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Biosynthetic Precursors of Deazaflavins†

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The incorporation of ^{13}C- and ^{14}C-labeled precursors into 5-deaza-7,8-didemethyl-8-hydroxyriboflavin (factor F_0) was studied with growing cells of *Methanobacterium thermoautotrophicum*. 5-Amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione was incorporated into the deazaflavin and into riboflavin without dilution. Tyrosine and 4-hydroxyphenylpyruvate were incorporated into the deazaflavin and into cellular protein. 4-Hydroxybenzaldehyde was not incorporated. A reaction mechanism is proposed for the formation of the deazaflavin chromophore from 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione and tyrosine or 4-hydroxyphenylpyruvate.

The green fluorescent coenzyme F_{420} (Fig. 1, compound 1) was discovered relatively recently in methanogenic bacteria by Wolfe and his coworkers (4). Its chromophore was identified by Eirich et al. (10) as 5-deaza-7,8-didemethyl-8-hydroxyriboflavin (compound 2, factor F_0). In methanogenic bacteria, coenzyme F_{420} serves as a redox cofactor in two-electron transfer reactions (14, 32). More recently, deazaflavins were also found in halobacteria (20) and in streptomycetes (7, 12, 21, 28). In *Streptomyces aureofaciens*, a deazaflavin coenzyme serves as a cofactor for the biosynthesis of tetracycline (21). A DNA photolyase with a deazaflavin chromophore in *Streptomyces griseus* has been described (12, 28).

Isotope incorporation studies with a variety of ^{13}C- or ^{14}C-labeled precursors such as guanine, adenosine, glycine, acetate, and pyruvate showed that the pyrimidine ring and the ribityl side chain of compound 2 are derived from a nucleotide precursor (11, 16, 25, 26). Feeding experiments with ^{13}C-labeled acetate and pyruvate showed identical labeling patterns in the benzenoid rings of the deazaflavin and tyrosine (11). Moreover, the labeling of C-5 of the deazaflavin chromophore followed the labeling of the β-side-chain carbon in tyrosine. These experiments suggested that a derivative of prephenate (such as 4-hydroxyphenylpyruvate or tyrosine) is a committed precursor of compound 2. On the other hand, 4-hydroxybenzoate was ruled out, since it should lead to a different labeling pattern at C-5 of compound 2.

On the basis of these data, Eisenreich et al. (11) proposed that the initial purine precursor is converted to 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione and that this intermediate is condensed with a prephenate derivative, yielding the deazaflavin system. In this paper we provide more direct evidence for this proposed sequence of events.

MATERIALS AND METHODS

Microorganism. *Methanobacterium thermoautotrophicum* Marburg (DSM 2133) was obtained from the Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany.

Culture medium. *M. thermoautotrophicum* was grown in a mineral salt medium as described previously (11).

Chemicals. The following reagents were purchased from the following suppliers: [U-^{14}C]4-hydroxybenzaldehyde (12.4 mCi/mmol) from Sigma, Taufkirchen, Germany; [U-^{14}C]tyrosine (486 mCi/mmol) and [1-^{14}C]ribose (60 mCi/mmol) from Amersham Buchler, Braunschweig, Germany; [1-^{13}C]ribose (99% enrichment) from Omicron Inc., South Bend, Ind.; and [6,8-$^{13}C_2$]tyrosine (99% enrichment) from Cambridge Isotope Laboratories, Woburn, Mass.

Preparation of [1-^{14}C]ribitylamine. [1-^{14}C]ribose (27.5 g, 0.18 mol, 0.110 μCi/mmol) was converted to [1-^{14}C]ribose oxime by previously published procedures (24). The yield was 26 g (0.16 mol, 0.105 μCi/mmol, 88.8%). The compound was converted to ribitylamine by hydrogenation over platinum (23, 24), which was obtained as an aqueous solution (0.11 mol, 68.8%).

Preparation of [1-^{13}C]ribitylamine. [1-^{13}C]ribitylamine was prepared from [1-^{13}C]ribose (2 g, 13.3 mmol) as described above. The yield was 10 mmol (87%).

