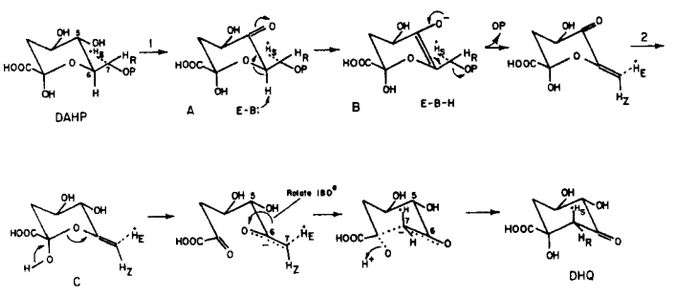


FIGURE 7. Possible overall mechanism for action of DHQ synthase. Note that in the final step, addition occurs from above the plane of the C=O bond.

In Figure 7, a basic group of the enzyme, E-B^-, was postulated for deprotonation at C-6. On the basis of work with carbacyclic phosphonate analogs of DAHP, it now appears that an enzyme group is not required and instead, the phosphate group of DAHP itself functions as the base.[105] The analogs were the homophosphonate (isosteric with DAHP) and phosphonate compounds A and B (Figure 8), and two unsaturated compounds (Figure 8C and D). While oxidation at C-5 and proton exchange at C-6 (using the pyranose numbering system) were theoretical possibilities with these analogs, the DHQ syn-

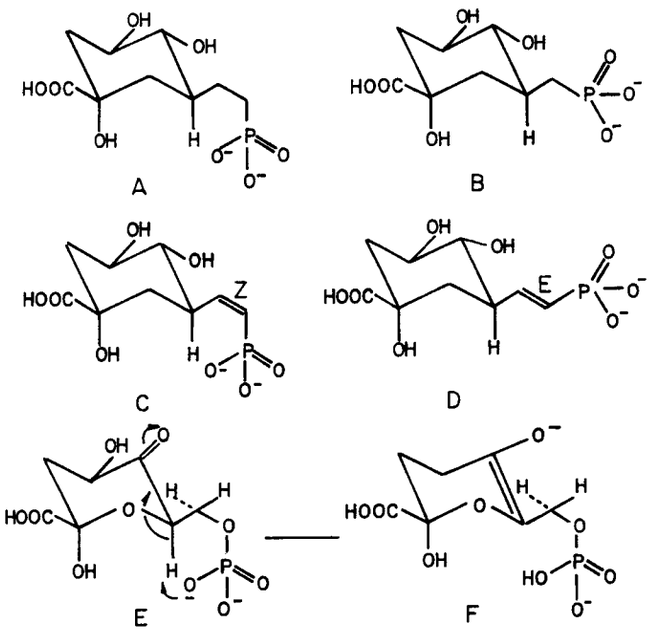


FIGURE 8. Carbacyclic phosphonate and homophosphonate analogs of DAHP. A modified version of the deprotonation possibility, shown in Figure 7 as involving an enzyme group, is also drawn here as E → F.

thase catalyzed elimination reaction of phosphate could not be completed (phosphonate present, not phosphate). The results of these experiments, using a DHQ synthase preparation from *E. coli*, were as follows:

Compound in Figure 8	Binding ability	Oxidation, C-5 OH	Proton exchange, C-6
A	Binds as well as DAHP	Yes	Yes
B	Binds very tightly[a]	Yes	No
C	Binds better than A	Yes	Yes
D	Binds weakly	No	No

[a] This is the best inhibitor yet discovered for this enzyme, K_i, about 0.8 n*M*.

These observations implied a crucial role for the positioning of the phosphonate group. In particular, proton exchange only occurred in the two structures (Figure 8A and C) where a phosphonate oxygen was close to the C-6 proton. In other words, the base responsible for proton abstraction was an oxygen of the phosphonate group. By extrapolation, it seemed likely that with the actual substrate, DAHP, the phosphate group played a similar role. Hence the second reaction step, shown previously as Figure 7, A → B, could be postulated as Figure 8, E → F. Attractive features of this proposal include:

1. The enzyme exploits one of the strongest bases available at physiological pH, namely $-OPO_3^{2-}$.
2. The enzyme avoids removal of the axially located C-6 hydrogen at a tertiary center, which is in a 1,3-diaxial situation with the axial C-2 OH group.
3. Deprotonation produces a better leaving group for the subsequent elimination of phosphate.

If this proposal and that suggesting that conversion of the *enol*pyranose, Figure 7C, to DHQ is nonenzymatic, are correct, the actual work required of the enzyme is vastly simplified. The number of required catalytic groups becomes reasonable and what at first appeared as a complex mechanism is ingeniously simple. Apart from binding substrate, metal, NAD^+, and possibly transition states, all that is required of enzyme catalysis is oxidation and reduction. The enzyme has been described as "a sheep in wolf's clothing".[105] The use of the substrate analogs noted here and others to elucidate the early and late steps of the DHQ synthase reaction mechanisms has recently been reported in detail.[106,107] While the rationalization of the seemingly complex chemistry is a major achievement, researchers are cautioned that a dilemma exists — how can all of the necessary catalytic groups, NAD^+, and a divalent cation be assembled at a single active site? The elegantly rational chemistry seemingly imposes impossible structural demands on the protein.

An interesting result was obtained with a carbacyclic analog of DAHP containing the normal phosphate group (Figure 9A). This material was a potential precursor for an analog (Figure 9B) of the reaction intermediate (Figure 9C). While it did function as an inhibitor,[107] on incubation with enzyme in the absence of DAHP it was converted to compound D (Figure 9).[107,108] This was consistent with the general theory just outlined and with the utilization of the α anomer of DAHP.

The C-3 fluoro analogs of DAHP were converted by a combination of DHQ synthase and DHQ dehydratase from *E. coli* to 6-fluoro-DHS (Figure 10). These analogs were prepared chemically[109] or enzymatically from the reaction of Z fluoro phospho*enol*pyruvate and E4P.[40]

Carbacyclic phosphonate analogs of DAHP (including the most powerful inhibitor thus far discovered, structure B, Figure 8), were discussed previously in connection with the reaction mechanism of DHQ synthase. Earlier, phosphonate and homophosphonate analogs with the pyranose ring structure had been

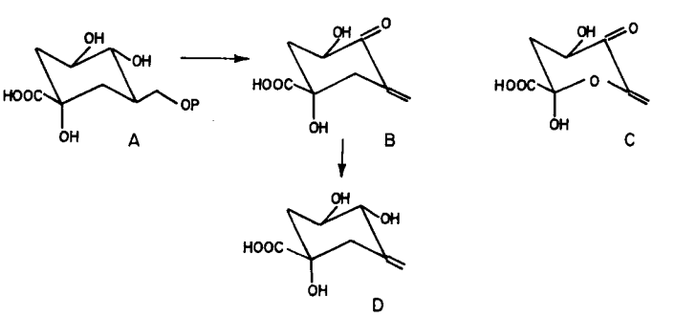


FIGURE 9. Utilization of a carbacyclic DAHP analog, A, by DHQ synthase.

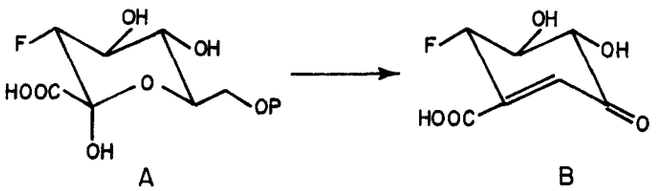


FIGURE 10. Conversion of 3-fluoro-DAHP to 6-fluoro-DHS by the combined action of DHQ synthase and DHQ dehydratase. The reaction is shown for the 3R,3F diastereoisomer of 3-fluoro-DAHP; the product, B, is 6S6F —DHS. The 3-S diastereoisomer behaved similarly.

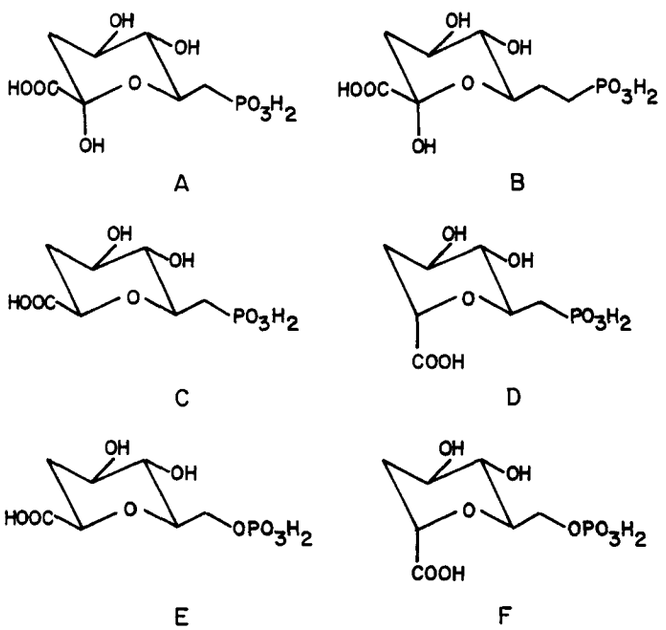


FIGURE 11. Pyranose analogs for DHQ synthase. A and B, respectively, are the phosphonate and homophosphonate structures corresponding to DAHP itself. C and D are "anhydrophosphonate" analogs, and E and F are "anhydrophosphate" analogs.

examined and both A and B of Figure 11 were claimed to inhibit *E. coli* DHQ synthase.[110,111] Reexamination with homogenous *E. coli* DHQ synthase and a direct assay method, indicated that A was a competitive inhibitor, but that B was without effect.[112]

Pyranose structures lacking the C-2 OH group also have been examined.[112] Three materials, Figure 11D, E, and F were competitive inhibitors of *E. coli* DHQ synthase, and C was without action. The inhibitory action of Figure 11E (K_i = 193 μM) was significantly less than that of F (K_i = 33 μM). Compounds A and D of Figure 11 were competitive inhibitors to a partially purified DHQ synthase of pea seedlings and Figure 11B and C were without action. The action of the nonisosteric phosphonate, Figure 11A, in postemergent treatment of seedlings of *Pisum sativum, Echinochloa crusgalli, Setaria viridis, Sorghum halepense,* and *Avena fatua* was investigated. With the exception of *E. crusgalli,* all of the plants showed up to a fourfold buildup of dephosphorylated DAHP (DAH), and discoloration and desiccation of plant tissue occurred. These "visual effects" were least for *P. sativum.*[113] DHQ synthase is also inhibited by the phosphonate compound, glyphosate.

1. DHQ Synthases from Bacteria and Plants

The monofunctional DHQ synthase from *E. coli* has been purified to homogeneity by several workers. An early purification (2840-fold) to homogeneity indicated M_r = 57,000.[114] This value now appears too high.

More recently, a 9000-fold purification was obtained from wild-type *E. coli* K12 (strain MM 294), but the preparation was not homogeneous.[115] Recombinant DNA technology was therefore used to produce a strain (*E. coli* JB12[pLC29-47]) with a 20-fold increase in DHQ synthase activity in crude lysates. A 550-fold purification yielded nearly 2 mg of almost homogeneous enzyme from 19 g of cells. Furthermore, by inserting the *aroB* gene from pLC29-47 behind a *tac* promoter in the plasmid pKK223-3, a subclone pJB14 was isolated and inserted in *E. coli* RB791. The transformant, *E. coli* RB791(pJB14), when induced with isopropyl β-D-thio-*galacto*-pyranoside, produced nearly 1000 times the yield of the wild-type strain. From lysates of this strain, homogeneous DHQ synthase was obtained by only two chromatographic steps. More than 100 mg of homogeneous enzyme could be obtained from 50 g of cells. The M_r of this preparation was 40,000 to 44,000.

Homogeneous DHQ synthase was also prepared from *E. coli* AB2826pGM107.[116] The amino acid sequence of 362 residues was determined by a combined nucleotide and direct amino acid sequencing strategy. The calculated M_r of 38,880 agrees well with that determined by Frost et al.[115]

An electrophoretically homogeneous preparation of plant DHQ synthase was obtained from *Phaseolus mungo* seedlings (4400-fold purification from an ammonium sulfate fraction of a crude extract). The preparation contained small amounts of Cu^{2+}. It was active without further metal additions, but was stimulated by both Co^{2+} and Cu^{2+}. The M_r was 67,000 (Sephadex G-100) and the minimum M_r was 43,000 (SDS-gel electrophoresis).[117] DHQ synthase has also been purified (4900-fold) from pea seedlings; the preparation was inhomogeneous under denaturing conditions.[113] The native enzyme had M_r = 66,000 by size exclusion chromatography and was apparently a dimer (subunit M_r = 33,000).

2. Preparation of DAHP

Many of the SHK pathway intermediates are not readily accessible, and DAHP is no exception. It has been obtained from organisms lacking DHQ synthase such as *E. coli* AB2847A (*aroB*$^-$).[102] Somewhat better yields were obtained from *E. coli* JB-5, but these could not be increased by transformation with

a multicopy plasmid (pKB-45) coding for DAHP synthase-Tyr.[115]

A detailed comparison has been made of various chemical and microbiological methods.[118] To produce DAHP as a substrate, the microbiological route is probably the best. By growth of *E. coli* JB-5 under defined conditions (initial growth in a rich medium, followed by transfer to a minimal medium), the combined yield of DAHP and DAH is 342 μmol/10^8 cells (the cell density is 10.5 × 10^8/l). The quantities of DAH produced microbiologically in both *E. coli* JB-5 and AB2847 are five to six times that of DAHP. To produce substrate DAHP analogs, chemical synthesis is recommended.

Immobilized enzymes have been assembled into a "reactor" for DAHP synthesis.[119] The arrangement required fructose and PEP as "substrates" and four immobilized enzymes (hexokinase, pyruvate kinase, transketolase, and DAHP synthase). The yield of "pristinely pure" DAHP was 85% based on fructose. Another enzymatic approach to DAHP synthesis used aldolase to catalyze C-4 to C-5 bond formation with correct stereochemistry. Unfortunately, much chemical manipulation and several chromatographic purifications were required. The 95% pure product was less active as a DHQ synthase substrate than authentic DAHP and probably contained an inhibitor.[120]

Two recent chemical syntheses of DAH (or its ester) have been achieved from D-arabinose with -OH groups protected by either acetone[121] or cyclohexanone.[122] DAH produced chemically or microbiologically can also be converted chemically to DAHP after formation of methyl(methyl-3-deoxy-D-*arabino*-heptulopyranosid)onate. The latter compound is made available by chemical synthesis.[123,124]

C. 3-Dehydroquinate Dehydratase

The enzyme EC 4.2.1.10 converts DHQ to DHS. It is, therefore, responsible for initiating the process of aromatization by introducing the first of three double bonds. Although often referred to as 3-dehydroquinase, the recommended name is 3-dehydroquinate dehydratase, abbreviated here as DHQ dehydratase.

The reaction is one of the relatively few enzymatic dehydrations known to proceed with a syn (cis) elimination of the elements of water (see Figure 12). To account for the observed syn stereochemistry, it was proposed that the H^+ and OH^- components were removed in separate steps. The proton is eliminated first and a carbanion intermediate is formed and stabilized as resonance forms.[125] Since $NaBH_4$ inactivates the enzyme in the presence, (but not the absence), of DHQ, the substrate may link covalently to the enzyme via a lys residue.[126] Furthermore, inhibition by the his-directed diethylpyrocarbonate suggested that an imidazole side chain carried out deprotonation.[126] This residue, known as B-H^+, could also be involved in expulsion of the hydroxyl ion (see Figure 12).

A skew-boat conformation provides the best spatial possibility for expulsion of the H_R proton at C-2, since it places H_R

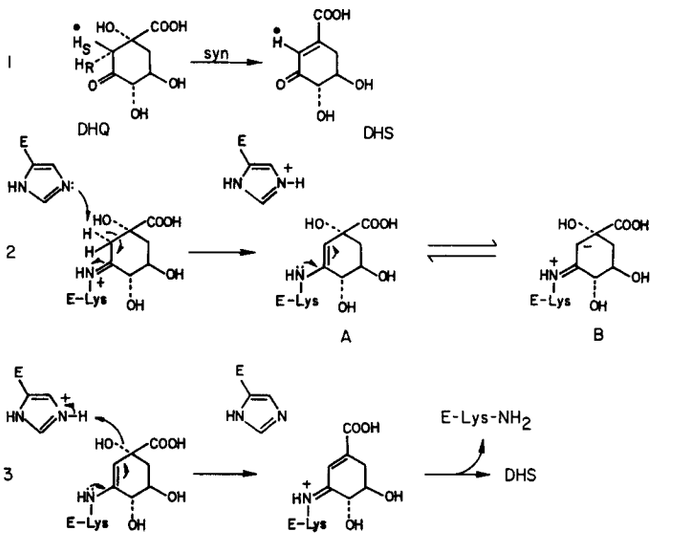


FIGURE 12. Reaction mechanisms for DHQ dehydratase. (Line 1) The overall syn elimination of the elements of water. (Line 2) Possible mechanism for proton removal by his residue of enzyme (E) with Schiff base formation by a lys residue (E-lys). Note the possibility for resonance stabilization of the carbanion intermediate as A ↔ B. (Line 3) Possible mechanism for removal of hydroxyl from intermediate A.

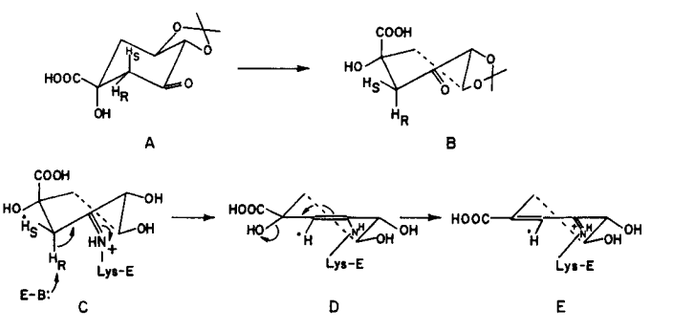


FIGURE 13. Conformational possibilities for DHQ dehydratase substrates. A → B represents the conformational change of the isopropylidene derivative of DHQ. C → D → E is a possible reaction mechanism with DHQ as substrate bound to the enzyme via a lys residue. In structure D, movement of the lone pair electrons from the nitrogen has been left out because of space limitations.

in an axial arrangement. This possibility was strengthened by the fact that the isopropylidene derivative, Figure 13A, was a substrate for DHQ dehydratase; conformational arrangements with H_R axial, other than the skew-boat (Figure 13B), are impossible for this substrate.[127] The sequence, C → D → E of Figure 13, appears likely to represent the conformational possibilities for the DHQ dehydratase reaction.

Three substrate analogs, with reactive functional groups, were examined as irreversible inhibitors of DHQ dehydratase.[128] A lys residue was suggested as a binding site for the —COO^- group; the analog, A, Figure 14, with a chloromethyl

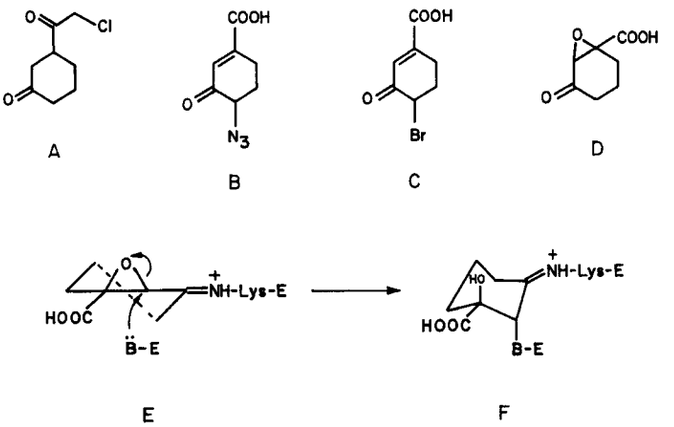


FIGURE 14. Substrate analogs of DHQ dehydratase. All of the compounds were examined in racemic form.

ketone group was inhibitory possibly by alkylation of the putative lys. Analog B, but not C, Figure 14, was also inhibitory as a photoaffinity label suggesting a possible role for a C-4 OH site.

In attempts to "label" the active site basic group (presumably his as already noted), the racemic epoxide (Figure 14D), was found to be inhibitory. Although the question of enantiomeric specificity was not resolved, the mechanism shown in Figure 14 (E → F) was proposed to account for the observed inhibition.[128] The structures shown were presumably chosen to accomodate the likelihood that the basic group is located in the active site below the plane of the cyclohexanone ring. This results in the C-2 configuration of the product D, Figure 14, being the inverse of that of the usual substrate. Clearly, further work with the epoxide inhibitor is desirable.

In *E. coli*, this monofunctional enzyme is encoded by the *aroD* gene, which has been precisely located and sequenced. In a 1.8-kb Cla 1 insert in the plasmid pKD201, the *aroD* sequence began with ATG (met) at position 703 and ended with a TGA (stop) codon at position 1423. The native enzyme was a dimer and the monomer had 240 amino acids; the predicted sequence was confirmed by N-terminal analysis of the first 17 amino acid residues. The enzyme was purified to homogeneity (4000-fold, 19%) from wild-type *E. coli*.[126] However, a higher yield (7 mg from 20 g of cells) was obtained from the overproducing strain *E. coli* AB 2848/pKD201.[129] In pea seedlings, DHQ dehydratase is present in intact chloroplasts and root plastids.[21]

D. Shikimate Dehydrogenase

Although often called shikimate oxido-reductase (SHORase), it seems more appropriate to use SHK dehydrogenase; this is the recommended Enzyme Commission name for EC 1.1.1.25 (shikimate:NADP$^+$ oxido-reductase). This dehydrogenase converts DHS to SHK by a straightforward reaction utilizing the H_A proton of NADPH (see Figure 1).

1. Monofunctional SHK Dehydrogenase from E. coli

A 20,000-fold purification from wild-type *E. coli* ATCC 14948 yielded electrophoretically homogeneous material. The SHK dehydrogenase was somewhat unusual in being monomeric.[130] A large-scale purification (10.5 mg homogeneous enzyme from 20 g of cells) was carried out with an *E. coli* strain (AB2834/p*I*A321) containing the *aroE* ORF under the control of the *tac* promoter in the expression vector pKK223-3.[131] The *aroE* gene was sequenced and on ORF of 846 bp was identified. The assigned sequence agreed with the partial amino acid sequence determined on the pure enzyme (the first 30 amino acid residues were determined unambiguously). Interestingly, the amino acid "fingerprint" (29 to 31 residues governed by a set of 11 rules) characteristic of an ADP-binding βαβ fold[132] was present between residues 121 and 151. This fold was probably the NADP$^+$ binding site.

Three other ketones were reduced by the *E. coli* enzyme.[133] The S enantiomer of the racemic 5-deoxy analog (Figure 15A) was an excellent substrate (k_{cat} = 75 s^{-1}) compared to DHS (k_{cat} = 100 s^{-1}). The R enantiomer (Figure 15B) was also reduced, but very slowly. The achiral analog lacking both OH groups, Figure 15C, and the S enantiomer of the dihydrodideoxy compound, Figure 15D, were also reduced slowly. In all cases, the configuration of the newly formed C-3 —OH group corresponded to that in SHK itself. The presence of a C-4 —OH, but not a C-5 —OH is important for this enzyme, and formation of the enzyme-substrate complex may involve hydrogen bonding with the C-4 —OH. The enzyme is enantioselective with respect to the racemic substrates, with a preference for the S configuration at C-1 and C-4.

2. Plant Enzymes

The following information relates to enzymes described simply as SHK dehydrogenases: bifunctional DHQ dehydratase-SHK dehydrogenases are present in plants such as spinach, pea seedlings, and corn (Section II.J.4).

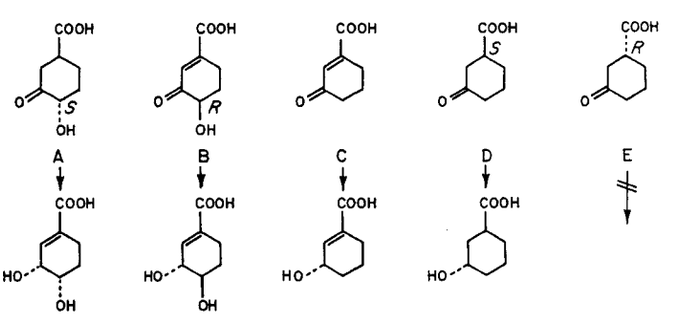


FIGURE 15. Substrate analogs of SHK dehydrogenase. There was no reduction of the (R) dihydrodideoxy compound E.

A partial purification of SHK dehydrogenase from tomato fruit yielded, on hydroxyapatite chromatography, evidence for two isozymes (such isozymes had been described earlier in other plant preparations).[134] The major fraction (11.5-fold purification) was not homogeneous; the M_r was estimated to be 73,000. Among a number of compounds, only protocatechuic acid functioned as an inhibitor. Hg, Zn, and Cu were inhibitory; a requirement of —SH groups for activity was established.

In Ponderosa pine needles, SHK dehydrogenase was a monomer and there were three allozymes produced by three different alleles in the population surveyed. Any given individual had a phenotype of one, or at most two, of the three allozymes.[135] SHK dehydrogenases were partially purified from larch and pine needles.[136,137] It was stated that in conifers (in contrast to all other plants) all three isozymes of SHK dehydrogenase were active, not only with NADP, but also with NAD. It was thought that the NAD-dependent SHK dehydrogenase catalyzed initial reactions of an alternative pathway for conversion of SHK to hydroxybenzoates.

The herbicide, Sandoz 6706[4-chloro-5-(dimethylamine)-2-(α, α, α-trifluoro-*m*-tolyl)-3(2H)-pyridazinone] increased SHK dehydrogenase activity in barley shoots (*Hordeum vulgare*), particularly with high intensity light.[138]

E. Shikimate Kinase

Shikimate kinase, EC 2.7.1.71 (ATP: shikimate 3-phosphotransferase), will be abbreviated here as SHK kinase. The reaction is an unexceptional phosphate transfer from ATP to the C-3 —OH group of SHK (see Figure 1). SHK kinase is known to exist in isozymic forms in *E. coli* and *S. typhimurium* and in complex forms in other organisms.[4,9] This behavior is somewhat unusual for an enzyme in the middle of a metabolic pathway, and no convincing explanation for it has been advanced. Weiss and Edwards[5] suggested the possibility of branching at SHK via unrecognized catabolic or anabolic pathways. Such biosynthetic branches are now known (see Section III.A.3), but apparently exist neither in *E. coli* nor *S. typhimurium*; the role of SHK kinase isozymes in the SHK branches remains enigmatic.

1. E. coli SHK Kinase II

Most recent investigations have focused on SHK kinase II, and SHK kinase I remains relatively unexplored. The structural gene for SHK kinase II in *E. coli* is *aroL* and its expression is under the control of the regulator gene, *tyrR*. The gene has been cloned and sequenced, and overexpression strains have been constructed. The enzyme has been purified by two groups of investigators.[140-142] From *E. coli* HW87/pMH423, an 81-fold purification gave homogeneous enzyme and from *E. coli* JP1680 (which contains the *aroL*$^+$ plasmid pMU377 and overproduces about 50-fold), a 73-fold purification gave nearly homogeneous material. The observed M_r values, about 20,000 to 22,000, agreed with the value of 18,937 calculated from the amino acid sequence; the enzyme is monomeric. The coding sequence contained 519 bp yielding a polypeptide with 173 amino acids. The amino acid sequence, determined for the first 24 residues, agreed with that predicted from the DNA sequence.

Enzyme activity was dependent on the presence of divalent cations, with Mg^{2+} being the most effective. The enzyme was not inhibited by a variety of aromatic amino acids and substituted benzoates. The affinities of the two kinases for SHK were very different: for SHK kinase I, >20 m*M*, and for SHK kinase II, 200 μ*M*. These values indicate that SHK kinase II functions in the biosynthetic SHK pathway.

The *aroL* gene in *E. coli* contranscribes with at least one other gene, *aroM*. The latter codes for a polypeptide of approximate M_r = 26,000 (225 amino acid residues). The function of this gene and its product remains unknown. The *aroL* gene of the commercially important *Brevibacterium lactofermentum* has been cloned.[143] Recombinant plasmids with this gene had elevated levels of SHK kinase. In addition, the *aroB* (encodes DHQ synthase) and *aroE* (encodes SHK dehydrogenase) genes were also present in these recombinant plasmids. The three genes were closely located on the DNA fragment in the order *aroL*, *aroB*, and *aroE*, forming a cluster on the chromosome.

2. Plant Enzymes

SHK kinase has been partially purified from *Phaseolus mungo* seedlings and *Sorghum bicolor*.[144,145] In both cases, activity was maximal at pH 8.6 to 9.0 and Mg^{2+} was required. The sorghum enzyme was inhibited by caffeate and less so by *p*-coumarate. A pea seedling enzyme was located in stromal preparations of washed chloroplasts.[21]

Thioredoxin stimulated SHK kinase from spinach chloroplasts and the effect was probably related to an observed dependence on light for aromatic amino acid biosynthesis.[146] Partially purified enzyme (36-fold, Sephadex G-75 chromatography) had an apparent M_r of about 27,000.

F. 5-*Enol*pyruvyl-Shikimate 3-Phosphate Synthase

The enzyme forming EPSP [5-*O*-(1-carboxyvinyl)-3-phosphoshikimate] is EC 2.5.1.19. The amended formal name in 1984 became phospho*enol*pyruvate:3-phosphoshikimate 5-*O*-(1-carboxyvinyl)transferase. Although the recommended name is 3-phosphoshikimate 1-carboxyvinyltransferase, the abbreviation EPSP synthase is common and is used here. The overall reaction between S3P and PEP is shown in Figure 1. This reaction has assumed considerable importance since EPSP synthase is the major target for inhibition by the broad spectrum, nonselective, postemergence herbicide, glyphosphate (the active component of the weed killer, Roundup®).

1. Reaction Mechanism

EPSP synthase has been intensively investigated; it seems convenient to discuss the recent results relating to mechanism

first. Two decades ago, Sprinson and his colleagues suggested an addition-elimination mechanism with formation of the "tetrahedral intermediate" (Figure 16A). If only transiently, the —CH_2 group of PEP and EPSP actually existed as a methyl.[4] The "Sprinson mechanism" was consistent with the observed cleavage of the C-O bond of PEP (rather than of the O-P bond). More recently, a mechanism involving an enzyme-substrate (PEP) complex was suggested.[147] The mechanism required the enzyme and PEP to form a complex (complex 1, Figure 16B) which added S3P, followed by phosphate elimination. Another possibility was the formation of a second complex (complex II, Figure 16C) by phosphate elimination, followed by the addition of S3P. The mechanism was based on the observed enzyme catalyzed exchange of ^3H from solvent ^3H_2O into PEP In the presence of the substrate analog, dideoxy-S3P. The exchange was, however, slower than the catalytic turnover.

The tetrahedral intermediate, Figure 16A, has now been isolated and characterized. Initial evidence came from very detailed studies using rapid chemical quench-flow kinetic methods.[148,149] Trapping experiments established that the kinetically preferred order of reaction was S3P binding first, followed by PEP binding. When EPSP formation was monitored with excess enzyme being mixed with a saturating level of S3P and a limiting level of PEP, a transient formation of pyruvate was observed (maximum at 10 ms; no pyruvate formation from either EPSP or PEP under the quenching conditions used). Pyruvate formation was ascribed to breakdown of an intermediate, labile under the acid quench conditions. Similar results were obtained with excess enzyme, EPSP, and a high concentration of phosphate, thus driving the reaction in reverse. The transient-state kinetic analysis led to the following scheme, with values being obtained for all 12 of the rate constants (I = Intermediate).

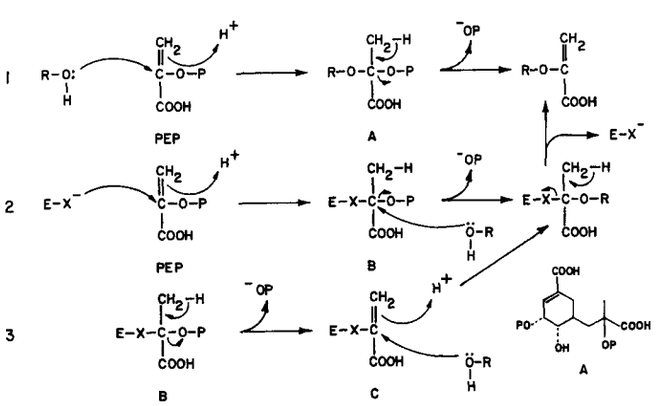


FIGURE 16. Reaction mechanism for EPSP synthase. (Line 1) The Sprinson mechanism with the tetrahedral intermediate, A. (Line 2) Possible involvement of group X on the enzyme. Complex I (structure B) can be attacked by S3P with loss of phosphate, or as shown on line 3, can lose phosphate to form complex II (structure C). ROH is S3P with the "exposed" OH group at C-5.

$$\begin{array}{ccccccc} & k_1[S3P] & & k_2[PEP] & & k_3 & \\ E \Leftrightarrow & E \cdot S3P & \Leftrightarrow & E \cdot S3P \cdot PEP & \Leftrightarrow & E \cdot I & \\ & k_{-1} & & k_{-2} & & k_{-3} & \\ & k_4 & & k_5 & & k_6 & \\ \Leftrightarrow & E \cdot EPSP \cdot P & \Leftrightarrow & E \cdot EPS\,P & \Leftrightarrow & E & \\ & k_{-4} & & k_{-5[P]} & & k_{-6[EPSP]} & \end{array} \qquad \text{(Scheme 3)}$$

Although the intermediate decomposed under acid conditions to pyruvate and S3P, it was stable to rapid denaturing of the enzyme under mild basic conditions (neat triethylamine). In this way, microgram amounts of intermediate were actually isolated; it was decomposed to EPSP by the action of EPSP synthase. The tetrahedral structure was deduced from ^1H NMR, ^{31}P NMR, and ^{13}C NMR observations.[150] This conclusion was confirmed by the failure of attempts to demonstrate partial reactions expected on the basis of the enzyme-substrate complex possibility.[151] Evidence from the use of phosphonate inhibitors (see later) suggests tentatively that the tetrahedral intermediate (Figure 16A) has the R configuration at the chiral center of the side chain. More recently, evidence was obtained for the existence of two enzyme-intermediate complexes by ^{13}C NMR spectroscopy. The complete structures of these complexes are not yet known.[152].

2. Stereochemistry of the EPSP Synthase Reaction

The accepted reaction mechanism requires (1), the addition of S3P and a proton to the double bond of PEP to form the tetrahedral reaction intermediate, and (2) elimination of PO_3H_2- and a proton from the intermediate to form the double bond present in the *enol*pyruvyl moiety of EPSP. In each case, the addition or elimination could proceed with either a syn or anti stereochemistry. Thus, one possibility is for an initial anti addition followed by syn elimination (see Figure 17). This diagram assumes "bulk" labeling with all isotopes (i.e., 100% ^2H, 100% ^3H), and that an isotope effect in the elimination leads to exclusive loss of ^1H. By consideration of all combinations of stereochemical options, the results shown in Figure 17 are obtained.

Location of hydrogen isotopes in the product was determined by conversion of the —CH_2-containing EPSP to a compound with a chiral methyl; the latter was analyzed in the usual way.[153] In practice, the EPSP was first converted to CHA (either using whole cells of *K. pneumoniae* or isolated CHA synthase). In the analytical procedures, a catalytic hydrogenation known to proceed specifically with syn stereochemistry was carried out generating chiral methyl groups as shown in Figure 18. The reduced material was then converted chemically to racemic lactate. Thus, starting with EPSP (figure 18A), the two lactates, Figure 18B and C, would be produced. Reaction of aliquots of these lactates in separate experiments with L- and D-lactate dehydrogenase yielded pyruvate samples, Figure 18D and E,

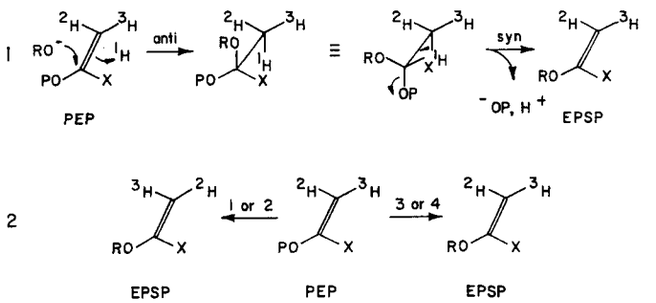


FIGURE 17. Stereochemical possibilities for EPSP synthase. X = —COOH, and RO^- is the ion from S3P. (Line 1) Consequences of anti addition and syn elimination. The equivalence symbol, ≡, is used to indicate a conformational change. (Line 2) Summary of all stereochemical possibilities. 1 = syn addition and elimination; 2 = anti addition and elimination; 3 = syn addition, anti elimination, 4 = anti addition, syn elimination.

