Advances in Enzymology and Related Areas of Molecular Biology, Volume 53
Edited by Alton Meister

BIOSYNTHESIS OF RIBOFLAVIN, FOLIC ACID, THIAMINE, AND PANTOTHENIC ACID

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CONTENTS

I. Introduction

There is still much that is not yet known about the biosynthesis of vitamins, even though the details of the pathways for the biosynthesis of other equally important biological substances such as amino acids, purines, and

pyrimidines have been known for some time. Incomplete knowledge about how the vitamins are made cannot be attributed to lack of effort, but rather to the difficulties encountered in such investigations. The vitamins are relatively complicated chemical compounds and since they are catalytic agents they are not made in large quantities. Thus the elucidation of the biosynthetic pathways of these substances represents a most challenging problem in metabolic biochemistry.*

II. Riboflavin

On the basis of their analyses of a number of riboflavin-requiring mutants of *Saccharomyces cerevisiae*, Bacher, Lingens, and colleagues (2-4) proposed a tentative pathway for the biosynthesis of riboflavin with guanine (or the nucleoside or a nucleotide of guanine) as the precursor. Since the evidence on which this tentative pathway was based, as well as other evidence indicating that a guanine compound is the precursor was thoroughly discussed in a previous review (1a), no further extensive discussion of this background material is presented here. Recent information has led to the modification and extension of the tentative pathway of Bacher and coworkers so that the enzymatic reactions that are now thought to result in the biosynthesis of riboflavin are as shown in Figure 1. In the presentation that follows, the evidence for the individual steps of this pathway are discussed.

A. GTP AS THE PRECURSOR

The first definitive evidence that the precursor guanine compound is a nucleoside or a nucleotide was provided by Mailänder and Bacher (5), who demonstrated that a mutant of *Salmonella typhimurium*, unable to interconvert guanine and guanosine, incorporated [ribose-^{14}C]guanosine into riboflavin and GMP without a dilution of radioactivity. This not only established that guanosine or a guanine nucleotide is the precursor, but it also indicated that the ribose portion of such a compound is retained and converted metabolically to the ribityl group of riboflavin. Supporting evidence was supplied by Mitsuda and coworkers (6,7), who concluded that GTP is the precursor through their analyses of the nucleoside and nucleotide pools of *Eromothecium ashbyii* under various conditions of flavinogenesis, and by Bresler et al. (8), who presented evidence for a role for a guanosine compound as the precursor in *Bacillus subtilis*. The

*Earlier reviews on the biosynthesis of the water-soluble vitamins have appeared (la, lb).

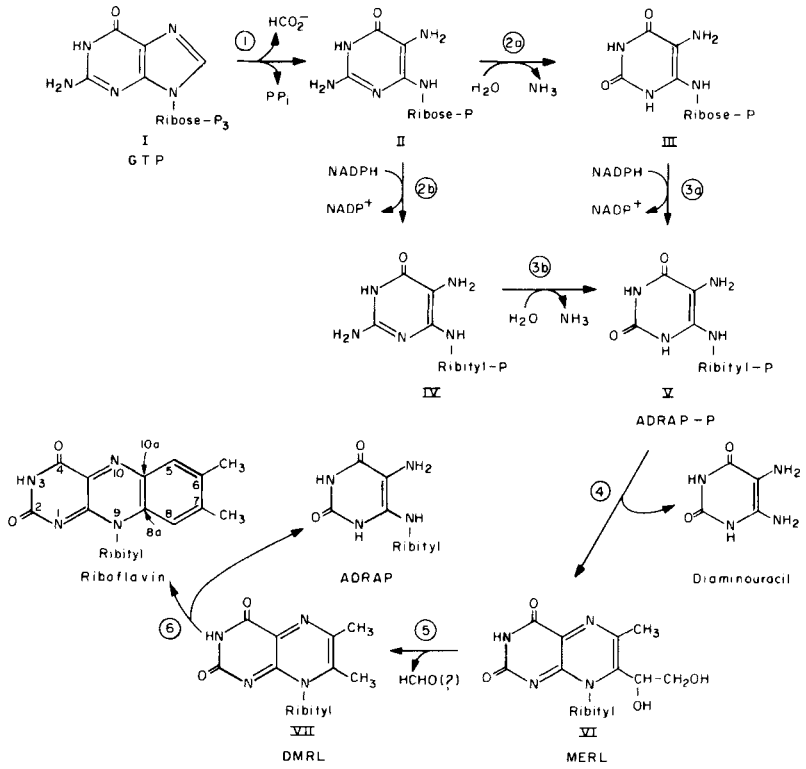


Figure 1. The enzymatic reactions thought to be involved in the formation of riboflavin. In reaction 4, 2 moles of **V** are thought to be needed for the synthesis of 1 mole of **IV** and 1 mole of diaminouracil. Similarly, in reaction 6, 2 moles of **VII** are known to be used for the production of 1 mole of riboflavin and 1 mole of ADRAP.

most conclusive evidence that GTP is the precursor was provided with the discoveries in *Escherichia coli* (9), *B. subtilis* (10), and the yeasts *Pichii guilliermondii* (11) and *Torulopis candida* (12) of enzymes that catalyze the removal from GTP of C-8 as formate. The first and most informative report about this enzyme was that of Foor and Brown (9), who purified the enzyme by 2200-fold from extracts of *E. coli* and determined that the products of its action are formate, pyrophosphate, and 2,5-diamino-6-oxy-4-(5′-phosphoribosylamino)pyrimidine (II, Fig. 1). They also described some of the properties of the enzyme and suggested it be called GTP cyclohydrolase II to distinguish it from a previously studied *E. coli*

enzyme, GTP cyclohydrolase, known to be involved in the biosynthesis of folic acid. At the time the report appeared the authors suggested that GTP cyclohydrolase II is a part of the riboflavin biosynthetic pathway, although no direct evidence was available to support the supposition. However, subsequent investigations, discussed in a later section of this chapter, have confirmed that this enzyme is indeed involved in the biosynthesis of riboflavin.

B. ORIGIN OF THE RIBITYL GROUP

When the subject of riboflavin biosynthesis was last reviewed (1a) the origin of the ribityl group of riboflavin had not yet been conclusively established. However, recent evidence has clearly shown that this group is derived from the ribose moiety of GTP. This has been established by studies on the incorporation of ^{14}C-labeled species of guanosine into riboflavin in *S. typhimurium* (5), *P. guilliermondii* (13,14) *E. ashbyii* (7,15), and *B. subtilis* (8) and decisively confirmed with the discoveries of enzymes from various sources that catalyze the reduction of the nucleotidyl ribose group (13,16,17). The earlier contention that ribitol and ribose are preferentially incorporated into the ribityl group of riboflavin (18-20) was based on studies with whole cells under conditions in which the effects of pool sizes were hard to interpret and thus the conclusions were suspect. In fact, Miersch, one of the advocates of a precursor role for ribitol (18,19), has recently stated that earlier observations were inconclusive (13,21) and, with collaborators, has done some of the recent work to indicate that the ribityl group is derived from the ribose of GTP (13,21).

If, as seems likely, reaction 1 of Figure 1 is involved in the biosynthesis of riboflavin, this reaction should theoretically be followed by deamination and reduction (the latter to convert the ribosyl-P group to ribityl-P). That these steps (in no particular order) occur was suggested by the work of Mitsuda and coworkers, who have identified 5-amino-2,6-dioxy-4-ribitylaminopyrimidine as a product accumulated by resting cells of the riboflavin overproducer *E. ashbyii* (22) and a riboflavin-deficient mutant of *B. subtilis* (23). Analyses of accumulation products of mutants of *S. cerevisiae* (2) and *P. guilliermondii* (13) have suggested that reduction precedes deamination in the pathway. However, a genetic and biochemical investigation of a series of riboflavin-requiring mutants of *B. subtilis* by Bresler et al. (24) indicated that in this organism deamination occurs before reduction. Investigations of these two (deamination and reduction) processes at the enzyme level have indicated that in *E. coli*, deamination definitely precedes reduction (16) (by reactions 2a and 3a, Fig. 1), where-

as in *P. guilliermondii* (13) and *Ashbya gossypii* (17) reduction takes place before deamination (reactions 2b and 3b). The most complete enzyme work has been done by Burrows and Brown (16) in *E. coli*. These workers separated the deaminase and the reductase, purified each by about 200-fold, and determined some of their properties. For both enzymes it was established that the dephosphorylated form of the substrate is not acted on by the enzyme and that the phosphate group is retained in the product. NADPH was needed for reductase activity to be expressed; NADH was utilized approximately 30% as well as NADPH. No extensive information was given about the reductase present in *P. guilliermondii* (13) and *A. gossypii* (17). In both systems work was limited primarily to observations on the existence of these enzymes in these organisms and the order in which the reductase and the deaminase functions in the biosynthetic pathway.