Preparation of [1′-^{14}C]5-nitro-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione. A solution of 5-nitro-6-chloro-2,4(1H,3H)-pyrimidinedione (21 g, 0.11 mol) (5, 6) in 200 ml of ethanol was added to a solution of 17 g of [1-^{14}C]ribitylamine (0.11 mol) (24) in 100 ml of water. The pH was adjusted to 8 by adding 1 M NaOH. The mixture was heated at 60°C for 1 h and kept at room temperature for 15 h. The pH was adjusted to 10.7 by adding 25% NH_4OH. The precipitate was removed by filtration. The solution was evaporated to dryness. The residue was dissolved in 100 ml of water and placed on a column of Dowex 1X8 (100-200 mesh, formate form, 3 by 50 cm). The column was developed with (i) 1 liter of water, (ii) 1 liter of 0.01 M formic acid, and (iii) 2 liters of 0.1 M formic acid. 5-Nitro-6-ribitylamino-2,4(1H,3H)-pyrimidinedione
was monitored by reversed-phase high-pressure liquid chromatography (HPLC) with a column of Nucleosil 10 C_{18} (4.55 by 250 mm) and an eluent containing 10 mM ammonium formate and 10 mM formic acid. The effluent was monitored photometrically (254 nm). Fractions containing 5-nitro-6-ribitylamino-2,4(1H,3H)-pyrimidinedione were pooled and concentrated to a small volume. The retention volume was 8 ml. The yield was 28.8 g (0.1 mol, 87.8 μCi/mol, 91%).

Preparation of [1′-^{13}C]5-nitro-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione. [1′-^{13}C]5-nitro-6-ribitylamino-2,4(1H,3H)-

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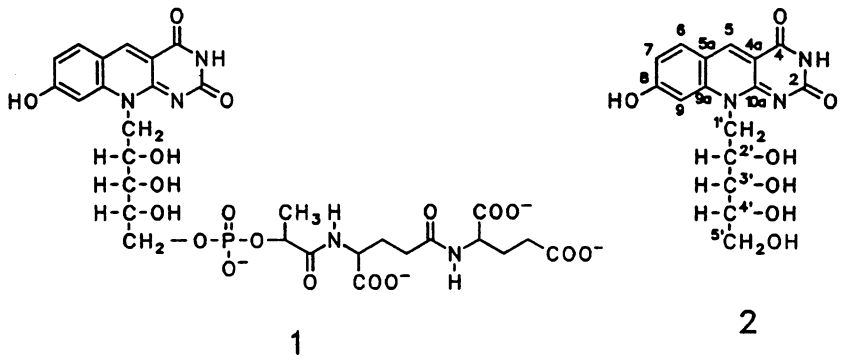
† Dedicated to Professor Helmut Simon on the occasion of his 65th birthday.



1 2

FIG. 1. Structures of coenzyme F_{420} (compound 1) and factor F_0 (compound 2).

pyrimidinedione was prepared from 10 mmol of [1-^{13}C]ribitylamine as described above. The yield was 1.03 g (3.37 mmol).

Preparation of [1′-^{14}C]5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione. An aqueous suspension of [1′-^{14}C]5-nitro-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (28.8 g, 0.1 mol) in 400 ml of water was hydrogenated over palladium on charcoal (50 mg) at room temperature and at atmospheric pressure. The reaction was complete after 15 h. The solution was passed through a nitrocellulose filter (0.45-μm pore size) and was used immediately.

Preparation of [1′-^{13}C]5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione. [1′-^{13}C]5-nitro-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (0.46 g, 1.5 mmol) was hydrogenated as described above. The aqueous solution was used immediately.

Purification of tyrosine transaminase. Tyrosine transaminase (EC 2.6.1.5) was partially purified from *Saccharomyces cerevisiae* S288C grown in medium containing glucose (1%), malt extract (0.3%), yeast extract (0.3%), and peptone (0.5%) (8, 27). Cells were passed through a French press at 63 MPa. The suspension was centrifuged, and the supernatant was dialyzed against 67 mM sodium phosphate (pH 7.2). Ammonium sulfate was added to 65% saturation, and the precipitate was discarded. The ammonium sulfate concentration was increased to 80%, and the precipitate was harvested by centrifugation and dissolved in water. The solution was dialyzed against 10 mM phosphate (pH 7.2) and then applied to a column of hydroxyapatite (2 by 2 cm, Bio-Gel HTP; Bio-Rad). The column was developed with a gradient of 10 to 500 mM phosphate buffer (pH 7.2). The specific activity of the partially purified enzyme was 2.7 μmol min^{-1} mg^{-1}.