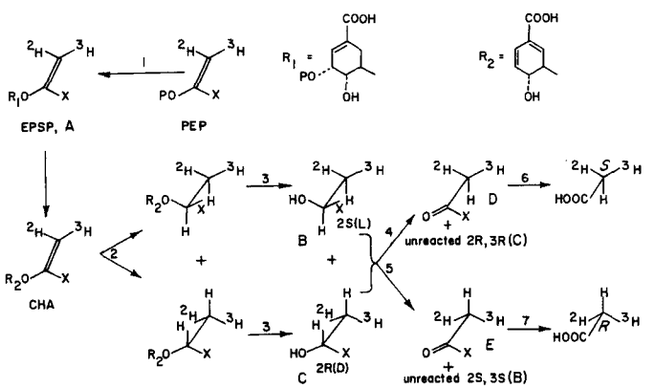


FIGURE 18. Stereochemical analysis of EPSP synthase. The process is assumed to start (reaction 1) with the conversion of (E)−[3-^{2}H, ^{3}H]-PEP to EPSP by anti addition, syn elimination (see also Figure 17, line 1); EPSP is further converted to CHA (see text). Reaction 2, catalytic hydrogenation with syn stereochemistry; reaction 3, conversion to racemic lactate; reaction 4, treatment with L-lactate dehydrogenase; reaction 5, treatment with D-lactate dehydrogenase; reaction 6, oxidation of pyruvate to acetate after removal of unreacted C; reaction 7, as 6, after removal of unreacted B.

with a methyl group as the sole center of chirality. After removal of unreacted lactate, the pyruvates were converted to acetate for final chirality analysis.

The formidable technical problems in carrying out this work were overcome by two groups of investigators.[154-156] It should be noted that the account given here is simplified and ignores these technical problems:

1. Unlike ^{2}H, which is readily available at close to 100 atom%, ^{3}H is used at tracer levels
2. Although there is an observed primary kinetic isotope effect in the deprotonation step of the EPSP synthase reaction, it does not lead to exclusive elimination of ^{1}H as illustrated here
3. Proton exchanges with solvent can occur and must be minimized

The observed stereochemical relationship is that from (E)-[3-^{2}H, ^{3}H] PEP the EPSP and CHA have E configuration in the ^{2}H, ^{3}H containing side chain. This implies either an anti/syn or syn/anti situation for the addition/elimination process. The anti/syn possibility is diagrammed in Figure 18 (it is not known to which "face" of the PEP double bond the addition takes place).

3. Amino Acids Involved in the Active Site

A surprisingly large number of amino acids have been implicated in the active site of EPSP synthase, either for catalytic activity or for binding of substrates and inhibitors. They include arg, glu, his, and lys; a cys residue is proximal to the active site, but is not essential for either catalysis or substrate binding. A clear picture of their roles must await determination of the three-dimensional structure of the enzyme. This section includes some work with the inhibitor, glyphosate (see Section II.F.7).

a. ARGININE

A possible role for a guanidino group in the active site of EPSP synthase from *K. pneumoniae* was suggested by an observed inhibition with the arg-directed reagent, phenylglyoxal.[157] More detailed experiments utilized homogeneous *Petunia hybrida* EPSP synthase, with either phenylglyoxal or 4-hydroxyphenylglyoxal.[158] The possibility that a cys residue was being modified by these reagents was ruled out. The modification of arg residues followed pseudo-first-order kinetics. A partial protection against the inhibition was obtained with S3P and complete protection with a mixture of S3P and glyphosate (no protection with glyphosate alone). Of three reactive arg residues, arg-28 and arg-131 were identified as two that became labeled when labeled phenylglyoxal was used. These two arg residues are conserved in all EPSP synthases thus far studied. Interestingly, the arg-28 residue is close to the lys-23 also identified as a reactive component in the petunia enzyme.

b. CYSTEINE

When EPSP synthase of *E. coli* was reacted with the thiol reagent, DTNB [5,5′-dithiobis-(2-nitrobenzoic acid)], two of six cys residues were modified, and with significant loss of enzyme activity. Disulfide bridges were not present. In the presence of S3P and glyphosate, one of the two cys residues reacted with DTNB but without loss of catalytic activity. By reaction of the DTNB-inactivated material with KCN, the -SH groups were converted to -SCN. Enzyme containing two such groups had activity comparable to that of native enzyme. Hence, it was con-

cluded that these reactive cys residues were not required for catalytic activity. The reactive cys residues were cys-288 and cys-408; the latter was protected from reaction with DTNB by S3P and glyphosate.[159] The *E. coli* enzyme was hardly affected at all by iodoacetamide and *N*-ethylmaleimide, but the enzyme from *K pneumoniae* was inactivated by the latter reagent.

c. GLUTAMATE

The EPSP synthase of *E. coli* was inactivated by 1-ethyl-3-(dimethylaminopropyl)carbodiimide in the presence of glycine ethyl ester. Complete inactivation required modification of four carboxyl groups, with only one, identified as glu-418, being essential for activity. Inactivation was prevented by preincubation of the enzyme with S3P and glyphosate and the binding of glyphosate to the modified enzyme was less than with the native protein. Although the exact role of this glutamate residue was not established, it is apparently located at or close to the glyphosate binding site.[160]

d. HISTIDINE

The his-directed reagent, diethylpyrocarbonate, inactivated EPSP synthase from *E. coli*, and the inactivation was prevented by the presence of substrates. The pH inactivation rate data implied involvement of a group with $pK_a = 6.8$; this was within the range expected for the nonprotonated form of his. Although complete inactivation required modification of four his residues, only one was critical for catalytic activity. This essential his residue reacted with diethyl pyrocarbonate twice as rapidly as did the nonessential his residues. Since the diethyl pyrocarbonate inactivated enzyme could still bind S3P and glyphosate, the his residue was assigned a role in catalysis but not in binding. This essential his may be the nucleophile required to form the tetrahedral reaction intermediate.[161]

e. LYSINE

EPSP synthase from *E. coli* was inactivated by pyridoxal phosphate, and substrates prevented inactivation. This and earlier work suggested a role for lys at the active site, with the usual Schiff base binding for pyridoxal phosphate. With 90% inactivation, about 1 mol pyridoxal phosphate was incorporated per mole of enzyme. Following borohydride reduction and tryptic digestion, a peptide was obtained containing the lys residue at position 22.[162] Confirmation of a role for lys-22 was obtained by site-directed mutagenesis at this position, using the *Petunia hybrida* enzyme as expressed in *E. coli*. In the petunia enzyme, lys-23 is equivalent to lys-22 in the sequence of the *E. coli* enzyme. Of three replacements, ala, arg, and glu, for lys-23, only the enzyme containing arg had any activity. The purified enzyme with arg-23 was less sensitive than wild-type enzyme (lys-23) to inactivation by pyridoxal phosphate. The arg-23 modified enzyme, but not the catalytically inactive ala-23 enzyme, was able to bind S3P. It is thus likely that a strongly cationic group, specifically lys-23 (petunia) or lys-22 (*E. coli*), has an important role in substrate binding.[163] The amino acid sequence around lys-22(23) and arg-28 is conserved in the bacterial, fungal, and plant enzymes. This region is presumably an important component of the active site.

4. EPSP Synthase from E. coli

The EPSP synthase from untransformed *E. coli* ATCC 14948 was purified (843-fold) to homogeneity as a monomer with $M_r = 55,000$ (exclusion chromatography) and 49,000 (polyacrylamide gel).[164] Moreover, the *aroA* gene was cloned and inserted into a multicopy plasmid. The transformed strain, *E. coli* AB2829/pKD501, overproduced the EPSP synthase about 100-fold, and was resistant to glyphosate. The purified, homogeneous enzyme could be obtained in milligram amounts by a procedure requiring only a 50-fold purification.[165] The monomeric enzyme had M_r ranging from 42,000 to 49,000.

The availability of the cloned *aroA* gene and milligram quantities of pure enzyme allowed the use of a combined amino acid and nucleotide sequencing strategy to derive the complete amino acid sequence of the polypeptide. The amino acid sequence to leu-44 was directly determined (with three unidentified residues). The predicted sequence from the DNA results corresponded to a polypeptide of 427 residues with calculated $M_r = 46,112$; this value agrees well with those just given for the isolated enzyme.[166] The EPSP synthase from *S. typhimurium* had the same length as that from *E. coli* and there was 11% divergence in the amino acid sequence.[167]

The *E. coli* EPSP synthase is apparently the first of the main trunk enzymes to be crystallized. The crystals ($1.5 \times 0.4 \times 0.4$ mm) were suitable for medium-resolution single-crystal X-ray diffraction studies.[168] It is hoped that avenues are opening for determination of the three-dimensional architecture of this and other SHK pathway enzymes.

In both *E. coli* and *S. typhimurium*, the expression of the *aroA* gene is linked to that of the *serC* gene.[169,170] The latter encodes for 3-phosphoserine aminotransferase, an enzyme in the serine biosynthetic pathway and that which is also required for pyridoxine biosynthesis. These two genes form an unusual "mixed function operon" with their products being involved in two separate pathways. The significance of the association is not immediately clear. One possibility is an involvement in enterobactin biosynthesis, for which both serine and chorismate are required. Enterobactin is an iron-binding compound (see Section III.D.3) and iron starvation causes derepression of several genes for proteins concerned in iron acquisition. The grouping of a SHK pathway gene and a gene involved in ser biosynthesis may allow coregulation according to the iron status of the organism. Strains of *S. typhimurium* with deletion mutations in the SHK (*aroA*) and purine biosynthetic pathways have been constructed as possible candidates for a per os live vaccine against typhoid fever.[171] Vaccine strains of *S. typhimurium* with stable mutations in *aroC* alone or in *aroC* and *aroA* together have also been constructed.[172]

5. EPSP Synthase from Other Bacteria

A purification of EPSP synthase from *K. pneumoniae* ATCC 8724 (>700-fold) yielded material at least 95% homogeneous[147] and from *K. pneumoniae* 62-1, a 3300-fold purification gave homogeneous protein stabilized by "Polybuffer 74" containing 1 m*M* dithiothreitol.[173] The enzyme consisted of a single polypeptide chain, M_r = 32,400 (Sephadex G-100 chromatography) and 42,900 (SDS-gel electrophoresis). A random sequential kinetic mechanism was likely for the forward reaction.

When the *aroA* locus of *Bordetella pertussis* was cloned into *E. coli*, the gene was expressed and complemented an *aroA* *E. coli* mutant. The gene sequence of 1329 nucleotides was determined, including the stop codon TGA, leading to a polypeptide of 442 amino acid residues. The deduced M_r was 46,688. There was considerable homology in the DNA sequence with those of other microorganisms.[174]

A partial purification (187-fold) of EPSP synthase from *B. subtilis* gave enzyme estimated to be 70% pure. Monovalent cations were required for activity, with NH_4^+ being the most effective.[175] This cation activation was in contrast to an anion requirement described for the *K. pneumoniae* enzyme. The NH_4^+-activated *B. subtilis* enzyme showed an increased sensitivity to glyphosate. Failure to recognize cation requirements for EPSP synthases in general may have given unreliable estimates of inhibition of glyphosate.

6. EPSP Synthases from Plants

Pea seedling EPSP synthase was purified (3150-fold) to electrophoretic homogeneity by a modification of the process used for the *E. coli* enzyme.[176] The monofunctional enzyme was monomeric with M_r = 50,000 (denatured) and 44,000 (gel permeation chromatography). The forward reaction was strongly inhibited by glyphosate.

With dark-grown seedlings of *Sorghum bicolor*, a 1300-fold purification was described in which three isozymes were apparently copurified. Two enzymes (II and III) were resolved from the purified preparation by high resolution anion exchange HPLC; small amounts of the third isozyme (I) were occasionally present. All three of these isozymes were detected in partially purified extracts of dark-grown *S. bicolor* shoots. Values for M_r ranged from 51,000 to 57,000. The EPSP synthase activity was inhibited by metals such as Cu^{2+}, Pb^{2+}, and Zn^{2+}, suggesting the need of —SH group(s) for activity.[177]

EPSP synthase activity of petunia was expressed at high levels in flowers. The developmental and tissue-specific regulation of the gene has been studied.[178] Other work with plant EPSP enzymes is discussed in the following section.

7. Inhibition of EPSP Synthase by Glyphosate

The mechanism of action of herbicides has enormous economic significance as well as intrinsic interest. Two general reviews on the action of herbicides on amino acid biosynthesis have appeared recently and should be consulted for more detail than can be given here.[179,180] Much attention has been focused on the widely used material, glyphosate [*N*-(phosphonomethyl)-glycine]. This broad-spectrum, nonselective, postemergence herbicide is present in Roundup® weed killer and is the subject of *The Herbicide Glyphosate*;[181] this book refers to some 7000 publications. The projected worldwide sales of glyphosate for 1986 indicated that it would become the first "one-thousand million dollar herbicide molecule".[181] In view of the economic significance of glyphosate, an enormous volume of work has been carried out. Only some of the recent developments of concern in biochemistry are discussed here.

The major target of this herbicide is EPSP synthase, although other enzymes of the SHK pathway are also inhibited. For example, in *Candida maltosa*, DAHP synthase-Tyr and DHQ synthase (see Sections II.A and II.B) are inhibited by glyphosate concentrations in the millimolar range, while EPSP synthase is inhibited in the micromolar range.[43]

Plants and cultured plant cells treated with glyphosate contain high levels of SHK. In plants, the glyphosate concentration to allow SHK to accumulate was lower in the light (0.01 m*M*) than in the dark (0.04 m*M*).[182]

With *K. pneumoniae*, growth in the presence of glyphosate leads to the excretion of S3P into the medium.[183] The effect of glyphosate on *E. coli*, *P. aeruginosa*, and *B. subtilis* has been compared in a study of the "energy-drain" effects of glyphosate in organisms with a diversity of regulatory pathways for the SHK pathway. Unlike the first two organisms, *B. subtilis* did not accumulate S3P.[62] Under similar conditions with glyphosate *C. maltosa* and other yeasts accumulated SHK and S3P in cells in roughly equimolar concentrations and excreted free SHK into the medium.[43,44]

The value of this herbicide would be even greater if crop plants resistant to glyphosate could be produced by genetic engineering. By transformation with vectors which lead to overproduction of wild-type EPSP synthase, or by production of enzymes with an altered affinity for glyphosate, glyphosate resistance has already been obtained in petunia, tobacco, and tomato plants. For more detail, other reviews should be consulted.[184,185] By May 1989, field tests of glyphosate-tolerant tomatoes had been carried out for 2 years and for 1 year on a rape variety (canola) producing an oil. The first field tests on glyphosate-tolerant cotton and soybeans were then being undertaken.[186]

The two methods for obtaining glyphosate tolerance are

1. Overproduction of EPSP synthase. In *E. coli* cells with a multicopy plasmid carrying *aroA* (*E. coli* 594/pMON4), overproduction of EPSP synthase (5- to 17-fold) gave an at least 8-fold increased tolerance to glyphosate.[187] This effect was also observed in another overexpression strain (*E. coli* AB 2829/pKD501) and in *K. pneumoniae*.[187,188] Plant cell cultures of *Petunia hybrida* have been made tolerant to glyphosate and show elevated levels of EPSP

synthase. Furthermore, as previously noted, glyphosate-resistant transgenic petunia plants have been obtained. This work represented a breakthrough to establishing selective herbicide resistance in economically important crop plants.[189]

2. Modification of EPSP synthase. A glyphosate-resistant phenotype of *S. typhimurium* was obtained by ethylmethane sulfonate mutagenesis.[190] The mutant *aroA* gene from *S. typhimurium* was cloned in *E. coli* and conferred resistance to that organism. The responsible mutation was a change from pro to ser at position 101. It resulted from a single point mutation in the aroA gene encoding EPSP synthase.[167] Expression of the mutant *S. typhimurium aroA* gene was obtained in tobacco plants and such transformed plants showed an increased tolerance to glyphosate.[191]

A glyphosate resistant EPSP synthase was isolated from a glyphosate-resistant strain of *K. pneumoniae;* immunological data indicated that the mutant and wild-type enzyme had similar, but not identical, antigenic determinants.[183]

In addition to cell cultures of *P. hybrida,* cell cultures of several plants (*Corydalis sempervirens, Daucus carota, Petunia hybrida*) which have been made resistant to glyphosate all show elevated levels (10- to 40-fold) of EPSP synthase. Elevated levels of EPSP synthase were also present in cultured cells of the glyphosate-tolerant strain of *Nicotiana tabacum* 17. In this case, the level of activity was about twice that of a glyphosate-sensitive line (W 38).[97] With *C. sempervirens,* glyphosate-resistant cultured cells overproduced EPSP synthase some 43-fold; the enzyme was purified (ca. 60-fold) to homogeneity. Enzymes from adapted and nonadapted cells showed no significant differences in kinetic properties and had identical values for M_r = 44,700.[192] The overproduction of EPSP synthase in glyphosate-tolerant cell suspension cultures of *C. sempervirens* was apparently not based on amplification of the corresponding gene. There was probably a reduction in the rate of proteolytic degradation of the enzyme.[193]

The glyphosate-resistant enzyme from *P. hybrida* MP4-G cells was also purified (82-fold) to homogeneity. It was a monofunctional monomer with M_r = 49,000 to 55,800. The specific activity of the MP4-G enzyme was 10- to 20-fold higher than that of enzyme from nonresistant MP4 cells. The two enzymes did not differ in their glyphosate sensitivity in kinetic experiments.[194]

A more efficient isolation of the latter enzyme was achieved by a small-scale high-performance chromatographic procedure. Initially, chromatography on a Pharmacia HR 10/10 MonoQ quaternary ammonium anion-exchange column was used. Several active fractions were obtained (charge modified derivatives or isozymes?); those of high activity were further chromatographed on a Bio-Rad TSK phenyl 5PW hydrophobic interaction HPLC column. On analytical isoelectric focusing, high activity fractions showed a single band.[195]

In a further development, the cDNA for mature petunia EPSP synthase, lacking a chloroplast transit sequence (see a later section), has been cloned into the plasmid pMON342 and then expressed in *E. coli*. Large-scale isolation of the enzyme from *E. coli* SR481/pMON342 (grown under high-density fermentation conditions) yielded 220 mg of enzyme from 1.2 kg of cells (112-fold purification). This material was essentially homogeneous on polyacrylamide gel electrophoresis (PAGE), with M_r = 44,000; this value was in agreement with that deduced from the DNA sequence.[196]

Similar cDNA clones encoding EPSP synthase in tomato and *Arabidopsis thaliana* have been isolated and sequenced.[197] The deduced amino acid sequences for the enzyme are very similar for all of the plants and amount to 445 residues. There is considerable homology with enzymes from bacteria, less so with enzymes from fungi. A complete exon-intron map of the EPSP synthase genes of tomato and petunia has been obtained and the expression of EPSP synthase has been analyzed in detail.[184]

In the plant EPSP synthases, a transit peptide sequence is involved, containing from 72 to 76 amino acids.[198] This sequence is necessary since the EPSP synthase is synthesized as a cytoplasmic precursor and then translocated to the stroma of plastids where cleavage to mature enzyme occurs.[199] It is of interest that a transit peptide sequence from *Petunia hybrida* can efficiently target a bacterial (*E. coli*) EPSP synthase to the chloroplast compartment. The transit peptide sequence is removed in the chloroplast to yield a fully active, glyphosate-resistant enzyme.[200]

Plasmid vectors that provide resistance against glyphosate have been constructed and have found applications in transforming genetically unlabeled laboratory, wild, and industrial strains of *S. cerevisiae*.[201]

Despite this volume of work, the mode of action of glyphosate is not entirely clear. Kinetic studies with the fungal EPSP synthase of the *arom* complex indicated a sequential kinetic mechanism with S3P binding first (see also earlier work on the tetrahedral intermediate). The inhibition by glyphosate was due to a specific and reversible interaction with the E-S3P complex to form a dead-end complex. Glyphosate did not bind to the free enzyme.[202] Similarly, kinetic studies of a partially purified enzyme from cells of *Nicotiana silvestris* were also consistent with competition between PEP and glyphosate for the E-S3P complex.[203]

On the basis of such observations, the ionic form of glyphosate was proposed as a transition-state analog of a protonated PEP carbanion.[180] This idea was developed before the unequivocal identification of the tetrahedral reaction intermediate (see an earlier section); the carbanion has not been identified as a component of the presently accepted reaction mech-

anism. Clarification of the precise inhibitory mechanism must await further observations.

A recent study has detailed the ^{13}C, ^{15}N, and ^{31}P NMR chemical shifts of glyphosate over the pH range -1.0 to $+13.0$.[204] Over the range from pH 0.0 to 9.0, glyphosate exists as a spirocyclic, rotationally restricted conformation with H bonds between oxygen anions (carboxylate, phosphonate) and ammonium ion protons. This conformation may be approximated by Figure 19A (the original paper must be consulted for precise descriptions of bond angles and distances). Below pH 0.0 (with net charge of $+1$) or above pH 11.5 (net charge of -3) the conformation is that of a linear structure. The P-C-N and N-C-C distances have increased and formation of a H bond between a phosphonate anion and ammonium proton would require the unlikely distance of about 2.8 to 2.9 Å (Figure 19B).

These observations were extended to the "dead-end" enzyme-S3P-glyphosate complex. Chemical shift perturbations were observed for the bound glyphosate and were attributed to changes in bond angles about the phosphonate, amine, and carboxyl moieties. The glyphosate was present in the protonated, ammonium ion form, but the carboxyl group was not protonated. The bound conformation was equivalent to that which would be observed with glyphosate itself in solution at pH 10.1 or pH -0.8. This conclusion indicates a generally linear conformation for the bound glyphosate.[204]

Two phosphonate analogs, Figure 20A and B, of the tetrahedral intermediate were synthesized. The R diastereoisomer

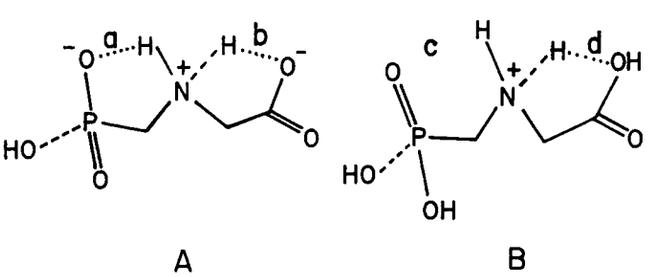


FIGURE 19. Conformations of glyphosate. A = approximate conformation over the pH range, 0.0 to 9.0; B = approximate conformation at pH <0.0 and pH >11.5, and for glyphosate bound to EPSP synthase. Bond lengths: a = 2.00 Å, b = 1.75 Å, c = 2.90 Å, d = 1.98 Å.

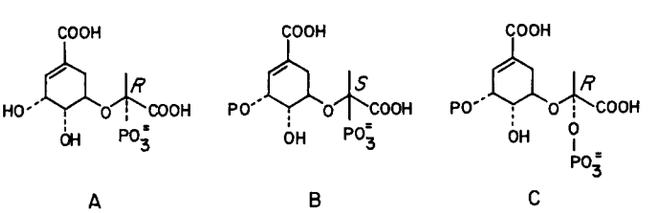


FIGURE 20. Phosphonate analogs of the EPSP synthase tetrahedral intermediate (A, B) and the configuration of the actual reaction intermediate, C.

(Figure 20A) showed K_i of 15 n*M* for *P. hybrida* EPSP synthase and was the most potent inhibitor so far reported. It was bound to the enzyme more tightly than glyphosate by an order of magnitude and more tightly than the S diastereoisomer (Figure 20B) by two orders of magnitude. Assuming that these inhibitors were bound with the phosphonate group in the site normally occupied by phosphate, it was predicted that the actual reaction intermediate, Figure 20C, had R chirality at the side chain chiral carbon.[205]

G. Chorismate Synthase

The conversion of EPSP to CHA is catalyzed by EC 4.6.1.4 [O^5-(1-carboxyvinyl)-3-phosphoshikimate phosphate lyase], hereafter abbreviated as CHA synthase. Since CHA is a cyclohexadiene structure, this enzyme introduces the second of the three double bonds which are necessary for formation of the benzene ring. The reaction is, formally, a 1,4 elimination of phosphate, and it occurs with loss of the C-6H_R proton. However, if a concerted mechanism is involved (as would be anticipated), elimination of phosphate ion and the C-6 proton would be expected to occur from the same side of the ring plane, and this would require elimination of the H_S proton.[3] Loss of the H_R proton would be possible, however, with a two-step process involving an enzyme linked intermediate (see Figure 21A).

An alternative possibility required a phosphate migration to form a transient intermediate, Figure 21B.[39] A subsequent anti elimination of phosphate would then be possible with the original H_R of EPSP. (Note that by the vagary of the sequence rule this proton is H_S in the intermediate B of Figure 21.) A stereospecific synthesis of the intermediate, termed *iso*-EPSP, was

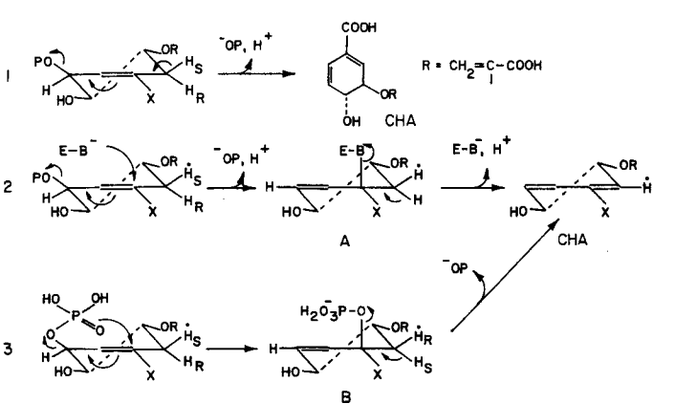


FIGURE 21. Reaction mechanism for CHA synthase. X = —COOH. (Line 1) Anticipated loss of H_S proton from EPSP for 1,4 elimination of phosphate. (Line 2) Loss of H_R proton by two-step process with enzyme-linked intermediate. (Line 3) Loss of H_R proton by two-step process involving phosphate group migration.

carried out from (−)-QA.[206] The *iso*-EPSP did not function as a substrate for *N. crassa* CHA synthase, but did behave as a competitive inhibitor (K_i comparable to that of K_m for the normal substrate). Although it was possible that productive binding of the *iso*-EPSP did not take place from the exterior of the protein, the two-step process with the enzyme-linked intermediate apparently remains the most likely explanation.

While it is manifest that the reaction involves no change in the oxidation state of the substrate, CHA synthase from various sources is unusual in requiring a reduced flavin cofactor, $FMNH_2$ or $FADH_2$, for catalytic activity. The enzymes thus far described fall into one of two groups:

1. In *E. coli* and *Pisum sativum* the CHA synthase is monofunctional and has no ability to generate the reduced flavin by oxidation of NADH or NADPH [e.g., F + NAD(P)H + $H^+ \rightarrow FH_2$ + NAD(P)$^+$; this reaction is described as "diaphorase" activity in several papers]. Thus, assay is possible only when reduced flavin is supplied and under anaerobic conditions. A rapid, simple assay for CHA synthase under these conditions has been developed.[207]
2. In *N. crassa* and *B. subtilis* the enzyme is bifunctional and contains a flavin reductase activity. The *N. crassa* reductase enzyme has absolute specificity for FMN and NADPH, and is apparently covalently associated with CHA synthase. The enzyme from this organism can utilize exogeneously supplied $FMNH_2$ or $FADH_2$ (see Section 11.K).

1. CHA Synthase from Bacteria

For *E. coli*, a plasmid carrying the *aroC* gene was constructed and the DNA sequence was determined. An ORF encoding a polypeptide chain of 357 residues was present; the calculated M_R for the protein was 38,183. The enzyme was purified (658-fold) to electrophoretic homogeneity from the overproducing strain, *E. coli* AB2849/pGM602 (yields 1.16 mg from 20 g cells). The first 30 amino acids from the N-terminal were sequenced and the sequence corresponded to that predicted from the DNA sequence.[208] The native enzyme was a tetramer. The purified enzyme had no detectable ability to utilize NADH to reduce flavin. Immobilized cells of *K. pneumoniae* 62–1 have been used for the sequential synthesis of CHA.[209]

2. CHA Synthase from N. crassa

In *N. crassa*, this enzyme is encoded by the *arom3* gene; nonhomogeneous preparations were obtained prior to 1975.[24] More recently, the *N. crassa* enzyme was found to be very susceptible to proteinases present in the organism. A purification (3210-fold) using "a full antiproteinase strategy" gave electrophoretically homogeneous material (0.42 mg from 90 g of freeze-dried powdered mycelium). This material had a specific activity (32 U/mg protein) more than 10-fold greater than that of earlier preparations. The native protein was a tetramer, with the subunit having M_r = 50,000. This enzyme, unlike that from *E. coli*, had an intrinsic ability to reduce flavin (specifically FMN) using NADPH. Presumably the presence of this second activity accounts for the larger size of the *N. crassa* enzyme compared to that of *E. coli* (M_r = 50,000 vs. 38,183). Although the complete amino acid sequence has not been determined, three peptides obtained by proteolysis showed significant homology with stretches of the *E. coli* sequence toward the C-terminal.[208]

3. Plant Enzyme

Prior to 1986, there were no reports of CHA synthase in any plants. This enzyme has now been detected in pea seedlings and has been shown to be located, to a major extent, in the chloroplast.[210]

H. Chorismate Mutase

1. Reaction Mechanism

The enzyme for the conversion of CHA to PPA (EC 5.4.99.5, chorismate pyruvate mutase) is abbreviated here as CHA mutase. In *E. coli* and some other bacteria, CHA mutase exists as a single bifunctional polypeptide with either prephenate dehydratase (EC 4.2.1.51; abbreviation, PPA dehydratase) or prephenate dehydrogenase (EC 1.3.1.12; abbreviation, PPA dehydrogenase). The CHA mutase-PPA dehydratase combination is encoded by the *pheA* gene and provides the precursor for phe; it has been referred to as P-protein. In a similar fashion, the CHA mutase-PPA dehydrogenase is encoded by the *tyrA* gene and leads to the tyr precursor; it has been termed T-protein. The bifunctional enzymes are discussed in Section 11.J. However, in dealing with the reaction mechanism of CHA mutase, some results obtained with bifunctional proteins are included here.

The C-1 to C-6 double bond rearranges during the CHA mutase reaction; PPA contains double bonds in positions 2,3 and 5,6 (assuming original SHK numbering). The reaction is a somewhat unusual example of a [3,3]-sigmatropic Claisen rearrangement in metabolism. Although it is often cited as the only such example, 4-amino-4-deoxy-CHA and –ICHA undergo similar rearrangements. Although a pericyclic mechanism was also proposed for PEP-phosphomutase,[211] it now appears there may be an alternate explanation.[212] As a first approximation, the CHA mutase rearrangement may be diagrammed as shown in Figure 22. Only (−)CHA functioned as a substrate for *E. coli* CHA mutase-PPA dehydrogenase and CHA mutase of *Streptomyces aureofaciens;* the (+) enantiomer did not inhibit the *E. coli* preparation.[213]

The reaction mechanism of this enzyme has received considerable attention. An important step was to determine the overall stereochemistry by tracing the fate of the $=CH_2$ group

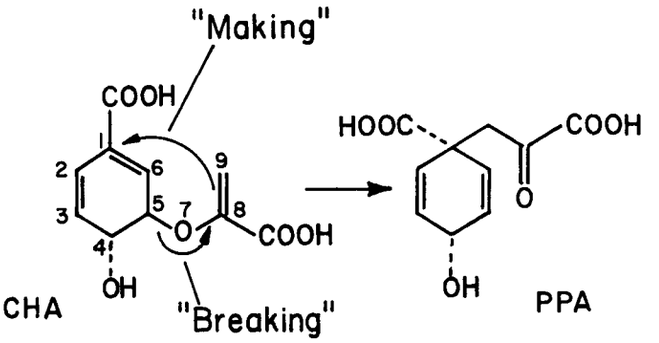


FIGURE 22. Conversion of CHA to PPA by CHA mutase. The "breaking" and "making" bonds are identified.

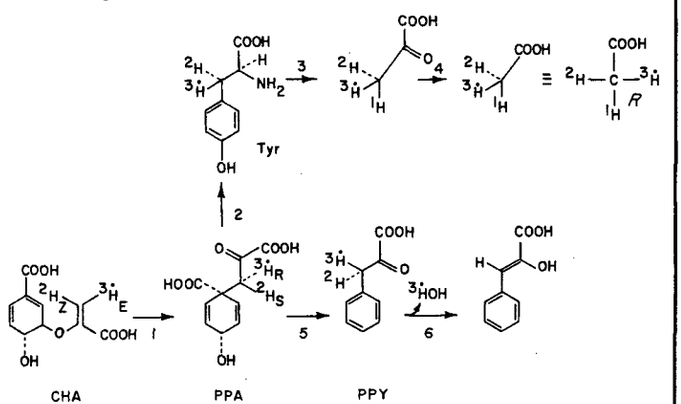


FIGURE 23. Stereochemistry of CHA mutase. Results from the two groups of investigators are shown here. The substrate CHA was prepared either chemically or from (E)-[3-^{2}H,^{3}H]-PEP via EPSP synthase. The enzymes and reactants were as follows: (1) chorismate mutase either as isolated bifunctional enzyme with PPA dehydrogenase or contained in whole bacterial cells. (2) PPA dehydrogenase, tyr aminotransferase. (3) Tyr phenl lyase. This reaction is known to proceed with retention of configuration. (4) 1, Lactate dehydrogenase and NADH, 2, $K_2Cr_2O_7/H^+$. (5) pH <6.0. (6) PPY tautomerase. This reaction is known to remove H_R to solvent.

hydrogens in the *enol*pyruvyl side chain of CHA during conversion to the —CH_2-group of the PPA side chain. The question is whether the H_R proton of the latter group derives from H_E or H_Z of the CHA methylene. In one set of experiments, stereospecifically labeled CHA prepared enzymatically from E-[3-^{2}H, ^{3}H]PEP (via EPSP synthase, see Section 11.F.2) was fed to *E. coli* cells. Cell protein was hydrolyzed for phe and tyr isolation, and tyr was degraded via pyruvate to acetate for chirality assay.[155,156] As shown in Figure 23, CHA mutase converts the H_E proton of CHA to H_R of PPA.

Similar results were obtained with either chemically synthesized, racemic CHA or enzymatically synthesized material.[214,215] In this work, conversion to PPA was carried out with *E. coli* CHA mutase-PPA dehydrogenase. The PPA was converted nonenzymatically (pH <6) to PPY and the latter was reacted with PPY tautomerase. The latter enzyme was known to release the H_R proton of PPY to the solvent (see Figure 23).

Although the enzyme catalyzed rearrangement rate is more than 1 million times faster at the active site than free in solution, the nonenzymatic process still occurs readily (e.g., pH 7.5, 60°C, 25 min). The nonenzymatic rearrangement proceeds with the same stereochemistry as does the enzyme catalyzed process.[216]

From these stereochemical observations a distinction can be made between two alternate structures for the reaction transition state. In this rearrangement, it is necessary to incorporate partial bonds between C-5 and O-7 in CHA (breaking bond) and between C-1 and C-9 in PPA (making bond). The transition state contains six atoms (5 C and 1 O) in a cyclic arrangement and can exist as either a chair- or boat-like conformation. As shown in Figure 24, the observed stereochemistry is consistent only with the utilization of a chair-like transition state. The transition state is actually somewhat asymmetrical, with the breaking C—O bond (about 0.145 nm) being significantly shorter than the making C—C bond.[217]

The same conclusion was reached earlier through the use of transition state structural analogs as inhibitors.[218] As a chair conformational analog, *exo*-6-hydroxybicyclo[3,3,1]nonane-1-*exo*-3-dicarboxylate (Figure 24A) was used and as an analog for the boat conformation, *exo*-6-hydroxybicyclo-[3,3,1]nonane-1-*endo*-3-dicarboxylate (Figure 24B). Of these two materials, only the *exo*-COOH isomer (Figure 24A) was an inhibitor.

To obviate the possibility that Figure 24A functioned via an energetically less favorable (less populated) conformation, an adamantane structure was tested with a "locked" chair-chair conformation. 6-Hydroxyadamantane-1,3-dicarboxylate (Figure 24C) had K_i similar to that of the *exo*-COOH nonane, indicating that the stable chair-chair conformation of the latter was responsible for the observed inhibition. From other work it was concluded that both the C-6 —OH and C-1 —COOH groups were important for binding to the active site.