From their analyses of various products accumulated by mutants of *B. subtilis*, Bresler et al. (24) have identified one such product as 5-amino-2,6-dioxy-4-ribuloseaminopyrimidine and suggest that this compound is an intermediate in the reduction of the ribosyl group to a ribityl group. In such a reaction the reduction could be viewed as occurring in two stages; the first is an Amadori-type rearrangement of the ribosyl group to a ribulose moiety, and the second is one in which the ribulose group would be reduced to a ribityl group in the presence, presumably, of NADPH. Burrows and Brown (16) found no evidence for such an intermediate in the system from *E. coli*, but neither can their work rule out such a possibility. Further work with this enzyme system is necessary to resolve this uncertainty.

The conclusion from the results of the enzyme work by Burrows and Brown (16) that the intermediates in the pathway are phosphorylated has received support from the finding by Logvinenko et al. (14) that incubation of dialyzed extracts of *P. guilliermondii* with GTP and NADPH resulted in the formation of significant quantities of phosphorylated 2,5-diamino-6-oxy-4-ribitylaminopyrimidine.

C. CONVERSION OF 5-AMINO-2,4-DIOXY-6-RIBITYLAMINOPYRIMIDINE (ADRAP) TO 6,7-DIMETHYL-8-RIBITYLLUMAZINE (DMRL)

The four carbons needed to convert ADRAP to DMRL ultimately become the methyl carbons and C-5, C-6, C-7, C-8, C-8a, and C-10a of the riboflavin molecule (see Fig. 1 for numbering system), since in the last step of the pathway riboflavin synthase catalyzes the removal of a four-carbon

unit from 1 mole of DMRL and the addition of this unit to a second mole of DMRL to yield riboflavin (25). The source of these carbons has been a subject of considerable interest and speculation. A case has been made for butaneione (diacetyl), but as Plaut (26) has pointed out the observed stimulation of riboflavin production by butanedione can be explained on grounds other than its having a role as the true precursor. Some of the data obtained from measurements of the incorporation of various radioactive compounds are consistent with the view that a pentose compound is the source of the four-carbon unit (see ref. 26 for a critical discussion). The recent comparison by Alworth et al. (27) of the incorporation into riboflavin of various labeled substances by *Propionibacterium shermanii* has supported the view that a pentose is the precursor, and this proposal is further supported by the observation of Bresler et al. (28) that a riboflavin-requiring mutant of *B. subtilis* accumulates and excretes a compound that they isolated and identified as 6-methyl-7-(1′,2′-dihydroxyethyl)-8-ribityllumazine (MERL, shown in Fig. 1 as VI). They suggested that MERL is formed from 2 moles of ADRAP, one used as the donor of a five-carbon unit (derived from the ribityl group) and one as the acceptor. They further suggested that through the loss of a one-carbon unit DMRL can be made from MERL enzymatically. This suggestion has received further support from a recent report by Hollander et al. (29), who found that in the presence of a dialyzed ammonium sulfate fraction prepared from an extract of *E. coli*, ADRAP can be converted to DMRL and riboflavin (the enzyme preparation contained riboflavin synthase) without the addition to the reaction mixture of any other carbon source. These workers also reported (*a*) that at least two enzymes are needed for the conversion of ADRAP to DMRL, (*b*) that pyridine nucleotide (NAD^+ was used most effectively) is required, and (*c*) that diaminouracil is also a product. The evidence that at least two enzymes are required is consistent with the observation in *S. cerevisiae* that two gene products are needed for the transformation of ADRAP to DMRL (4).

Since, at least with the enzymes studied from *E. coli*, the phosphate ester of ADRAP (ADRAP-P) is known to be the biosynthetic product (16), but either free ADRAP or the phosphate ester can be converted to DMRL (29), the tentative conclusion is that ADRAP-P is dephosphorylated either before or during the action of the enzymes that convert ADRAP to DMRL. An alternative explanation is that the phosphate group is retained to yield the diphospho ester of MERL, which in turn would be expected

to be converted to the phosphate ester of DMRL. This possibility cannot yet be rigorously ruled out, although it seems unlikely, since ADRAP is such a good substrate and the phosphate ester of DMRL is known not to be a substrate for riboflavin synthase (31), the terminal enzyme that catalyzes the conversion of DMRL to riboflavin.

Considerations of the chemistry involved in the formation of MERL from 2 moles of ADRAP suggest that the ribityl group of 1 mole of ADRAP is oxidized to a five carbon diketo compound, (CH_3-C(=O)-C(=O)-CH(OH)-CH_2OH), which in turn reacts with another mole of ADRAP to yield MERL. Neither the mechanism of this reaction nor that of the putative enzymatic reaction for the conversion of MERL to DMRL has been established, but the reported involvement of NAD^+ in catalytic quantities in the overall conversion of ADRAP to DMRL (29) suggests that an oxidative reaction takes place (perhaps in the formation of MERL), followed by a reductive reaction to regenerate NAD^+, perhaps in the conversion of MERL to DMRL. The latter reaction theoretically should require a reduction. The study of the mechanisms of these purative enzymatic reactions must await further work with the use of enzymes in more purified states.

D. CONVERSION OF 6,7-DIMETHYL-8-RIBITYLLUMAZINE TO RIBOFLAVIN

The conversion of DMRL to riboflavin is catalyzed by the enzyme known as riboflavin synthase (or riboflavin synthetase). This enzyme is known to occur in a variety of microorganisms (26) and has been purified from several. The mechanism by which it uses 2 moles of DMRL to make a mole of riboflavin has been elucidated, especially by the elegant work of Plaut and coworkers, and has been reviewed in detail by Plaut (26). The most interesting recent development related to riboflavin synthase has come from the studies of Bacher and collaborators on the enzyme from *B. subtilis*, an organism in which the biosynthesis of this enzyme is known to be controlled by repression (32,33). Two riboflavin synthases of different molecular weights were found in extracts of a derepressed mutant of *B. subtilis* (31). The light enzyme was found to be a trimer of identical subunits (termed α subunits), and the heavy enzyme (representing only 20% of the total riboflavin synthase activity) contained 1 mole (three subunits) of light enzyme and approximately 60 β subunits (33). Only preparations containing the light enzyme possessed riboflavin synthase activity. Bacher and Mailänder (34) have recently isolated riboflavin-requiring mutants

missing the β subunits, but containing the α subunits. These mutants possess normal riboflavin synthase activity and can grow without riboflavin if the medium is supplemented with butanedione (diacetyl). The authors conclude that the mutants are missing one or more enzymes necessary for the conversion of a precursor (probably ADRAP) to 6,7-dimethyl-8-ribityllumazine (DMRL). The probable explanation for the observed growth in the presence of butanedione is that this compound reacts nonenzymatically with ADRAP to yield DMRL, and the latter compound is then converted to riboflavin by the action of riboflavin synthase (i.e., the light enzyme present in the mutant). Thus these observations suggest that in *B. subtilis* a portion, at least, of riboflavin synthase is associated with another protein thought to have enzyme activity in the catalysis of a previous reaction of the biosynthetic pathway. Now that more information is available about the pathway, and methods are available to test for activities of the other biosynthetic enzymes, it should be possible to test this hypothesis directly.

In the conversion of 2 moles of DMRL to riboflavin, 1 mole of 5-amino-2,6-dioxy-4-ribitylaminopyrimidine (ADRAP) is also thought to be produced, as a by-product. Since the evidence is strong that this compound is also an intermediate in the biosynthetic pathway, the question arises as to whether, after its production as a by-product, it is reutilized for the production of DMRL. No firm evidence exists for enzymatic reutilization, but Mitsuda et al. (34a) have shown that with the use of a partially purified riboflavin synthase from *E. ashbyii* the by-product is reutilized, provided butanedione is supplied in the reaction mixture. This can be explained as a nonenzymatic reaction of the butanedione with ADRAP to yield DMRL and thus should not be considered as evidence for the enzymatic utilization of the by-product, but it is an important observation because it confirms that the by-product is ADRAP. Since there is no reason to believe that it is different from the ADRAP produced biosynthetically, it seems likely that the by-product can thus enter into the biosynthetic pathway and be converted to DMRL and riboflavin.