Preparation of [U-^{14}C]4-hydroxyphenylpyruvate. [U-^{14}C]4-hydroxyphenylpyruvate was prepared from [U-^{14}C]tyrosine by treatment with tyrosine transaminase from *S. cerevisiae*. The reaction mixture contained 4 nmol of pyridoxal phosphate, 1 μmol of α-ketoglutarate, 10 μl of tyrosine transaminase solution, 100 nmol of glutathione, 2 U of glutamate decarboxylase type V from *Escherichia coli* (Sigma), and 530 nmol of [U-^{14}C]tyrosine in 300 μl of 67 mM potassium phosphate (pH 6.9). The mixture was incubated for 17 h at 37°C. The reaction was terminated by adding trichloroacetic acid and then heating at 100°C for 2 min. The mixture was applied to a reversed-phase HPLC column (Nucleosil 10 C_{18}, 4.5 by 250 mm). The eluent contained 30 mM formic acid. The effluent was monitored photometrically (280 nm). The retention volume of 4-hydroxyphenylpyruvate was 32 ml. Fractions were concentrated by evaporation under reduced pressure. The yield was 45%.

Bacterial culture. *M. thermoautotrophicum* was grown at 65°C as described previously (11). The fermenter was gassed with a mixture of 80% H_2 and 20% CO_2 at a rate of 250 ml/min per liter of culture medium. The pH was kept at 6.8 by adding 20% Na_2CO_3. ^{13}C- or ^{14}C-labeled compounds were added to the culture when the cell optical density at 660 nm had reached a value of 0.3 (1-cm light path). The fermenter was kept in the dark to avoid photodecomposition of coenzymes. Fermentation was continued until cells reached the stationary phase. The cells were harvested aerobically by centrifugation.

Assay of tyrosine aminotransferase. Cells of *M. thermoautotrophicum* were harvested anaerobically at the end of the exponential growth phase. The cell mass (6.5 g) was suspended in 18 ml of 0.1 M potassium phosphate (pH 7.1) containing 0.2 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. The suspension was passed through a French pressure cell at 124 MPa. The suspension was centrifuged at 4°C and at 38,000 × g (Sorvall SS34 rotor). Enzyme assays were performed within 5 to 7 h after cell lysis in an anaerobic chamber containing a mixture of 95% N_2 and 5% H_2. Assay mixtures contained 5 mM tyrosine, 1.33 mM pyridoxal phosphate, 0.33 M glutathione, 3.33 mM α-ketoglutaric acid, 2 U of glutamate decarboxylase type V from *E. coli*, and 100 μl of cell lysate in a total volume of 1 ml. Reaction mixtures were incubated at 37 or 65°C for 1 h. Assay mixtures were analyzed by reversed-phase HPLC with a column of Nucleosil 10 C_{18} (4.5 by 250 mm) and an eluent containing 0.1 M ammonium formate and 30 mM formic acid. The effluent was monitored fluorometrically (excitation wavelength, 290 nm; emission wavelength, 345 nm). The retention volume of 4-hydroxyphenylpyruvate was 14 ml.

Isolation of metabolites. Riboflavin and factor F_0 were isolated and purified to constant specific activity by published procedures (11). Tyrosine was obtained after acid hydrolysis of the cell mass as described previously (11).

NMR spectroscopy. ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at 8.46 T with an AM 360 NMR spectrometer (Bruker, Karlsruhe, Germany). Factor F_0 was measured in deuterated/dimethyl sulfoxide containing 0.5% phosphoric acid. Tyrosine was measured in 0.1 M NaOD. All measurements were performed at 27°C under identical conditions. The following acquisition and processing parameters were used: 64 K data set; pulse width, 30° (2 μs); scan interval, 2.5 s; spectral range, 14.7 kHz; composite pulse ^{1}H-decoupling; line broadening, 1 Hz.