An involvement of the —COOH groups of CHA and possibly the —OH group was also suggested since CHA mutase from *Streptomyces aureofaciens* Tu 24 was inhibited by in-

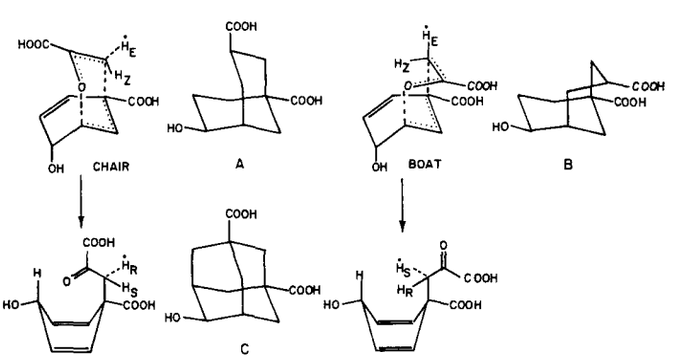


FIGURE 24. Transition state structures and inhibitors for CHA mutase. Since CHA mutase converts the H_E proton of CHA to H_R of PPA, only the chair transition state is likely.

organic anions and by simple aromatic acids (2-,3-, and 4-hydroxybenzoates, and most effectively 4-hydroxyisophthalate).[219] However, it now appears that the —OH group is not involved, at least for *E. coli* CHA mutase-PPA dehydrogenase.[220] This conclusion stems from two facts. First, the racemic methyl ether of CHA (i.e., with $—OCH_3$ replacing —OH) was a reasonable substrate with $k_{cat}/k_{uncat} = 2.0 \times 10^4$ (compare 2.3×10^6 for (−)-CHA). Second, the rearrangement of CHA without the C-4 —OH group (Figure 25A), was accelerated at least 100-fold by CHA mutase-PPA dehydrogenase (Figure 25, Reaction 1). This work was somewhat complicated by the thermal instability of this analog; in buffered ^2H_2O solution ($p^2H = 7.2$) at 30°C, the half-lives for rearrangement to Figure 25B, and elimination (aromatization to benzoate) were 3.5 and 8.0 min, respectively (compare for (−)-CHA, 935 and 8400 min). However, the presence of enzyme enhanced the extent of rearrangement relative to elimination. With [S]:[E] = about 90, an approximately 47% enhancement of rearrangement over the thermal process was obtained. This value increased with the increase in [E]; at [S]:[E] = 30, the enhancement was 100%. Thus, under these conditions of high enzyme concentration, the compound was completely metabolized by the enzyme.

While the conditions just described are unphysiological and far removed from the usual [S]:[E] ratios of conventional enzymology, nevertheless they do establish that the C-4 —OH group is not required for catalysis. Enhancement of the rearrangement did not occur with thermally denatured enzyme so the effect was clearly due to a catalytic protein.

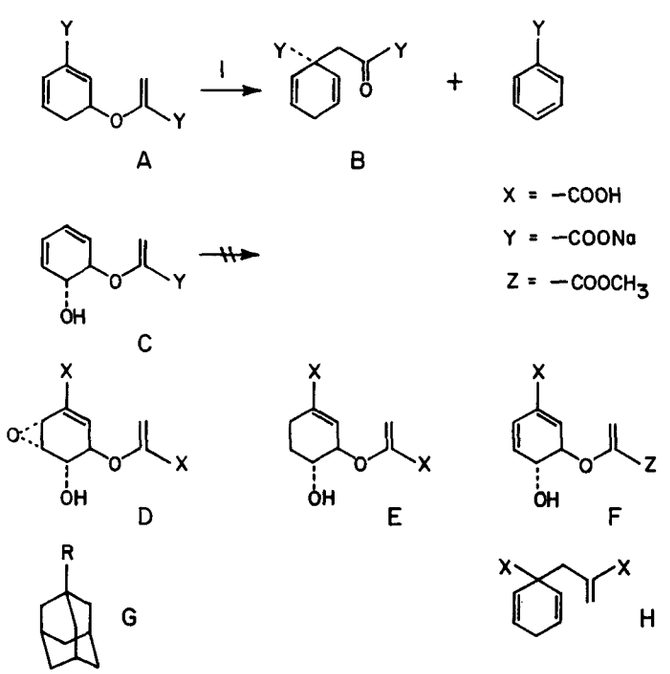


FIGURE 25. Alternate substrates and inhibitors for CHA mutase. (See text for descriptions.)

Since a CHA analog lacking the —COOH group (Figure 25C) was not a substrate, it was concluded that the only functional groups required for CHA mutase catalyzed rearrangement are the two carboxyl groups. It is still possible that H-bond formation with the C-4 —OH group enhances stabilization of the transition state and contributes to the rate acceleration observed with CHA itself.

The epoxide, Figure 25D, and 5,6-dihydro-CHA, Figure 25E, were competitive reversible inhibitors; in addition, the methyl ester, Figure 25F, was neither a substrate nor an inhibitor.[221] Citrate and L-2-hydroxyglutarate inhibited CHA mutase-PPA dehydratase, and it was proposed that these two materials were also transition state analogs. The closest relationship was seen with L-2-hydroxyglutarate; perhaps significantly, this was the more effective of the two inhibitors.[222]

Utilization of adamantane derivatives has been further explored in more recent work, using *K. pneumoniae* CHA mutase-PPA dehydrogenase. The 1-phosphonate derivative (Figure 25G), R = $—PO_3^{2-}$, was more effective than those previously described. For substituents at the 1 position, the inhibitory activity decreased in the following sequence:

$$—PO_3^{2-} \gg —P(OCH_3)O_2^- > —COO^- > —CH_2COO^- > —SO_2^- > —SO_3^-$$

The activity of the phosphonate derivative seems to indicate that binding does not necessarily require —OH and —COOH groups.[223] PPA has been known as an inhibitor for some time and Figure 25H, [2-(1-carboxy-1,4-dihydrobenzyl)acrylic acid] was also effective.

Other potential inhibitors have been examined, including one in which a nitronate moiety was substituted for the carboxyl derived from PEP. Contrary to expectation, it did not prove to be a good inhibitor. Figure 26 summarizes the results with materials ranging from poor to very effective with respect to inhibitory action. The *endo* diacid (3-*endo*-8-*exo*-8-hydroxy-2-oxabicyclo-[3.3.1]non-6-ene-3, 5-dicarboxylate; Figure 26A) is, so far, the most potent inhibitor known for CHA mutase. The adamantane inhibitors affect both of the activities of the CHA mutase-PPA dehydrogenase enzyme.[224,225] A cycloheptadiene ring analog has also been synthesized and is a moderate inhibitor of *E. coli* CHA mutase-PPA dehydrogenase.[226]

Old and new evidence relating to the mechanism of CHA mutase has been considered in detail.[227] Secondary tritium isotope effects were determined for the C-1 to C-9 making bond and the C-5 to O breaking bond of CHA in both the enzymatic and nonenzymatic process. The substrates were either [9-^{3}H, 7-^{14}C]CHA or [5-^{3}H, 7-^{14}C]CHA. For the nonenzymatic conversion (pH 7.5, 60°C), $k_H/k_T = 1.149$ for bond breaking (with 5-^{3}H-labeled material) and 0.992 for bond making (with 9-^{3}H-labeled material). It was concluded that the C—O bond

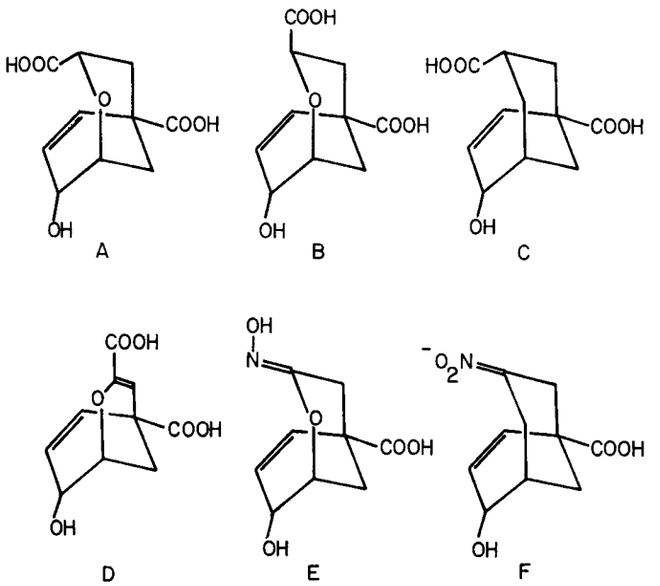


FIGURE 26. Transition state analogs as CHA mutase inhibitors. A is, so far, the most potent inhibitor known; B, C, and D were less tightly bound; and E and F were poor inhibitors. Note that C and F are carbacyclic compounds.

was about 40% broken at the transition state, but the new C—C bond was not then detectably formed.

On the other hand, for the enzymatically catalyzed rearrangement, isotope effects for bond making (C—C) and bond breaking (C—O) were unity within experimental error. It was suggested that the isotope effects were suppressed in the enzymatic process and that a rate-limiting transition state preceded an isotopically sensitive rearrangement step.[227] The secondary tritium isotope effect at C-4 was investigated with [4-^{3}H, 7-^{14}C]CHA. A small inverse k_H/k_T value of 0.96 was observed. In addition, a solvent isotope effect, k_{H_2O}/k_{T_2O} = 2.2 was established for the enzymatic reaction. For the nonenzymatic process the solvent isotope effect was 0.95.[228]

A further fact to be taken into account is that in water and methanol solutions, the favored conformer of CHA is that with the —OH group at C-5 and pyruvyl group at C-4 in the pseudoequatorial arrangement. For the reaction to occur, both of these groups must become axial. The conformational equilibrium is, however, rapid on the NMR time scale.[229]

Four *a priori* mechanistic possibilities have been appraised in the light of the mass of data.[228] Although the very detailed arguments cannot easily be summarized, the favored interpretation is a heterolytic cleavage of CHA between C-5 and the ether oxygen atom (see Figure 27). An enzyme nucleophilic group is proposed to initiate attack at C-5 (after conformational isomerization of CHA to the diaxial structure). Not the least of the attractions of this mechanism is that it "gives the enzyme something more to do than merely bind to the appropriate conformation of the substrate. . .". It is also in harmony with

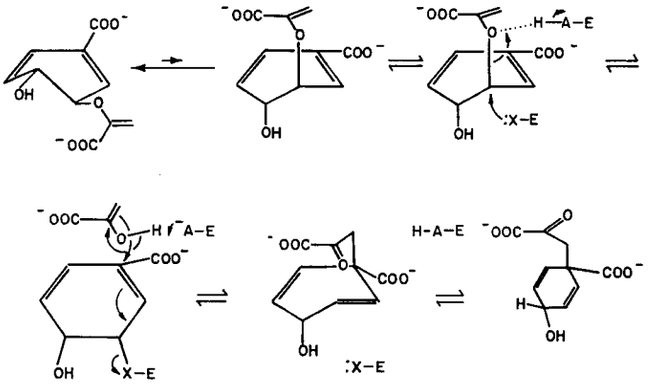


FIGURE 27. Reaction mechanism for CHA mutase. Possible binding to the enzyme by the C-4 OH group and —COO^{-} groups is not indicated.

the observed tritium isotope effects. Moreover, the substantial solvent hydrogen isotope effect is indicative of protonic movement as required by general acid and/or general base catalysis in the proposed mechanism.

No suggestions were put forward as to the identity of the postulated nucleophile X and H-A. Early work with the CHA mutase-PPA dehydratase enzyme clearly indicated a role for a lys residue. The only other residues for which experiments suggest a role in the mutase reaction are tyr and trp. Unfortunately, there have been apparently no explorations of a role for his. It should be noted that the same bifunctional enzyme showed a velocity for the mutase reaction (in acetate/phosphate/borate) buffer which increased only slightly over the pH range from 6.5 to 9. It was suggested that enzyme residues ionizing in the pH range from 5 to 9 were unlikely to be involved at the catalytic site.[222]

Groups at Cornell and Indiana Universities have jointly reported on detailed studies of the nonenzymatic rearrangement of CHA and many related compounds.[230] The polar medium (water) in which the rearrangement is normally carried out was found to be a significant factor for ease of reaction. The work generally supports the conclusion that the C—O bond undergoes substantial dissociation before the new C—C bond is formed. There is some disagreement between the two groups as to a dipolar vs. radical structure for the transition state. Also considered was the fact that the rearrangement is accompanied by elimination so that CHA, for instance, gives rise to both PPA and 4-hydroxybenzoate. The two processes do not appear to be linked mechanistically, but the two respective transition states must be very similar. As with rearrangement, the elimination process involves extensive C—O bond cleavage as the initial event.

Racemic and (−) forms of (Z)-9-methyl-CHA (Figure 28A)[20] underwent an uncatalyzed Claisen rearrangement at pH 7.5 and 30°C with a half-life of 5.7 h forming "methylprephenate" (Figure 28B) and the decarboxylation product (Figure 28C).

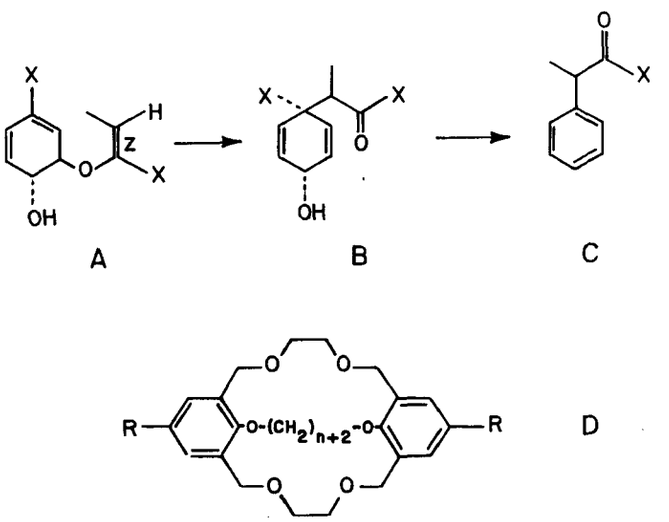


FIGURE 28. Use of 9-methyl-CHA in CHA mutase reaction, and systems designed to mimic the active site. X = —COOH. For the D structures, n = 1 and R = $—CH_3$; with n = 3, R = H, $—CH_3$, —Cl, —Br, or $—OCH_3$.

The (Z)-9-methyl-CHA was a limited substrate for CHA mutase. The ratio $k_{catalyzed}/k_{uncatalyzed}$ was 4.2×10^4 (compare 1.5×10^6 for (−)CHA). Evidence was obtained for a chair-like transition state in these enzymatic reactions.

Molecular systems have been constructed which, it was hoped, would mimic the properties of the active site of CHA mutase. This work remains in a preliminary phase; however, binding of substituted *p* phenols ($R\text{-}C_6H_4\text{-}OH$, R = H, Cl, NO_2) and 2,2,2-trifluoroethanol to cryptands of the type shown as Figure 28D was observed.[231]

In another direction, a monoclonal antibody was generated that catalyzed the rearrangement of CHA to PPA. The antibodies were elicited to the transition state analog, Figure 26A (the most potent inhibitor yet known). The antibody apparently provided an environment which was complementary to the conformationally restricted transition state. The rate acceleration, $k_{antibody\ catalysis}/k_{thermal\ rearrangement}$, was 1×10^4 (10°C, pH 7.0) compared to a value of 3×10^6 for enzymatic catalysis under the same conditions.[232] Another antibody was constructed using the same transition state analog as the hapten. In this case the rate acceleration was 1×10^2 (25°C, pH 8.0). Only (−)-CHA, and not the (+) enantiomer was a substrate for this antibody catalysis, confirming the "enzyme-like nature of this tailored antibody binding site."[233]

2. Monofunctional CHA Mutase in Microorganisms

a. BACTERIA

CHA mutase, purified to homogeneity (2200-fold) from *Streptomyces aureofaciens* (prior to 1975) had the useful property of being remarkably heat stable. Following inactivation at 100°C, reactivation to between 30 and 100% of original activity was possible. The M_r for the native enzyme (probably a trimer, subunit M_r = 14,500) was in the range 51,000 to 63,000.[234]

Wild-type *P. aeruginosa* contained a low level of a monofunctional CHA mutase activity which was product inhibited by PPA, as well as a bifunctional CHA mutase-PPA dehydratase (P protein) as major component. The monofunctional enzyme was designated CHA mutase-F. In a leaky, phe-requiring mutant, *P. aeruginosa* PAT 1051, the CHA mutase-F was the only mutase activity present.[29,235] CHA mutase-F was detected in *Erwinia* sp., in *Serratia marcescens*, and perhaps surprisingly, in *S. typhimurium*.[235]

A dissociable CHA mutase from *Brevibacterium flavum* had unique properties. It was strongly inhibited by phe and tyr, and the inhibition was overcome by trp. It contained two nonidentical components A and B. A was a tetramer of subunits of M_r = 55,000 and B a dimer of subunits with M_r = 13,500. Moreover, A was a bifunctional protein also containing DAHP synthase activity.[236,237] CHA mutase activity was present in a number of spore-forming organisms of the order *Actinomycetales*. Inhibition was observed with tyr (all cases) and trp (most cases).[238]

b. YEASTS

The monofunctional CHA mutase of *S. cerevisiae* was subject to feedback inhibition by tyr, but was strongly activated by trp in a unique control mechanism. The wild-type *ARO7* gene was cloned and sequenced; an ORF of 771 bp encoded a polypeptide of 256 amino acid residues (including initiating met) with M_r = 29,750. There was no homology between the yeast CHA mutase and the N-terminal CHA mutase activities of the two bifunctional enzymes of *E. coli*. Mutant CHA mutases unresponsive to tyr and trp, exhibited a 10-fold increase in enzyme activity. The mutation resulted from a single base pair exchange causing a thr to ile substitution in the C-terminal portion of the enzyme.[239]

The yeast, *Pichia guilliermondii*, yielded a mutant with a complete loss of sensitivity to phe and a considerable loss of sensitivity to trp.[240] In addition, in the absence of trp, the CHA mutase activity of the mutant was sevenfold higher than that of wild-type. The changes are similar to those just noted in *S. cerevisiae*. A partially purified CHA mutase (M_r = 63,000) from *Candida maltosa* was inactive unless trp was present. Isozymic forms were not observed. It was similar to a preparation from *Hansenula henricii*.[241]

c. CHA MUTASE IN PLANTS

It has been recognized for some time that plants frequently contain more than one form of CHA mutase. Most attention has focused on two species: CHA mutase-1, a chloroplast enzyme, activated by trp, and inhibited by phe and tyr; and CHA mutase-2, a cytosolic enzyme, which is not regulated by ar-

omatic amino acids. Although early evidence for a third, unstable isozyme had been largely discounted, a recent paper reported the separation of three forms of CHA mutase from *Solanum tuberosum* by DEAE cellulose chromatography in the Pipes buffer system;[242] other buffers did not yield this level of resolution. Two of the forms, CHA mutase-1A and CHA mutase-1B, were activated by trp and inhibited by phe and tyr. The third form, CHA mutase-2, was not affected by the aromatic amino acids. The 1A and 1B forms both had M_r = 60,000 and appeared to be artifacts generated by the anionic buffer. Previously, an inducible, aromatic amino acid-sensitive form of potato CHA mutase was purified (1033-fold);[243] it was not examined for homogeneity.

Purification of CHA mutase isozymes from mung bean seedlings and sorghum was achieved prior to 1975.[24] Electrophoretic homogeneity has apparently been obtained only with CHA mutase-1 of sorghum (1389-fold purification). This regulated isozyme was possibly a dimer with native M_r = 56,000, and subunit M_r = 36,5000. The purified CHA mutase-2 isozyme (1018-fold, M_r = 48,000) still showed a minor component on electrophoresis.[244] These two isozymes from *Sorghum bicolor* were immunologically distinct. It is not yet known whether the two have evolved from a common ancestor or whether the two genes have evolved independently.[245]

Many plants contain the two isozymic forms.[246] Although in some cases the unregulated cytosolic species had not been detected, the existence of this isozyme in one of the exceptions, *Solanum tuberosum*, has now been documented.[22,242] The ratio of mutase activities was also examined in response to wounding. The CHA mutase-1:CHA mutase-2 ratio was 2:1 in fresh and 9:1 in aged tuber discs. In contrast to the high activity of CHA mutase-1 in aged discs, CHA mutase-2 comprised the majority total activity in green leaves (CHA mutase-1:CHA mutase-2 = 1:4). There is apparently an organ-specific regulation of the expression of the two isozymes. This work provided further evidence for the general existence of the "dual-pathway" arrangement.

Two CHA mutase isozymes were partially purified from *Nicotiana sylvestris*. CHA mutase-1 (M_r = 52,000) was the major fraction in protoplastids of suspension cultures or chloroplasts of green leaves; it was subject to feedback inhibition by tyr. CHA mutase-2 (M_r = 65,000) was a cytosolic enzyme uninhibited by tyr.[247,248] A detailed consideration of the differential allosteric regulation of the two isozymes indicated that the chloroplast enzyme, CHA mutase-1, was concerned with aromatic amino acid biosynthesis, while the cytosolic isozyme, CHA mutase-2, was involved with secondary metabolite biosynthesis.[249]

I. Isochorismate Synthase

This important enzyme is formally classified as an isomerase (EC 5.4.99.6); the reaction, however, involves addition and elimination of $-OH^-$ (see Figure 29). It is only recently that

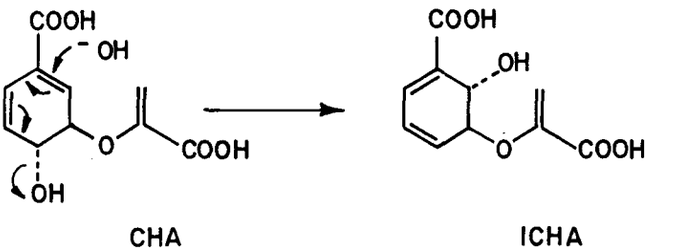


FIGURE 29. Addition-elimination reaction mechanism for ICHA synthase.

the situation was clarified with respect to *entC*, the gene encoding ICHA synthase in *E. coli*. It now appears that four genes required for enterobactin synthesis (Section II.D.3) are linked in an iron-regulated polycistronic operon, *entCEBA(P15)*; P15 is an uncharacterized protein, with M_r = 15,000. The complete nucleotide sequence of *entC* has been determined recently; an 1173 bp ORF encodes for a 391-residue protein, M_r = 42,917.[250] There is considerable homology with two other CHA utilizing proteins, *trpE* and *pabB*.[250,251]

Prior to 1990, the polypeptide product of *entC*, ICHA synthase, had been only partially purified, using *K. pneumoniae* as the source. Some improvements in the standard procedure were reported.[252] ICHA synthase has now been purified (12-fold) to homogeneity from an overexpression *E. coli* strain (K38/pGP1-2/pJLT5053).[253] A yield of 11 mg was obtained from 3 l of cells. The subunit M_r was 43,000 in excellent agreement with the value predicted from the nucleotide sequence. The incoming hydroxyl group was shown to derive from water, rather than by intramolecular transfer from CHA.

J. Bifunctional Main Trunk Enzyme Activities

1. CHA Mutase-PPA Dehydratase of E. coli

Bifunctional CHA mutase-PPA dehydratase of *E. coli* was purified (250-fold) to electrophoretic homogeneity by use of a Sepharosyl-phe affinity column in the presence of 0.4 *M* NaCl (pH 8.2). NaCl was inhibitory to both enzyme activities and increased the sensitivity of the enzyme to phe inhibition.[254] This purification method has been used by many other investigators; there are apparently no reports of improved methods, for example, using overexpression strains. The *pheA* gene, encoding this bifunctional protein, has been sequenced, and specifies a protein of 386 residues with M_r = 43,111 (includes first met residue, but not formyl). There is some homology between the CHA mutase-PPA dehydratase and CHA mutase-PPA dehydrogenase enzymes.[54]

All possible classes of *E. coli* mutants have been obtained lacking either dehydratase activity, mutase activity, or both. In a detailed study, a strain of *E. coli* lacking CHA mutase-PPA dehydrogenase was first isolated and subjected to further mutagenesis (it was required that no mutase activity, other than

that associated with the dehydratase be present). It was possible to obtain a protein (>95% pure) lacking PPA dehydratase; the minimum M_r was 40,000 (similar to that of wild-type enzyme). Attempts to purify proteins with only PPA dehydratase, or with neither activity, were not successful since the proteins did not bind to the Sepharosyl-phe affinity column. The mutation to the dehydratase negative condition had little structural effect on the mutase site, consistent with the two sites being separate. A reactive cys (see later) was still present, and a thr residue was implicated in the dehydratase activity.[255]

The smallest size of the CHA mutase-PPA dehydratase is probably a dimer of two identical subunits (M_r = 43,000). At pH 8.2, a concentration-dependent self-association takes place, and a dimer and a tetramer coexist in a rapid reversible equilibrium. Further association to a higher polymeric form is also possible. At pH 7.4, the major species is probably an octamer. The addition of the feedback inhibitor, phe, or an increase in ionic strength also causes an increase of higher polymer forms at pH 8.2. A variety of effects (e.g., the allosteric kinetics, inhibitor binding) are probably all consequences of self-association and/or isomerization of the enzyme.[256,257]

The values of pK_M for CHA in a citrate/phosphate/borate buffer differed significantly from those obtained in acetate/phosphate/borate solutions, particularly in the pH 6 to 8 region. Further work showed that several di- and tricarboxylic acids were inhibitors and in a differential manner as shown below (see also Reaction Mechanism):

	% Inhibition	
	Mutase	**Dehydratase**
Citrate	28 (competitive)	5 (noncompetitive or uncompetitive)
cis-Aconitate	51 (partially competitive)	11 (competitive)
2-Hydroxyglutarate	38 (−)	82 (−)

− = not determined.

These results are consistent with the existence of two separate activity sites for CHA mutase and PPA dehydratase. Since all these inhibitors have an effect on both activities, there may be some interconnection between the sites.[222] There was no evidence for channelling of PPA from one site to the other.[258]

The effects of specific chemical modification of amino acid residues in purified CHA mutase-PPA dehydratase (*E. coli*) are as follows:

1. *Cysteine*. The cys-directed reagent, 5,5′-dithiobis(2-nitrobenzoate) (DTNB), inhibited the dehydratase activity completely at a ratio of 1.2 mol of —SH per mole of enzyme subunit but gave only a 5% inhibition of CHA mutase activity. Reaction of two further moles of —SH per mole of enzyme subunit raised the mutase inhibition to 30%. This reagent caused significant changes in the protein secondary structure (α-helical content increased from 25 to 28%), probably in the environment around one or more tyr residues. Addition of PPA (competitive inhibitor of the CHA mutase activity) to the modified enzyme decreased the helical content to that of native enzyme. Such addition also decreased or eliminated the reactivity of the —SH group with DTNB. The differential inactivation of the two activities provided further evidence for separate or slightly overlapping sites.[259] Similar results obtained with *N*-ethylmaleimide and tetrathionate led to the conclusion that there was no functional role for cys-SH at the mutase active site.
2. *Tyrosine*. Modification by tetranitromethane of 2 tyr residues per enzyme subunit again gave a differential inactivation; dehydratase activity was lost completely, but only 30% of mutase activity.[260]
3. *Lysine*. 2,4,6-Trinitrobenzene sulfonate gave complete inactivation of mutase activity, and only a 20% loss of dehydratase activity.[260]
4. *Tryptophan*. On reaction with dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium bromide, there was partial loss of both activities and a desensitization of both to inhibition by phe. The possible role of trp residues was unclear.[260]

In summary, all of these results indicated separate or only slightly overlapping sites for the two activities.

Four sulfoxide analogs of tetrahydro- and dihydro-PPA have been prepared. All behaved as modest competitive inhibitors of *E. coli* CHA mutase-PPA dehydratase with no significant differences in the affinity.[261] It is unlikely, therefore, that they bind as substrate analogs.

2. CHA Mutase-PPA Dehydratase from Other Organisms

The bifunctional enzyme from *Acinetobacter calcoaceticus* was purified to electrophoretic homogeneity, purification being 714-fold (CHA mutase) and 885-fold (PPA dehydratase). The native enzyme was a dimer (subunit M_r = 45,000); in the presence of phe or tyr, there was a twofold increase in M_r of the native enzyme. Phe was a relatively poor noncompetitive inhibitor of CHA mutase, and a strong inhibitor of PPA dehydratase activity. CHA mutase activity was feedback inhibited (competitively) by reaction product, PPA. PPA dehydratase, but not CHA mutase activity, was stimulated by high ionic strength which reduced the observed positive cooperative binding of PPA. Tyr activated the PPA dehydratase activity by lowering K_M for PPA (without affecting the cooperativity).[262,263]

A CHA mutase-PPA dehydratase bifunctional protein was present in *Neisseria gonorrhoeae*.[28] Both enzyme activities

were inhibited by phe and the PPA dehydratase was strongly activated by tyr.

3. CHA Mutase-PPA Dehydrogenase of E. coli

CHA mutase-PPA dehydrogenase was first purified to homogeneity (65-fold with respect to mutase activity) from *E. coli* JP232 in 1971.[264,265] The native enzyme was dimeric with M_r = 82,000. A homogeneous dimeric protein has been obtained from *E. coli* JP 2312 (a regulatory mutant), with specific activity threefold higher than that prepared earlier.[266] Kinetic data suggested a rapid-equilibrium, random mechanism with two dead-end complexes (E-NADH-PPA and E-NAD-HPP).[267] Another purification from *E. coli* JP2319 (contains the *tyrR* 370 and *trpS* 378 alleles) exploited the fact that growth with limiting amounts of trp gave the enzyme at levels of 50- to 100-fold those of wild-type strains. High glycerol concentrations (20 to 50% v/v) promoted stability.[268]

Recombinant DNA techniques were used to produce high yields of homogeneous CHA mutase-PPA dehydrogenase (up to 4% of cell dry weight). *E. coli* K12 was transformed with a multicopy plasmid, pKB45, to yield strain KB9397 which gave 55 mg of pure enzyme from 20 g of cells. Since plasmid loss from this strain occurred if tetracycline was omitted from the growth medium, a second strain, JFM30, with tyr prototrophy was constructed. The enzyme yield from this strain was comparable to that from KB9397 and the strain was used successfully over a 2-year period. Enzyme levels were 5000-fold higher than those of wild-type strains and 6-fold higher than those of regulatory mutants. The enzyme was identical to that from a *tyrR* strain.[269]

The *tyrA* gene has been sequenced and leads to a calculated length of 373 amino acid residues for CHA mutase-PPA dehydrogenase. The calculated M_r = 42,042 is consistent with those actually observed. *N*-Formyl met was removed following translation. In the first 54 amino acids of the *tyrA* gene product, 22 residues were identical with and 4 were similar to those of the *pheA* gene product. The N-terminal third of each protein contains the (common) mutase activity and the remainder either the PPA dehydrogenase or PPA dehydratase activity.[54] In addition to *tyrA*, the tyr operon contains a second structural gene, *aroF*, encoding DHA synthase-Tyr.

By *in vitro* mutagenesis of plasmid DNA containing the *tyrA* gene, followed by subsequent transformation of *E. coli* strains, six mutants were obtained producing a mutase positive-dehydrogenase negative enzyme and a protein lacking both activities. The loss of PPA dehydrogenase activity was apparently due to a marked reduction in its ability to bind NAD^+. The isoelectric point of this mutant enzyme was lower than that of wild-type enzyme, consistent with the alteration of a single amino acid residue concerned with NAD^+ binding. This alteration did not affect the interaction of the enzyme with CHA, PPA, and tyr. Definitive conclusions about the relationship of the active sites for the activities were not possible.[270] More recently, fragments of the *tyrA* gene have been engineered to express either CHA mutase or PPA dehydrogenase activities without the other.[271]

a. SITE RELATIONSHIPS FOR CHA MUTASE-PPA DEHYDROGENASE

Although there appears to be a close relationship between the two enzyme sites, the situation is not as straightforward as that for CHA mutase-PPA dehydratase. In early work, the experimental data for the time course of the overall reaction were compared to computer simulations derived from one- and two-site models. In connection with other observations, the two-site model was regarded as unlikely and the rather unusual conclusion was drawn that the two different reactions occurred at a single site.

This hypothesis was subsequently modified since malonate at low concentrations prevented reaction of the enzyme with PPA but not CHA; diethyl malonate, however, prevented binding of both substrates. It was then suggested that the two sites shared some common features and overlapped. More recent work on the end-product inhibition by tyr in the presence of NAD^+ supported this idea. Tyr at 100 μM inhibits both activities differentially — 95% for PPA dehydrogenase and a maximum of 45% for CHA mutase. Enzyme affinity for tyr was increased by NAD^+ and affinity for NAD^+ was increased by tyr. These effects resulted from enzyme aggregation with NAD^+ and the preferential binding of tyr to a tetramer. Derivatives of tyr substituted at position 3 were also inhibitory, 3-fluoro-DL-tyr more so than tyr. Tyramine, HPP, and D-tyr were poor inhibitors for the PPA dehydrogenase. It was concluded that for binding, the L configuration was necessary and both the α-COO^- and $-NH_3^+$ were required.[272]

Although CHA and adamantane derivatives were linear competitive inhibitors (both reactions) under Michaelis-Menten kinetic conditions, this was not so with lower substrate concentrations and/or higher inhibitor concentrations.[273] Moreover, positive cooperativity for substrate binding in the absence of inhibitors occurred with a larger range of substrate concentrations. Clearly, the enzyme does not exhibit properties of an allosteric protein. Addition of albumin activated both CHA mutase and PPA dehydrogenase and there was a change to Michaelis-Menten kinetics.

This welter of kinetic information supported a close spatial relationship between two interacting active sites. Further support was the finding of three unique cys-containing sequences in each subunit of *E. coli* CHA mutase-PPA dehydrogenase. One particularly important cys was essential for both activities.[268] PPA protected the enzyme from inactivation by DTNB and the combination of NAD^+ and tyr was most effective in reducing the rate of inactivation and in protecting the enzyme activities. These results were consistent with two enzyme activities located at closely situated and interacting sites. It will be useful to study the two separate activities which can pre-

sumably now be obtained from the separated gene components of *E. coli*.[271]

4. DHQ Dehydratase-SHK Dehydrogenases from Plants

It has been recognized for some time that DHQ dehydratase-SHK dehydrogenase were usually associated together in higher plants. Such a bifunctional enzyme was isolated earlier from the moss, *Phycomitrella patens* and purified (about 1300-fold) to homogeneity. The two enzyme activities were associated with a single polypeptide (M_r about 48,000). In some cases the bifunctional enzyme occurred as isozymic forms.[274] One complex was purified from deveined spinach leaves (60 μg from 8 kg leaves). The preparation gave "essentially one protein band" on SDS-PAGE. However, under nondenaturing conditions, a pattern of four bands was obtained; these were believed to be charge isomers and did not differ significantly in M_r (59,000 to 67,000, depending on electrophoretic conditions). The two enzymatic activities were clearly associated with a single protein located in the stroma.[275]

Isozymic forms of SHK dehydrogenase were found in chloroplast extracts of pea seedlings. The enzyme was encoded by the nuclear gene *Skdh 1*.[276] Other workers found that SHK dehydrogenase of pea chloroplasts (probably a bifunctional complex) yielded two major bands on polyacrylamide gradient gel electrophoresis.[277] One band had M_r of about 60,000; the other had M_r of about 110,000. Both consisted of several sub-bands apparently due to a time-dependent disaggregation. These molecular size variants of SHK dehydrogenase were possibly degradation products of a labile, multiprotein complex closely related to the *arom* protein (see Section II.L).[278]

A definitely bifunctional protein was purified (6500-fold) to homogeneity from pea seedlings.[279] The enzyme was monomeric with M_r = 59,000. This value was close to the sum of the subunit M_r values of the two separable enzymes from *E. coli* — DHQ dehydratase = 29,000 and SHK dehydrogenase = 32,000 (Section II.C and II.D.1). Thus, the plant bifunctional enzyme may have been formed by the fusion of two genes, similar to those that can be separated in *E. coli*. Three isozymic forms existed, two of which were chloroplastidic. Other workers have also observed an extraplastidic SHK dehydrogenase in pea plants; both intra- and extraplastidic activities were regulated by light intensity and the availability of mineral nutrients.[278]

A different situation was found in extracts from corn seedlings. A DHQ dehydratase-SHK dehydrogenase ("DHQase 1") was accompanied by a DHQ dehydratase-QA dehydrogenase ("DHQase2").[280] The enzymatic activity for QA required NAD and was strongly activated by the presence of SHK. Either the two activites of "DHQase2" were associated as a complex or as a bifunctional polypeptide (for QA dehydrogenase, see Section III.J.2.a).