E. REGULATION OF THE BIOSYNTHESIS OF RIBOFLAVIN

No published evidence has yet appeared for control of the riboflavin pathway by feedback regulation of any of the enzymes involved. However, convincing evidence for regulation of the pathway by coordinate repression in *B. subtilis* has been reported by Bacher and coworkers (31,33,34) and

by the extensive investigations of Bresler and his collaborators reported in a series of papers on the "riboflavin operon" (8,10,24,28,32,35-48). The latter workers have mapped the regulatory and structural genes involved and their work has also been of great significance in the elucidation of individual steps of the biosynthetic pathway (discussed earlier). Of particular interest are their recent investigations (47), which have indicated the existence in *B. subtilis* of two operator genes, one ($ribO_e$) that regulates the transcription of the gene coding for GTP cyclohydrolase II, the first enzyme of the biosynthetic pathway, and a second (ribO) that may regulate the transcription of the rest of the genes of the pathway. Riboflavin is known to be the effecter that interacts with the regulator protein (product of ribO) to shut off the synthesis of riboflavin synthase (45), and the authors suggest that an intermediate in the biosynthetic pathway is the effector that represses the transcription of the gene coding for GTP cyclohydrolase II upon interaction with the $ribO_e$ product. Hence the coordinate action of the two regulatory gene products would be expected to repress the synthesis of the first enzyme and the last enzyme, and perhaps all of the enzymes of the pathway.

III. Folic Acid

The biosynthesis of folic acid and related compounds was reviewed in detail in 1971 (49,50). However, to present a complete picture of the subject, aspects of material included in those reviews is briefly presented here along with more recent material, especially regarding the formation of polyglutamates of folate.

A. ENZYMATIC SYNTHESIS OF DIHYDROPTEROIC ACID AND DIHYDROFOLIC ACID

The enzymatic reactions known to be involved in the formation of dihydropterioc acid (H_2pteroate) and dihydrofolic acid (H_2folate) are shown in Figure 2. Substitution of *p*-aminobenzoylglutamic acid for *p*-aminobenzoic acid in reaction B allows the synthesis directly of H_2folate, although evidence presented later suggests that *p*-aminobenzoate is the physiological substrate. The identification of the precursor pteridine compound as 2-amino-4-oxy-6-hydroxymethyl-7,8-dihydropteridine (hereafter abbreviated as H_2pterin-CH_2OH) resulted primarily from the early work of Shiota and collaborators (51,52) with an enzyme system from

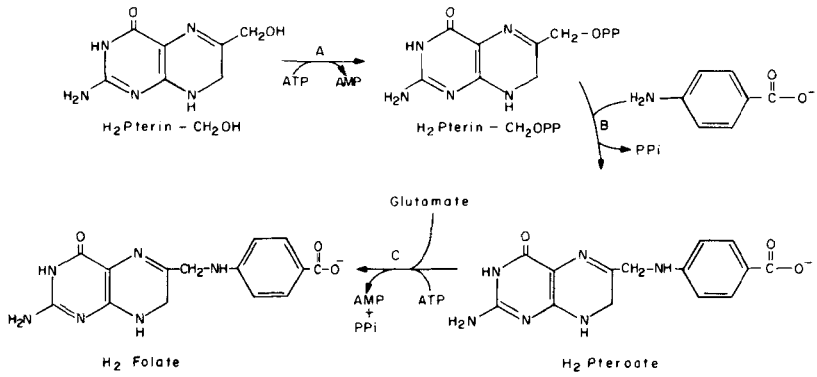


Figure 2. The enzymatic synthesis of H_2pteroic acid and H_2folic acid.

Lactobacillus plantarum and of Brown and coworkers (53-55), who used an *E. coli* system. Later work established that H_2pterin-CH_2OH is also the precursor in a variety of other microorganisms (56-60) and in plants (61-67). The ATP requirement for the enzymatic formation of the H_2pteroate (from *p*-aminobenzoate) or H_2folate (from *p*-aminobenzoylglutamate) (53,68) suggested that a phosphorylated form of the pteridine substrate might be an enzymatic intermediate and stimulated Shiota et al. (69) to prepare and test the phosphorylated compounds H_2pterin-CH_2OP and H_2pterin-CH_2OPP. They found that only the later compound (i.e., the pyrophosphoester) could be used in the *L. plantarum* system in the absence of ATP. Somewhat later, Weisman and Brown (54) made the same observation in the *E. coli* system and this was followed by findings indicating that the pyrophosphoester is an intermediate in all systems that have been studied (see refs. 49 and 50).

The identification of H_2pterin-CH_2OPP as an intermediate indicated that two enzymes are involved: one to catalyze the formation of the intermediate and a second needed for the formation of H_2pteroate from *p*-aminobenzoate and the intermediate. The initial evidence for the existence of two enzymes (54) was followed by the separation of the two enzymes present in *E. coli* (70) and in *L. plantarum* (71) and descriptions of their properties (70-72). The enzyme that catalyzes the production of H_2pterin-CH_2OPP (reaction A, Fig. 2) has been purified 400-fold from *E. coli* and has been shown to be relatively stable to heating at 100°C and to have a molecular weight of approximately 15,000 (70). In reaction B, catalyzed by H_2ptero-

ate synthetase, Mg^{2+} is required (70,73) and H_2pteroate and inorganic pyrophosphate are produced in equimolar quantities (70,75). H_2Pteroate synthetase activity has been found in many microorganisms (60,70-78) and in plants (64,66,67).

In all systems studied, H_2pteroate synthetase can use either *p*-aminobenzoate or *p*-aminobenzoylglutamate as substrates to yield, as products, H_2pteroate or H_2folate, respectively. Since *p*-aminobenzoate is used more effectively as substate in all systems and since no credible evidence exists for the formation of *p*-aminobenzoylglutamate as a biosynthetic product, it seems very likely that H_2 pteroate is a biosynthetic intermediate and that *p*-aminobenzoylglutamate is not. Support for this conclusion has been provided by the discovery in a number of microorganisms (79) and in plants (62,67,80) of an enzyme named H_2folate synthetase, which catalyzes reaction C in Figure 2.

The utilization of *p*-aminobenzoate or *p*-aminobenzoylglutamate has been shown to be competitively inhibited by sulfonamides with the use of H_2pteroate synthetase preparations from *E. coli* (81), *Diplococcus pneumoniae* (56,58) and *Veillonella* (75), a fact that explains the well-known effects of sulfonamides as bacteriostatic agents. Since the work of Brown (81) and Shiota et al. (75) shows that sulfonamides are substrates for H_2pteroate synthetase, the old view that sulfonamides are competitive inhibitors of the utilization of *p*-aminobenzoate has had to be modified to indicate that this is really a case of the sulfonamide competing with *p*-aminobenzoate as a substrate.

B. BIOSYNTHESIS OF THE PTERIDINE PORTION OF FOLID ACID

Folic acid is only one of a number of naturally occurring pteridines. Before enzyme work was undertaken, a great deal of evidence, largely the result of investigations on the incorporation of ^{14}C-labeled compounds, had accumulated to indicate that purine (or a derivative) is the precursor of all naturally occurring 2-amino-4-hydroxypteridines, including the pteridine portion of folic acid. The reader is directed to earlier reviews (49,50) for a more complete presentation of this background material. Evidence derived primarily from investigations with enzyme preparations has clearly indicated that GTP is the purine compound used as precursor and that the initial reaction in the biosynthesis of all 2-amino-4-hydroxy-pteridines is the removal from GTP of C-8, as formate, and the formation of the triphosphoester of 2-amino-4-oxy-6-(trihydroxypropyl)-7,8-dihydro-