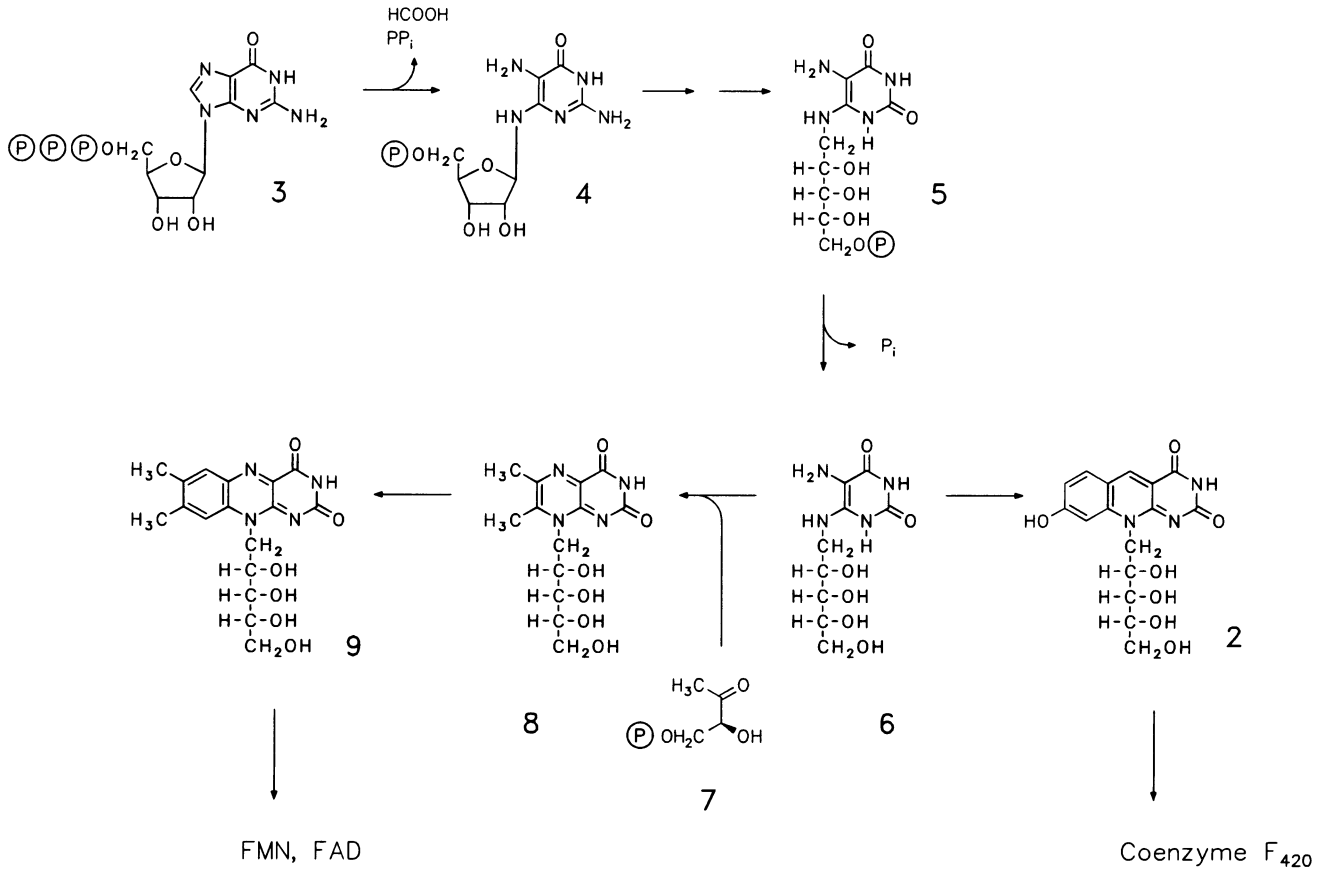


FIG. 2. Biosynthesis of flavins and deazaflavins.

RESULTS

Incorporation of pyrimidine precursor. Naturally occurring flavins and deazaflavins show close structural similarity. More specifically, the structural motifs of the pyrimidine ring and the position 10 side chain are identical. It is also well established that these modules that occur in both types of coenzymes are derived from a purine nucleotide (11, 16, 26). Thus, it appeared plausible that the biosynthesis of flavins and deazaflavins could proceed by a common pathway, and the established riboflavin precursor 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (compound 6) or its 5'-phosphate (compound 5) appeared as a potential branching point (Fig. 2). To test this hypothesis, we added [1'-^{14}C]5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione to a growing culture of *M. thermoautotrophicum* (18 liters) at a concentration of 5.8 mM. During the subsequent growth period, the culture developed an intense green fluorescence. At the end of the logarithmic growth phase, the culture medium contained about 30 mg of factor F_0 (compound 2) per liter as compared with about 3 mg of factor F_0 per liter in standard cultures without the added compound 6.