5. Other Polyfunctional Enzymes

In addition to the *arom* complexes (Section II.L), certain polyfunctional enzymes in *B. subtilis* are discussed separately (Section II.K). A bifunctional DAHP synthase-CHA mutase occurs in *Brevibacterium flavum*. Purification gave an apparently homogeneous enzyme with M_r = 250,000; an −SH group was essential for DAHP synthase, but not for CHA mutase activity.[236] The complex subunit nature of this preparation is discussed in Section II.J.2.a).

K. Polyfunctional Enzymes in *B. subtilis*

It is convenient to discuss separately certain polyfunctional enzyme complexes of *B. subtilis*. In this organism, genes encoding biosynthetic enzymes tend to be more highly clustered than is the case in *E. coli* and the *aro* and *trp* genes may be part of a "supra operon".[281]

Between 1972 and 1978, some enzymes of the SHK pathway in this organism were studied using strain WB 2802 (a prototrophic derivative of *B. subtilis* 168).[282] A bifunctional DAHP synthase-CHA mutase was purified to homogeneity (160-fold) as a single protein; M_r of monomer = 38,500. It was concluded that both enzyme activities resided on a single polypeptide chain. Various polymers, up to a tetramer, could be formed. DAHP synthase activity was sensitive to feedback inhibition by PPA or CHA. This bifunctional protein had the remarkable ability to activate SHK kinase. The latter enzyme was obtained during chromatography with only a trace of activity. Full kinase activity was restored on mixing with the DAHP synthase-CHA mutase complex.

On mild proteolysis of the bifunctional protein, CHA mutase activity was removed, leaving a protein with only DAHP synthase. The data suggested that the CHA mutase active site was identical to the feedback inhibition site for DAHP synthase. In addition, under these conditions, the ability to activate SHK kinase was lost. There appeared to be two distinct sites for CHA mutase activity and for activation of SHK kinase, and these sites were apparently located in close proximity on the polypeptide chain.

In summary, this organism contained a trifunctional enzyme complex of DAHP synthase, SHK kinase, and CHA mutase, with the first and third of these activities being located on a single, bifunctional polypeptide chain.

Another trifunctional complex contained two of the remaining SHK pathway enzymes (DHQ synthase and CHA synthase) and, in addition, a NADPH-dependent flavin reductase catalyzing the following reaction

$$NADPH + H^+ + FMN\ (FAD) \rightarrow NAD^+ + FMNH_2\ (FADH_2)$$

The CHA synthase activity was purified (about 139-fold) to electrophoretic homogeneity and had M_r = 24,000 (SDS-gel electrophoresis). It required for activity the NADPH-dependent

flavin reductase, flavin (FMN or FAD), NADPH (NADH was inactive), and Mg^{2+}. The flavin reductase, also purified to homogeneity, had M_r = 13,000 (SDS-gel electrophoresis).

The *B. subtilis* DHQ synthase was purified as a complex in association with the two enzymes just described (CHA synthase and NADPH-dependent flavin reductase). The DHQ synthase was only active in association with the CHA synthase. However, as just implied, the flavin reductase could be separated from the complex which then retained DHQ synthase activity. Both NAD^+ and Co^{2+} were required for DHQ synthase activity.

These results agree with the observation that single-step mutants lacking CHA synthase activity also lacked DHQ synthase activity. However, single-step mutants lacking DHQ synthase activity had normal levels of CHA synthase activity. The three enzymes, DHQ synthase, CHA synthase, and NADPH-dependent flavin reductase, were located on separate polypeptide chains, but associated together to form a trifunctional enzyme complex.

There appears to have been little further work on this interesting system. Clearly, gene cloning and sequencing would be very helpful in unravelling this complex situation. It is encouraging that a gene amplification of a unit containing, *inter alia*, the SHK kinase structural gene (*aroI*) has now been reported. In strains NMM13 and NMM14 the SHK kinase enzyme activity was four times higher than that of the original strain, NA64.[283]

Although it was stated earlier that *B. subtilis* contained a bifunctional DAHP synthase-CHA mutase, this is probably true only for certain strains such as 168. In the wild-type Marburg strain, the enzymes are monofunctional.[284] Both of these enzymes have been partially purified and have identical M_r of 180,000 (tetrameric). The monofunctional enzyme is inhibited by PPA only; the bifunctional by both PPA and CHA. To account for the formation of the bifunctional DAHP synthase-CHA mutase, the following explanation was proposed. Each subunit of the monofunctional enzyme type has two distinct domains — a compact one with the active site, and a smaller, more exposed area containing a site for the feedback inhibitor, PPA. CHA does not bind at this site. However, mutagenesis leads to alteration of the binding site (change of a single amino acid?), now allowing the binding of both CHA, and preferentially of the transition state intermediate for the reaction, CHA → PPA. That is to say, the bifunctional enzyme arose by artificial generation as a result of mutation.

The significance of these enzyme complexes for regulation is not clear. It should also be noted that very different results have been obtained with *Bacillus licheniformis*. In this organism, the three SHK pathway enzymes just discussed were easily separated and did not form complexes.

L. The *arom* Complexes

In some organisms, the five enzymes catalyzing the conversion of DAHP to EPSP are specified by gene clusters, and the enzymes are present as a complex, pentafunctional polypeptide. The polypeptide is referred to as the *arom* protein and has been the subject of a general review.[285] These complexes have been studied in fungi, particularly *N. crassa* and *A. nidulans*, in yeasts such as *S. cerevisiae* and *Schizosaccharomyces pombe*, and in *Euglena gracilis*.

In general, the *arom* polypeptides and the five separable enzymes of *E. coli* are homologous. Thus, the complexes may have arisen by fusion of ancestral *E. coli*-like genes. If so, at least some of the functional regions of the *arom* proteins should have maintained a degree of structural autonomy, and might be isolated by limited proteolysis. In a detailed study of the proteolysis of the *arom* protein from *N. crassa*, a stable polypeptide of M_r = 68,000 was obtained.[286] The best proteolytic agents were trypsin and subtilisin, but chymotrypsin and papain were also used. A short, exposed loop in the *arom* polypeptide was apparently particularly susceptible to proteolysis. The isolated fragment resisted further proteolysis.

The M_r = 68,000 fragment of the original *arom* protein contained DHQ dehydratase and SHK dehydrogenase activities; as demonstrated in this article, these are the penultimate and final activities at the C-terminal end of the *arom* protein. Even after denaturation with SDS or 8 *M* urea, the fragment peptide refolded and regained some of the SHK dehydrogenase activity. It is striking that these two enzyme activities also occur together as bifunctional protein in some yeasts, the moss *Phycomitrella patens*, and in several plants (see Section II.J). Other proteolytic fragments have been obtained from the *N. crassa arom* protein but are not yet well characterized.[285] A truncated peptide with EPSP synthase and DHQ dehydratase activities was obtained from the *A. nidulans arom* protein.

In *S. cerevisiae*, the *ARO1* gene encodes the *arom* pentafunctional polypeptide. The expression of this gene was under the "general control" mechanism, with a response to limitation in the amino acid supply. The length of the mRNA molecule (after removal of the poly A tail) was around 5000 nucleotides. In the nontranslated 5′-flanking sequences of the gene, there were multiple copies of the hexanucleotide, TGACTC. This short, repeated DNA sequence is characteristic of a number of co-regulated genes under general control and functions as a binding site for the regulatory GCN4 activator protein (see also work on the *ARO3* gene product, DAHP synthase-Phe).[287]

The individual *S. cerevisiae* genes and enzymes are as follows: *ARO1C*, DHQ synthase; *ARO1E*, DHQ dehydratase; *ARO1D*, SHK dehydrogenase; *ARO1B*, SHK kinase; and *ARO1A*, EPSP synthase. The entire nucleotide sequence (4767 bases, including the stop codon TAG) was determined and corresponded to a polypeptide of 1588 amino acid residues with a calculated M_r = 174,555.[288] This value is close to that of 159,698 for the sum of the masses of the five separable enzymes of *E. coli*. The total number of amino acids for the sum of the *E. coli* enzymes is 113 smaller than for the *S. cerevisiae* protein. Many of the extra residues in the *S. cere-*

visiae protein occur in regions linking the catalytic domains. On comparison of *E. coli* and *arom* protein sequences, clear homologies were found between the sequence of each *E. coli* enzyme and a region of corresponding length in the *arom* protein. The first 392 residues were homologous with the *E. coli* DHQ synthase; thereafter, the enzyme order was EPSP synthase, SHK kinase, DHQ dehydratase, and, at the C-terminus, SHK dehydrogenase. Observed homologies ranged from 38% for EPSP synthase to 21% for DHQ dehydratase. Features of the individual enzymes of the *S. cerevisiae arom* protein are

1. *DHQ synthase*. There are two very highly conserved subdomains (residues 100-213 and 258-387) compared to the *E. coli* enzyme. One of these regions includes the βαβ nucleotide-binding fold.
2. *EPSP synthase*. This domain, located between residues 404 and 866, is well conserved. The homology to the *E. coli* enzyme is 38% and to that of *A. nidulans*, 55%; there are two very well-conserved subdomains. There is a highly conserved cys (cys-853 in *S. cerevisiae*) in all sequences examined. Although pro 101 is apparently important in the bacterial enzymes (glyphosate resistance in *S. typhimurium* involves the change, pro → ser), it is replaced by phe in *S. cerevisiae* (position 505) and in *A. nidulans*. It appears that pro cannot be an essential feature in glyphosate sensitivity.
3. *SHK kinase II*. This enzyme, located from residue 887 to 1059, shows less homology in comparison with *E. coli*. There is one well-conserved region (895-909) which has sequence homology with ATP binding sites in some other enzymes (phosphofructokinase and adenylate kinase).
4. *DHQ dehydratase*. Lys-1227 is likely involved in imine formation, and a his residue for proton abstraction is probably his-1198. This residue is also conserved in the corresponding *A. nidulans arom* protein domain.
5. *SHK dehydrogenase*. Although the *E. coli-S. cerevisiae* homology is 25%, the *A. nidulans* SHK dehydrogenase has diverged substantially (15% homology with *E. coli* and 27% homology with *S. cerevisiae*).

In *N. crassa*, the five genes encoding the *arom* protein are *arom2*, DHQ synthase; *arom9*, DHQ dehydratase; *arom1*, SHK dehydrogenase; *arom5*, SHK kinase; and *arom4*, EPSP synthase. Reinterpretation of early genetic evidence indicates that the activities are located on the polypeptide in the same sequence as in *S. cerevisiae*.[286] At least part of the *arom* cluster gene of *N. crassa* has been cloned into a λ-phage vector and was expressed in *E. coli*. The insert was 4.0 kb, and thus smaller than the size expected for the complete gene (between 4.5 and 4.6 kb). A low level of (biosynthetic) DHQ dehydratase activity was present. The absence of part of the gene may have changed the tertiary structure of the protein, and hence lowered the expected specific activity for the enzyme.[289]

The *arom* protein of *N. crassa*, purified to electrophoretic homogeneity (730-fold with respect to DHQ synthase activity), was a dimer of two identical subunits, each with M_r = 165,000. To obtain a protein of this size, purification had to be carried out rapidly in the presence of proteinase inhibitors. There was a single Zn^{2+} per subunit, and this metal (and NAD) was required for DHQ dehydratase activity. This Zn^{2+} requirement is interesting, since the *E. coli* DHQ synthase probably requires Zn^{2+} *in vivo* rather than Co^{2+}.

The *arom* locus of *A. nidulans* was cloned in an *E. coli* bacteriophage vector. Analysis of 6.5 kb of unique sequence revealed a single ORF of 4812 bases (including TAA as a stop condon). The inferred M_r of the pentafunctional polypeptide was 175,101 (1603 amino acid residues). There was considerable homology (from 15 to 36%) with the individual *E. coli* enzymes.[288,290-292] The physical positions of the DNA sequences for the *arom* polypeptide were determined and subfragments were expressed in appropriate *E. coli aro* mutants. The *E. coli* and *A. nidulans* enzymes may have arisen by divergent evolution from common ancestral sequences; the *A. nidulans arom* locus probably derived by multiple gene fusion.[293]

The *aro3* gene from *Schizosaccharomyces pombe* was cloned into the *E. coli* plasmid pBR322. The gene cluster specified five enzymatic activities in the same order as in *N. crassa* (different from that of *S. cerevisiae*).[294]

The *Euglena gracilis arom* protein was purified (about 2000-fold) and estimated to have M_r = 249,000.[295]

III. BRANCHES FROM THE MAIN TRUNK COMPONENTS

The many branching possibilities occurring along the SHK pathway are examined under the following headings: A, from pre-SHK intermediates and SHK; B, from S3P, EPSP, and CHA; C from PPA; and D, from ICHA. An overview schematic figure for each category is identified in each heading.

A. Branches from Pre-Shikimate Intermediates and Shikimate (Figure 30)

1. The Role of DAHP and DHQ

a. mC_7N UNITS DERIVED FROM 3-AMINO-5-HYDROXYBENZOATE

Branching from DAHP or DHQ can give rise to either 3-hydroxy-5-aminobenzoate, or to 3-aminobenzoate. These compounds are the precursors to a particular metabolic moiety, found in many antibiotics, etc., with seven carbon atoms and one nitrogen atom. In particular, this unit is a characteristic feature of the ansamycins. These compounds have a long aliphatic ansa bridge of polyketide origin joining two positions

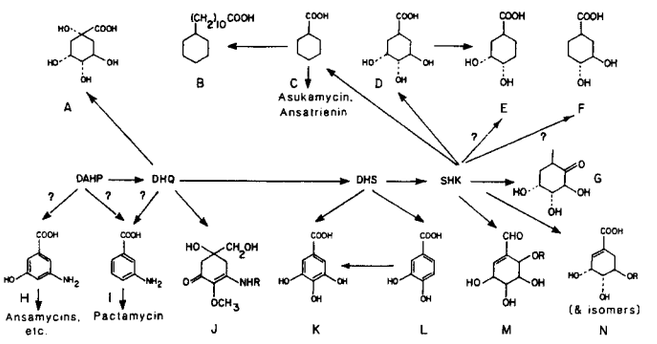


FIGURE 30. Branches from the SHK pathway at DAHP, DHQ, DHS, and SHK. Saturated structures (cyclohexanes) are grouped at the top of the figure.

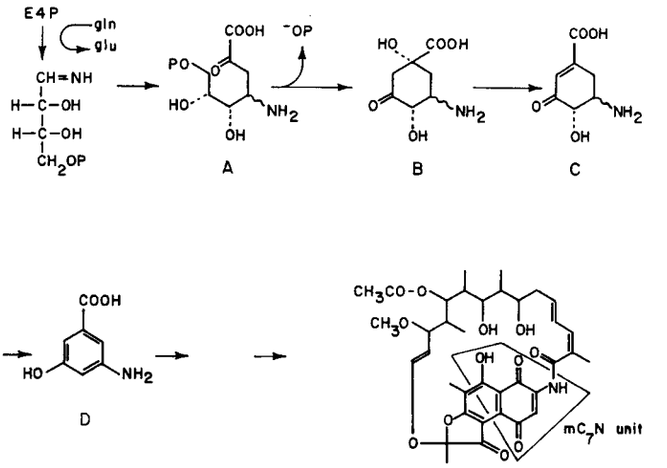


FIGURE 31. Possible pathway for biosynthesis of 3-amino-5-hydroxybenzoate (D) and its role in ansamycin formation. A = "amino-DAHP", B = "amino-DHQ", C = "amino-DHS". The ansamycin shown is rifamycin S. In the formation of A, a molecule of PEP is, of course, required. B → C and C → D both require loss of water. There is no information about configurations at the chiral centers carrying the putative —NH_2 group. The remainder of the ansamycin molecule derives as a polyketide from acetate and propionate units, and also requires *S*-adenosylmethionine for formation of the methoxyl group.

of a nucleus. The nucleus can be either naphthalenoid (rifamycins, streptovaricins, tolypomycins, halomycins, naphthomycin, actamycin, rubransarols) or benzenoid (geldanamycin, herbimycins, macbecins, ansatrienins, maytansoids, ansamitocins). They have many biological properties, including useful or potentially useful antibiotic actions, and antiviral and antitumor activities. All contain a six-membered carbocyclic ring with a seventh carbon and a nitrogen atom arranged in an *m* disposition. This unit is referred to as the mC_7N unit, or simply the C_7N unit. A similar unit is present in other natural products.

Despite the structural similarities, there a number of means for producing these units, including some which are not SHK pathway related. For more detail and leading references, other reviews should be consulted.[7,296,297] The detailed biosynthetic scheme for many ansamycins presented by Ghisalba[297] needs some revision in light of studies on the origin of oxygen atoms in rifamycins B, O, and S.[298]

Genetic and tracer experiments have established an important role for 3-amino-5-hydroxybenzoate as one source of mC_7N units. It probably acts as a "starter" unit for the polyketide chain assembly, presumably as its CoA derivative. Compounds identified as utilizing 3-amino-5-hydroxybenzoate include rifamycins, actamycin, ansamitocin, mitomycin, geldanomycin, naphthomycin, porfiromycin, and ansatrienins; the utilization of this unit is exemplified for the case of rifamycin S in Figure 31.

In these compounds, labeled SHK does not function as a precursor for the mC_7N unit. While this failure could be attributed to permeability problems, this was not so for ansatrienin formation by *Streptomyces collinus*. This compound contains, in addition to the mC_7N unit, a cyclohexane carboxylate unit; labeled SHK is converted to the latter unit but not the former. Such evidence points strongly to a pre-SHK compound. One possibility, DHQ, was not incorporated into mitomycin.[299] Although obvious reactions would lead from either DHQ or DHS to 3-amino-5-hydroxybenzoate, they imply transamination at the C-3 carbonyl of these compounds. It is known, however, that the N atom is introduced on the carbon atom which corresponds to C-5 of SHK. Another possibility is the formation of a 5-DHQ (3-DHQ → QA → 5-DHQ).[300]

Perhaps more likely is formation of 4-deoxy-4-amino-DAHP ("amino-DAHP").[297] A DAHP synthase containing a further subunit with the ability to transfer the gln-NH_2 group would smoothly yield amino-DAHP.[296] Subsequent reactions, akin to those of the SHK pathway itself, would yield 5-amino-DHS, and hence 3-amino-5-hydroxybenzoate (see Figure 31). Although speculative, the route may be supported by the fact that the amide nitrogen of gln is the best nitrogen donor for rifamycin biosynthesis.

When [carboxy-^{13}C]-3-amino-5-hydroxybenzoate was fed to cultures of *Streptomyces verticillatus*, [6-methyl-^{13}C]-porfiromycin was formed. Recovered 3-amino-5-hydroxybenzoate showed a significant reduction in ^{13}C content (from 85 to 52 atom% excess) establishing this compound as a new, naturally occurring aromatic amino acid.[301] It has actually been isolated from a strain of *Nocardia meditteranei* blocked in rifamycin production.[302]

Addition of 3-amino-5-hydroxybenzoate to an actamycin fermentation increased the yield of antibiotic, thus supporting a role for it in the biosynthesis of actamycin. Similar additions of the 4-chloro, 6-chloro-, and *N* and *O*-methyl derivatives repressed actamycin biosynthesis and did not lead to production of structurally modified actamycins.[30]

b. mC_7N UNITS FROM 3-AMINOBENZOATE

During formation of pactamycin by *Streptomyces pactum*, 3-aminobenzoate was incorporated as an mC_7N unit.[304] A pos-

sible route to 3-aminobenzoate would be by transamination of DHS or DHQ (Figure 32).

c. mC_7N UNITS NOT DERIVED FROM SHK INTERMEDIATES

For convenience, pathways not involving SHK intermediates will be noted briefly. The mC_7N units of asukamycin (see Section III.A.3) and manumycin (Figure 32B) may originate in a Krebs' cycle C_4 moiety (possibly succinate) and a C_3 unit from a triose pool (possibly glycerol).[296,305] Interestingly, 3-aminobenzoate addition to *Streptomyces parvulus* suppresses manumycin formation and leads to a new metabolite (Figure 32C) lacking the characteristic epoxide structure and with a fully aromatic ring system.[306] The valienamine component of acarbose and validamycin A arises from a $C_3 + C_2 + C_2$ condensation[296,307] and the mC_7N unit of kinamycin has a polyketide origin via 4-amino-2-hydroxytoluate.[308]

d. QUINATE PATHWAY

Some account is given here of processes involving QA. This acid occurs commonly in plants either free or in combined structures as esters and may be present in amounts as high as 2 to 10% of the dry weight of leaves of higher plants. It has been known for considerably more than 100 years that QA is converted to hippuric acid in man; this transformation is confined to man and Old World monkeys. It requires formation of benzoate followed by conjugation with glycine to hippurate.

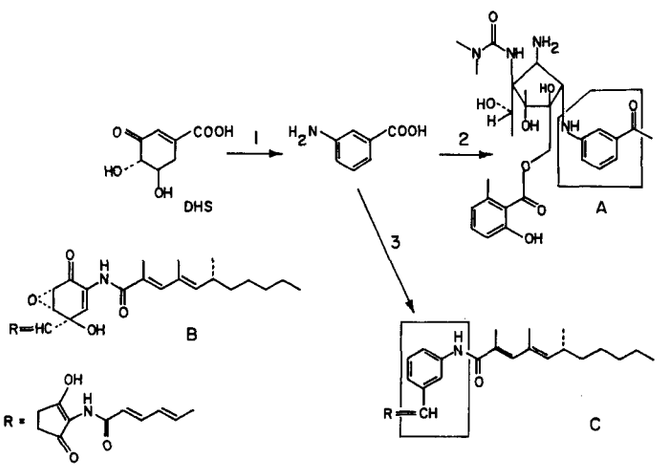


FIGURE 32. Utilization of 3-aminobenzoate. The top line shows a possible route from DHS to 3-aminobenzoate and its subsequent incorporation into pactamycin, structure A. The mC_7N unit in pactamycin appears to be C_8 at first glance; however, the $—CH_3$ attached to the carbonyl derives from acetate. Compound B, manumycin (from *S. parvulus*) contains an mC_7N unit not derived from the SHK pathway. This organism, however, utilizes added 3-aminobenzoate, forming structure C, as described in the text. The mC_7N units are highlighted by boxes. Reaction 1, transamination and loss of 2 H_2O; reaction 2; biotransformation by *S. pactum;* reaction 3; biotransformation by *S. parvulus*.

Cyclohexane carboxylate is an intermediate and is formed by the action of intestinal bacteria, with the further aromatization to benzoate carried out by animal enzymes. Thus, Rhesus monkeys treated with neomycin to suppress gut bacteria show a considerable diminution in overall formation of benzoate. The conversion presumably occurs as follows:[309,310]

$$QA \rightarrow DHQ \rightarrow DHS \rightarrow SHK \rightarrow \rightarrow \text{Cyclohexane carboxylate} \rightarrow \rightarrow \text{Benzoate} \rightarrow \text{Hippurate}$$

The steps to DHS constitute part of the "QA pathway" which is discussed here with reference to plants and various microorganisms (see Figure 33).

The role of QA in plants is somewhat engimatic. One possibility is that QA functions as a reservoir to provide SHK pathway intermediates under certain conditions. However, tracer experiments have established that the QA pool is metabolically active with a rapid turnover.[26] Although QA could be formed by a branch from the main trunk SHK pathway at the level of DHQ, some evidence has suggested an independent pathway for QA biosynthesis. In tobacco cell cultures, under shoot-forming and nonshoot-forming conditions, there was a greater rate of net synthesis of QA than SHK from [^{14}C]glucose. These results were held to indicate that QA was possibly a regulatory component in the SHK pathway.[311]

For QA formation from the SHK pathway, a QA dehydrogenase would be necessary. Following early reports of the isolation of such preparations from plants more detailed work

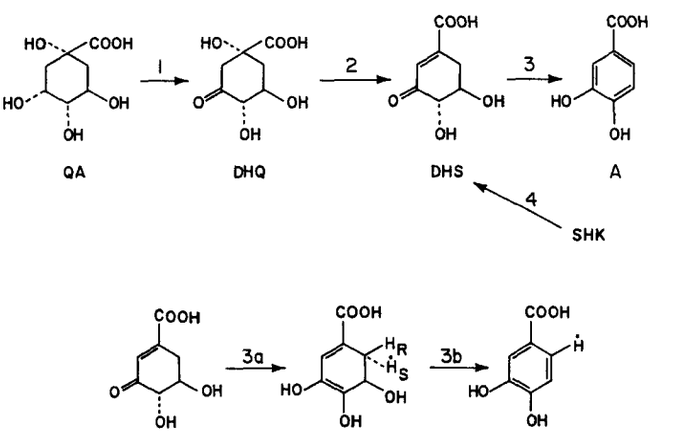


FIGURE 33. The quinate pathway. The enzymes are as follows: 1, QA dehydrogenase; 2, DHQ dehydratase; 3, DHA dehydratase; 4, SHK dehydrogenase. Protocatechuate (A) can undergo further catabolic reactions to form 3-ketoadipate. A mechanism for the observed syn elimination of the elements of water by DHS dehydratase is also shown at the bottom of the figure as a two-step process, 3a and 3b.

has been undertaken. In shoot-forming tobacco cell tissue cultures, there was a rapid and significant increase in QA dehydrogenase activity, in the direction, QA → SHK, from days 3 to 12. This activity was higher in shoot-forming tissue than in nonshoot-forming tissue. QA dehydrogenase was present in extracts from etiolated pine seedlings; these preparations converted labeled QA to protocatechuate, gallate, and vanillate.[312,313]

QA dehydrogenase (EC 1.1.1.24 — NAD dependent) from carrot cell suspension cultures had the unique property of being subject to covalent modification. The enzyme (M_r = 42,000) was active when phosphorylated and inactive when dephosphorylated. Reactivation of dephosphorylated enyzme required a Ca^{2+} calmodulin-dependent protein kinase and ATP. The calmodulin was not a tightly bound subunit of the protein kinase. Cyclic adenosine monophosphate (cAMP) had no effect on the rate of phosphorylation.[314] The QA dehydrogenase activity decreased in carrot cell suspension cultures supplemented with a Ca^{2+} ionophore and EGTA (ethylene glycol bis-[amino ethyl ether]NN″-tetraacetic acid). It was believed that intracellular Ca^{2+} controlled the ratio of active:inactive QA dehydrogenase by way of a two-cycle cascade:[315] (1) activation-deactivation of calmodulin-dependent protein kinase, and (2) phosphorylation-dephosphorylation of QA dehydrogenase. The protein kinase activity was ATP-Mg^{2+} dependent with a maximum pH of 8.0 The protein phosphatase activity was inhibited by NaF and enhanced by Mg^{2+} over a broad pH range (and even at the low temperature of 10°C).[316]

A further complexity with the carrot cell QA dehydrogenase was the finding that in dark-grown cells, the enzyme behaved as a dimer of M_r = 110,000 with two subunits, M_r = 42,000 and 60,000. The latter, apparently a regulatory subunit, contained a Ca^{2+} binding site. The QA dehydrogenase of dark-grown cells was not subject to phosphorylation-dephosphorylation and was activated directly by Ca^{2+} by a process not involving calmodulin.[317]

A catabolic pathway for QA metabolism to protocatechuic acid in microorganisms has received considerable attention and can only be considered briefly here. Three enzymes are needed for the conversion to protocatechuate, and in the following order: QA dehydrogenase, DHQ dehydratase, and DHS dehydratase (see Figure 33). The conversion of DHQ to DHS occurs, of course, as part of the main trunk SHK pathway. However, the organisms to be discussed, *N. crassa* and *A. nidulans*, contain a biosynthetic enzyme (B-DHQ dehydratase) and, in addition, a separate inducible catabolic enzyme (C-DHQ dehydratase), which is distinguished genetically and physically from the B-DHQ dehydratase. The genes for the B-DHQ dehydratase are part of the *arom* gene cluster. In *N. crassa* and *A. nidulans* the catabolic genes are also organized as the *qa* and *QUT* clusters. The structural genes encoding the enzymes and regulatory genes are as follows:[318,319]

	N. crassa	*A. nidulans*
QA dehydrogenase	*qa-3*	*QUT*B
C-DHQ dehydratase	*qa-2*	*QUT*E
DHS dehydratase	*qa-4*	*QUT*C
Regulatory genes	*qa-1F, qa-1S*	*QUT*A, *QUT*D, *QUT*G, *QUT*R

I. The *QUT* Cluster in *A. nidulans*

The *QUT* gene cluster of *A. nidulans* has been subjected to extensive genetic analysis. The gene order is

*QUT*C. . . .*QUT*D (permease), *QUT*B, *QUT*E, *QUT*A, *QUT*R (repressor)

This order is different from that in *N. crassa*.[319,320] The structural genes have been cloned in *E. coli*. The *QUT*D gene function (permease) has been located within the cloned gene cluster and corresponds to a region with sequence homology to the *N. crassa qa-Y* gene.[321]

The determined DNA sequence of *QUT*B contained an ORF of 993 nucleotides coding for a protein of inferred M_r = 36,000. There was significant homology with the SHK dehydrogenase of the *arom* protein of this organism and the QA dehydrogenase of *N. crassa*.[292] The nucleotide sequence of *QUT*E showed an ORF of 462 nucleotides encoding a protein of 153 residues, M_r = 16,505.[322] There was extensive homology with the *N. crassa* protein, but none with the B-DHQ dehydratase of the *arom* protein.

II. The *qa* Cluster in *N. crassa*

In *N. crassa* the tightly linked *qa* gene cluster has the following gene order: *qa-1, qa-3, qa-4, qa-2*. The entire DNA sequence of the cluster has been determined.[318] There are seven closely adjacent genes in a segment of about 17.3 kb; about 60% of the *qa* cluster consists of coding sequences. The individual components for the three enzymes of interest are described separately. The role of the regulatory genes (*qa-1f* and *qa-1s*) has been reviewed.[323] There are two additional presumptive *qa* genes, *qa-X* and *qa-Y;* their functions are not yet completely clear.

The first enzyme in the pathway, QA dehydrogenase (NAD dependent), is apparently responsible not only for the conversion QA → DHQ, but also for the conversion of SHK to DHS. It is more appropriately referred to as QA(SHK) dehydrogenase. The SHK activity is presumably necessary since SHK as well as QA can serve as sole carbon source for fungal growth. The enzyme is a polypeptide of 321 amino acid residues, with a calculated M_r = 35,210.[318] It has been purified to homogeneity from *N. crassa* M18. Although the enzyme is a monomer, M_r = 41,000, three species (probably charge isomers) were detected by electrophoresis under nondenaturing conditions. The enzyme had a single binding site for both substrates. This inducible catabolic QA(SHK) dehydrogenase of *N. crassa*

is homologous with the *E. coli* SHK dehydrogenase. This homology is 19%, but in the N-terminal region, up to residue 153 of *E. coli*, it increases to 27%.[131]

It is of interst that the catabolic QA(SHK) dehydrogenase is NAD-dependent, whereas the biosynthetic SHK dehydrogenase requires NADP. Moreover, other catabolic-QA(SHK) dehydrogenases have been described which utilize neither of the pyridine nucleotides and are likely to require flavin and cytochrome components. For example, *Acinetobacter calcoaceticus (Moraxella calcoacetica)* contained an NADP-dependent SHK dehydrogenase (presumably biosynthetic) and a second dehydrogenase, not pyridine nucleotide-dependent, which was assigned a catabolic role.[324] Utilization of NAD by plant SHK dehydrogenases has been described in Section II.D.2.

The structural gene (*qa-2*) for *N. crassa* C-DHQ dehydratase has been cloned and was expressed in *E. coli* (the *qa-2* cluster genes, other than *qa-2* were not expressed in *E. coli*).[325] The C-DHQ dehydratase contained 173 amino acids and had M_r = 18,270. Since the native wild-type enzyme had M_r of about 222,000, it must be composed of 12 identical subunits.[318] The enzyme has been purified either from *N. crassa* M18 (lacking QA dehydrogenase) or from *E. coli* SK2884 containing appropriate plasmids with the *qa-2* gene. The apparently pure native enyme from *N. crassa* was a mixture of intact and proteinase-cleaved enzyme monomers. With the enzyme from *E. coli*, some monomers were also truncated, but to a lesser extent. The protein sequence data of the bacterially expressed enzyme corresponded to the true N-terminal sequence deduced from the nucleotide sequence of the *qa-2* gene. As a result of the proteinase activity, there was no homology for the N-terminal amino acid sequences of the fungal and bacterially expressed enzymes.[326]

A 20- to 50-fold increase in the expression of C-DHQ dehydratase in *E. coli* was obtained when the bacterial strains were deficient in polynucleotide phosphorylase; however, the level of the chromosomally encoded B-DHQ dehydratase was not affected. There are apparently structural differences between the eukarytoic and prokaryotic mRNAs.[327]

The *qa-4* gene of *N. crassa* encodes a polypeptide of 359 amino acids with M_r = 40,000; the purified DHS dehydratase was a monomer. The sequence determined from the DNA data agreed with a partial amino acid sequence determined from the N-terminus.[328] DHS dehydratase was very susceptible to heat denaturation, but was stabilized by Mg^{2+}; maximum catalytic activity required the presence of Mg^{2+} or other divalent cations. The removal of the elements of water was a syn elimination (see Figure 33).[329]

e. CYCLOHEXENES

A number of cyclohexene metabolites have a structural relationship with DHQ, except that the DHQ -COOH group has apparently been reduced to $-CH_2OH$.[330] The mycosporines contain amino acid related moieties, and are produced by fungi and a surprising number of marine organisms. Typical examples are mycosporine glutaminol (Figure 34A) and iminomycosporine-gly (Figure 34B). A possible precursor (Figure 34C) of these compounds has been isolated from fish eggs; a further material named gadusol (Figure 34D) is also present in cod roe[331] and other fish eggs.[330]

Some evidence implicates DHQ in biosynthesis of mycosporine glutaminol in *Tricothecium roseum*.[332] QA addition stimulated metabolite formation in this organism, and [U-^{14}C]-DHQ was predominantly incorporated in the C_6-C_1 unit. Some radioactivity in the glutaminol portion apparently arose from acetate formed via the ketoadipate pathway. A possible biosynthetic sequence is shown in Figure 34 (DHQ → C → A).

This possible pathway from DHQ is unlikely to be involved in the formation of gadusol and related compounds in fish eggs since fish require essential amino acids in their diet and presumably lack the basic SHK pathway. The fish may degrade dietary mycosporines by removal of the N containing unit. If so, the amount of mycosporines consumed by cod must be considerable; their roes contain 4 g gadusol per kilogram dry weight.