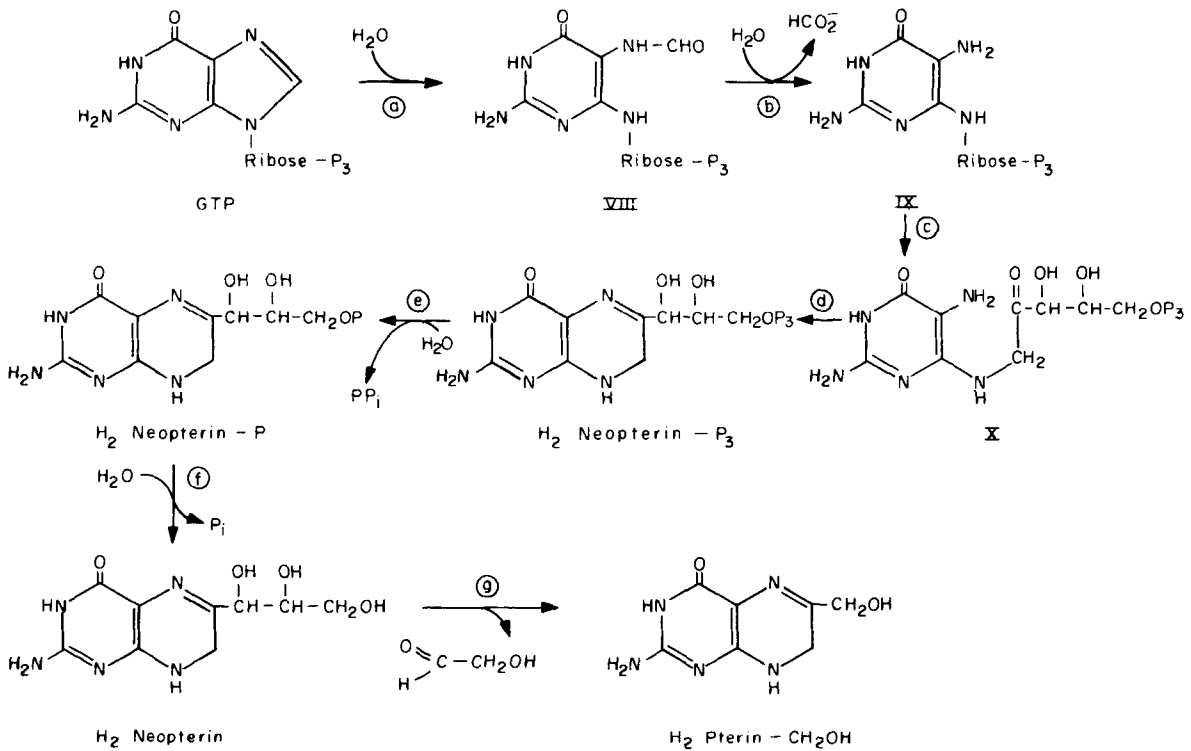


Figure 3. The biosynthetic pathway for the conversion of GTP to H_2pterin-CH_2OH. P_3 is an abbreviation for a triphosphate unit.

pteridine. This compound, which has been given the trivial name of dihydroneopterin triphosphate (hereafter abbreviated as H_2neopterin-PPP, see Fig. 3), has been established as a common intermediate in the biosynthesis of all 2-amino-4-hydroxypteridines. Since a discussion of what is known about the biosynthesis of all of these pteridines is outside the scope of this chapter, in the following presentation only those aspects of pteridine biosynthesis directly related to the formation of the pteridine portion of folic acid are discussed.

Figure 3 contains the set of reactions responsible for the enzymatic conversion of GTP to H_2pterin-CH_2OH, the pteridine known to be used for the direct enzymatic formation of H_2pteroate and H_2folate. The preliminary enzyme work indicated that a guanosine compound is used for folate formation in an *E. coli* system (82,83), an *L. plantarum* system (84), and spinach (63). From their investigations on the incorporation into folate of ^{14}C from various species of ^{14}C-labeled guanosine, Reynolds and Brown (83) concluded that C-8, C-4′, and C-5′ of guanosine are removed during this overall conversion, and they proposed a tentative biosynthetic pathway that was consistent with these observations and similar to a pathway that had been proposed earlier (85,86) for the biosynthesis of pteridine pigments in insects. This hypothetical pathway included as one of the putative intermediates 2-amino-4-oxy-6-(trihydroxypropyl)-7,8-dihydropteridine (H_2neopterin), a substance that had originally been reported to occur in *E. coli* [as a phosphate ester (87)] and in the larvae of honeybees (88). The work of Jones et al. (89,90) established a role for H_2neopterin as an intermediate with the observation that in the presence of extracts of *E. coli* it can be produced from GTP (90) and can be converted to H_2pteroate (89) through the intermediary formation of H_2-pterin-CH_2OH (90).

That GTP is the guanosine compound that functions as precursor was established almost simultaneously from independent investigations conducted with enzyme preparations from several sources (63,84,91,94). Burg and Brown (95) purified by 800-fold the enzyme (from extracts of *E. coli*) that catalyzes the removal of formate from GTP and suggested it be called "GTP cyclohydrolase." These workers identified the products of the action of this enzyme as formate and H_2neopterin-PPP and determined some of its properties. Shiota et al. (52) found that these same products are formed from GTP with an enzyme prepared from *L. plantarum* and suggested that the enzyme be called "dihydroneopterin triphosphate

synthetase." More recently, Yim and Brown (96), helped considerably by the application of an affinity chromatography step devised by Jackson et al. (97), purified GTP cyclohydrolase from *E. coli* to homogeneity and reported that it consists of four identical subunits, each of 51,000 molecular weight, and that each subunit contains two identical polypeptide chains. The catalytically active form of the enzyme (molecular weight of 210,000) can be dissociated into the inactive subunits by subjecting the enzyme to a relatively high concentration (0.3 *M*) of KCl. The enzyme is quite stable to heating, with a half-life of 7 min at 87°C. The evidence is clear that the *E. coli* enzyme is a single protein (96), but GTP cyclohydrolase activity prepared from several other microorganisms [*L. plantarum* (98), *Comamonas sp.* (99), and *Streptomyces rimosus* (100)] apparently exists as multiple forms of differing molecular weights.

Although theoretical considerations suggest that the conversion of GTP to H_2neopterin-PPP proceeds in a stepwise manner, as shown in Figure 3, there is no evidence that more than one enzyme is involved in this transformation in any system studied. In fact, the evidence is quite convincing, at least in *E. coli*, that only one enzyme is involved. Thus, if **VIII**, **IX**, and **X** of Figure 3 are intermediates they would be expected to exist as enzyme-bound intermediates. There is some indirect evidence for the formation of such intermediates. For example, 2-amino-5-formamido-6-(5′-triphosphoribosyl)amino-4-oxypyrimidine (**VIII**, Fig. 3) has been prepared and shown to be converted to formate and H_2neopterin-PPP in the presence of extracts of *L. plantarum* (101,102) and the purified enzyme from *E. coli* (96). Also, evidence that **X** may be involved in the reaction sequence was provided by Wolf and Brown (103), who found that incubation of 7-methyl-GTP with GTP cyclohydrolase from *E. coli* resulted in the formation of a compound that they identified as methylated **X**. The conclusion drawn from this observation is that 7-methyl-GTP underwent the equivalent of reactions a, b, and c in Figure 3, but that the presence of the methyl group prevented ring closure (reaction d, Fig. 3). These observations provide indirect evidence that **VIII**, **IX**, and **X** are involved in the reaction sequence and, since they cannot be isolated as free intermediates, the resultant conclusion is that they exist as enzyme-bound intermediates.

The initial reports on the existence of enzymes in *E. coli* (90) and chinese cabbage (63) that catalyze the conversion of H_2neopterin to H_2pterin-CH_2OH (reaction g, Fig. 3) were followed by purification of the enzyme from *E. coli* (104) and the determination of some of its properties.

The enzyme was named "dihydroneopterin aldolase" (104), since the reaction it catalyzes is similar to those reactions catalyzed by aldolases. The enzyme is heat stable (no loss of activity results from heating at 100°C for 5 min) and does not use neopterin, tetrahydroneopterin, H_2neopterin monophosphate, or H_2neopterin triphosphate as substrate in place of H_2neopterin. The products of its action on H_2neopterin were identified as 2-amino-4-oxy-6-hydroxymethyl-7,8-dihydropteridine (H_2pterin-CH_2-OH) and glycolaldehyde, as shown in Figure 3.

Since H_2neopterin-PPP is the product of action of GTP cyclohydrolase and since dihydroneopterin aldolase will use only H_2neopterin as substrate, the complete dephosphorylation of H_2neopterin-PPP is clearly a necessary step in the folate biosynthetic pathway. Suzuki and Brown (105) discovered in *E. coli* an enzyme, which they named "dihydroneopterin triphosphate pyrophosphohydrolase," that catalyzes the removal as inorganic pyrophosphate of two of the three phosphate groups of H_2neopterin-PPP to yield the monophosphoester (H_2neopterin-P) as the other product. This enzyme is apparently specific for H_2neopterin-PPP (e.g., none of the nucleoside triphosphates can be used as substrate) and requires Mg^{2+} for activity. No evidence has been obtained for the existence of a specific enzyme for the removal of the third phosphate group (to generate H_2neopterin from H_2neopterin-P, a necessary step in the pathway) although it is known that this can be accomplished, at least in *E. coli*, through the action of nonspecific phosphomonoesterases (105).

C. PTEROYLPOLYGLUTAMATES

Pteroylpolyglutamates (also sometimes called "folylpolyglutamates") is a general term applied to a variety of folate compounds containing two or more glutamate residues in γ-peptide linkages. Since the initial report on the natural occurrence of these polyglutamates (106), subsequent analyses have shown that nearly all intracellular folate occurs as polyglutamates, ranging from 2 glutamate residues up to 12 (with 4–6 residues the predominant form), depending on the cells analyzed. Additional complexities that make accurate determinations of the number of glutamate residues difficult are: (*a*) the pteroyl group of all these polyglutamate forms can exist naturally also in the dihydro and tetrahydro forms and (*b*) each polyglutamate can also exist in several known naturally occurring forms with various one-carbon groups attached. These include the following derivatives of tetrahydropteroylpolyglutamates: 5-methyl, 5-formyl,

10-formyl, 10-hydroxymethyl, 5-hydroxymethyl, 5,10-methylene, and 5, 10-methenyl. Recent advances in methodology (107-109) have allowed analyses for the number of glutamate residues in the various naturally occurring forms to be more credible, and the reader can anticipate, in the near future, extensive reexaminations of various tissues and microorganisms based on the newer methods.

The literature on the *in vivo* production of pteroylpolyglutamates from folate is extensive and is not reviewed here. The present discussion concentrates, instead, only on the enzymatic work on the formation of these substances. The first report on the enzymatic formation of pteroylpolyglutamates was made by Griffin and Brown (79), who in their studies on the enzymatic production of H_2folate also observed that in the presence of an extract of *E. coli*, tetrahydrofolate (H_4folate) could be converted in small quantities to substances that appeared to be H_4pteroyldiglutamate and H_4pteroyltriglutamate. Almost 10 years later Gawthorne and Smith (110) presented the first evidence for the enzymatic production of pteroylpolyglutamates in a mammalian system. They found with an enzyme preparation from sheep liver that polyglutamates could be produced from H_4PteGlu*, 5-formyl-H_4PteGlu*, or 5-methyl-H_4PteGlu*. ATP, Mg^{2+}, K^+, and glutamate were all required in the system. These workers did not determine the number of glutamate residues present in the products, but they concluded that glutamates are added one at a time in the system, since γ-glutamylglutamate could not replace glutamate as substrate.

In 1975 Masurekar and Brown (111) extended the previous investigations (79) on the *E. coli* system. They purified an enzyme that catalyzes the production of a pteroyldiglutamate and established that the enzyme uses 10-formyl-H_4PteGlu as substrate very effectively (K_m of less than $2\mu M$) and 5,10-methylene-H_4PteGlu and H_4PteGlu (K_m of 10-12 μM for each) less effectively. No other folate compound was active as substrate and no other amino acid, including γ-glutamylglutamate, could replace glutamate as substrate. ATP, Mg^{2+}, and K^+ (or NH_4^+) were absolute requirements. A single product was formed that was identified as a diglutamate. No evidence was obtained for the formation of a triglutamate (or higher polyglutamates) with this enzyme.

Taylor and Hanna (112,113), have studied the enzymatic formation of pteroylpolyglutamates with extracts of cells (ovary and lung) from the

*PteGlu is the abbreviation for pteroylglutamic acid, and H_2PteGlu and H_4PteGlu represent the dihydro and tetra forms of this substance, respectively.

Chinese hamster and have presented evidence that a single enzyme is responsible for catalyzing the stepwise addition of up to three glutamate residues to H_4PteGlu (112). Naturally occurring folate compounds used as substrate were, in decreasing order of effectiveness, H_4PteGlu, 5-formyl-H_4PteGlu, and 5-methyl-H_4PteGlu. PteGlu (folate) also appeared to be a substrate when crude extracts were used, but this activity was not evident when a 25-fold purified enzyme preparation was used. These were the only folate compounds tested as substrates. L-Glutamate was used exclusively as the second substrate and, as expected, ATP (or dATP or GTP) and Mg^{2+} were required. An interesting observation is that at a relatively high (100 μM) concentration of H_4PteGlu only one product was observed, which was identified as the diglutamate (H_4PteGlu$_2$); however, at lower substrate concentrations (5 μM) a second product appeared, and when the concentration was progressively lowered (down to 1 μM) the amounts of H_4PteGlu$_2$ formed decreased, the amounts of the second compound increased, and a third compound was detected in small amounts. The second and third compounds were identified as the triglutamate (H_4PteGlu$_3$) and tetraglutamate (H_4PteGlu$_4$). These investigators also reported that mutants of these cells, which have a triple nutritional requirement for adenosine, glycine, and thymidine, are missing this polyglutamate synthetase; however, revertants obtained by growth in a medium deficient in adenosine, glycine, and thymidine were found to possess enzyme activity (112,113). The failure of the mutants to grow in the presence of PteGlu and in the absence of adenosine, glycine, and thymidine indicates that in these cells the formation of pteroylpolyglutamates is necessary, but it is not known whether the necessity is for the production of a coenzyme form of folate that is used much more effectively than is the corresponding monoglutamate in the synthesis of adenosine, glycine, and thymidylic acid, or whether the effect is to keep folate compounds from leaking out of the cells by the conversion of the monoglutamate to polyglutamates. A combination of these two explanations also seems possible. Ample evidence exists from work with individual enzymes from a variety of sources that tetrahydropteroylpolyglutamates are used as coenzyme in certain processes either exclusively (114-117) or more effectively (118-125) than is H_4folate.

Recently, McGuire et al. (126) conducted a thorough study of the enzymatic synthesis of pteroylpolyglutamates with rat liver preparations and found that this system and the hamster system of Taylor and Hanna (112) have some characteristics in common. For example, H_4PteGlu is the most effective substrate in both systems and both exhibit an inverse

relationship between substrate concentration and glutamate chain length of the product; that is with the rat liver enzyme at high concentrations H_4PteGlu the predominant product was H_4PteGlu$_2$ and at relatively low concentrations a mixture of products was observed, consisting of polyglutamates with up to four glutamate residues. With the rat liver enzyme all folate compounds tested were used as substrate, including PteGlu and H_2PteGlu, but the most effective substrate was H_4PteGlu, with 10-formyl-H_4PteGlu next (88% of the activity of H_4PteGlu). 5-Methyl-H_4PteGlu and 5-formyl-H_4PteGlu were poor substrates. When H_4PteGlu$_4$ was added as substrate, the pentaglutamate was synthesized in small quantities, but no evidence for the formation of a hexaglutamate from the pentaglutamate was obtained, although the pentaglutamate clearly was bound to the enzyme, since it was inhibitory. In fact, the authors suggest that the inhibiting action of the pentaglutamate [reported to be the predominant form of folate compounds in rat liver (127-129)] may be important in the regulation of the synthesis of the pteroylpolyglutamates in rat liver. In the rat liver system the evidence is consistent with the existence of a single enzyme that catalyzes the stepwise addition of glutamate residues up to the pentaglutamate, and in this respect this system resembles the hamster system.

The recent work of Shane et al. (130) and Shane (131,132) provides new and important information about the enzymatic synthesis of pteroylpolyglutamates in microorganisms. Shane (131) has purified (to about 95% purity) a pteroylpolyglutamate synthetase from *Corynebacterium sp.* and reported on many of its characteristics. Absolute specificity for L-glutamate was observed, but the enzyme was able to use a broad range of folate compounds as substrate; H_4PteGlu and 5,10-methylene-H_4PteGlu were most effective. Somewhat surprisingly, H_2PteGlu, H_2pteroate, and H_4pteroate were reasonably good substrates and the authors suggest that in this organism dihydrofolate synthetase and polyglutamate synthetase activities are associated with the same protein. 5-Methyl-H_4PteGlu was a poor substrate and activity with PteGlu was vanishingly small. The evidence indicated that only one glutamate was added in all cases, even with relatively low concentrations of folate substrate, except with H_4pteroate and 5,10-methylene-H_4PteGlu. With H_4pteroate as substrate, a mixture of H_4PteGlu and H_4PteGlu$_2$ was detected; very small quantities of the triglutamate, as well as large amounts of the diglutamate, were produced from 5,10-methylene-H_4PteGlu. In the presence of either 5,10-methylene-

$H_4PteGlu_2$ or 10-formyl-$H_4PteGlu_2$ small quantities of the corresponding triglutamates were detected as products. These results led the author to conclude tentatively that, as in the mammalian systems, in *Corynebacterium sp.* a single enzyme is reponsible for the stepwise addition of glutamate residues to account for the conversion of H_4pteroate (or H_2pteroate) to $H_4PteGlu_3$. The *Corynebacterium* enzyme resembles the enzyme from *E. coli*, studied by Masurekar and Brown (111), in that K^+ is required and the major activity is for the addition of a single glutamate to the folate substrate. It should be noted that the possiblity that the *E. coli* enzyme can use a pteroyldiglutamate cannot be discounted, since the enzyme was not tested with such a substrate. One difference between the two bacterial enzymes is that the *Corynebacterium* enzyme is active with a broader range of compounds than is the *E. coli* enzyme; however, the two are similar in that both use $H_4PteGlu$, 10-formyl-$H_4PteGlu$, and 5,10-methylene-$H_4PteGlu$ quite effectively. The two bacterial enzymes appear to have more characteristics in common than either has in common with the mammalian (rat liver and hamster) enzymes that have been studied extensively.