2. The Role of DHS

a. GALLIC ACID

Gallic acid (Figure 30K), often present in derivatized form as esters with glucose, other polyols, QA, and SHK, is a common plant component. Several biosynthetic pathways have been proposed, including a direct dehydrogenation from DHS. Evidence for the latter route is available for the mold, *Phycomyces blakesleeanus*, and in several plants.[333] It is said to

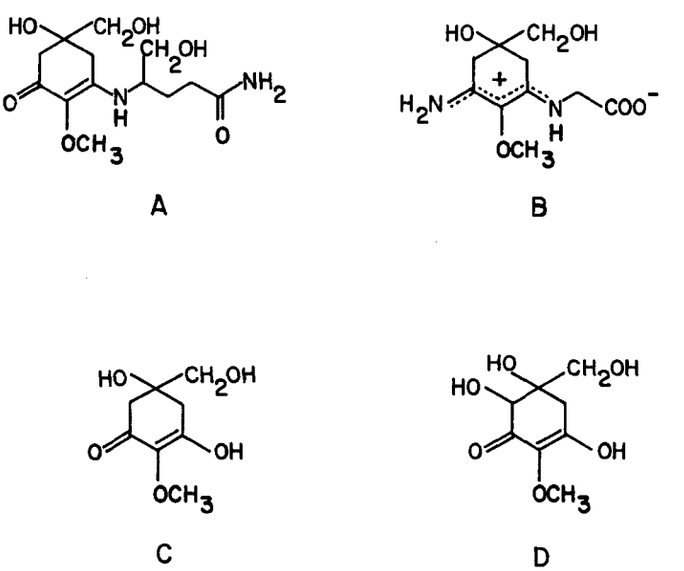


FIGURE 34. Cyclohexenes possibly formed from DHQ. A = mycosporine glutaminol; B = iminomycosporine-gly; C = possible precursor of A and B, isolated from fish eggs; D = gadusol.

be the only example of a plant hydroxybenzoate derived directly from an alicyclic precursor. It thus holds a somewhat enigmatic position since these plant hydroxybenzoates are generally derived from catabolic breakdown of C_6-C_3 materials. Gallate is formed by hydroxylation of protocatechuate in *Pelargonium hortorum*.[4]

b. PROTOCATECHUIC ACID

Protocatechuic acid (Figure 30L) another common plant component, is generally assumed to be derived from DHS by a DHS dehydratase.[334] There is little direct evidence for this assumption. However, glyphosate-treated cells of *Quercus robus* and *Rhus typhina* leaves accumulate protocatechuate, presumably as a backup product from the inhibition of EPSP synthase.[335]

There is, however, considerable evidence for the formation of protocatechuate from DHS in microorganisms, and work in *Neurospora* has been extensively reviewed.[5] Preparations of DHS dehydratase from *N. crassa* were discussed in connection with the QA pathway. SHK metabolism by intestinal bacteria leads to catechol as follows: SHK → DHS → protocatechuate → catechol. This conversion has also been demonstrated using pure cultures of *Lactobacillus plantarum* utilizing either SHK or QA.[336]

Four benzenoid compounds (moskachans A through D) have been isolated from *Ruta angustifolia* and were described as SHK metabolites (without experimental evidence). They may arise from protocatechuate.[337]

3. The Role of SHK

In 1980, Weiss and Edwards[5] commented on the peculiarities of SHK kinase (see Section II.E) and said that "it may be permissible to speculate that perhaps some as yet unrecognized biosynthetic or degradative pathway branches off from shikimate. The recent work on ω cyclohexyl fatty acids . . . may suggest that the search for products of such a pathway need not necessarily be restricted to aromatic compounds." It is gratifying that considerable information is now available on the production of cyclohexane carboxylate (and derived compounds), some cyclohexanone derivatives, and other products, all of which are derived from SHK.

a. CYCLOHEXANE CARBOXYLATE

Several natural products contain cyclohexane rings which do not arise by reduction of aromatic systems such as phe or benzoate. Certain dihydroxycyclohexane carboxylates are discussed later in connection with dihydro-SHK. The materials, ansatrienin and asukamycin (Figure 35A and B, respectively), contain both a cyclohexane carboxylate unit (Figure 30C) and an mC_7N unit; they have already been mentioned with regard to the latter. The cyclohexane carbonyl unit of these structures may originate from SHK by the reaction sequence of Figure 35. For further processing, formation of the CoA ester of cyclohexane carboxylate is assumed.[296]

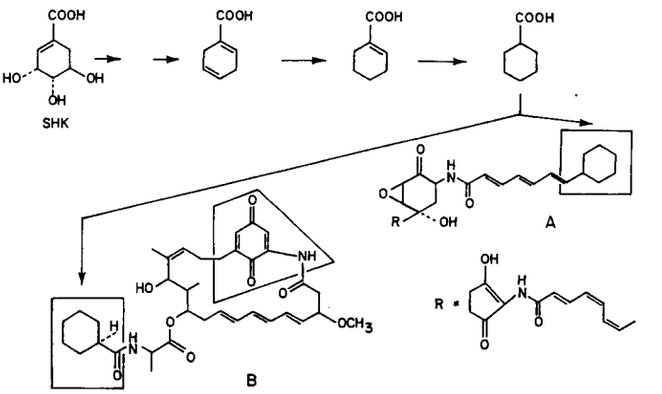


FIGURE 35. Formation of cyclohexane carboxylate and related compounds. A = asukamycin; B = ansatrienin. The unit in the irregularly shaped box of B is derived from 3-amino-5-hydroxybenzoate. The C_7N unit of A is not derived from the SHK pathway.

In formation of the symmetrical cyclohexane system, both of the hydrogen atoms originally at C-6 of SHK are lost and C-2 of SHK becomes C-6 of the cyclohexane carboxylate (using sn numbering). The sequence shown in Figure 36 may lead to ansatrienin biosynthesis in *Streptomyces collinus*.[296]

It had been known for some time that addition of SHK (or QA, see Section II.A.1.d) to the diets of rats gave rise to an increased production of hippurate (the glycine conjugate of benzoate). This results from metabolism of SHK initially to cyclohexane carboxylate by intestinal bacteria; the cyclohexane carboxylate is then aromatized to benzoate by mammalian enzymes and is excreted as hippurate. Although it was suggested[310] that dihydro-SHK was an intermediate to the formation of cyclohexane-carboxylate, this possibility should be reexamined in the light of the pathway just discussed in *Streptomycetes*.

b. OMEGA-CYCLOHEXYL FATTY ACIDS

Acids of this kind (Figure 30B) occur in various acidophilic-thermophilic bacteria and in two strains of the mesophile, *Curtobacterium pusillum*. Early work established a role for cyclohexane carboxylate, itself derived from SHK. The CoA ester of cyclohexane carboxylate functions as a "starter" for polyketide elongation via malonyl CoA. Some information is available concerning the fatty acid synthase of *C. pusillum*.[338] The stereochemistry of the process was studied using [6,6-^{2}H2]-glucose as a precursor for SHK.[339] Evidence was obtained for the pathway shown in Figure 37. Apparently there is no evidence for involvement of intermediates used for ansatrienin biosynthesis.

c. DIHYDRO-SHK AND DIHYDROXYCYCLOHEXANE CARBOXYLATES

It was already noted that *Lactobacillus plantarum* converts added SHK or QA to protocatechuate. In addition, this organ-

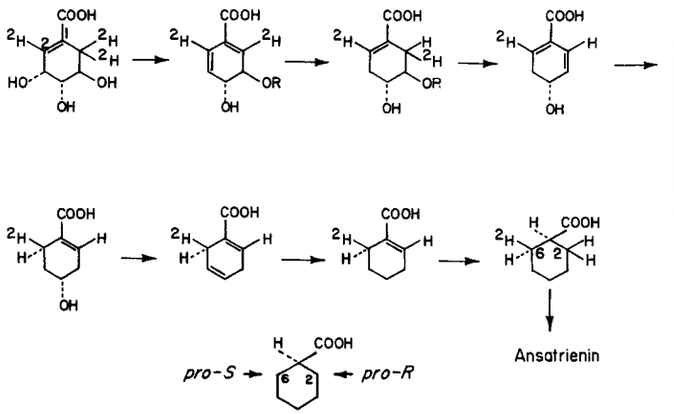


FIGURE 36. Stereochemistry of conversion of ^2H-labeled SHK to cyclohexane carboxylate. R = —H or —C(=CH_2)COOH.

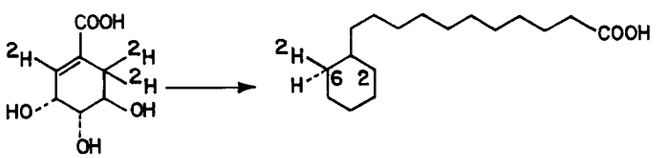


FIGURE 37. Formation of ω-cyclohexyl fatty acids from SHK. The enzyme used was a fatty acid synthase preparation from *Curtobacterium pusillum*. The labeled SHK was derived biosynthetically from [6,6-$^2H_2$]glucose.

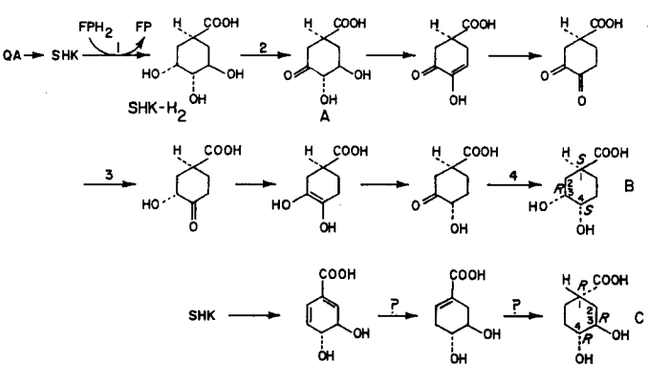


FIGURE 38. Formation of dihydro-SHK and certain dihydroxycyclohexane carboxylates by bacteria. FP = a flavoprotein component. B = (3R,4S)-dihydroxycyclohexane-(1S)-carboxylate; C = (3R,4R)-dihydroxycyclohexane-(1R)-carboxylate. 1, SHK reduction with FPH_2; 2, "hydroaromatic dehydrogenase" and NAD^+; 3 and 4, "hydroaromatic dehydrogenase" and NADH. A possible route to C is shown on the bottom line.

ism and *L. pastorianus* reduce added SHK to dihydro-SHK (Figure 30D and Figure 38). In *L. plantarum*, dihydro-SHK undergoes further extensive metabolism to (3R,4S)-dihydroxycyclohexane-(1S)-carboxylate (Figure 38B). Evidence has been obtained for all of the reactions shown in Figure 38; all were represented as reversible. The enzyme, NAD-dependent "hydroaromatic dehydrogenase", has been isolated from *L. plantarum* and purified to some extent.[340] It catalyzes oxidation step 2; two of the reductions, steps 3 and 4, shown in Figure 38; and the reaction, QA → DHQ. The possible presence of other dehydrogenases was not totally excluded. The reduction of SHK to dihydro-SHK, Figure 38, step 1, was attributed to a flavoprotein. The initial product of the oxidation of dihydro-SHK, 3,4-dihydroxy-5-oxocyclohexane-1-carboxylate (Figure 38A), was isolated from *Acetomonas oxydans*.[341]

When SHK was administered to rats, two isomers of 3,4-dihydroxycyclohexanecarboxylate were identified in feces. These products were assumed to arise from the action of intestinal bacteria by an unspecified mechanism.[310] One was the (3R,4S)-dihydroxycyclohexane-(1S)-carboxylate (Figure 38B) just described as a product of SHK metabolism by *L. plantarum*. It may, therefore, have been produced by the pathway shown in Figure 38. The other isomer was (3R,4R)-dihydroxycyclohexane-(1R)-carboxylate. This product could have been derived by the reduction of an intermediate produced during ansatrienin biosynthesis (Figure 36A). Other mechanisms are, of course, possible.

d. CYCLOHEXANE AND CYCLOHEXENE STRUCTURES WITH THE SHK —COOH GROUP REDUCED

Some compounds appear to derive from SHK by addition of a fourth oxygen atom at the original C-6 of SHK. More intriguing is that the —COOH group has been reduced, either to —CHO, —CH_2OH, or —CH_3, showing a complete series for reduction of —COOH to —CH_3 (see Figure 39).

Figure 39A, (R = H), rancinamycin III, was produced by growth of *Streptomyces lincolnensis* on sulfur-depleted medium. It was actually a mixture of four components with stereochemical differences. Also produced under these conditions were rancinamycins I and II, which have acyl groups (either four or five carbons) substituted on the new hydroxyl function. The authors suggested "that the chirality at C-3, C-4 and C-5 in the rancinamycins remains the same as it does in shikimic acid". Unhappily, they drew structures based on the unnatural enantiomer of SHK.[342]

Figure 39B is a metabolite (KD 16-U1) of *Steptromyces filipiensis;* the configuration at C-6 (based on C=O = 1) was not determined.[343] Figure 39C was isolated as an inhibitor of

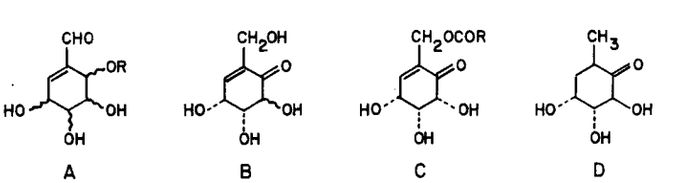


FIGURE 39. Cyclohexane and cyclohexene structures with an apparent reduction of the SHK —COOH group. A, R, =H, rancinamycin III; B, metabolite KD 16-U1; C, a glyoxalase inhibitor: D is derived from various *Streptomycetes*.

glyoxalase I from *Streptomyces griseosporeus* (R = —CO—CH=CH—CH_3). In this case the configuration of the C-5 SHK position had apparently undergone inversion.[344] The compound with a fully reduced SHK —COOH (Figure 39D) was isolated from *Streptomyces phaeochromogenes* and *S. albus*.[345]

Several of the metabolites considered in connection with Figure 30 also occur as esters or acyl derivatives. As noted, QA esterified with caffeic acid is chlorogenic acid. Gallate esterifies with glucose, other polyols, QA, and SHK. SHK itself occurs as acyl derivatives (Figure 30N) with various acids such as caffeic, 3-ethyl *cis*-crotonate and 3-hydroxy-3-methylglutarate.[346]

B. Branches from S3P, EPSP, and CHA (Figures 40 and 41)

Owing to the number of interesting and important nitrogen-containing metabolites derived from CHA, the overall schematic for this section uses two figures. Figure 40 includes the non-nitrogenous metabolites from S3P, EPSP, and CHA. Although reductiomycin contains nitrogen, the nitrogen atom is not related to the SHK pathway; hence, reductiomycin is included in Figure 40. Figure 41 illustrates the nitrogenous metabolites originating in CHA.

1. Non-Nitrogenous Metabolites (Figure 40)

a. THE ROLE OF S3P

The methyl ester of 3,4-anhydro-SHK (Figure 42A) was isolated from the fungus *Chalara microspora*. A nucleophile, X^-, probably attacked S3P with elimination of HX (see Figure 42).[347] Another epoxide, chaloxone, accompanied methyl 3,4-anhydro-SHK.[348] While this compound may also be a SHK metabolite,[349] there is no proof.

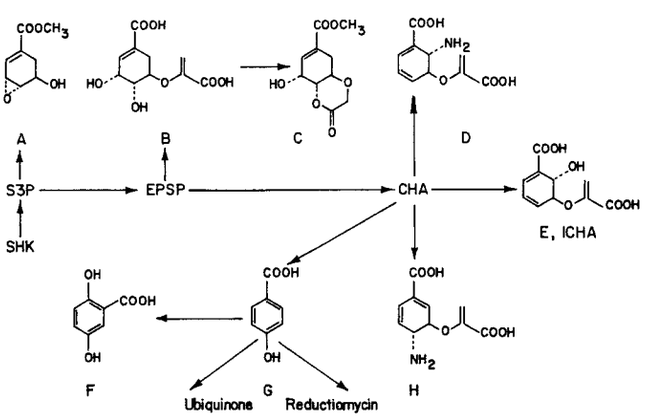


FIGURE 40. Branches from S3P, EPSP, and CHA forming non-nitrogenous products. A, methyl ester of 3,4-anhydro-SHK (see Figure 42); B = 5-*enol* pyruvyl-SHK (see Figure 43); C = methyl 5-lactyl-SHK lactone (see Figure 43); D = 2-amino-2-deoxy-ICHA (see Figure 41); E = ICHA (see Figure 41); F = gentisate; G = 4-hydroxybenzoate (see Figures 44 and 45); H = 4-amino-4-deoxy-CHA (see Figure 41).

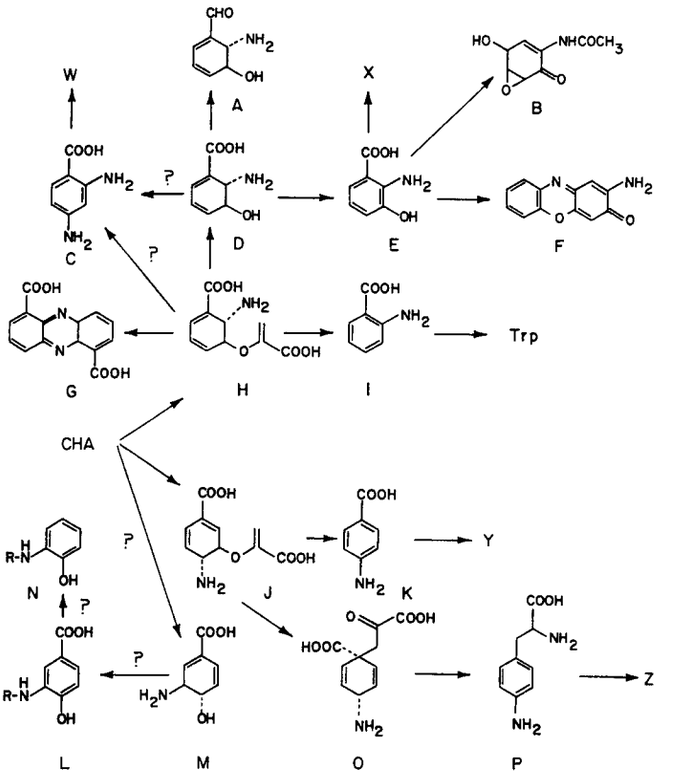


FIGURE 41. Branches from CHA forming nitrogenous products. A = 6-amino-5-hydroxy-1,3-cyclohexane-1-carboxaldehyde; B = metabolite LL-C10037α; C = 4-aminoanthranilate (see Figure 56); D = 2,3-dihydro-3-hydroxyanthranilate; E = 3-hydroxyanthranilate; F = 2-aminophenoxazinone; G = phenazine-1,6-dicarboxylate (see Figure 55); H = 2-amino-2-deoxy-ICHA; I = anthranilate; J = 4-amino-4-deoxy-CHA; K = 4-aminobenzoate; L = 3-acetamido-4-hydroxybenzoate; M = 3-amino-4-hydroxybenzoate (see Figure 62); N = 2-acetamidophenol; O = "prephenate-like" product formed from J; and P = L-(4-aminophenyl)alanine. The formation of further products is indicated as follows: W = formation of streptonigrin; X = formation of oryzoxymycin; Y = formation of folate and *N*-(γ-L-glutamyl)-4-hydroxyaniline; Z = formation of chloramphenicol and obafluorin.

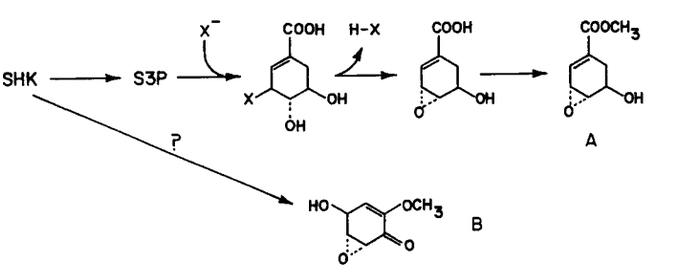


FIGURE 42. Products derived from S3P. A = methyl ester of 3,4-anhydro-SHK; B = chaloxone.

b. THE ROLE OF EPSP

It is of interest that EPSP had at one time been regarded as "the branch point compound", terminating the main trunk of the aromatic biosynthetic pathway.[39] 5-*Enol*pyruvyl-SHK itself (Figure 43A) occurs naturally and is presumably derived by dephosphorylation of EPSP. It has been synthesized chemically.[16] 5-*Enol*pyruvyl-SHK has been suggested as a likely precursor of methyl 5-lactyl-SHK lactone (Figure 43B) from *Penicillium* sp K-114.[350] It is not known in what sequence the necessary reactions of reduction, lactonization, and methylation occur.

c. NON-NITROGENOUS COMPOUNDS DERIVED FROM CHA

I. Ubiquinone Biosynthesis

A great many quinones (benzo-, naphtho-, anthra-) occur naturally, some having vital biological functions. Thomson's[351] important sourcebook provides descriptions of newly isolated materials. It will, doubtless, lead to much biosynthetic speculation and experimentation.

One of the functionally important benzoquinones is ubiquinone (Q-n, where n indicates the number of isoprenyl residues). The term actually embraces a series of materials that differ in the length of the isoprenyl side chain and its degree of unsaturation. CHA is the precursor for 4-hydroxybenzoate, itself an important intermediate in the biosynthesis of Q in microorganisms. In *E. coli*, the pathway to Q has been well characterized for some time,[352] both in terms of genetics (there are eight genes for the conversion of CHA to Q) and biochemistry (see Figure 44). The initial step, CHA → 4-hydroxybenzoate,

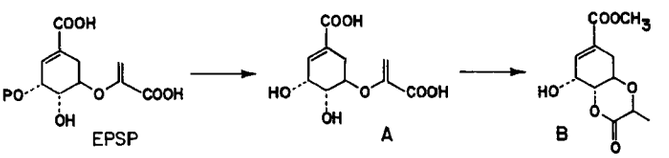


FIGURE 43. Formation of 5-*enol*pyruvyl-SHK (A) and methyl 5-lactyl SHK lactone (B).

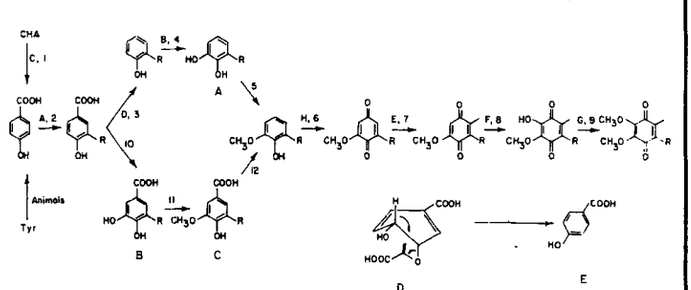


FIGURE 44. Biosynthesis of ubiquinones. The *E. coli* pathway is indicated with the necessary genes; to save space; *ubi* has been omitted in the genetic designations. The eukaryote variation is shown as B → C. SAM = *S*-adenosylmethionine; SA = *S*-adenosylhomocysteine.

requires pyruvate elimination, possibly by a concerted mechanism (Figure 44, D → E). Of the compounds shown in Figure 44, A is the least well characterized, and mutants defective in its methylation have not been identified.

The *ubi* genes of *E. coli* have apparently not been sequenced and little is known of the control mechanisms for Q biosynthesis in this organism. Recently, the expression of the *ubiG* gene has been studied with the aid of a *ubiG-lacZ* fusion.[353] The expression of *ubiG* was higher under aerobic than anaerobic conditions, and the presence of glucose in the culture medium decreased transcription from the gene. The *ubiG* transcription was probably modulated positively by the cAMP receptor protein-cAMP complex. *E. coli* strains with resistance to streptomycin and to phleomycin and bleomycin were apparently deficient in the *ubiF* gene.[354,355]

Eukaryotes have a variation in the early steps of the biosynthetic pathway as shown in Figure 44, B → C.[356] In yeast, Q biosynthesis is regulated by glucose at this step.[357] In animals, 4-hydroxybenzoate is obviously not derived by the CHA pathway; instead it is formed from tyr.[356] In view of this fact, it is pointless to attempt[357] to describe Q as a "vitamin".

A remarkably high level of Q-10 production has been attained in tobacco cell suspension cultures by a multiple cloning technique. The only organic substrate in the culture medium is sucrose, but it is not known whether tyr degradation is the source of 4-hydroxybenzoate. The strain Z8A-3B-22 produces 1.85 mg Q-10 per gram dry weight of cells. This cell line has possible industrial applications.[359]

II. Biosynthesis of Reductiomycin

Reductiomycin, a metabolite of *Streptomyces xanthochromegenus*, contains a dihydrofuranylacrylic acid unit that is apparently derived from CHA via 4-hydroxybenzoate (or its aldehyde). No utilization of tyr could be detected.[296] [^{13}C-COOH]-4-Hydroxybenzoate, when administered to cultures of the microorganism, gave reductiomycin with isotope at the predicted position. There are two possible pathways (Figure

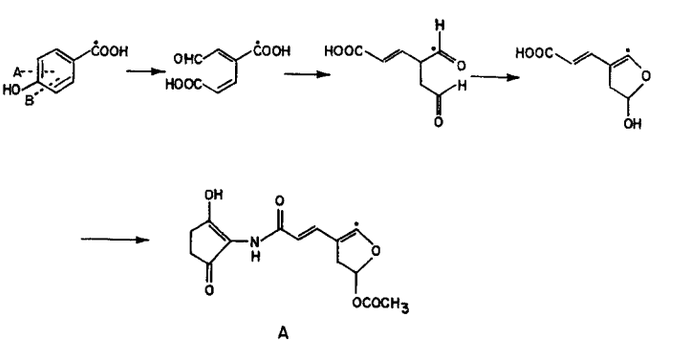


FIGURE 45. Biosynthesis of reductiomycin (A). Cleavage of the benzene ring of 4-hydroxybenzoate could occur at either position A or B. Only the A possibility is indicated here. The C_5N unit derives from 5-aminolevulinate.

45) for the cleavage of the symmetrical 4-hydroxybenzoate which could be distinguished by the use of labeled glycerol (Figure 45 shows only one possibility). Although cleavage of a benzene ring is somewhat unusual, it has been well documented in several cases. This particular process is reminiscent of that involved in penicillic acid biosynthesis. Moreover, the benzene ring of gentisaldehyde undergoes cleavage during patulin biosynthesis.

4-Hydroxybenzoate can also be derived from cyclohexane carboxylate during the growth of *Corynebacterium cyclohexanicum*. In this catabolic pathway it undergoes further hydroxylation to 3,4-dihydroxybenzoate. The latter is formed by several microorganisms and the enzyme, 4-hydroxybenzoate hydroxylase has been purified to homogeneity.[360] Strains of *Bacillus* that utilize 4-hydroxybenzoate convert it to gentisate (Figure 40F). The introduction of the second —OH group is accompanied by a carboxyl group migration.[361]

2. Nitrogenous Metabolites (Figure 41)

CHA can undergo amination with the formation of either 2-amino-2-deoxy-ICHA (Figure 40D) or 4-amino-4-deoxy-CHA (Figure 40H). From these two intermediates, many other varied nitrogenous compounds derive, including important primary metabolites such as trp and folate, and secondary metabolites including antibiotics. The aromatic compounds derived from 2-amino-2-deoxy-ICHA characteristically have —NH_2 *ortho* to —COOH, the prototype being anthranilic acid (2-aminobenzoate). The compounds derived from 4-amino-4-deoxy-CHA characteristically have —NH_2 *para* to —COOH, the prototype being 4-aminobenzoate.

a. 2-AMINO-2-DEOXY-ICHA AND DERIVED METABOLITES

The compound referred to here as 2-amino-2-deoxy-ICHA is formally *trans*-6-amino-5-[(1-carboxyethenyl)oxy]-1,3-cyclohexadiene-1-carboxylate. It has now been definitely characterized as an intermediate in the biosynthesis of anthranilic acid and, as such, plays an important role in trp biosynthesis. Therefore, it is considered first of all with reference to trp.

The lengthy branch from CHA to trp (Figure 46) has been intensively investigated, particularly with respect to regulation and genetics. There is considerable variation from microorganism to microorganism with respect to the organization of the genes of the trp biosynthetic pathway.[362] Some gene products are enzymatically bi- or trifunctional, and some enzyme activities require two gene products. The number of genes involved is either four (*A. nidulans, Coprinus* sp., *N. crassa, Schizosaccharomyces pombe*), five (*S. cerevisiae, E. coli*), six (*Serratia marcescens, B. subtilis*), or seven (*Pseudomonas putida, Brevibacterium lactofermentum*).

The genes encoding the necessary enzymes in the enteric bacteria, *E. coli* and *S. typhimurium*, are contiguous and constitute the deservedly famous "trp operon". This operon is one of the most studied groups of anabolic genes in these two

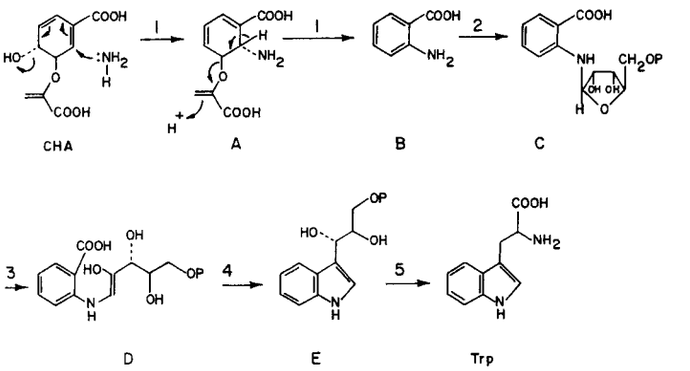


FIGURE 46. The biosynthesis of trp from CHA. A = 2-amino-2-deoxy-ICHA; B = anthranilate; C = phosphoribosylanthranilate; D = 1-(2-carboxyphenylamino)-1-deoxyribulose 5-phosphate; E = indoleglycerol phosphate. 1, ASI (AS II required if gln is the amino donor)—from A → B, pyruvate is lost; 2, anthranilate phosphoribosyl transferase (AS II)—requires phosphoribosylpyrophosphate and eliminates pyrophosphate; 3 phosphoribosylanthranilate isomerase; 4, indoleglycerol phosphate synthase-reaction with loss of CO_2; 5, Trp synthase—requires ser and eliminates glyceraldehyde 3-phosphate. Structure D may also exist as the keto isomer.

organisms and other enteric bacteria. The nucleotide sequence of the entire operon (five genes, *trpE, trpD, trpC, trpA,* and *trpB*) is known for both *E. coli* and *S. typhimurium*.[363,364] In *B. subtilis* a *trp* operon of six structural genes has been sequenced.[365] This operon is part of a *trpEDCFBA-hisH-tyrA-aroE* or even larger cluster with *aroFBH* at the 5′ end.[281] The whole *trp* operon has also been sequenced in *Brevibacterium lactofermentum;* the 7725 bp fragment of DNA contains seven ORFs corresponding to the genes *trpL, trpE, trpG, trpD, trpC, trpB,* and *trpA*.[366]

In view of the existence of many reviews emphasizing genetics and regulation,[367-371] it would be presumptious to attempt a general treatment here. Attention is focused on the biochemistry of trp biosynthesis. The following recent papers may be noted for convenience: the study of *trp* genes in *Pseudomonas acidovorans*[372] and in *Zymomonas,*[373] and the regulation of trp biosynthesis in *Caulobacter crescentus*.[374]

Of the five *E. coli* genes, two specify a protein with bifunctional enzymatic activity, and two specify different subunits of a single enzyme. In biosynthetic sequence, they are as follows:

Gene	Enzyme activity	EC no.
trpE	Anthranilate synthase I[a]	EC 4.1.3.27
trpD	Anthranilate phosphoribosyl transferase[b]	EC 2.4.2.18
trpC	Bifunctional phosphoribosylanthranilate isomerase-indoleglycerol phosphate synthase	EC 4.1.1.48
trpA, trpB	A and B protein components of trp synthase	EC 4.2.1.20

[a] Activity with NH_2 as amino donor.
[b] This component also required for overall anthranilate synthase activity when gln is the amino donor.

In yeast, phosphoribosylanthranilate isomerase and indoleglycerol phosphate synthase are monofunctional enzymes specified by two genes. The yeast pattern is shown below; all of the genes have been sequenced. Other eukaryotic organisms have been reviewed:[368]

Yeast gene	Enzyme	Sequence Ref.
TRP2	Anthranilate synthase	375
TRP4	Anthranilate phosphoribosyl transferase	376
TRP1	Phosphoribosylanthranilate isomerase	377
TRP3	Indoleglycerol phosphate synthase	375
TRP5	Tryptophan synthase	378

Although, of course, trp is formed in plants, the enzymes are not as well characterized as in microorganisms.[25] The 4-chloro derivative of trp occurs in pea seed protein as a minor component. It is probably the precursor for 4-chloroindole-3-acetic acid (a natural auxin). Elucidation of the mechanism for introduction of the chlorine atom would be of considerable interest.[379]

I. Anthranilate Synthase (AS)

This first enzyme in the pathway from CHA to trp is strongly inhibited by trp. Either NH_3 or gln may function as the donor of the amino group.

$$\text{CHA} + NH_3 \rightarrow \text{Anthranilate} + \text{Pyruvate}$$

$$\text{CHA} + \text{Gln} \rightarrow \text{Anthranilate} + \text{Pyruvate} + \text{Glu}$$

2-Amino-2-deoxy-ICHA (Figure 46A) had been considered a likely intermediate in the AS reaction. It was synthesized in either (+) or racemic forms and was enzymatically competent in the AS reaction.[380,381] Since overall, protonation of the *enol*-pyruvol side chain occurs on the *re* face,[156] the reaction, 2-amino-2-deoxy-ICHA → pyruvate + anthranilate, may be concerted (Figure 47, A → B + C).

Direct evidence for the participation of 2-amino-2-deoxy-ICHA in the overall process has not been obtained. However, using a lactyl-CHA analog (Figure 47D), evidence was obtained for the actual accumulation of the corresponding intermediate (Figure 47E) to about 15 mol% during the reaction. The V_{max} with this lactyl analog was 3% of that with CHA. The product, Figure 47E, was converted by AS to anthranilate in the absence of NH_3. The (S)-lactyl analog was also a substrate for AS having V_{max} = 4.4% of that with CHA.[382] The cycloheptadiene analog of CHA was a good inhibitor of AS (as it was of 4-aminobenzoate synthase).

2-Amino-2-deoxy-ICHA is rather unstable and undergoes the nonenzymatic rearrangement (20 h, room temperature) shown as Figure 47, A → F; the latter compound is an AS inhibitor. This rearrangement resembles that of ICHA to isoprephenate.

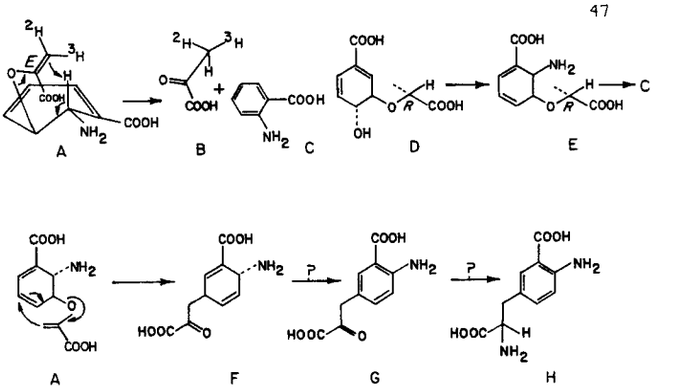


FIGURE 47. Formation of anthranilate from 2-amino-2-deoxy-ICHA and analogs; rearrangement of 2-amino-2-deoxy-ICHA. A = 2-Amino-2-deoxy-ICHA; B = pyruvate; C = anthranilate; D = lactyl analog of CHA; E = lactyl analog of 2-amino-2-deoxy-ICHA; F = rearrangement product of 2-amino-2-deoxy-ICHA; G = 3-(4-amino-3-carboxyphenyl)pyruvate; H = 3-(4-amino-3-carboxyphenyl)alanine.

It is a process that had been proposed some time ago as a route to 3-(4-amino-3-carboxyphenyl)alanine (Figure 47H) via a keto acid (Figure 47G).[383] Although this amino acid is apparently not known as a natural product, it has been synthesized as the racemate.[384]

Bacterial AS has two subunits. Component I(AS I) catalyzes the overall conversion, CHA + NH_3 → anthranilate + pyruvate, while component II(AS II) has a gln amidotransferase activity (thus enabling gln to be used as the amino donor). In the AS-gln reaction, gln binds to cys 84 of AS II. AS II is often fused as a multifunctional protein with other trp biosynthetic enzymes.

One of the best studied preparations of AS is that present in *S. typhimurium* where AS II also carries an anthranilate phosphoribosyl transferase (PRT) activity.[385] The intact native AS-PRT complex is a tetramer (M_r = 228,000) containing two molecules each of AS I (M_r of monomer = 57,000) and AS II (M_r of monomer = 56,900). Proteolytic digestion of this $\alpha_2\beta_2$ intact complex leads to an "AS partial complex" (M_r = 156,00), which contains an amino-terminal fragment of AS II. The AS II so modified lacks PRT activity. Both of the complexes and also the individual subunits (obtained from appropriate *trpE* and *trpD* mutant strains) have been purified to homogeneity.[385] Thus, from *S. typhimurium* TB1409/pSTP89, a two-step purification gave homogeneous preparations of the intact AS-PRT complex (purification of 20.3-fold with respect to AS activity, 17.8-fold with respect to PRT activity). Enzyme preparations from other organisms have also been well described.[9,386,387]

The kinetics and metal-binding properties of AS preparations from *S. typhimurium* were recently investigated. Metals interact at the active site with CHA but no gln.[388]

In *N. crassa,* AS II (glutamine amidotransferase) is part of a trifunctional polypeptide with phosphoribosylanthranilate isomerase and indoleglycerol phosphate synthase. This polypeptide is encoded by the gene, *TRP1,* which has been cloned in *E. coli* and sequenced.[389] The multifunctional tetrameric AS complex of this organism was degraded by elastase to two fragments. One of them possessed AS activity, the other had both indoleglycerol phosphate synthase and phosphoribosylanthranilate isomerase activity. For a detailed model of the AS complex the original paper should be consulted.

In plants, AS apparently lacks a subunit structure, and only gln serves as the amino donor.[25] Two isozymes of AS were partially separated from extracts of plants and cultured cells of *Nicotiana tabacum.*[390] One form was resistant to feedback inhibition by trp and was localized in the cytosol of cultured cell protoplasts; the other was trp sensitive and located in the particulate fraction. In earlier work, two forms of AS were obtained from 5-methyl-trp susceptible and -resistant cultured cells of *Solanum tuberosum.*

The genes, *trpE* and *trpD,* have been sequenced as part of the *trp* operon in *E. coli* and *S. typhimurium.* The DNA sequences of these genes show considerable homology with only a 12.5% difference in amino acid composition.[364,391]

As already noted, the nucleotide sequence of yeast *TRP2* (encoding AS I) has been deterrmined. In this organism, the AS activity is located as a multifunctional, hetero-oligomeric enzyme with indoleglycerol phosphate synthase.[375] The AS gene of *Rhizobium meliloti* has been cloned in *E. coli* and its sequence determined.[392] Similarly, the *trpE* gene of the thermophile, *Thermus thermophilus* (encodes AS I), has been cloned and sequenced.[393]

II. Anthranilate Phosphoribosyl Transferase (PRT)

As already noted, in *E. coli* and *S. typhimurium* the phosphoribosylation of anthranilate with phosphoribosyl pyrophosphate (see Figure 46) is catalyzed by the AS II component (*trpD* gene product). This protein was purified (136-fold for the dimeric form) from *S. typhimurium* TB41 to about 95% homogeneity.[385] From an overproducing (400-fold) strain of *S. cerevisiae,* PRT has also been purified 15.8-fold to near homogeneity (95%). The enzyme was a dimer (M_r = 83,000) of identical subunits, with a sequential catalytic mechanism. This purified enzyme is of value for *in situ* generation of the unstable compound, phosphoribosylanthranilate.[394]

III. Phosphoribosylanthranilate Isomerase (PRAI)

Phosphoribosylanthranilate isomerase converts phosphoribosylanthranilate (Figure 48A) to 1-(2-carboxyphenylamino)-1-deoxyribulose 5-phosphate (Figure 48C). This reaction (see Figure 48) is a practically irreversible Amadori rearrangment. In enteric bacteria, it occurs with indoleglycerol phosphate synthase (IGPS) activity on a single polypeptide chain of M_r = 49,500 (452 amino acid residues). A 39-fold purification from *E. coli* W3110 gave pure product which could be crystallized and for which the three-dimensional structure was determined.[395] The two catalytic activities occur as N-terminal IGPS, residues 1 to 255, and C-terminal PRAI, residues 256 to 452. These activities reside on distinct functional domains of similar folding — that of an eightfold parallel β-barrel with α-helices on the outside connecting the β-strands. Both active sites are in depressions on the surface of the domains created by the outward curving loops between the carboxyl termini of the β-sheet strands and the subsequent α-helices. The active sites do not face each other so that channeling of substrate between them is probably not possible. The advantage gained by the gene fusion appears to be the mutually stabilizing interactions between the two functional domains.

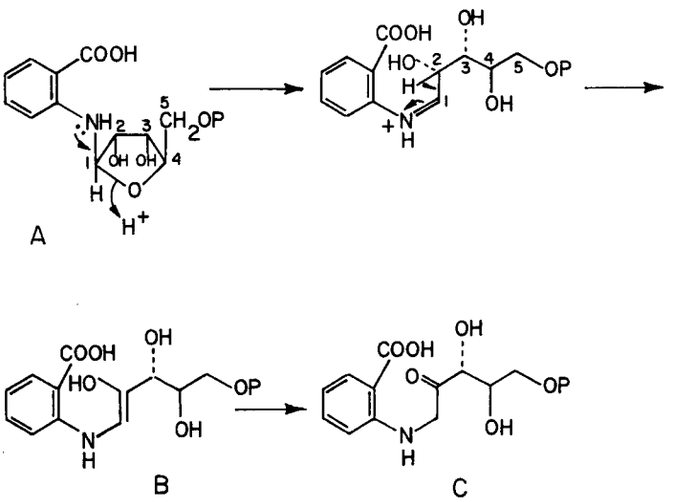


FIGURE 48. Action of phosphoribosyl anthranilate isomerase in the formation of *enol*-1-carboxyphenylamino-1-deoxyribulose 5-phosphate. The rearrangement of phosphoribosylanthranilate (A) leads to the *enol* product, B; tautomerization of the keto structure [1-(2-carboxyphenylamino)-1-deoxyribulose 5-phosphate] presumably occurs spontaneously.

The *TRP1* gene of *Kluyveromyces lactis* has been cloned and analyzed.[396] The role of the *TRP1* gene in yeast trp biosynthesis has been investigated.[397]

IV. Indoleglycerol Phosphate Synthase (IGPS)

This enzyme catalyzes the cyclization of 1-(2-carboxyphenylamino)-1-deoxyribulose 5-phosphate to indoleglycerol phosphate (see Figure 49). As noted, IGPS is associated with PRT as a bifunctional protein in enteric bacteria. *B. subtilis* and *Pseudomonas putida* have two separate enzymes for these activities; *Brevibacterium flavum* has a multienzyme complex.

In yeast, IGPS is part of a multifunctional hetero-oligomeric enzyme encoded by *TRP3* and *TRP2*; nucleotide sequences have been determined.[375] In *A. nidulans,* IGPS is part of a

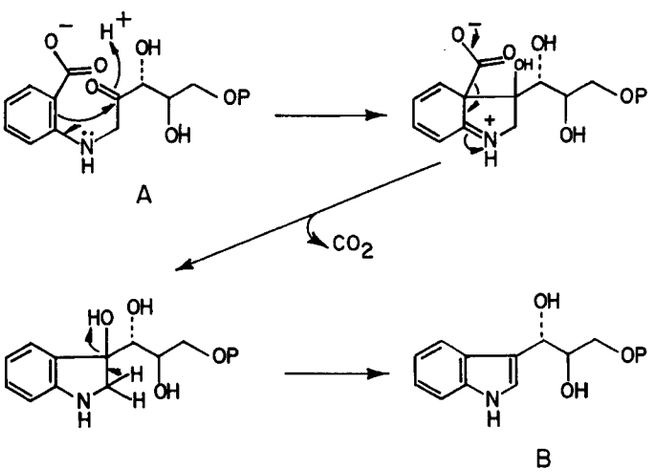


FIGURE 49. Possible reaction mechanism for indoleglycerol phosphate synthase. The substrate [1-(2-carboxyphenylamino-1-deoxyribulose 5-phosphate], A, is converted to indoleglycerol phosphate, B, with loss of CO_2.

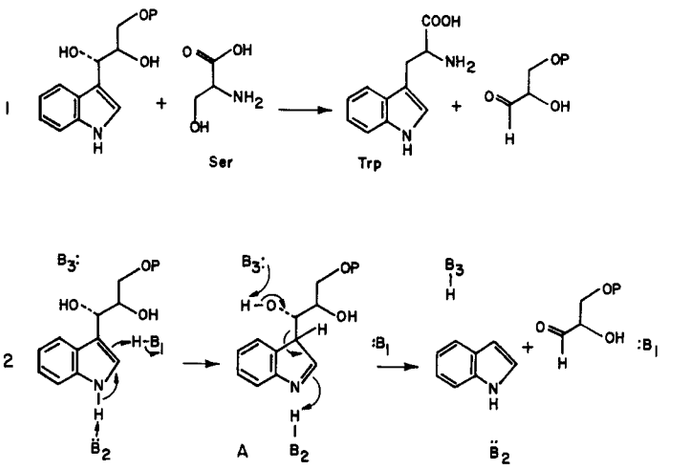


FIGURE 50. Reaction mechanism for trp synthase. (Line 1) Overall reaction for trp formation. (Line 2) Formation of indole; three enzyme bound groups, B_1, B_2, and B_3 are postulated (see text).

trifunctional polypeptide that catalyzes the first, third, and fourth steps of trp biosynthesis. The genes have been sequenced.[398] In *N. crassa,* IGPS is part of a "postchorismate multienzyme complex" also containing AS and PRAI activities. A purification procedure for this complex (M_r = 240,000) has been described.[399]

v. Trp Synthase

Despite the already enormous number of publications relating to this much-studied enzyme, a landmark paper, published in 1988, will likely trigger a new avalanche of information. This publication provides the complete three-dimensional structure of crystalline trp synthase from *S. typhimurium*. It is indicative of the volume of the literature that this single paper lists 94 references.[400] All that can be attempted here is a brief statement of the present picture with emphasis on the complex chemistry of the catalytic reactions. The "charmed history" of trp synthase has been reviewed.[401]

This description focuses on the enzyme from either *E. coli* or *S. typhimurium*. For the present purpose, it is necessary to know that the holoenzyme is a tetramer of two dissimilar subunits ($\alpha_2\beta_2$); the individual polypeptides are also described as the A and B proteins (there is more detail in a later section.) The overall reaction is that of Figure 50. It is catalyzed by the tetrameric holoenzyme or by the isolated β_2 dimer. Trp synthase catalyzes many other reactions such as β-eliminations and replacements, transaminations, and racemizations; these reactions are not considered here.

The reaction catalyzing synthesis of trp is a two-step process; however, indole is not normally released as an intermediate. The two separate reactions are as follows:

a. Indoleglycerol phosphate → Indole + Glyceraldehyde 3-phosphate
b. Indole + Ser → Trp + H_2O

Reaction a is catalyzed by the α-subunit (or by pyridoxal free tetramer) and b by the β_2-subunit (or tetramer) in presence of pyridoxal phosphate.

Reaction a is essentially an aldol cleavage, probably facilitated by three catalytic groups using "push-pull" general acid-base catalysis (see Figure 50). Of the three groups shown, there is strong evidence that B_3 is the carboxylate group of glu 49 of the α-subunit. This is the group catalyzing the actual cleavage following formation of a tautomeric structure, Figure 50A. A second catalytic group, B_2 is very likely asp 60.[402-404] This group is involved in removing the hydrogen on N-1 of the indole ring; B_1 protonates the indole ring at C-3 so that these two groups cooperate to form the tautomer structure (Figure 50A). Asp 60 can be replaced by glu with retention of some catalytic activity.

A tyr residue, 175, is located near the active site and its —OH group might have some role; since it can be replaced by phe it is not essential. Replacement of tyr 175 by cysteine caused loss of activity as did replacement of gly 211 by glu. Somewhat surprisingly, the double mutant containing both of these changes (tyr → cys at 175, and gly → glu at 211) had some activity. This was attributed to maintenance of the proper geometry of substrate binding rather than to compensatory effects on catalysis.[403]

The α-subunit of trp synthase from *E. coli, S. typhimurium,* and five interspecies hybrids was examined by equilibrium and kinetic methods with respect to the urea-induced unfolding of the protein. The proteins all followed the same folding mechanism and this behavior was consistent with the postulate that

folding mechanisms are conserved in homologous proteins. This conservation is apparently an important evolutionary pressure.[405]

In reaction b between indole and ser, the β-OH group of ser is replaced by an indole residue. Configuration is retained at the β-position during this replacement for reaction either with indole or indoleglycerol phosphate. This simple statement neglects the technical difficulties of this elegant work; some 14 enzyme-catalyzed reactions were involved.[2,406] Any detailed mechanism must account for this overall stereochemistry (Figure 51).

The ser first reacts with (enzyme bound) pyridoxal phosphate (PLP) to form the aldimine structure usual in these reactions. Each β-subunit contains 1 mol of bound PLP. Following loss of the β-OH group, replacement by indole occurs (Figure 52); the trp-PLP aldimine forms pyridoxamine phosphate and trp in the usual way.

The intermediate indolenine, Figure 52A, was believed to have the S configuration since the (αS,3S) diastereoisomer of 2,3-dihydro-L-trp (Figure 53A) was a potent inhibitor of trp synthase, whereas the other diastereoisomer (αS,3R, Figure 53B) was much less potent. The dihydro-L-trp is a reaction intermediate analog so the indolenine (Figure 52A) was postulated to have 3S configuration. It is of interest that the (αS,3R) diastereoisomer (and not the αS,3S) was inhibitory to another enzyme, tryptophanase.[407]

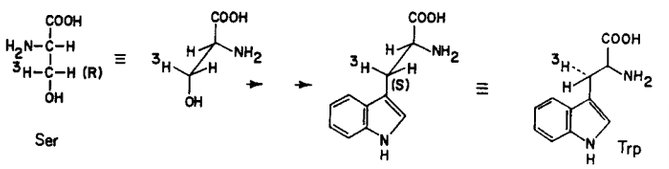


FIGURE 51. Stereochemistry of replacement of ser —OH group by indole during action of trp synthase. The R to S change is a result of the operation of the sequence rule; there is actual retention of configuration.

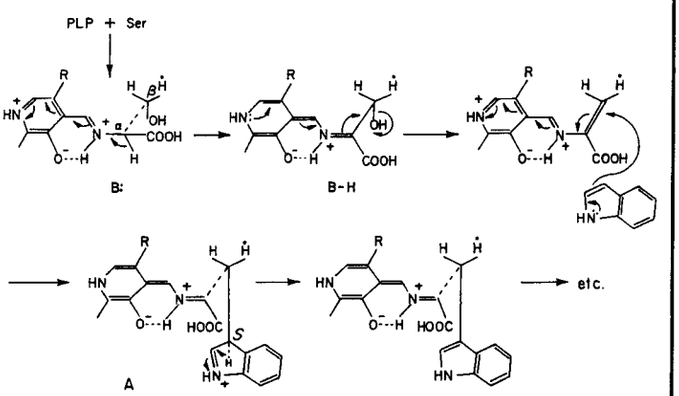


FIGURE 52. Role of pyridoxal phosphate in trp synthase reaction. PLP = pyridoxal phosphate; R = —CH_2OP; "etc," indicates breakdown to trp and pyridoxamine phosphate.

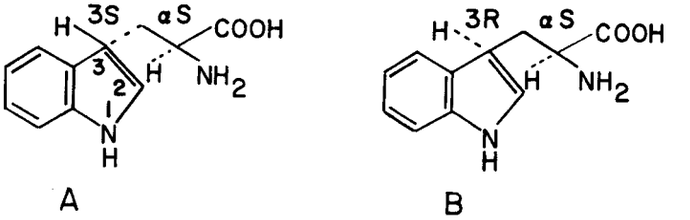


FIGURE 53. Diastereoisomers of 2,3-dihydro-trp.

This mechanism is a simplified one in that the actual interaction of the β-subunit and PLP (presumably lysine bound) was ignored. By the use of various substrate analogs and by kinetic analysis[408,409] evidence was obtained for a very complex series of transient intermediates.[410-414]

Although early work had suggested roles for his 86, arg 148, and cys 230 in the reactions of the β-subunit, these conclusions have been discounted.[415] In the wild-type β-subunit, lys 87 forms the Schiff base with PLP. Amino acid substitution of lys 87 yields a catalytically inactive protein which does, however, bind PLP, α-subunit, and ser. In the homodimeric form of trp synthase from *N. crassa*, the lys that binds PLP is located in the same relative position as is that in the yeast and *E. coli* enzymes.[416]

A new example of the versatility of trp synthase has been discovered.[417] The holo $\alpha_2\beta_2$ complex was converted to apo tetramer by removal of PLP. This apo $\alpha_2\beta_2$ complex was found to catalyze a "half-transaminase" reaction (see Figure 54) between indole-3-pyruvate and pyridoxamine phosphate, forming PLP and trp.

$$\text{Pyridoxamine-P} + \text{Indole-3-pyruvate} \rightarrow \text{PLP} + \text{Trp}$$

The product of this process, trp, is not a natural substrate for trp synthase and pyridoxamine phosphate would normally be formed during a "complete-transaminase" reaction and recycled by the second half-reaction back to PLP. The PLP formed in this process is not released from the enzyme, and hence the reaction is stoichiometric with respect to the apo $\alpha_2\beta_2$ complex used initially.

Trp synthase holoenzyme and the individual subunits have been purified from many organisms.[418] Thus to cite only one example, a 7.4-fold purification of the tetrameric complex from *E. coli* strain W3110 gave homogeneous material which could be crystallized (and with a yield of 2.85 g from 500 g of bacterial cell paste). The two subunits may be conveniently prepared from the tetrameric complex (which is more stable than the two separate subunits).[419]

As indicated earlier, the three-dimensional structure of crystalline *S. typhimurium* trp synthase tetramer has been determined at 2.5 Å resolution.[400] The four polypeptide chains were arranged nearly linearly in the order αββα as a complex of

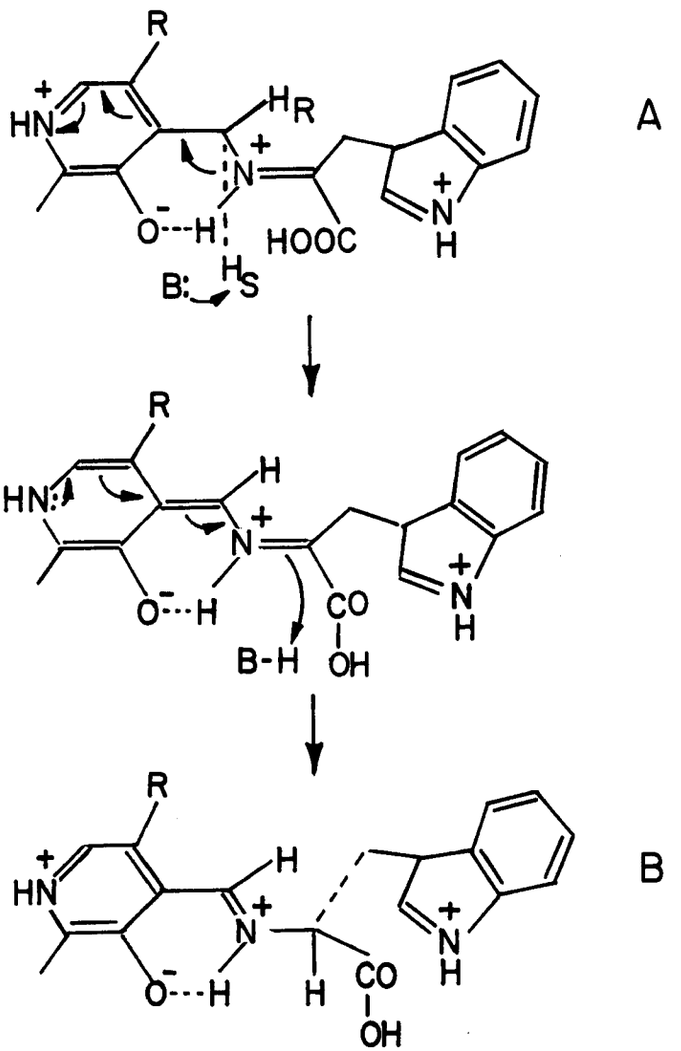


FIGURE 54. "Half-transaminase" reaction of trp synthase. The structure, A, is formed from pyridoxamine phosphate and indole-3-pyruvate by action of apo $\alpha_2\beta_2$-trp synthase. The final structure, B, breaks down to PLP and trp.

length 150 Å. The overall polypeptide fold of the α-subunit was that of an eightfold α/β barrel. The β-subunit contained two domains of nearly equal size folded similarly as helix/sheet/helix structures. The PLP binding site was deep within the interface between the two β-subunit domains. The active sites of neighboring α- and β-subunits were separated by a distance of about 25 Å; a tunnel connected these active sites and its diameter was that of indole. This tunnel was postulated to play a role in facilitating the diffusion of indole from its point of production (α-subunit active site) to its site of utilization (β-subunit active site). Hence, free indole is not liberated during the action of trp synthase.

A third enterobacterial *trpA* sequence has been determined, that of *K. aerogenes (sic — K. pneumoniae?)* There is considerable homology with the sequences in *E. coli* and *S. typhimurium*.[420]

In *S. cerevisiae*, trp synthase is a homodimer of two subunits of M_r = 76,000 (this is slightly larger than the sum of the A and B chains of the *E. coli* enzyme). It is likely a protein in which the A and B domains, characteristic of *E. coli*, have undergone fusion. The two segments are joined by a connecting region of 28 residues. Overall, the protein probably contains 706 residues (an initial met is assumed to be removed by processing). The structural gene, *TRP5*, has been sequenced in part and completely.[420,421] There is considerable homology between the yeast trp synthase and the A and B chains of the enzyme from enteric bacteria. One unusual feature is that the A and B coding regions are fused in the order A followed by B. In all other prokaryotes so far examined, the two structural genes are in the same operon and that for the B chain precedes that for the A chain.

The *N. crassa trp-3* sequence (encoding trp synthase) has strong homology to yeast *TRP5* polypeptide (A domain, 54% and B domain, 75%), but less so to *E. coli trpA* polypeptide (31% identity) and *trpB* polypeptide (50% identity).[422]

The nucleotide sequences of the *trpBA* genes of the archebacterium, *Methanococcus voltae* have been determined. There were significant homologies with the amino acid sequences of trp synthase from other sources.[423] In *P. aeruginosa*, the *trpB* and *trpA* genes are not part of an operon. They are separate from the other structural genes of the trp pathway and undergo regulation by induction rather than by repression. They are transcribed in the order *trpB-trpA*. The DNA sequences of the two genes have been determined along with flanking sequences. Again, there are considerable homologies with other trp synthases.[424]

The trp synthase β-subunit of *E. coli* and other organisms shows significant homology to the PLP dependent *O*-acetylserine sulfhydrase A[425] and to threonine synthase.[426] These homologies are consistent with similarities in the catalytic processes of these enzymes. The enzymes may have evolved from a common ancestor.

vi. Phenazines

There are more than 50 bacterial phenazine pigments (Figure 41) representing "every colour of the visible spectrum".[427] They have a long history since the blue-colored pyocyanine has been known since 1859. Phenazines often show antibiotic properties and an ability to intercalate with double stranded DNA. Phenazine 1-carboxylate biosynthesis is important for the biological control of take-all disease in wheat root.[428] In biosynthetic terms, it is likely that they all derive from phenazine-1,6-dicarboxylate (see Figure 55) via the SHK pathway. Since neither anthranilate nor 3-aminobenzoate is involved, the anthranilate precursor, 2-amino-2-deoxy-ICHA, may represent the branch point.[429]

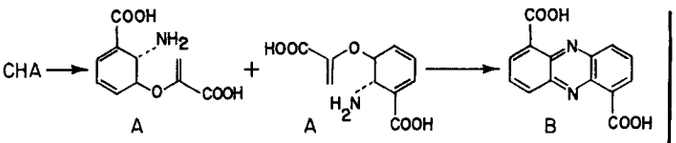


FIGURE 55. Possible biosynthetic pathway from 2 mol 2-amino-2-deoxy-ICHA, A, to phenazine-1,6-dicarboxylate, B. The latter compound is the likely precursor to other phenazines.

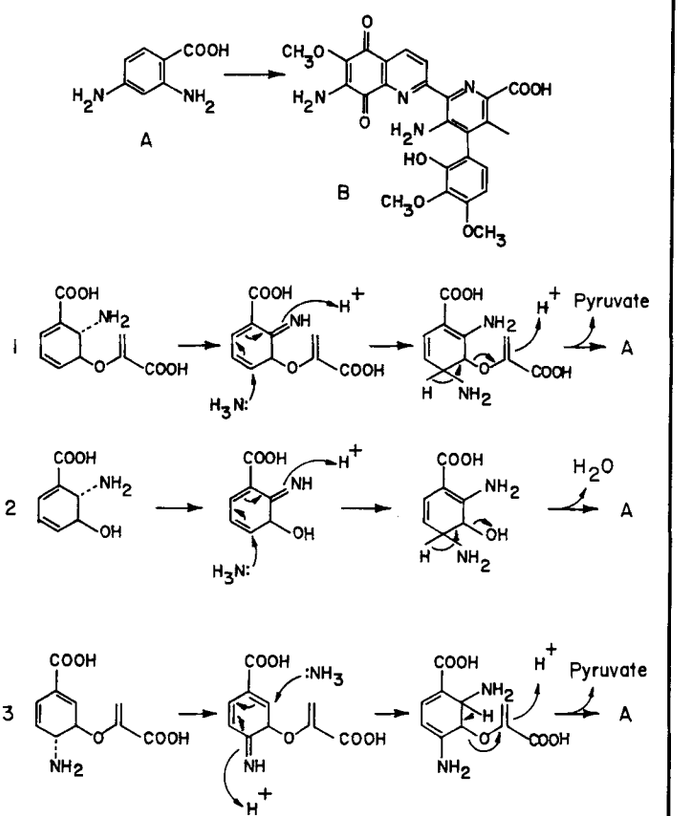


FIGURE 56. Role of 4-aminoanthranilate, A, in streptonigrin biosynthesis. Three possible mechanisms for 4-aminoanthranilate synthesis are shown.

vii. Streptonigrin

Streptonigrin (see Figure 41), an anticancer antibiotic from *Streptomyces flocculus*, has one SHK derived ring, probably via 4-aminoanthranilate. The latter amino acid was isolated from *S. flocculus* cultures. Although the precise origin of 4-aminoanthranilate is unknown, it could originate from intermediates derived from CHA.[430,431] Either 2-amino-2-deoxy-ICHA (Figure 56B), or 2,3-dihydro-3-hydroxyanthranilate (Figure 56C), or 4-amino-4-deoxy-CHA (Figure 56D) could be the precursor.

viii. 2,3-Dihydro-3-Hydroxyanthranilate

The compound, *trans*-2,3-dihydro-3-hydroxyanthranilate (Figure 41D) has been known for some time to be a metabolite from *Streptomyces aureofaciens*.[5] It is likely to be derived from 2-amino-2-deoxy-ICHA.

ix. 3-Hydroxyanthranilate

It was suggested[383] that *trans*-2,3-dihydro-3-hydroxyanthranilate was a precursor for 3-hydroxyanthranilate (Figure 41E). The lactic acid ester of 3-hydroxyanthranilate is the antibiotic, oryzoxymycin.[432] The aldehyde derived from 3-hydroxyanthranilate, 6-amino-5-hydroxy-1,3-cyclohexadiene-1-carboxaldehyde (Figure 41A), is the antibiotic, P-3355, produced by *Streptomyces amylovorus*.[433]

Although 3-hydroxyanthranilate is a precursor to the actinomycins, it is derived in those cases from trp. However, *Brevibacterium iodinum* produces not only the phenazine compound, iodinin, but in addition, small amounts of 2-aminophenoxazinone (Figure 41F). While this compound shows a structural relationship with the actinomycins, the 3-hydroxyanthranilate precursor is apparently not derived from trp. For production of 2-aminophenoxazinone, labeled SHK was a more effective precursor than trp and trp addition did not decrease incorporation of label from SHK into the metabolite.[429] Hence, it is likely that in this organism the 3-hydroxyanthranilate is derived from 2-amino-2-deoxy-ICHA via the dihydro-3-hydroxyanthranilate.

More recently, 3-hydroxyanthranilate was established as a precursor of the antibiotic, LL-C10037α (Figure 41B) produced by *Streptomyces* LL-C10037.[349] It was proved that the —NH_2 group was introduced at the position corresponding to C-6 of SHK. The steps beyond 3-hydroxyanthranilate have been investigated.[434] A second hydroxyanthranilate, with the OH group at position 6, is derived from ICHA and is discussed later.

b. 4-AMINO-4-DEOXY-CHA AND DERIVED METABOLITES

In addition to the amination of chorismate at its position 2 (= position 6 of SHK) as in the formation of anthranilate, CHA also undergoes a process leading to production of 4-aminobenzoate; this represents formally —OH loss and amination at position 4. 4-Aminobenzoate is an important intermediate for the formation of folate[435] and some other compounds. This review uses 4 rather than *para* to describe the amino group location; unfortunately, the relevant gene names use the abbreviation PABA for 4-aminobenzoate.

The biosynthesis of 4-aminobenzoate is not yet completely understood. Enzyme preparations converting CHA to 4ABA have been purified to some extent. In *E. coli*, the (holo) enzyme named as 4ABA synthase contains a large subunit (4ABA synthase I, M_r = 53,400, encoded by *pabB*) and a small subunit (4ABA synthase II, M_r = 212,700, encoded by *pabA*). Nucleotide sequences have been determined for *pabB* in *E. coli*, *S. typhimurium*, and *K. pneumoniae*.[436,437] Similarly, *pabA* nucleotide sequences have been determined for *E. coli*, *S. typhimurium*, *K. pneumoniae*, and *Serratia marcescens*.[438,439]

There is considerable homology between these sequences and those of the AS components I and II. Apparently, a common ancestor existed for the genes encoding 4ABA synthase and AS.

The large *E. coli* subunit has been partially purified (9-fold) to 25 to 30% homogeneity from an overexpressing strain.[382] The tandem overexpression of both *pabA* and *pabB* gene products has also been reported together with modest purification of the products.[440]

Some other organisms contain a similar two-subunit enzyme, but in *Streptomyces griseus* this is not the case. In this organism, the partially purified enzyme could not be separated into two fractions, and was determined to have an approximate M_r = 50,000.[441]

Although attractive mechanisms exist for formation of 4ABA from ICHA, 4ABA synthase I from the strain *E. coli* BN116, containing the plasmid pAS4, failed to produce 4ABA from ICHA and NH_3.[442] The use of CHA rather than ICHA has been confirmed in *K. pneumoniae* and *Streptomyces* sp.[443]

The holoenzyme, 4ABA synthase, forms an intermediate, likely pre-aromatic, and a separate enzyme (tentatively termed X, M_r = 49,000) converts the intermediate to 4ABA (see Figure 57).[444] The 4ABA synthase II component is a gln amidotransferase subunit and can be omitted from incubation mixtures if NH_3 is supplied. It is apparently responsible for amide transfer to 4ABA synthase component I. The two components must interact physically in order for 4ABA synthesis to occur.

The only material securely identified as an intermediate in 4ABA bioynthesis is 4-amino-4-deoxy-CHA (Figure 57A) synthesized as the racemate.[442] 4-Amino-4-deoxy-CHA is converted enzymaticaly to 4ABA and is possibly the substrate for "enzyme X". If so, the remaining problem is how is CHA converted to 4-amino-4-deoxy-CHA without the intervention of ICHA? The antranilate precursor, 2-amino-2-deoxy-ICHA, might be produced and converted to 4-amino-4-deoxy-CHA by either a sigmatropic shift, an addition-elimination reaction, or a carboxyl group migration.[443]

CHA + NH_3 → 2-amino-2-deoxy-ICHA → 4-amino-4-deoxy-CHA → 4ABA

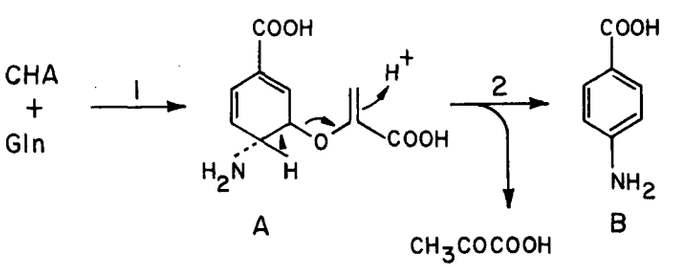


FIGURE 57. Formation of 4-aminobenzoate by action of 4-ABA synthase. Reaction 1 requires 4-ABA synthase I and II, and reaction 2 requires the putative enzyme "X".

This proposal presumably requires at least three enzymes if NH_3 is used, four if gln is the amido donor. However, the evidence so far available suggests a maximum of three for the reaction with gln. A simpler reaction possibility would require a nucleophilic group, X^-, on 4ABA synthase I (See Figure 58).[445]

Several compounds have been examined for inactivation and inhibition of 4ABA synthase. A cycloheptadiene structure, Figure 59A, was the most potent inhibitor with K_i = 230 μM (K_M for NH_3 dependent reaction with CHA = 42 μM).[382] As already noted, this material also inhibits CHA mutase and AS. Moreover, the glycolyl ether, Figure 59B, was actually a better substrate than CHA itself (V_{max} = 140% of that with CHA). The R and S lactyl ethers, Figure 59C and D, were also substrates, but with V_{max} values reduced, respectively, to 13 and 5.4% of that with CHA.[382]

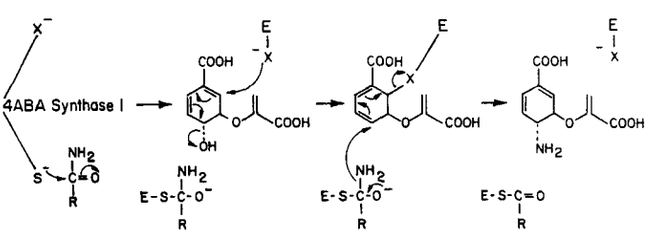


FIGURE 58. Possible mechanism for formation of 4-amino-4-deoxy-CHA. R = —$(CH_2)_3$COOH. The 4-ABA synthase I is postulated to have a basic group, X^-, and a cys residue as shown at the left. Subsequently in this figure, 4-ABA synthase I is represented by E. Initially, the cys residue combines with gln; subsequently the group X^- interacts at position 2 of CHA and the "E-CHA" complex is further attacked by the enzyme-bound gln at position 4. 4-ABA synthase II could be involved in these later reactions, and X could be associated with it instead of 4-ABA synthase I.

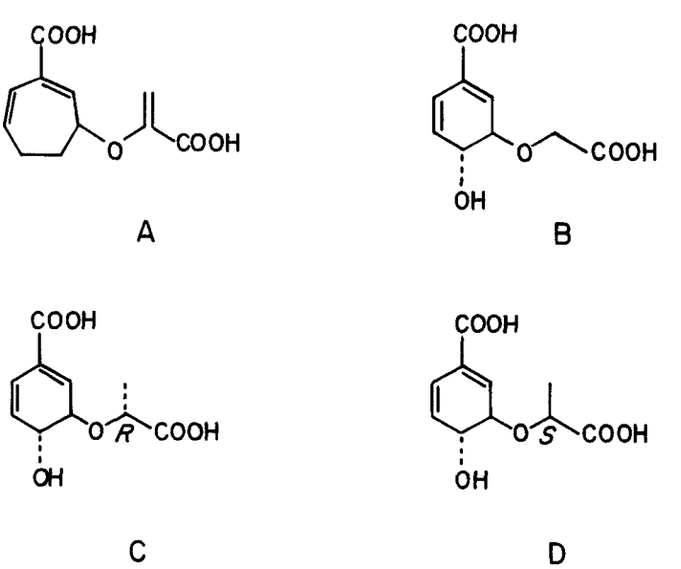


FIGURE 59. Inhibitors and substrates for 4-ABA synthase.

In addition to its important role in the formation of folate, 4ABA may also be involved in the biosynthesis of methanopterin. This cofactor, produced by methanogenic bacteria such as *Methanobacterium thermoautotrophicum*, contains a "pentitylaniline" unit which apparently derives from an intact pentose chain, and 4ABA which has undergone decarboxylation.[446]

I. *N*-(γ-L-Glutamyl)-4-Hydroxyaniline and Related Compounds

Compounds derived from 4ABA occur in the mushroom, *Agaricus bisporus*. One is the unusual metabolite, *N*-(γ-L-glutamyl)-4-hydroxyaniline (Figure 60A). Neither labeled PPA nor anthranilate were used as precursors for this compound; however, excellent incorporation of ^{14}C-labeled CHA and 4ABA was observed.[447] Hence, 4ABA is likely the direct precursor; it undergoes a hydroxylation accompanied by decarboxylation (compare the "pentitylaniline" unit just discussed for methanopterin).

$$4ABA + NAD(P)H + O_2 \rightarrow 4\text{-Hydroxyaniline} + NAD(P)^+ + H_2O$$

The enzyme, 4ABA hydroxylase, which requires FAD, was purified to homogeneity and utilized the H_A proton of reduced pyridine nucleotide.[448,449] Further metabolism of *N*-(γ-L-glutamyl)-4-hydroxyaniline leads to the hydroxyazaquinone, Figure 60B. This material is an inhibitor of some enzymes requiring —SH groups at the active site.[450]

II. Chloramphenicol, Obafluorin, and Related Compounds

Formation of chloramphenicol requires L-(4-aminophenyl)alanine which probably derives from 4-amino-4-deoxy-CHA. As noted, 4-amino-4-deoxy-CHA rearranged spontaneously to the corresponding "prephenate-like" structure (see Figure 61). This process, and the further conversion to L-(4-aminophenyl)-alanine (Figure 61C) is catalyzed by an "arylamine synthase" system of *Streptomyces venezuelae*.[442] The

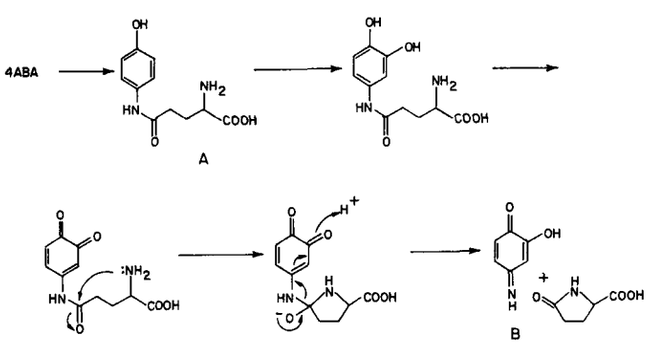


FIGURE 60. Formation of *N*-(γ-L-glutamyl)4-hydroxyaniline, A, and related compounds. In the formation of B, pyroglutamate (5-oxoproline) is also produced.