The preliminary reports on the presence of two enzymes in *Neurospora crassa* (133), one for the synthesis of $H_4PteGlu_2$ and a second for the conversion of this diglutamate to longer chain lengths, and the apparent inability of the *E. coli* enzyme to catalyze the synthesis of anything other than the diglutamate has suggested that prokaryotes and lower eukaryotes require at least two enzymes for the formation of pteroylpolyglutamates, whereas the evidence seems clear that in mammalian systems only a single enzyme is necessary. However, Shane (131) has suggested, on the basis of the work with the *Corynebacterium* system, that this hypothesis is probably not correct and that the microbial systems probably also accomplish the synthesis of the polyglutamates with a single enzyme. Further work with microbial systems is necessary to clarify this point.

IV. Thiamine

Thiamine, also known as vitamin B_1 and aneurin, contains pyrimidine and thiazole moieties that are formed by independent biosynthetic pathways and then used for the biosynthesis of the vitamin and its coenzyme form, thiamine pyrophosphate. Since the enzymatic steps involved in the conversion of the pyrimidine and thiazole moieties (hereafter referred to simply as "pyrimidine" and "thiazole") to thiamine and thiamine pyro-

phosphate were elucidated some time ago and this subject was discussed completely in previous reviews on thiamine in 1971 (134) and 1975 (135), this chapter concentrates only on the newer information, largely derived from the incorporation of isotopically labeled compounds, on the biogenesis of pyrimidine and thiazole.

A. BIOGENESIS OF THE PYRIMIDINE MOIETY

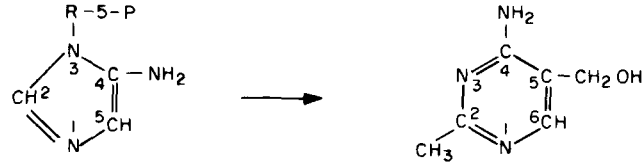
Although the biosynthetic pathway for the formation of pyrimidine has not yet been completely elucidated, enough is known to eliminate the possibility that this pyrimidine is made by the same pathway by which pyrimidines found in nucleic acids are made. Goldstein and Brown (136) found that neither pyrimidines (uracil and orotic acid) nor pyrimidine precursors (aspartic acid and CO_2) are incorporated into the pyrimidine of thiamine, and other work discussed later, has indicated that the two pathways are dissimilar.

One of the precursors of pyrimidine has been established as formate by work from several laboratories showing that exogenous formate is incorporated into pyrimidine by growing cells of bacteria with very little dilution in specific radioactivity (136-138). Formate is known to be incorporated exclusively into C-2 of the pyrimidine ring by *E. coli* (139) and by *S. typhimurium* (138). Other compounds reported to be efficiently incorporated into pyrimidine are glycine and acetate (136). Tomlinson (140) has concluded from experiments with *B. subtilis* that C-2 of acetate is the precursor of the methyl group on the 2-position of the pyrimidine ring. However, some doubt has arisen about the significance of the incorporation of acetate from the findings of Kumaoka and Brown that administration to *E. coli* of nonradioactive formate significantly diminished the incorporation of radioactive acetate (139).

Some of the most important information on the biogenesis of pyrimidine was provided by Newell and Tucker (141-143), who demonstrated a close biosynthetic relationship between purines and the pyrimidine of thiamine. The isolation of mutants of *S. typhimurium* that require both a purine and the pyrimidine of thiamine as a result of a single mutation (144) suggested that a portion of the biosynthetic pathway for purines also functions in the biosynthesis of pyrimidine, a hypothesis consistent with the observed labeling by formate and glycine. Newell and Tucker showed that 4-aminoimidazole ribonucleotide is a common intermediate in the biosynthesis of purines and the pyrimidine of thiamine with the demon-

stration that this substance can satisfy simultaneously the requirement for purine and the pyrimidine of thiamine in a mutant of *S. typhimurium* selected for permeability to aminoimidazole ribonucleoside. Furthermore, they showed that radioactive aminoimidazole ribonucleoside was converted by this mutant to pyrimidine without significant dilution of radioactivity and that although methionine is required for the conversion of aminoimidazole ribonucleoside to pyrimidine no carbons from methionine are incorporated. Finally, they established that both carbons of glycine are incorporatted into the pyrimidine. The observation that no carbon from methionine (including the methyl group) is incorporated (142) is consistent with the earlier observation of Goldstein and Brown (136) that methionine is not incorporated. The function of methionine in this process remains unexplained.

Although the reactions whereby aminoimidazole ribonucleotide (or the nucleoside) might be converted to pyrimidine remain unknown, some recent results provide information about how this may happen. Estramareix and Lesieur (138,145) found that C-1 and C-2 of glycine become C-4 and C-6, respectively, of pyrimidine (see Fig. 4 for numbering system), and White and Rudolph.* (146) showed that the nitrogen of glycine becomes nitrogen



Aminoimidazole Ribonucleotide

Figure 4. The biogenesis of the pyrimidine portion of thiamine from the precursor 4-aminoimidazole ribonucleotide.

*The analytical techniques used by these workers in the course of their study of the biogenesis of thiamine (146-149) are especially noteworthy. They have developed methods of quantitatively converting cellular thiamine, without the addition of carrier amounts of the vitamin, to volatile derivatives of the pyrimidine and thiazole moieties that can then be purified and analyzed by the sequential method of gas chromatography-mass spectroscopy. By the application of this technique it is possible to analyze the incorporation of ^{13}C, ^{2}H, and ^{15}N from various suspected precursors of the vitamin into many of the individual positions of the thiazole and pyrimidine rings simultaneously.

1 of pyrimidine. These results suggest that the imidazole ring is opened between C-4 and C-5. This reaction is then followed by (*a*) the insertion of a two-carbon compound to form C-5 of the pyrimidine ring and the hydroxymethyl group attached to the ring at that position, (*b*) the removal of the ribose phosphate unit, and (*c*) the addition of a methyl group (see Fig. 4). The source of the methyl group is particularly puzzling, since it is not derived from methionine (136,142) or from the one carbon (formate) pool (139). The source of C-5 and its substituent hydroxymethyl group is also unclear.

There are some observations that suggest that yeast and bacteria use different pathways for the production of pyrimidine. David et al. (150) reported that C-4 [not C-2 as in bacteria (138,139)] is derived from formate. In addition, there is some evidence to suggest that glycine is not a precursor of the pyrimidine in yeast. Linnett and Walker (151) found no significant incorporation of [^{15}N]glycine, and more recently, White and Spenser (152) could detect no incorporation of [2-^{14}C]glycine in two strains of *S. cerevisiae*. Although it is clear that in yeast the pathway of biosynthesis of the pyrimidine of thiamine is different from that of the pyrimidines of nucleic acids (153), more work is required to clarify the biogenesis of the pyrimidine in these organisms.

Finally, Diorio and Lewin (154,155) have reported that certain thiamine-requiring *Neurospora* mutants produce the 5-aminomethyl and 5-formyl derivatives of pyrimidine and have suggested that these compounds may be intermediates in the biosynthesis of the 5-hydroxymethylpyrimidine compound, the substrate for the enzymatic synthesis of thiamine. A previous report by Camiener and Brown (156) that the aminomethylpyrimidine can be converted to the hydroxymethylpyrimidine by cellfree extracts of yeast provides some support for this suggestion. More information is needed about the identity of the ultimate precursor of C-5 and the hydroxymethyl group to evaluate this possibility.

B. BIOGENESIS OF THE THIAZOLE MOIETY

Some progress has been made in recent years toward the identification of precursors of the thiazole moiety. However, because investigations in this area have thus far been limited to radioactive and stable isotope incorporation experiments (with their concomitant difficulties in interpretation), only tentative conclusions can be drawn about the pathway. Much recent experimental effort has been focused on the origin of C-2 of thiazole (for numbering system, see Fig. 5). Early investigations with both

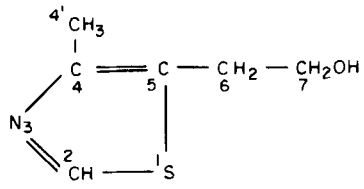


Figure 5. 4-Methyl-5-(β-hydroxyethyl) thiazole, the "thiazole" moiety of thiamine.

yeast and bacteria (153,157-159) implicated methionine as the source of both C-2 and the sulfur atom of the ring, in agreement with a speculative scheme for thiazole biosynthesis suggested by Harrington and Moggridge (160). However, the significance of these observations is questionable, since the levels of incorporation of [^{14}C] methionine were quite low and, indeed, other investigators have found that [^{14}C] methionine is not significantly incorporated into thiazole (151,152,161-164). The previous view (165) that cysteine might provide both sulfur and carbons for thiazole synthesis now appears not to be very likely on the basis of more recent work from a number of laboratories indicating that carbons of cysteine are not incorporated into thiazole (162-166).