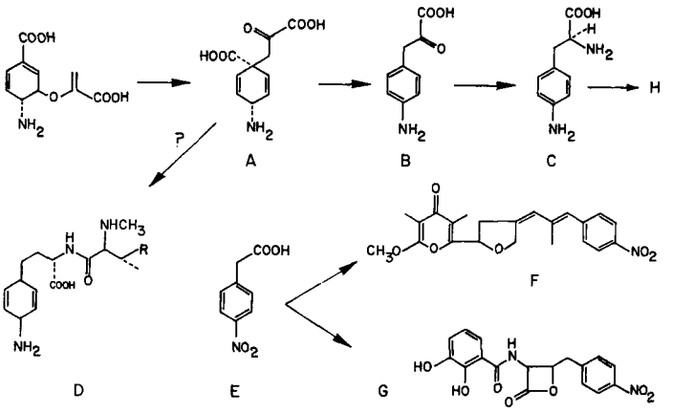


FIGURE 61. Formation of chloramphenicol and other compounds from L-(4-aminophenyl)alanine. In stravidin (D), R = —C_2H_5. The starting material is 4-amino-4-deoxy-CHA. C = L-(4-aminophenyl)alanine; E = 4-nitrophenylacetate; F = aureothin; G = obafluorin; H = chloramphenicol.

role of the carbon source in regulating chloramphenicol biosynthesis by *Streptomyces venezuelae* has been investigated in detail in both batch and continuous cultures.[451] During the nitrogen-starvation-induced trophophase-idiophase transition, "carbon catabolite repression" regulates enzyme synthesis, but does not establish the timing of chloramphenicol biosynthesis. Moreover, this mechanism does not function for nitrogen-sufficient growth with excess glucose.

L-(4-Aminophenyl)alanine, Figure 61C, has been isolated from seeds and leaves of *Vigna vexillata*. Tracer studies rule out a pathway via phe or tyr. Labeled SHK, however, was well incorporated and the results are compatible with the proposed role of 4-amino-4-deoxy-CHA.[383] The "PPA-like" compound, Figure 61A, is a possible precursor of part of the structure of stravidin, Figure 61D, from *Streptomyces avidini*.[452]

L-(4-Aminophenyl)alanine is also a precursor to aureothin (Figure 61F)[453] and obafluorin (Figure 61G)[454] Obafluorin is a β-lactone antibiotic produced by *Pseudomonas fluorescens*. The L-(4-aminophenyl)alanine in the latter case is converted to 4-nitrophenylacetate (Figure 61E), and this compound was also isolated from the organism.[454]

III. Amination of CHA at Position 5

3-Acetamido-4-hydroxybenzoate (Figure 41L and Figure 62A) has been isolated from cultures of *Pseudomonas cepacia* and may derive from CHA by amination at position 5 as shown in Figure 62.[455] 2-Acetamidophenol, (Figure 41N and Figure 62B) has been isolated from *P. aeruginosa*. It could be derived from 3-acetamido-4-hydroxybenzoate, but a possible role for 3-hydroxyanthranilate cannot be ruled out. Interestingly, the iron-containing nitrosophenol, ferroverdin, contains a 3-nitroso-4-hydroxybenzoate moiety.[456]

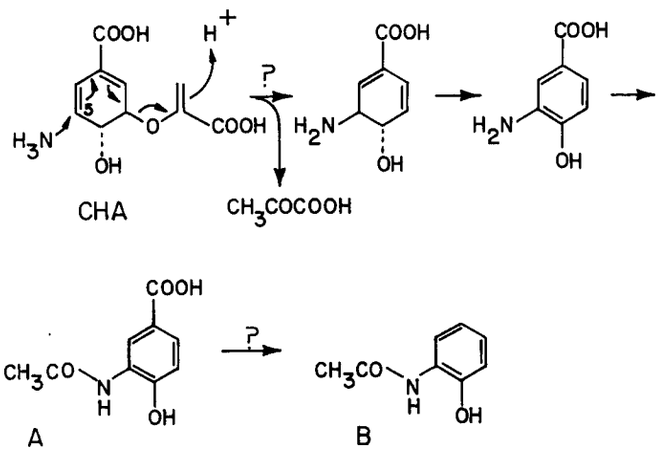


FIGURE 62. Possible amination of CHA at position 5. A = 3-acetamido-4-hydroxybenzoate; B = 2-acetamidophenol.

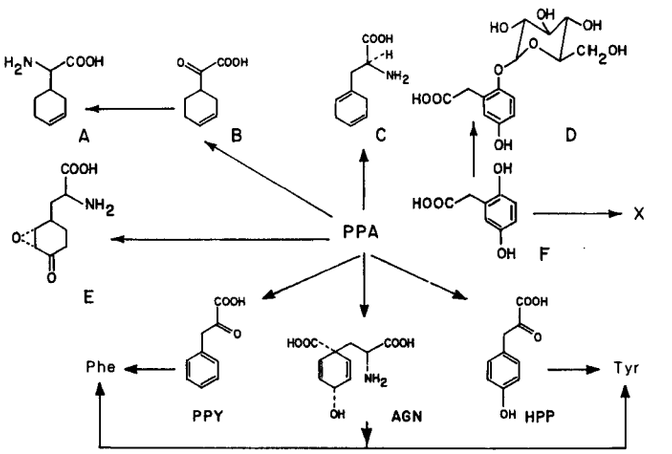


FIGURE 63. Branches from PPA. A = cyclohexenylglycine; B = ketomycin; C = 2,'5'-dihydro-phe; D = phaseolidin; E = anticapsin; F = homogentisate; X = formation of plastoquinones and tocopherols.

C. The Branches from Prephenate (Figure 63)

1. The Biosynthesis of Phe and Tyr

The branching from PPA is extensive, leading not only to phe and tyr, but to a number of secondary metabolites (see Figure 63). The bifunctional enzymes, CHA mutase-PPA dehydratase and CHA mutase-PPA dehydrogenase were considered in Section II.J.1 and II.J.2. In addition to the "classical" pathways to phe and tyr via respectively, phenylpyruvate (PPY) and 4-hydroxyphenylpyruvate (HPP), alternative routes via arogenate (AGN) have assumed considerable importance (see Figure 64). In early work, AGN was named as "pretyrosine"; rigorous confirmation of the proposed structure for this compound was obtained in 1980[457] and it has been synthesized chemically as the optically pure (+) enantiomer.[458]

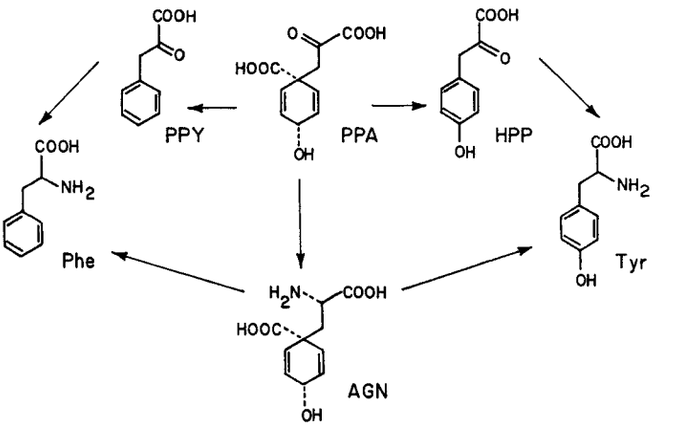


FIGURE 64. The biosynthetic routes to phe and tyr from PPA.

The AGN route was described in 1974 for tyr biosynthesis in cyanobacteria, particularly *Agmenellum quadruplicatum* and *Anacystis nidulans*, in what must be considered a landmark paper.[459] The impact of this observation steadily increased and by 1981 AGN was recognized as the sole precursor to both phe and tyr in *Euglena gracilis*.[460] Moreover, it now appears likely that AGN may be the major, if not exclusive, precursor for phe and tyr in higher plants.[461]

Both pathways to phe require at one stage a dehydratase enzyme to catalyze a dehydration coupled to a decarboxylation (in the "classical" pathway, PPA → PPY + H_2O + CO_2; in the AGN pathway, AGN → Phe + H_2O + CO_2). For the pathways to tyr, a dehydrogenase activity is required that is accompanied by decarboxylation. These enzymes show various substrate specificities, and for the dehydrogenases, cofactor specificities. The nomenclature used to describe the various enzymes is still in a somewhat fluid state, but the following appears to be emerging:

Dehydratases

1. PPA dehydratase (EC 4.2.1.51)	PPA specific, does not use AGN, various activators and inhibitors known
2. AGN dehydratase	AGN specific, does not use PPA, feedback inhibited by phe
3. Cyclohexadienyl dehydratase	Uses either PPA or AGN, not inhibited by phe

Dehydrogenases

1. PPA dehydrogenase (uses NAD, EC 1.3.1.12; uses NADP 1.3.1.13)	PPA specific, does not use AGN
2. AGN dehydrogenase (EC 1.3.1.43)	AGN specific, does not use PPA, feedback inhibited by tyr
3. Cyclohexadienyl dehydrogenase	Uses either PPA or AGN, and either NAD or NADP specific

The situation is complex and considerable work will be required before a more complete understanding is gained. Moreover, ambiguities can arise from the presence of enzyme mixtures. Not all of the types listed above are well characterized by, for instance, homogeneous enzyme preparations. In fact, relatively few of the enzymes have been purified to homogeneity. Some technical pitfalls have not always been avoided. For instance, a homogeneous protein obtained from *Phenylobacterium immobile* was described as AGN dehydrogenase; PPA was not a substrate.[462] However, the crucial proof of tyr formation was not given so that some other dehydrogenase may have been purified.[463] It is particularly important to note that AGN preparations may be contaminated with PPA or unknown compounds that may behave as dehydrogenase substrates.[464]

In terms of *in vivo* enzyme utilization, the following combinations have been observed:

1. Only the classical pathway via PPY and HPP is used for both phe and tyr, e.g., *E. coli, B. subtilis*. Despite the similarity in the post-PPA pathway, these two organisms differ considerably in the pre-PPA components. In *E. coli,* the PPA utilizing enzymes exist as the bifunctional complexes with CHA mutase. In wild-type *B. subtilis,* the CHA mutase is monofunctional, and in *B. subtilis* strain 168 it is bifunctional with DAHP synthase (see Section II.K). The classical dual pathway is used by *N. crassa*; however, triple blocked mutants of this organism (blocked in biosynthesis of phe, tyr, and trp) accumulate AGN essentially as a "dead-end" metabolite (see Section III.C.1.h).
2. Only the AGN pathway for both phe and tyr is used, e.g., *Euglena gracilis,* probably higher plants.
3. The classical pathway via PPY is used for phe biosynthesis and the AGN pathway is used for tyr biosynthesis, e.g., cyanobacteria, glutamic acid bacteria, various *Streptomyces*[465,466] and various *Actinomycetales*.[238]
4. The classical pathway via HPP is used for try biosynthesis and the AGN pathway for phe biosynthesis, e.g., *Pseudomonas diminuta*.
5. Both complete pathways coexist, e.g., *Pseudomonas aeruginosa,*[67,75] *Xanthomonas campestris, Neisseria gonorrhoeae,*[28] *Claviceps* sp. (forms phe from both AGN and PPY, tyr preferentially from AGN).[467]

Although many organisms contain both PPA dehydratase and cyclohexadienyl dehydratase there are apparently no examples of organisms with two of the dehydrogenases.

As already noted, the regulation of the pathways for biosynthesis of the aromatic amino acids, beginning with DAHP synthase, has received considerable attention; the situation is, of course, made more complicated by the existence of the AGN pathway. An excellent overview of the postprephenate pathways includes an analysis of technical problems.[464]

It is of interest that partially purified, bifunctional CHA mutase-PPA dehydrogenases from *K. pneumoniae* and *E. coli* were able to utilize AGN. The formation of tyr from this substrate was demonstrated. The utilization of both PPA and AGN was inhibited by tyr.[468] Other microbial enzymes, not occurring in association with a totally different enzymatic activity, are now considered briefly; they may show substrate ambiguities as already mentioned. The role of AGN in plants is considered in Section III.C.1.g.

a. PPA DEHYDRATASE

The formation of PPY from PPA is catalyzed by EC 4.2.1.51, prephenate hydrolyase (decarboxylase), generally referred to as PPA dehydratase. This enzyme has wide distribution in microorganisms,[469] but no PPA dehydratase activities have been described from higher plants.[91]

PPA dehydratase has been purified (10,000-fold) to electrophoretic homogeneity from *B. subtilis* NP1.[470] This enzyme was activated, *inter alia,* by met and leu and was strongly inhibited by phe, trp, and structural analogs of these amino acids. The presence or absence of such activator molecules considerably influenced the polymerization level of the molecule; forms with M_r of 35,000 (monomer), 55,000 (dimer), and 210,000 (octamer) were all observed. In the presence of activators (met, leu, PPA) the maximally activated octamer was present and was converted reversibly to the variably activated dimer by inhibitors (phe, trp). Heating at 32°C in the absence of effector molecules formed the inactive monomer.

This observed regulation by metabolites of seemingly unconnected pathways (met, leu) has been termed "metabolic interlock". The utilization of an "interlock" type of PPA dehydratase is apparently characteristic of Gram-positive bacteria. This interlocking pattern was also observed when the PPA dehydratase activity of an extreme-halophile archaebacterium was investigated.[471] The enzyme was stabilized by high salt concentrations (>2.0 *M* NaCl) with maximal activity at 3.0 *M* NaCl. Of the three aromatic amino acids, phe was strongly inhibitory, tyr was a fairly good activator, and trp was inhibitory in a complex fashion. Moreover, met, leu, and ile were activating effectors.

Highly purified (2000-fold) PPA dehydratase, with no AGN activity, was obtained from *Microtetraspora glauca.*[472] Phe, tyr, and trp were feedback inhibitors and the native enzyme had M_r = 110,000.

The PPA dehydratase of *Flavobacterium devorans* was purified (43-fold) and the estimated value of M_r was 135,000.[473] In contrast to the *B. subtilis* enzyme just described, this enzyme was activated by phe, tyr, and trp. Several spore-forming organisms of the order *Actinomycetales* contain a PPA dehydratase activity that is usually inhibited by phe; in a few cases with these organisms, tyr was a strong activator.[238]

The *pheA* gene (encoding PPA dehydratase) of the commercially important *Corynebacterium glutamicum* has been cloned and sequenced. An *E. coli pheA* auxotroph was com-

plemented when the gene was cloned in both orientations in the *E. coli* vector pUC8. The structural gene was located in a 1070 bp ORF; the enzyme had 315 amino acid residues with M_r = 33,740. The predicted gene product had a significant homology (26%) with the C-terminal region of *E. coli* CHA mutase-PPA dehydratase. These results confirmed the localization of the PPA dehydratase activity to the C-terminal two thirds of the *E. coli* enzyme (see Section II.J.1) and confirmed that there were separate CHA mutase and PPA dehydratase activities in *C. glutamicum*.[474]

In *Candida maltosa*, PPA dehydratase was also separable from CHA mutase and had M_r = 88,000. The dehydratase activity was stimulated by trp and its methylated analogs.[241]

b. AGN DEHYDRATASE

AGN dehydratase has not yet been purified to homogeneity. An approximately fivefold purification has been described from *Pseudomonas diminuta* 13184. The enzyme from strain 11568 was strongly inhibited by phe.[475,476] It was until recently difficult to assay this enzyme even though four methods were available.[476] A simpler method, based on the following two reactions, has now been introduced.[477] The formation of PPY in this assay is measured at 320 nm under basic conditions.

1. AGN $\xrightarrow{\text{AGN Dehydratase}}$ Phe

2. Phe + 2-ketoglutarate $\xrightarrow{\text{Aromatic aminotransferase from Acinetobacter calcoaceticus}}$ PPY + NH_3

c. CYCLOHEXADIENYL DEHYDRATASE

A cyclohexadienyl dehydratase utilizing either PPA or AGN is present in *Xanthomonas campestris*, *P. aeruginosa* and other *Pseudomonads*, *Serratia marcescens*, and *Erwinia* sp.[235] In contrast to the previously described AGN dehydratase, this enzyme is not inhibited by phe. The purification method used for AGN dehydratase of *Pseudomonas diminuta* was applied to *P. aeruginosa* cyclohexadienyl dehydratase; the extent of purification was not given.[475]

d. PPA DEHYDROGENASE

The dehydrogenases converting PPA to HPP are either NAD specific [EC 1.3.1.12, prephenate:NAD^+ oxidoreductase (decarboxylating)] or NADP specific [EC 1.3.1.13, prephenate:$NADP^+$ oxidoreductase (decarboxylating)]. They are described here in general as PPA dehydrogenases. They have been investigated in several microorganisms and show a wide variety of requirements with respect to NAD or NADP.[8,478] With the exception of some beans, PPA dehydrogenases have not been found in plants.

The PPA dehydrogenase of *Alcaligenes eutrophus* ATCC 17699 was purified (740-fold), but homogeneous product was not obtained.[478] The enzyme was inhibited by product HPP and structural analogs. *Candida maltosa* contains an NAD-dependent PPA dehydrogenase with M_r = 75,000. The enzyme, which has not been purified, was inhibited by tyr.[241]

e. AGN DEHYDROGENASE

The Enzyme Commission number EC 1.3.1.43 has been assigned to NAD-specific AGN dehydrogenase. The systematic enzyme name is 3-(1-carboxy-4-hydroxycyclohexa-2,5-dien-1-yl)-L-alanine:NAD^+ oxidoreductase. Unhappily, the recommended name is pretyrosine dehydrogenase, which uses the abandoned term for AGN. Since there is wide variation in the cofactor specificities for the AGN dehydrogenases,[8,463] and since some enzymes should be classified as cyclohexadienyl dehydrogenases, revision of the formal nomenclature is needed.

Two preparations of AGN dehydrogenase have been purified to homogeneity. The caveat has been entered that in neither case was tyr formation demonstrated.[463] A 95-fold purification of an unstable enzyme was obtained from *Streptomyces phaeochromogenes*. The homogeneous enzyme consisted of two identical subunits each of M_r = 28,100. The native enzyme was estimated to have M_r = 66,300. It was NAD specific and there was no inhibition by PPA.[466] Another NAD-dependent AGN dehydrogenase was purified 81-fold from *Phenylobacterium immobile*. The enzyme was a dimer (M_r = 69,000) of two identical subunits (M_r = 37,700). PPA was not a substrate and was, in fact, inhibitory.[462]

AGN dehydrogenase was partially purified from three species of coryneform bacteria (*Corynebacterium glutamicum*, *Brevibacterium flavum*, *Brevibacterium ammoniagenes*). These organisms contained no PPA dehydrogenase activity.[479]

f. CYCLOHEXADIENYL DEHYDROGENASE

PPA dehydrogenase and AGN dehydrogenase activities have not been separated in organisms such as *P. aeruginosa*, which have dual pathways. Single dehydrogenase proteins with dual specificity are presumably involved and are appropriately termed cyclohexadienyl dehydrogenases. NAD-specific cyclohexadienyl dehydrogenase activity also has been demonstrated in *Microtetraspora glauca*[472] and *Neisseria gonorrhoeaea*.[28] NADP-specific cyclohexadienyl dehydrogenase is present in *Acinetobacter calcoaceticus*.[59]

g. THE ROLE OF AGN IN PLANTS

By 1986 it was clear that the AGN pathway to tyr played a major role in plants. With the exception of mung bean[480] and possibly other bean species,[481] PPA dehydrogenase (mandatory for the classical pathway to tyr) had not been detected in plants. Hence, the only possibility for tyr biosynthesis was via AGN dehydrogenase.[461]

It may seem surprising that in that same year it could be stated that "the specific enzymological route of L-phenylalanine biosynthesis has not been established in any higher plant sys-

tem";[461] however, AGN dehydratase activity has now been detected unequivocally (and partially purified) in cultured-cell populations of *Nicotiana silvestris* and washed spinach chloroplasts.[461] A major experimental problem was that high levels of protease activity also gave rise to the production of phe in enzyme incubation mixtures. For *N. silvestris*, addition of protease inhibitors (leupeptin, pepstatin) was helpful, and with spinach, washed chloroplasts did not present this problem. Both of the enzymes were activated by tyr and inhibited by phe. Most of the AGN dehydratase activity from spinach was present in the chloroplast compartment.

Perhaps it should be reemphasized that PPA dehydratase activity for phe biosynthesis by the classical pathway was never detected in any plant. It seems most likely that the AGN pathway is generally used by higher plants for the synthesis of both phe and tyr. The only known exception to this statement appears to be for the developmental stage of seed germination in mung bean and possibly other beans such a soy and wax. It is not totally clear whether mung beans have separate enzymes or a single enzyme of the cyclohexadienyl type with dual specificity.

In plants, a partial purification (816-fold) of an NADP-dependent AGN dehydrogenase was obtained from cell cultures of *Nicotiana silvestris*. This preparation was inhibited by tyr.[481] Another plant AGN dehydrogenase was purified (10.5-fold) from etiolated *Sorghum bicolor* seedlings with stabilization by 20% ethylene glycol and 0.1% mercaptoethanol.[482] Gradient elution from a DEAE-cellulose column gave a single peak of NADP-dependent AGN dehydrogenase activity with 93% recovery. Electrophoretic homogeneity was not established. The AGN dehydrogenase was strongly inhibited by tyr and was not affected by phe, trp, and PPA. There was no PPA dehydrogenase activity. A low degree of purification of NAD-dependent AGN dehydrogenase from corn has been reported.[483] This NAD-dependent enzyme is an exception to the fact that all other photosynthetic eukaryotes have NADP-linked enzymes.

A sixfold purification of AGN dehydratase from etiolated seedlings of *Sorghum bicolor* has been reported, but the enzyme was not a homogeneous protein. The enzyme was inhibited competitively by phe and was stimulated by tyr. There was no evidence for any PPA dehydratase activity.[484]

h. AGN-RELATED COMPOUNDS AND ENZYMES

A triple blocked mutant of *N. crassa*, ATCC 36373, lacks PPA dehydratase, PPA dehydrogenase, and AS; it is, therefore, unable to convert PPA to either PPY or HPP. It accumulates PPA, AGN, and two other cyclohexadienyl compounds (see Figure 65). One of these new materials is derived by cyclization of AGN (Figure 65B) to a spiro-γ-lactone (Figure 65C); this lactone is named spiro-arogenate (SPN). In SPN, C-8 has the (expected) L (= S) configuration and as with AGN itself, the configuration at C-1 is reasonably assumed to be the same as in PPA. SPN production is catalyzed by a putative spirase.[485,486] Tracer experiments with [3-^{3}H]SHK in *N. crassa* 36373 established that, as expected, AGN appears prior to SPN.[487]

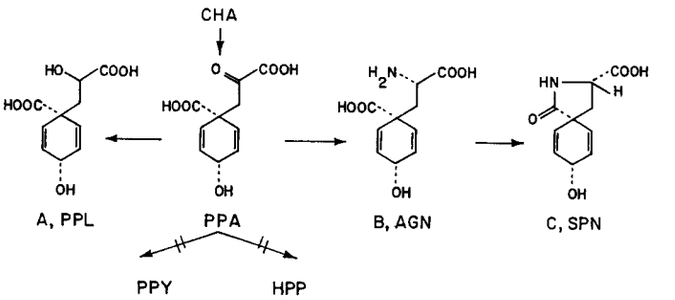


FIGURE 65. Formation of spiro-AGN and prephenyllactate in triple blocked mutant of *N. crassa*.

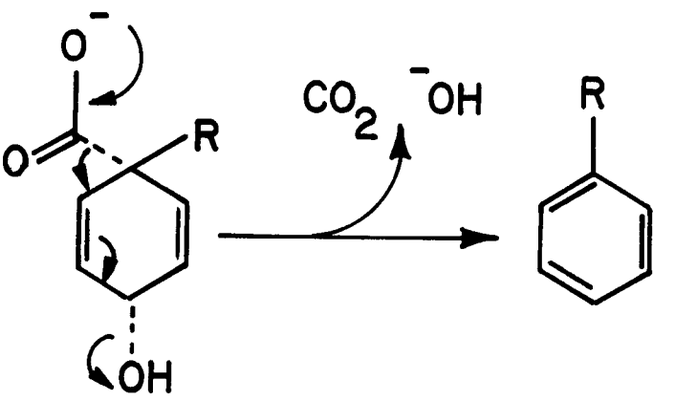


FIGURE 66. Aromatization of cyclohexadienyl compounds. If R = —$CH_2COCOOH$, the conversion is PPA → PPY; if R = —$CH_2CHOHCOOH$, the conversion is PPL → phenyllactate; if R = —CH_2CHNH_2COOH, the conversion is AGN → phe.

The second cyclohexadienyl structure, Figure 65A, is formed by a reduction of the PPA carbonyl group and is termed prephenyllactate (PPL). In PPL the configuration at C-8 is R. Clearly, a putative dehydrogenase is required for this reaction. Under mildly acidic conditions, the cyclohexadienyl structures are aromatized (see Figure 66) with the following order; PPL > PPA > AGN > SPN.[489] PPL functions as a substrate for two enzymes; *K. pneumoniae* cyclohexadienyl dehydrogenase forming 4-hydroxyphenyllactate and *K. pneumoniae* cyclohexadienyl dehydratase forming phenyllactate. The bifunctional CHA mutase-PPA dehydratase of this organism, however, does not use PPL as a substrate.[488]

AGN can be converted to SPN by heating at pH 7.5 to 12 and 100°C. These conditions argue forcibly that the putative spirase must exist for the *in vivo* conversion. The reverse process, SPN → AGN requires pH >12 and 100°C. For a summary of these processes, see Figure 67.

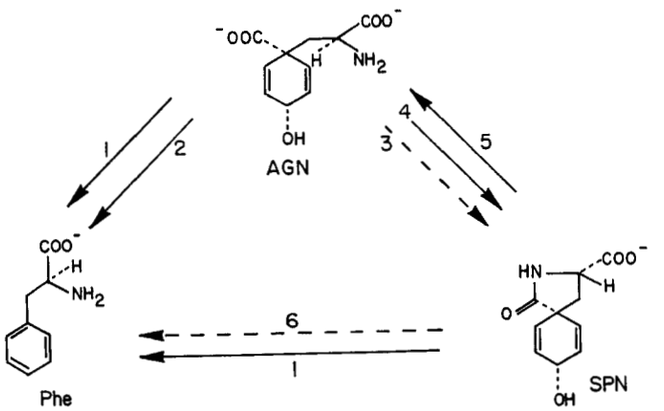


FIGURE 67. Interconversion of AGN and spiro-AGN and formation of phe. Reaction 1, H^+; reaction 2, AGN dehydratase; reaction 3, AGN spirase; reaction 4, pH 7.5 to 12.0, 100°C; reaction 5, pH >12, 100°C; reaction 6, SPN dehydratase.

I. PPA AMINOTRANSFERASE

PPA aminotransferase catalyzes the conversion of PPA to AGN and is thus an important enzyme. Electrophoretically homogeneous preparations have been obtained for *Nicotiana silvestris*.[490,491] The highly purified native enzyme had M_r = 220,000, with apparently two dissimilar subunits (M_r values of 44,000 and 57,000). These data suggested an $\alpha_2\beta_2$ structure. A near homogeneous preparation of PPA aminotransferase (41-fold purification) was obtained from *Anchusa officinalis*.[492] In *Sorghum bicolor* PPA aminotransferase was present in epidermal and mesophyll protoplasts and bundle sheath cells, and was predominantly located in the plastid.[493,494]

In general, these enzymes are characterized by a high pH optimum (pH 8.0 to 9.0), by heat stability, and by a strong substrate specificity for PPA. This specificity is in contrast to microbial enzymes which show multispecific accommodation of substrates. The heat stability was exploited to obtain a selective assay for this activity in suspension-cultured cells of *N. silvestris*.[495]

J. AMINOTRANSFERASES

There are four amino transferases in *E. coli* that catalyze terminal steps in amino acid biosynthesis. Of these, aromatic amino acid transferase (EC 2.6.1.57) apparently has the major role for phe and tyr biosynthesis.[496] An asp amino transferase (EC 2.6.1.1) has a broad specificity and will utilize PPY and HPP, but has a lower affinity for these materials than does the aromatic amino acid transferase. These two enzymes are encoded by the genes, *tyrB* and *aspC*, which have both been sequenced.[497,498]

The aromatic amino acid transferase is a polypeptide of 397 residues. The *tyrB* and *aspC* gene products (396 residues) are related proteins and show significant homologies. The *tyrB* gene is part of the *tyrR* regulon. The operator locus for *tyrB* has been identified and its expression found to be controlled at the transcriptional level by the TyrR protein with tyr as a co-repressor.[499] These regulatory aspects have been reviewed by Pittard.[9] The aromatic amino acid transferase has been purified (750-fold) to homogeneity from *E. coli*, as has the *aspC* gene product (213-fold). Both of these enzymes are homodimers with native M_r in the range from 42,000 to 45,000.[496]

In *P. aeruginosa*, not only are there dual enzymatic sequences to both phe and tyr, but in addition, this organism possesses five aromatic amino acid transferases.[500] Two enzymes, AT-1 and AT-2, were easily separated by gel filtration. It was concluded from phenotypic and enzymological characterization of appropriate mutants that AT-2 (M_r = 50,000) had the major role for biosynthesis of phe and tyr, while AT-1 (M_r = 64,000) was involved with asp and glu biosynthesis. A less well-characterized enzyme, AT-4 (M_r = 200,000), was also assigned an *in vivo* role for phe and tyr biosynthesis.

In *B. subtilis*, there are two aromatic amino acid transferases, one of which is also responsible for transamination of imidazole acetol phosphate to histidinol phosphate (required in his synthesis). The gene involved in the synthesis of the dual function transaminase, *hisH*, lies in the middle of a gene cluster concerned with biosynthesis of amino acids.[501]

Aromatic amino acid transferases are not well described in plants, and apparently none have been purified to homogeneity.[502]

2. Products for Homogentisate (via Hydroxyphenylpyruvate)

a. PLASTOQUINONES AND TOCOPHEROLS

Homogentisate (Figure 63F) is an important intermediate for the biosynthesis of plastoquinones (abbreviated as PQ-n where n defines the number of isoprenyl residues in the side chain), tocopherols, and related compounds (see Figure 63).[503,504] It occurs naturally as its β-D-glucoside (phaseoloidin) in the plant *Entada phaseoloides*.[505] Homogentisate can be derived from PPA via HPP; 4-hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27) catalyzes a complex reaction of hydroxylation and —CH_2COOH group migration with retention of configuration (see Figure 68).[506]

At the moment it is not entirely clear how plants in general derive HPP for homogentisate biosynthesis. It has been stated that "it is likely that homogentisate is formed directly from 4-hydroxyphenylpyruvate (HPP) straight through the shikimate pathway".[507] The necessary dioxygenase enzyme was located predominantly in chloroplasts. The envelope membranes had the highest specific activity for this enzyme, but the majority of the activity was located in the stroma. The separate reactions, shown below, were demonstrated in preparations of envelope membranes.

HPP → Homogentisate → 2-Methyl-6-phytylquinol

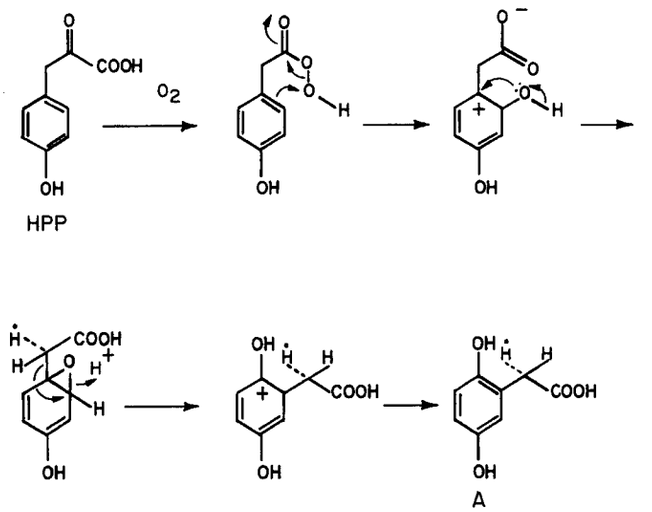


FIGURE 68. Stereochemistry of HPP dioxygenase. A = homogentisate.

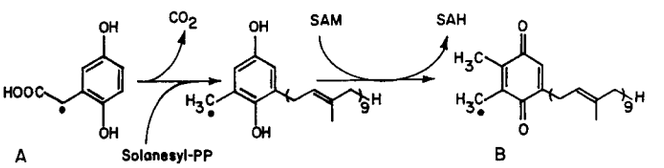


FIGURE 69. Formation of plastoquinone (PQ-9,B) from homogentisate, A.

Utilization of tyr was regarded only as a bypass. As demonstrated here, all of the enzymes for the last steps of tocopherol and PQ biosynthesis are localized on the inner envelope membrane.[508]

In contradiction to these conclusions, it now appears that most plants synthesize tyr via the AGN pathway and do not have PPA dehydrogenase activities (PPA —//→ HPP). Hence, they must derive HPP from tyr (PPA → AGN → tyr → HPP).

In biosynthesis of the tocopherols and PQ, the —CH_2COOH group decarboxylates to provide one of the characteristic methyl groups (the others originate in *S*-adenosylmethionine). As an example, the formation of PQ-9 occurs as shown in Figure 69. The decarboxylation was found to proceed with retention of configuration when chloroplasts of *Raphanus sativus* synthesized tocopherols and PQ. The process was believed to involve a quinone intermediate of unknown structure with respect to presence or absence of further substituents (see Figure 70).[509]

There is general agreement that in chloroplast preparations (e.g., from lettuce, spinach) the biosynthesis of α-tocopherol is as shown in Figure 71.[508,510,511] While 2-methyl-6-phytyl-benzoquinol (Figure 71A) appears to be the sole product derived from homogentisate in these preparations, isomeric forms exist in *Scenedesmus obliquus* and elsewhere.[512] There may be a second, extra-chloroplastidic pathway for tocopherol biosynthesis. The situation remains confusing and the original literature should be consulted for more details. The difficulties in elucidating the pathway(s) of tocopherol biosynthesis have been reviewed.[513]

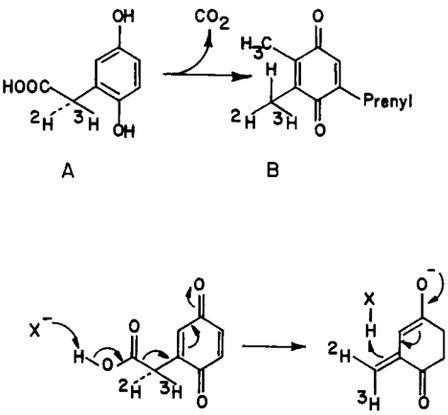


FIGURE 70. Possible mechanism for decarboxylation of labeled homogentisate, A, to form PQ, B. In the detailed reaction sequence at the bottom, possible ring substituents have not been indicated.

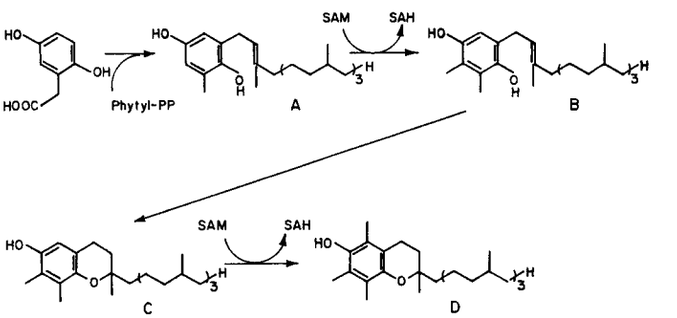


FIGURE 71. Biosynthesis of α-tocopherol. SAM = *S*-adenosylmethionine; SAH = *S*-adenosylhomocysteine. A = 2-methyl-6-phytyl-benzoquinol; B = 2,3-dimethyl-6-phytyl-benzoquinol; C = γ-tocopherol; D = α-tocopherol.

At the enzyme level, the γ-tocopherol methyltransferase (γ → α-tocopherol, Figure 71, C → D) from chromoplasts of *Capsicum anuum* was purified to electrophoretic homogeneity (M_r = 33,000 by SDS-PAGE). In the absence of detergents, higher molecular weight aggregates were formed.[514]

b. OTHER PRODUCTS FROM PPA

Tracer and genetic studies have indicated that the unusual amino acid, anticapsin (Figure 63E), derives from the SHK pathway via PPA. Anticapsin is a component, together with ala, of the dipeptide antibiotic, bacilysin, produced by *B. subtilis*.[515] Another antibiotic is 2′,5′-dihydro-phe (Figure 63C), produced by *Streptomyces arenae* and *Erwinia amylovora;* this compound is also necrotoxic to pear cells.[516] Tracer studies have established PPA (but not 5,6-dihydro-PPA) as a biosynthetic intermediate (see Figure 72).[517]

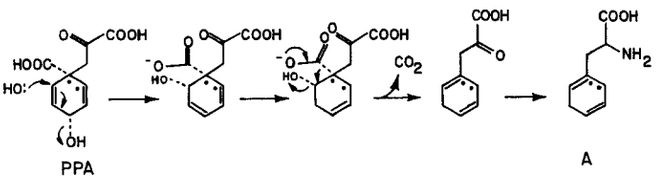


FIGURE 72. Biosynthetic pathway for formation of 2′,5′-dihydro-phe, A.

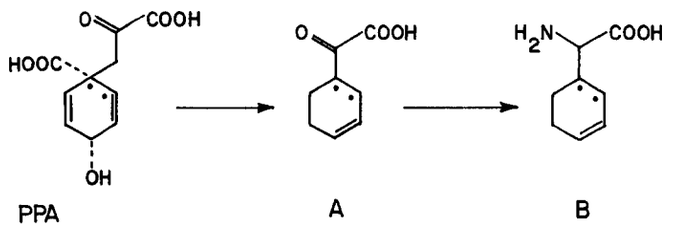


FIGURE 73. Biosynthesis of ketomycin, A, and cyclohexenylglycine, B, from PPA.

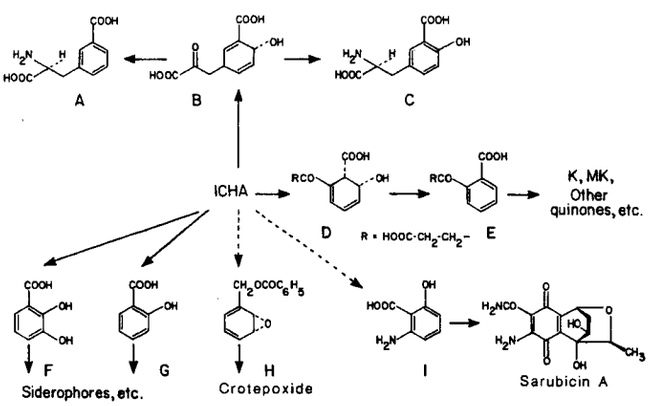


FIGURE 74. Branches from ICHA. A = 3(3-carboxyphenyl)alanine; B = iso-PPA; C = 3(3-carboxy-4-hydroxyphenyl)alanine; D = SHCHC; E = OSB; F = 2,3-dihydroxybenzoate; G = salicylate; H = epoxide intermediate; I = 6-hydroxyanthranilate.

The keto acid, ketomycin (Figure 63B), is an antibiotic produced by *Streptomyces antibioticus*. Biosynthetic tracer experiments have indicated roles for CHA and PPA. Neither phe nor 2′,5′-dihydro-phe were utilized. The utilization of PPA proceeded in a stereospecific manner (see Figure 73).[518] Ketomycin inhibits Gram-negative bacteria and *B. subtilis* by virtue of its conversion to 3-cyclohexenylglycine (Figure 63A); this amino acid is known to occur in some *Streptomycetes*.[517]

D. Branches from ICHA (Figure 74)

1. Naphthoquinones

In addition to the benzenoid quinones, ubiquinone and plastoquinone, another group of functionally important quinones is based on a naphthalenoid nucleus. These quinones are phylloquinone (abbreviated K, found in plants and some photosynthetic organisms) and the bacterial demethylmenaquinones (abbreviated DMK-n, where n indicates the number of isoprenoid residues in the side chain) and menaquinones (MK-n). The distribution of these quinones in microorganisms[520] and their biosynthetic pathways[352,521] have been reviewed extensively.

These naphthoquinones originate from ICHA, with important roles for the benzenoid aromatic, *o*-succinylbenzoate (OSB), its CoA derivative, and the naphthalenoid aromatic, 1,4-dihydroxy-2-naphthoate (DHNA) (see Figure 75). Evidence for the assigned structure of the CoA derivative of OSB has been published.[522,523]

In general, the reactions of MK biosynthesis are reasonably straightforward and bear some ressemblance to those of Q biosynthesis (prenylation, nuclear methylation). The most intriguing step is the conversion of ICHA to OSB: this process also requires the presence of 2-ketoglutarate and thiamin pyrophosphate (TPP). The isolation of the intermediate, 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC, see Figure 75), is consistent with the fact that two genes, *menD* and *menC* are involved in *E. coli*. It is likely that a decarboxylase activity converts 2-ketoglutarate to the TPP anion of succinic semialdehyde; this decarboxylase activity is distinct from that of the 2-ketoglutarate dehydrogenase complex.[524,525] Succinate itself can replace 2-ketoglutarate for MK biosynthesis in membrane preparations from *Micrococcus leisodeikticus*.[526] As indicated in Figure 76, the TPP-succinic semialdehyde presumably attacks ICHA at the 6 position, and following loss of TPP and pyruvate, SHCHC is formed. This

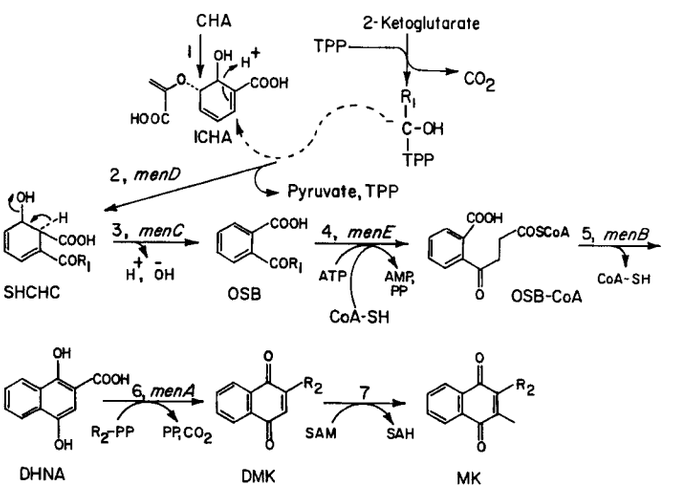


FIGURE 75. Menaquinone biosynthesis from ICHA. R_1 = HOOC—CH_2—CH_2—; R_2 = prenyl; TPP = thiamin pyrophosphate. The enzymes are as follows: 1, ICHA synthase, 2, SHCHC synthase; 3, OSB synthase; 4, OSB-CoA synthase, 5, DHNA synthase; 6, prenyltransferase; 7, methyltransferase.

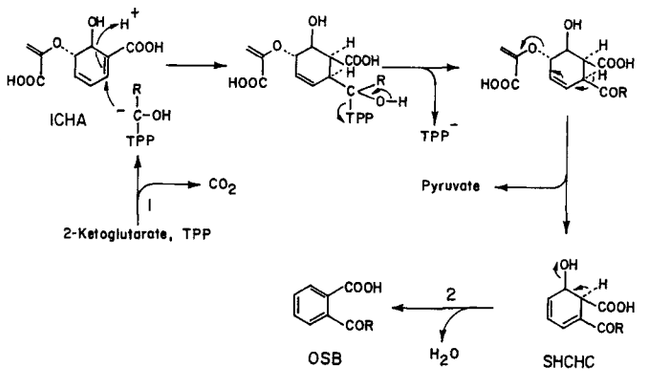


FIGURE 76. Postulated reaction mechanism for SHCHC synthase. Reaction 1 = ketoglutarate decarboxylase activity. R = HOOC—CH_2—CH_2—; TPP = thiamin pyrophosphate.

reaction is catalyzed by SHCHC synthase, the protein product of the *menD* gene. SHCHC is then dehydrated to OSB by OSB synthase, the protein product of the *menC* gene (SHCHC synthase and OSB synthase, were referred to earlier as OSB synthase I and II).

OSB is converted to OSB-CoA by the enzyme, OSB-CoA synthase in an ATP-dependent reaction. This enzyme, the protein product of *menE*, has been purified almost to homogeneity.[521] OSB-CoA is cyclized to DHNA (*menB* gene); in this process, the 3′-HR proton is retained (Figure 75).[527] The final steps of MK biosynthesis require prenylation accompanied by decarboxylation, and methylation of the so-formed DMK.

As just summarized, MK biosynthesis in *E. coli* requires five genes. One of them, *menD*, has been cloned, sequenced, and used in the construction of an overexpression strain for the first enzyme (SHCHC synthase) of the biosynthetic pathway.[528] Some initial purification of SHCHC synthase has been possible.[529]

The regulation of the MK biosynthetic pathway in *E. coli* is not well understood. The naphthoquinone level (sum of DMK and MK) has been known for some time to increase under anaerobic conditions. This anaerobic derepression of naphthoquinone biosynthesis is not regulated by FNR (the product of the *fnr* gene). Growth with fumarate or dimethylsulfoxide leads to MK as the predominant component, while with nitrate, DMK predominates.[530] The *men* locus from *B. subtilis* has been cloned and investigated in some detail. The analysis indicates a gene and transcription order of promoter-*menC,D-menE-menB*. The *men* cluster is expressed in the form of at least one polycistronic message, indicating the presence of a *men* operon.[531] Some nucleotide sequences have been determined and expression has been studied with *men′-lacZ* gene fusion.[532]

Plants contain phylloquinone (K) rather than MK; the only difference is that of a phytyl rather than a longer polyprenyl side chain. K biosynthesis follows the same pattern as does that of MK, but less detailed information is available. The formation of OSB and DHNA has been observed recently in enzyme extracts from cells of *Euglena gracilis*.[533,534] It is also known that DHNA undergoes phytylation with phytyl diphosphate in the envelope membrane of spinach chloroplasts.[535] The terminal step, methylation, occurs in the thylakoid membranes of chloroplasts; addition of soluble stroma protein is necessary.[536] Similar results have been reported for preparations from *Capsicum annuum* fruits.[537] Some plants contain MK-1, and a different biosynthetic route may be involved in its formation (see following section).

OSB is also an important intermediate for the production of some plant naphthoquinones (other than K), anthraquinones, and other products. In the biosynthesis of K, MK, lawsone (2-hydroxynaphthoquinone), and some anthraquinones (e.g., alizarin), no symmetrical intermediates are involved between OSB and the final naphthoquinones. However, for juglone (5-hydroxynaphthoquinone) biosynthesis, a symmetrical intermediate, possibly 1,4-naphthoquinone, is involved; this subject has been reviewed.[521,538]

In the formation of K and MK, addition of the phytyl or prenyl group occurs on the carbon atom derived from C-2′ of OSB (= C-2 of DHNA; see Figure 75). This C-2′ position of OSB also adds a single isopentenyl unit in the formation of some metabolites by callus tissues of *Catalpa ovata*. Many publications[539] in this area from Inouye's laboratory can only be briefly summarized here. Two of the observed metabolites are DMK-1 (= deoxylapachol) and MK-1 (see Figure 77I and J). It appears that the usual MK intermediate, DHNA, is not involved in the formation of these and related metabolites; instead, OSB cyclizes to carboxyoxotetralone (Figure 77D), and this latter compound undergoes prenylation to form the 2S enantiomer of 2-prenyl-2-carboxy-4-oxo-1-tetralone (Figure 77E).[540] Decarboxylation then leads to 2R-catalponone (Figure

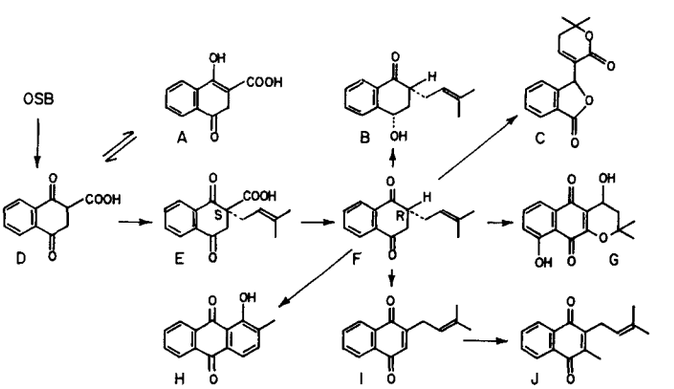


FIGURE 77. Metabolites formed by callus tissues of *Catalpa ovata*. A = tautomeric form of D; B = catalponol; C = catalpalactone; D = carboxy-4-oxo-1-tetralone; E = prenyl-carboxy-4-oxo-1-tetralone; F = catalponone; G = dihydroxylapachone; H = 1-hydroxy-2-methylanthraquinone; I = deoxylapachol (DMK-1); J = MK-1.

77F); this compound is definitely implicated as an intermediate in the biosynthesis of catalpalactone (Figure 77C), catalponol (Figure 77B), 4-9-dihydroxy-α-lapachone (Figure 77G), 1-hydroxy-2-methyl-anthraquinone (Figure 77H), DMK-1 (Figure 77I), and MK-1 (Figure 77J).[540,541] To some extent, a complex metabolic grid is involved with alternate routes possible to some of the compounds.

A prenylation pattern which is different from that observed with MK and the *Catalpa ovata* metabolites occurred in anthraquinone synthesis by some other plants of the family *Rubiaceae* (e.g., *Rubia tinctorum, Galium mollugo, Morinda lucida*). In these plants the process required DHNA. In anthraquinone-producing cell suspension cultures of *Galium* sp., ICHA was the OSB precursor.[542] An OSB-CoA ligase, obtained from anthraquinone-producing *Galium mollugo* cell suspension cultures, produced the "aliphatic" OSB-CoA ester, identical to that of bacteria.[543] After DHNA formation, a single isopentenyl unit was added at C-3 (of DHNA) and the —COOH at C-2 was retained (forming Figure 78A). Subsequent reactions gave lucidin (Figure 78B). This pathway was reinforced by isolation of related compounds (Figure 78C and D) from intact plants and cell suspension cultures of *Galium mollugo*.[544]

An interesting development has been the preparation of photoheterotrophic and photoautotrophic cell suspension cultures derived from a *Morinda lucida* plant. In this plant, anthraquinone glycosides are found in roots and these materials were absent from the photoautotrophic cultures. Lipoquinones, normally leaf constituents, were present in equivalent amounts in the photoautotrophic cultures and were less abundant in the photoheterotrophic cultures. When either culture type was kept in the dark in the presence of sucrose, abundant anthraquinone synthesis resulted, coincident with a disappearance of lipoquinones. Reflecting the situation in the intact plant, anthraquinone biosynthesis correlated with heterotrophy, lipoquinone synthesis with autotrophy.[545]

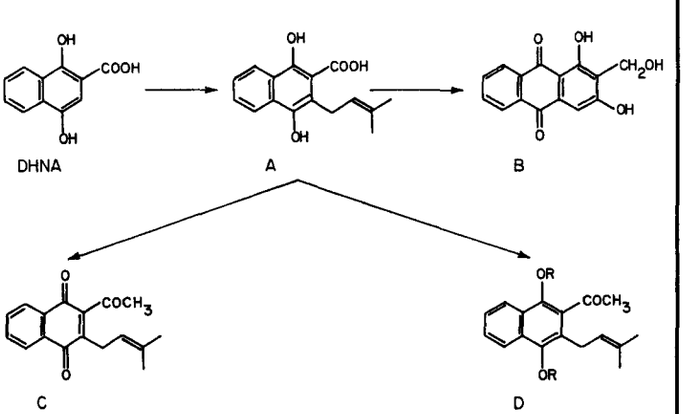


FIGURE 78. Formation of lucidin and related compounds. A = prenyl-DHNA; B = lucidin; C, D, further metabolites formed by *Gallium mollugo;* R = glucose.

A further variation for OSB prenylation has emerged for the biosynthesis of naphthoquinones and anthraquinones in cell cultures of *Streptocarpus dunnii (Gesneriaceae).* Anthraquinone formation requires the key intermediate, 2-prenyl-1,4-naphthohydroquinone (Figure 79A), which apparently undgoes a $—CH_3 \rightarrow —CH_2OH$ conversion prior to cyclization to 2-methylanthraquinone (Figure 79C). Other anthraquinones with a further —OH derive from 2-methylanthraquinone.[546] Figure 79A, is, of course, the quinol form of DMK-1. In *Catalpa ovata,* this material is said to be formed by the catalponone pathway. However, for the *S. dunnii* situation, the key intermediate derives either via DHNA or via the catalponone route. Whatever the precise route, the initial prenylation step for these anthraquinones is at the carbon atom equivalent to the C-2′ position of OSB.

In contrast, certain naphthoquinones (Figure 80) are unambiguously assigned a route via DHNA and lawsone (Figure 80A). The latter is converted to its 2-prenyl ether (prenylation

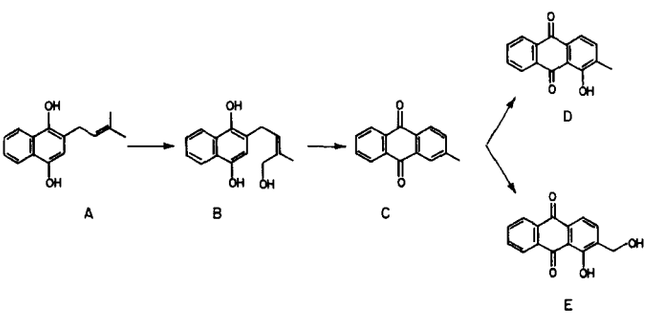


FIGURE 79. Quinone biosynthesis in cell cultures of *Streptocarpus dunnii.* A = 2-Prenyl-1,4-naphthoquinone; B = putative hydroxylation product of A; C = 2-methyl-anthraquinone; D, E, further metabolites formed from C.

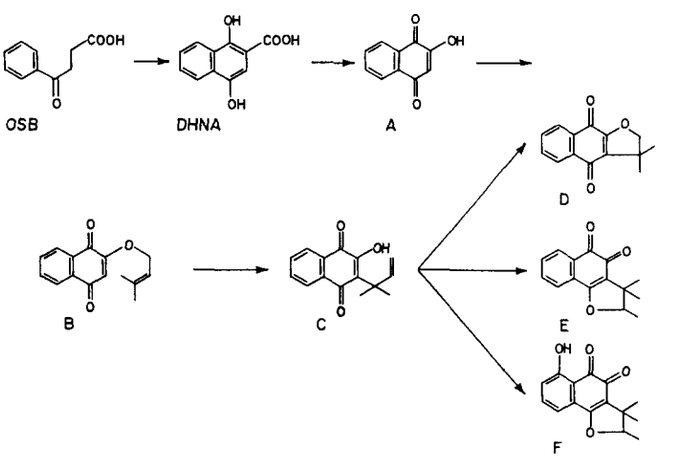


FIGURE 80. Role of lawsone in formation of further naphthoquinones. A = lawsone; B = 2-*O*-prenyl-lawsone; C = 2-hydroxy-3-(1,1-dimethylallyl)-1,4-naphthoquinone; D, E, F, further metabolites of C.

on the oxygen; Figure 80B) and a Claisen-type rearrangement leads to 2-hydroxy-3-(1,1-dimethylallyl)-1,4-naphthoquinone (Figure 80C), which is then utilized for the formation of other naphthoquinones. The net result of the Claisen rearrangement is to place the prenyl group at the carbon atom equivalent to C-3′ of OSB.

While the work of Inouye and his colleagues in this general area is impressive and apparently unimpeachable, independent confirmation of the catalponone pathway is desirable.

2. The Alkaloid, Shihunine

Another OSB-derived product is the unique alkaloid, shihunine, produced by *Dendrobium pierardii*. The biosynthetic pathway proposed for formation of this phthalidopyrrolidine is shown in Figure 81.[547] It is interesting that the first isolation of SHK itself from any *Orchidaceae* plant has been reported for *Dendrobium fuscens*.[548]

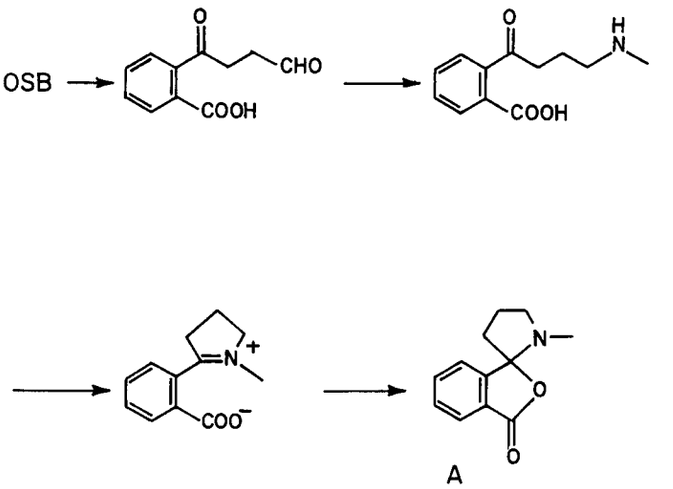


FIGURE 81. Biosynthesis of the orchid alkaloid, shihunine.

3. Siderophores from ICHA

Many metabolites which chelate iron are synthesized by microorganisms often under conditions of iron deprivation. These materials, termed siderophores, usually contain an aromatic hydroxy acid as part of a complex structure. Two of these acids, salicylate (Figure 74S) and 2,3-dihydroxybenzoate (Figure 74F), derive from ICHA. Although there are many siderophores of different structural types, evidence for biosynthetic routes is limited.

a. THE ROLE OF SALICYLATE

Mycobacterium smegmatis produces a siderophore, mycobactin S, which contains a salicylate unit; free salicylate is also secreted into the culture medium. It has been known for some time that all seven carbon atoms of SHK were incorporated intact into the salicylate units.[549] This incorporation is assumed to proceed as follows: SHK → CHA → ICHA → Salicylate (Figure 82). Cell-free extracts of *Mycobacteria* that produce salicylate are known to convert ICHA to salicylate in the absence of NAD^+; CHA was less effective as a precursor.[550] Other mycobactins, e.g., mycobactin P (from *M. phlei*), contain the polyketide derived 6-methylsalicylate; extracts from *M. phlei* do not catalyze salicylate formation.[550] In the case of *M. fortuitum*, both acids are present; evidence was obtained that in this organism the salicylate was ICHA derived, and the 6-methylsalicylate was polyketide derived.[551] Extracellular iron-binding materials (exochelins) occur in *Mycobacteria*.[552]

Pyochelin, a siderophore of certain *Pseudomonads* (*P. aeruginosa, P. cepacia, P. fluorescens*) has a unique chemical structure (Figure 83A); it contains a salicylate unit. Mutants of *P. aeruginosa* have been obtained which require salicylate for pyochelin biosynthesis; moreover, labeled salicylate was incorporated into the pyochelin structure as predicted. The salicylate was assumed to originate as previously described.[553]

Although not a siderophore, the antibiotic thermorubin (Figure 83B), produced by *Thermoactinomyces vulgaris*, is also salicylate derived. Of the 29 carbon atoms in this compound, 22 had a polyketide origin. The remaining seven came from salicylate, which presumably functioned as a "starter" via its CoA derivative. Since the salicylate unit was clearly not acetate derived, it presumably originated via ICHA.[554]

b. THE ROLE OF 2,3-DIHYDROXYBENZOATE (DHBA)

Dihydroxybenzoic acid (DHBA, Figure 74) is present in many siderophores.[555] Only the prototype, enterobactin (= enterochelin), produced by *E. coli* and other enteric bacteria is considered here with an emphasis on the formation of DHBA. DHBA (Figure 84A) derives from 2,3-dihydro-2,3-dihydroxybenzoate. Three genes (*entC, entB, entA*) are involved in the conversion from CHA; three or four further genes, *entD–F(G)*, form proteins that convert DHBA to enterobactin (Figure 84C). Although there has been some confusion and controversy, it now appears that four genes are linked in an iron-regulated *entCEBA* (P15) polycistronic operon (P15 is an uncharacterized protein, M_r of about 15,000).[556] This area of work is currently very active.

entC. The protein product of this gene catalyzes the conversion of CHA to ICHA and was discussed in connection with the main trunk of the SHK pathway. It is now stated that *entA* protein does not contribute to ICHA synthase activity.[557]

entB, entA. The polypeptide product of *entB* is probably best named 2,3-dihydro-2-3,-dihydroxybenzoate synthase (EC 3.3.2.1, recommended name, isochorismatase). The reaction is formally

$$\text{ICHA} + H_2O \rightarrow \text{2,3-Dihydro-DHBA} + \text{Pyruvate}$$

Overexpression of the *entB* gene has been obtained and the enzyme has been purified to homogeneity.[558]

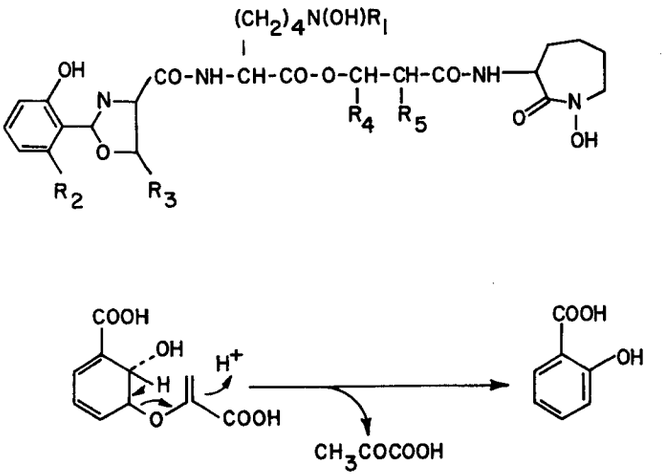


FIGURE 82. Structure of mycobactins and action of salicylate synthase. For A, R_1 = $CH_3(CH_2)_nCH{=}CHCO{-}$, n = 10, 12, 14, 16; R_2 = R_3 = R_5 = H; R_4 = CH_3. For B, R_1 = $CH_3(CH_2)_nCH{=}CHCO{-}$, n = 10, 12, 14, 16; R_2 = CH_3, R_3 = H; R_4 = C_2H_5; R_5 = CH_3.

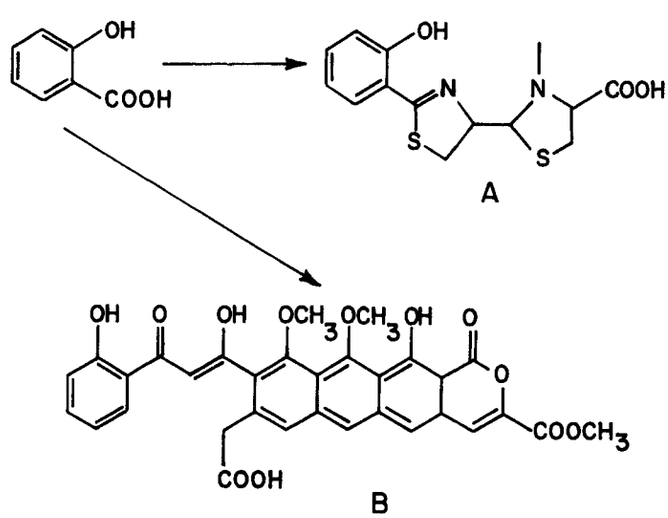


FIGURE 83. Salicylate role in formation of pyochelin, A, and thermorubin, B.

The polypeptide product of *entA* is a dehydrogenase oxidizing 2,3-dihydro-DHBA to DHBA with a requirement for NAD^+ (EC 1.3.1.28, 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase). The *entA* polypeptide, 2,3-dihydro-DHBA dehydrogenase, has been overproduced and purified to electrophoretic homogeneity. The native enzyme had M_r = 210,000 and was unusual among dehydrogenases in being octameric. Contrary to previous suggestions, this enzyme had no ICHA synthase activity.[559]

A nucleotide sequence of 2137 bp was determined and contained ORFs for *entB*, *entA*, and the 15 kDa protein.[556] Other workers sequenced a 3.25-kb fragment encoding the carboxy terminus of *entE*, the entire *entB*, *entA* regions and the 15 kDa polypeptide.[559] For convenience, it may be noted that *entD* and *entE* have also been sequenced.[560-562] The enzyme, DHBA-AMP ligase has been purified to homogeneity.[563]

It is appropriate to note that DHBA is widely distributed in various bacteria and fungi. In most cases, the biosynthetic pathway has not been determined. In addition to derivation via ICHA, DHBA can also be obtained from salicylate, anthranilate, 3-hydroxyanthranilate, and benzoate.[5]

4. Plant Epoxides

The plant products, crotepoxide (from *Croton macrostachys*), senepoxide (from *Uvaria catocarpa*), and pipoxide (from *Piper hookeri*) have been postulated to originate from ICHA by way of an epoxide (Figure 74H). A possible pathway for crotepoxide biosynthesis is shown in Figure 85.[564]

5. 6-Hydroxyanthranilic Acid

6-Hydroxyantharnilic acid is required for the biosynthesis of the antibiotic, sarubicin (a product of *Streptomyces helicus*), and ICHA has been postulated as its precursor.[565] Two biosynthetic pathways are possible (see Figure 86), depending on whether the oxidation step occurs before or after removal of the pyruvoyl unit.[566] The initial atack by NH_3 (or gln) is reminiscent of that by the TPP-succinic semialdehyde anion in MK biosynthesis.

6. 3-Carboxy Aromatic Amino Acids

Four 3-carboxy-substituted aromatic amino acids are known in various plants and there is good evidence that ICHA is the precursor for all of them. Experiments with labeled SHK indicate that the 3-carboxy group in 3-(3-carboxy-4-hydroxyphenyl)alanine (Figure 87C), and 3-(3-carboxyphenyl)-alanine

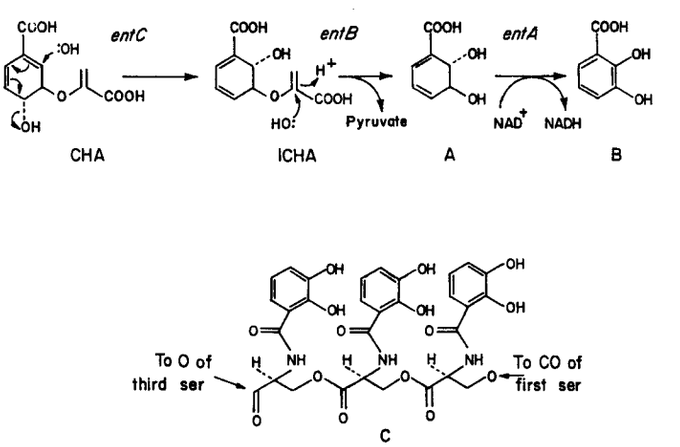


FIGURE 84. Formation of 2,3-dihydroxybenzoate (B) and structure of enterobactin (enterochelin). In the conversion of ICHA to A (dihydro-dihydroxybenzoate), an intermediate is presumably involved.

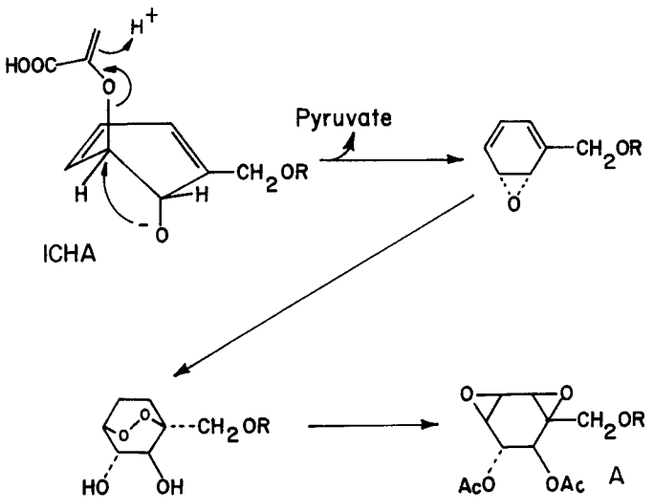


FIGURE 85. Possible formation of crotepoxide, A, from ICHA. R = $—COC_6H_5$; Ac = $—COCH_3$.

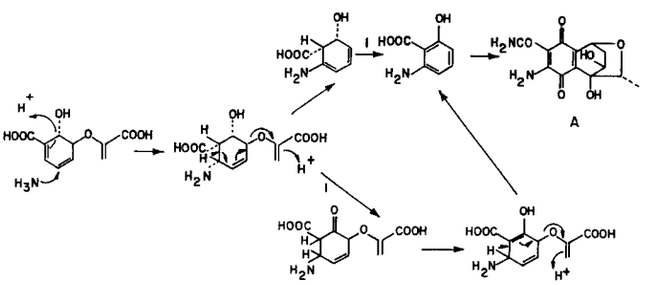


FIGURE 86. Biosynthesis of sarubicin, A, via 6-hydroxyanthranilate. 1 = loss of 2H.

(Figure 87E), derives from the carboxyl group of SHK with the *pro*-6-S hydrogen atom being retained.[567] These results are rationalized by a postulated rearrangement of ICHA to a 1,4-cyclohexadiene structure (Figure 87A), appropriately termed iso-PPA. The corresponding phenylglycine derivatives (Figure 87H) (R = H and OH) can apparently be derived from either the amino or keto acids via the mandelate structures (Figure 87F).[568] These glycine derivatives are isolated from plants as partially racemized D enantiomers.

ACKNOWLEDGMENT

I am indebted to Christine Berliner for much valuable assistance in connection with citations and figures.

ADDENDUM

Work on the SHK pathway continues at a vigorous pace. The following papers were published after the completion of this review in November 1989. They are listed with reference to the appropriate section of the review.

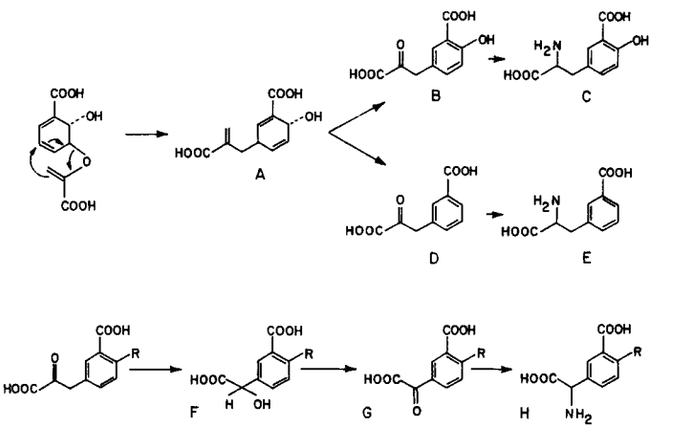


FIGURE 87. Biosynthesis of 3-carboxy aromatic amino acids. In all cases, R = H or OH. A = iso-PPA; B = 3-(3-carboxy-4-hydroxyphenyl)pyruvate; C = 3-(3-carboxy-4-hydroxyphenyl)alanine; D = 3-(3-carboxyphenyl)pyruvate; E = 3-(3-carboxyphenyl)alanine. The phenylglycine derivatives, H, could be derived from the keto acids, B or D, by way of the mandelates, F, and substituted phenylglyoxylates, G.

Section I:

Sutherland, J. K., Watkins, W. J., Bailey, J. P., Chapman, A. K., and Davies, G. M., The synthesis of 6α and 6β-fluoroshikimic acids, *J. Chem. Soc., Chem. Commun.*, 1386, 1989.

Section II.A:

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Dyer, W. E., Weaver, L. M., Zhao, J., Kuhn, D. N., Weller, S. C., and Herrmann, K. M., A cDNA encoding 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase from *Solanum tuberosum* L., *J. Biol. Chem.*, 265, 1608, 1990.

Baasov, T. and Knowles, J. R., Is the first enzyme of the shikimate pathway, 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (tyrosine sensitive), a copper metalloenzyme?, *J. Bacteriol.*, 171, 6155, 1989.

Section II.C:

White, P. J., Young, J., Hunter, I. S., Nimmo, H. G., and Coggins, J. R., The purification and characterization of 3-dehydroquinase from *Streptomyces coelicolor*, *Biochem. J.*, 265, 735, 1990.

Section II.F:

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Section III.A.1.d:

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Section III.B.:

Hagervall, T. D., Jonsson, Y. H., Edmonds, C. G., McCloskey, J. A., and Bjork, G. R., Chorismic acid, a key metabolite in modification of tRNA, *J. Bacteriol.*, 172, 252, 1990.
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Section III.B.2:

Crawford, I. P., The tryptophan paradigm, *Ann. Rev. Microbiol.* 43, 567, 1989.
Picknett, T. M., Saunders, G., and Holt, G., Expression of the *trpC* gene from *Penicillium chrysogenum* in *Aspergillus nidulans*, *Biotechnol. Appl. Biochem.*, 11, 464, 1989.
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Section III.C:

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Section III.D.3:

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Added in Proof: A review of the 1988 literature was published in June, 1990. **Dewick, P. M.,** *Natl. Prod. Rep.*, 7, 195, 1990.

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