Iwashima and Nose (167) obtained the first evidence suggesting that either phenylalanine or tyrosine might be a precursor of thiazole. They noted that the addition of phenylalanine to the growth medium caused repression of the synthesis of thiamine by a strain of *E. coli* and that this effect was not relieved by the pyrimidine portion of thiamine, but was reversed by the presence of any one of the following: tyrosine, shikimic acid, tryptophan, and thiazole. One interpretation of this observation is that phenylalanine represses the synthesis of tyrosine, which could be a precursor of thiazole. Subsequent evidence from several laboratories has supported this interpretation. Estramareix and Therisod (168) found that a tyrosine auxotroph of *E. coli* incorporated ^{14}C from either [U-^{14}C]-tyrosine or [2-^{14}C] tyrosine into C-2 of thiazole. Bellion et al. (163) observed similar results in *S. typhimurium* and White and Rudolph (147) have shown that *E. coli* B cells incorporate [^{15}N] tyrosine into thiazole. In the latter work, since the observed dilution of the ^{15}N-label (2.5-fold) in thiazole was found to be the same as the dilution of ^{15}N found in the tyrosine of the proteins, the incorporation was concluded to be consistent with the direct utilization of tyrosine for the formation of thiazole. In further studies, White (148) has identified 4-hydroxybenzyl alcohol as a metabolite of tyrosine in *E. coli* grown in the absence of thiamine or thia-

zole. The amount of 4-hydroxybenzyl alcohol produced was equivalent to the normal cellular content of thiamine. Growth of the cells in the presence of increasing amounts of thiamine led to a corresponding decrease in the amount of 4-hydroxybenzyl alcohol produced. These results are consistent with a role for tyrosine in the biogenesis of thiazole. Another experiment done in the course of this work also suggests the chemical nature of the cleavage reaction tyrosine must undergo in its conversion to thiazole. Incubation of the cells with [3,3-^{2}H] tyrosine led to the formation of 4-hydroxybenzyl alcohol that retained both deuterium atoms, a result that implies the cleavage of the bond between C-2 and C-3 of tyrosine is not of the reverse-aldol type, facilitated by prior hydroxylation at C-3 of tyrosine, but rather probably occurs with the elimination of an unstable quinone methide that then reacts with water to yield the observed product.

Estramareix and Therisod (169) have recently isolated from incubation mixtures of cells of *E. coli*, derepressed for thiamine biosynthesis, a new compound capable of supporting the growth of a thiazole-requiring strain of *E. coli*, which they have identified as 5(β-hydroxyethyl)-4-methylthiazole-2-carboxylic acid (or thiazole-2-carboxylate). The evidence that this compound is related to thiazole biosynthesis is: (*a*) it was excreted only by wild-type cells derepressed for thiamine biosynthesis; (*b*) a tyrosine auxotroph of *E. coli* synthesized the compound only in the presence of exogenous tyrosine and under conditions of derepression; and (*c*) incubation of cells of this auxotroph in the absence of tyrosine but in the presence of thiazole gave thiamine but not thiazole carboxylate. In addition, it was shown that thiazole carboxylate contains both C-1 and C-2 of tyrosine, but not C-3. However, these workers suggest that this compound is probably not an intermediate in thiazole production, since neither it nor a phosphorylated derivative is readily decarboxylated in the presence of cellfree extracts of strains prototrophic for thiamine. They believe it could be a closely related derivative of the biosynthetic intermediate. In this connection, they have prepared and tested a number of dihydro derivatives of thiazole carboxylate, but none of these was readily converted to thiazole. If, as seems likely from the results reported, this compound is derived from a precursor of thiazole, then the fact that it still contains C-1 of tyrosine suggests that elimination of 4-hydroxybenzyl alcohol occurs prior to loss of C-1 as CO_2. Therisod et al. (170) have identified another compound, a 6-hydroxy derivative of thiazole, that is excreted by *E. coli* cells that have been derepressed for thiamine bio-

synthesis and is also thought to be connected with thiazole production. Roles for these two substances (or closely related compounds) as intermediates in thiazole formation are not yet established and more work is required before definitive statements can be made.

Linnett and Walker have shown that in yeast [^{14}C] glycine is efficiently incorporated into thiazole (151) and (*a*) incubation of the cells with [^{15}N] glycine leads to significant incorporation of ^{15}N into thiazole, and (*b*) radioactivity from [2-^{14}C] glycine is specifically incorporated into C-2 of thiazole (160). These results are in conflict with the results of Johnson et al. (153), who reported that [methyl-^{14}C, ^{35}S] methionine is incorporated by yeast into thiazole, with retention of the ^{14}C/^{35}S ratio, but with significant dilution in specific radioactivity of the substrate. White and Spenser (152) have recently repeated these incorporation experiments with the same strain of *S. cerevisiae* used by Johnson et al. (153) and found that apparent incorporation of [methyl-^{14}C] methionine could be accounted for as a contaminant in the isolated thiazole, whereas, the incorporation of [2-^{14}C] glycine into C-2 of thiazole was real. Interestingly, no incorporation into thiamine was observed when the yeast was grown in the presence of [2-^{14}C] tyrosine. These observations seem to indicate that yeast and bacteria use different amino acids as precursors of the C-2-nitrogen portion of the thiazole ring, but White and Spenser (171) have pointed out that the observations do not necessarily imply that the pathways in bacteria and yeast are fundamentally different. The same carbon-nitrogen unit could be visualized as being transferred from both tyrosine and glycine to form thiazole. In the case of glycine only decarboxylation is required to yield this unit; for tyrosine, an additional step is required to eliminate the aromatic ring as 4-hydroxybenzyl alcohol. If the elimination of the side chain occurs before decarboxylation, as suggested by recent data (170), tyrosine would thereby be converted to glycine. To account for the observation that, with bacteria, tyrosine is incorporated into thiazole without significant dilution (147,168), it must be assumed that the putative glycine derived from tyrosine remains sequestered until reaction with another of the precursors of thiazole. That glycine might be involved in thiazole biosynthesis in bacteria was suggested earlier by the observations of Iwashima and Nose (172,173) that in a temperature-sensitive mutant of *E. coli*, which at the nonpermissive temperature requires thiazole for growth on glucose but not on glycerol, glycine can partially replace the requirement for thiazole under the nonpermissive conditions (172). In

addition, they showed that incubation of cells of this mutant in the presence of [2-^{14}C] glycine led to incorporation of glycine into both the pyrimidine and thiazole portions of thiamine (173). The ability of tyrosine to serve as a precursor of thiazole in this mutant was not tested.

Information about the precursors of the five-carbon chain C-4′, C-4, C-5, C-6, C-7) of thiazole in *E. coli* has been obtained by White (149), who presented evidence from the incorporation of deuterated carbohydrates and related compounds that a two-carbon unit, comprising C-4′ and C-4 of thiazole, is derived from pyruvate and that the remaining three-carbon unit (C-5, C-6, and C-7) comes from a three-carbon sugar, possibly glyceraldehylde-3-phosphate. White postulates that pyruvate and glyceraldehyde-3-phosphate are first condensed in an acyloin-type reaction to yield a five-carbon sugar that might then react with tyrosine and a sulfur compound in an undefined series of steps to yield thiazole. White and Spenser (171) have investigated this problem in yeast by growing *S. cerevisiae* in the presence of tracer amounts of ^{14}C-labeled hexoses, followed by isolation and chemical degradation of the thiamine produced. Although their experiments were not as complete as those of White (149) and no data were presented on the efficiency of incorporation of radioactivity from [^{14}C] glucose into thiazole, the fact that radioactivity from [2-^{14}C] glucose was incorporated equally into C-4′ and C-4 of thiazole suggests that, in yeast, pyruvate may not be a direct precursor of thiazole. More work needs to be done both in yeast and bacteria before a pathway can be proposed.

The precursor of the sulfur atom of the thiazole ring is still not known with certainty. Early experiments of Hitchcock and Walker (157) with yeast implicated methionine as the most likely source of the sulfur atom. In apparent agreement with these results, Johnson et al. (153) reported (also with yeast) that [^{35}S] methionine was incorporated more efficiently into thiazole than was [^{35}S] cysteine. White and Spenser (152), however, have suggested that the incorporation of methionine noted by Johnson et al. (153) resulted from the presence of a radioactive impurity in their preparations of isolated thiamine. There is evidence indicating that in bacteria cysteine can be used more effectively than methionine as the source of the sulfur atom. Estramareix et al. (164) found with a methionine auxotroph of *E. coli* that the incorporation of [^{35}S] sulfate into thiamine was lowered by the presence of cysteine or glutathione, but was unaffected by the presence of methionine or homocysteine, and Bellion

and Kirkley (174) reported that in *S. typhimurium* the incorporation of [^{35}S]cysteine was not diminished by the presence of nonradioactive methionine, homocysteine, or glutathione. Thus the experimental observations suggest that either cysteine or H_2S (which could be produced from cysteine) is the most likely precursor of the sulfur atom of thiazole.

V. Pantothenic Acid

Although pantothenic acid cannot be synthesized by animals, most microorganisms are able to make it from the precursors, pantoic acid and β-alanine. Animals and microbes can convert pantothenic acid to coenzyme A, the metabolically active form of the vitamin. The pathway for this enzyme conversion was elucidated some time ago and has been reviewed several times (1,175,176). Since most of the recent information on coenzyme A biosynthesis is concerned with the formation of the precursors of pantothenic acid, this chapter concentrates on the enzymatic synthesis of these precursors, pantoic acid and β-alanine.

A. PANTOIC ACID

The set of enzymatic reactions for the biosynthesis of pantoic acid is shown in Figure 6. That 2-keto-4-hydroxy-3,3-dimethyl butyric acid (ketopantoate) is a precursor was first suggested by the observation (177) that it could be reduced to pantoate by yeast. Confirmatory evidence was supplied by Lansford and Shive (178), who described a class of pantoate-requiring mutants of *E. coli* that can utilize ketopantoate in place of pantoate. The enzyme that catalyzes the reduction of ketopantoate to pantoate has been found in *Saccharomyces cerevisiae* and *E. coli* (179); it has been purified from *S. cerevisiae* (180) and some of its properties have been described (180-183). Enzymes specific for the reduction of

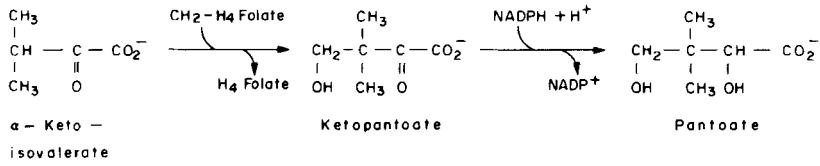


Figure 6. The enzymatic synthesis of pantoic acid. CH_2-H_4folate is an abbreviation for 5,10-methylenetetrahydrofolic acid (active formaldehyde).

ketopantoyllactone to pantoyllactone also occur in yeast and bacteria (179-183), but it has been suggested that these enzymes are not involved in the pantothenate pathway, but rather are concerned with the formation specifically of pantoyllactone, a compound that may be important in cytokinesis (181).

That the precursor of ketopantoic acid is α-ketoisovaleric acid was first suggested by Maas and Vogel (184), who found that whole cells of a strain of *E. coli* can synthesize ketopantoate and pantoate from α-ketoisovaleric acid, whereas a particular pantoate-requiring mutant cannot. The observation that *Bacterium linens* requires either pantoic acid or *p*-aminobenzoic acid as a growth factor (185,186) suggested that tetrahydrofolic acid, the metabolically active form of *p*-aminobenzoate, might be involved in the conversion of α-ketoisovalerate to pantoate. The report by McIntosh et al. (187) that α-ketoisovaleric acid can be converted to ketopantoate in the presence of cellfree extracts of *E. coli* only if formaldehyde is also supplied as substrate seemed to support the view that tetrahydrofolate participates in the overall reaction. However, when the enzyme that catalyzes the condensation of formaldehyde and α-ketoisovalerate was partially purified from extracts of *E. coli*, no role for tetrahydrofolate was observed (187). Since the K_m values for formaldehyde (0.01 *M*) and α-ketoisovalerate (0.1 *M*) are so high, the physiological significance of the reaction seemed questionable. Snell and coworkers (123,188) reinvestigated ketopantoate synthesis and found two different enzymes that carry out the synthesis of ketopantoic acid in *E. coli*. One is similar to that studied by McIntosh et al. (187) and the second, ketopantoate hydroxymethyltransferase, catalyzes the tetrahydrofolate-dependent formation of ketopantoate. Since Teller et al. (188) found that (*a*) the transferase is absent from a ketopantoate auxotroph (*E. coli* 99-4), whereas the tetrahydrofolate-independent activity is found in the same amounts in the auxotroph as in wild-type *E. coli*, and (*b*) the Michaelis constants of the transferase for its substrates are all within the physiological range, the conclusion was drawn that the tetrahydrofolate-dependent enzyme is the one involved in the biosynthesis of pantothenate. The function of the tetrahydrofolate-independent enzyme is the one involved in the biosynthesis of pantothenate. The function of the tetrahydrofolate-independent enzyme remains unknown.

Powers and Snell (123) have purified the ketopantoate hydroxymethyltransferase to homogeneity from extracts of *E. coli* and have shown: (*a*)

that it requires Mg^{2+} for activity; (*b*) that its properties indicate it is a class II aldolase; and (*c*) that polyglutamate forms of tetrahydrofolate are used more effectively as a coenzyme than is tetrahydrofolate. The additional observation that pantoate, pantothenate, and coenzyme A are all allosteric inhibitors of transferase activity suggests that feedback inhibition is an important mode of regulation for this pathway. Regulation by repression of enzyme synthesis appears not to occur (123,188).

Aberhart (189) has recently obtained data on the stereochemistry of the *in vivo* addition of the hydroxylmethyl group to α-ketoisovalerate. Cells of a valine-isoleucine auxotroph of *E. coli* were incubated in the presence of [4-^{13}C]-(2*RS*,3*S*)valine and β-alanine. The pantoate synthesized by the cells during the incubation was isolated as pantoyllactone and analyzed by ^{13}C-NMR. The spectrum of the product was consistent with inversion of configuration at the carbon of the chirally-labeled α-ketoisovalerate (derived from the added valine by transamination) during its conversion to ketopantoic acid. These results have not yet been confirmed with the use of the purified transferase.

B. β-ALANINE

β-Alanine is known to be produced (*a*) as a degradation product of uracil in animals (190-192), plants (193), and some microorganisms (194) and (*b*) by transamination of malonylsemialdehyde (produced from propionic acid) (197-201). However, β-alanine used for the biosynthesis of pantothenic acid is now known to be made by α-decarboxylation of asparatic acid. Indirect evidence that aspartate is the precursor was supplied by the early findings that aspartic acid, β-alanine, or pantothenic acid can reverse the growth inhibitory action of cysteic acid and hydroxyaspartic acid (202,203) and D-serine (200,204,205) in bacteria. Other relevant early evidence is that whole cells of a variety of microorganisms are known to be able to convert aspartate to β-alanine (206-210). That aspartate can be converted enzymatically to β-alanine has been demonstrated by Williamson and Brown (211), who purified to apparent homogeneity from extracts of *E. coli* an enzyme that catalyzes the α-decarboxylation of L-aspartic acid to yield CO_2 and β-alanine. The most compelling evidence that this decarboxylase is the enzyme responsible for the formation of β-alanine needed for the biosynthesis of pantothenic acid is that it is missing in a mutant strain of *E. coli* (M99-2) that requires either β-alanine or pantothenate as a nutritional factor, but is present in the wild-type strain and in a

revertant strain of the mutant (211). Other evidence in support of this conclusion is that the action of the decarboxylase is strongly inhibited by D-serine, β-hydroxyaspartic acid, and L-cysteic acid, three compounds that are known to interfere with the synthesis of pantothenic acid in bacteria (200,202-204). Cronan (212) independently discovered this enzyme in *E. coli* and, with the use of mutants and a partially purified preparation of the enzyme, showed that decarboxylase activity is associated with the genetic locus *pan D* and that this gene is closely linked with *pan B* (coding for ketopantoate hydroxymethyltransferase) and *pan C* (coding for pantothenate synthetase).

One of the most interesting aspects of the structure of the aspartate-α-decarboxylase is that it contains a pyruvoyl residue as a prosthetic group (211) rather than pyridoxal phosphate, the coenzyme present in most other bacterial amino acid decarboxylases. The presence of a covalently bound pyruvate residue implies that some post-translational processing of the protein must occur. Two possibilities for this processing can be imagined. Either the conversion of proenzyme to active enzyme could be catalyzed by a separate "activating" enzyme or, alternatively, the conversion might be affected by the proenzyme itself in an autocatalytic process. Snell and coworkers have shown that the second type of processing mechanism is responsible for production of the pyruvoyl residue of histidine decarboxylase of *Lactobacillus* 30a (213). Further work, involving biochemical and possibly genetic analyses, is necessary to elucidate the mechanism of formation of the pyruvoyl group of the aspartate decarboxylase.

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