Riboflavin and factor F_0 were isolated from the culture by chromatographic procedures and purified to constant specific activity by published procedures (11). Radioactivity from the ^{14}C-labeled pyrimidine was incorporated into both riboflavin and deazaflavin without significant dilution (Tables 1 and 2).

As a consequence of the high concentration of compound 6 added to the culture fluid, the specific radioactivity of proffered compound 6 and isolated factor F_0 were low. To obtain additional information, we performed a similar feeding experiment with compound 6 labeled with ^{13}C in the 1' position. Whereas radiolabeling gives only the bulk radioactivity of the isolated metabolite and is very sensitive to minor impurities, ^{13}C NMR can locate the label to an individual carbon atom and is therefore insensitive to minor impurities. Since the incorporation rate of compound 6 was high, as judged on the basis of the ^{14}C experiment, we decided to use the tracer at a low ^{13}C enrichment of 10% in an attempt to optimize the dynamic range of the ^{13}C NMR acquisition. We expected that this would enable us to observe the natural ^{13}C abundance of the unlabeled carbon atoms together with the signal of the enriched carbon atom.

TABLE 1. Incorporation of ^{14}C-labeled precursors by growing cultures of *M. thermoautotrophicum*

Precursor	Sp act (μCi/mmol)			
	Precursor	Riboflavin	Factor F_0	Tyrosine[a]
[1'-^{14}C]compound 6	0.082	0.102	0.061	ND[b]
[U-^{14}C]4-hydroxybenzaldehyde	1,150	ND	0.7	ND
[U-^{14}C]tyrosine	47.8	ND	1.4	1.6
[U-^{14}C]4-hydroxyphenylpyruvate	41.3	ND	3.1	3.3

[a] Isolated from bacterial cell mass after hydrolysis.
[b] ND, not determined.

TABLE 2. Isotope incorporation into metabolites from *M. thermoautotrophicum*

Precursor	Relative sp act[a] (%)		
	Riboflavin	Factor F_0	Tyrosine
[1′-^{14}C]compound 6	124	74	ND[b]
[1′-^{13}C]compound 6	ND	100	ND
[U-^{14}C]tyrosine	ND	2.9	3.4
[6,8-$^{13}C_2$]tyrosine	ND	7.1	8.8
[U-^{14}C]4-hydroxyphenylpyruvate	ND	7.6	7.9
[U-^{14}C]4-hydroxybenzaldehyde	ND	0.06	ND

[a] Relative specific activity = 100 × (specific activity of isolated metabolite/specific activity of proffered precursor).
[b] ND, not determined.

[1′-^{13}C]5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (10% ^{13}C enrichment) was added to a growing culture of *M. thermoautotrophicum* (300 ml) at a concentration of 5.0 mM. At the end of the growth phase, factor F_0 was isolated from the culture medium and analyzed by ^{13}C NMR spectroscopy (Fig. 3). The ^{13}C NMR signals of the deazaflavin compound 2 were assigned previously by two-dimensional NMR methods (11). When the ^{13}C NMR spectrum of the factor F_0 sample from the fermentation with [1′-^{13}C]5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinione was compared with a ^{13}C NMR spectrum of factor F_0 with natural ^{13}C abundance, it was found that the 1′ carbon atom of the fermentation product had a ^{13}C abundance of 10%. Thus, the experiment confirmed that the pyrimidine was incorporated into factor F_0 practically without dilution. It follows that compound 6 is the committed precursor of both riboflavin and the deazaflavin chromophore.

Incorporation of shikimate derivatives. The second question addressed in this study concerned the committed precursor of the benzenoid ring in the deazaflavin chromophore. It was already known that the origins of the benzenoid rings in flavins and deazaflavins are different. The xylene ring of riboflavin is derived from two pentose phosphate moieties via the carbohydrate 3,4-dihydroxy-2-butanone 4-phosphate (compound 7) in fungi, eubacteria, and archaebacteria (11, 19, 29, 30). On the other hand, the origin of the phenolic ring

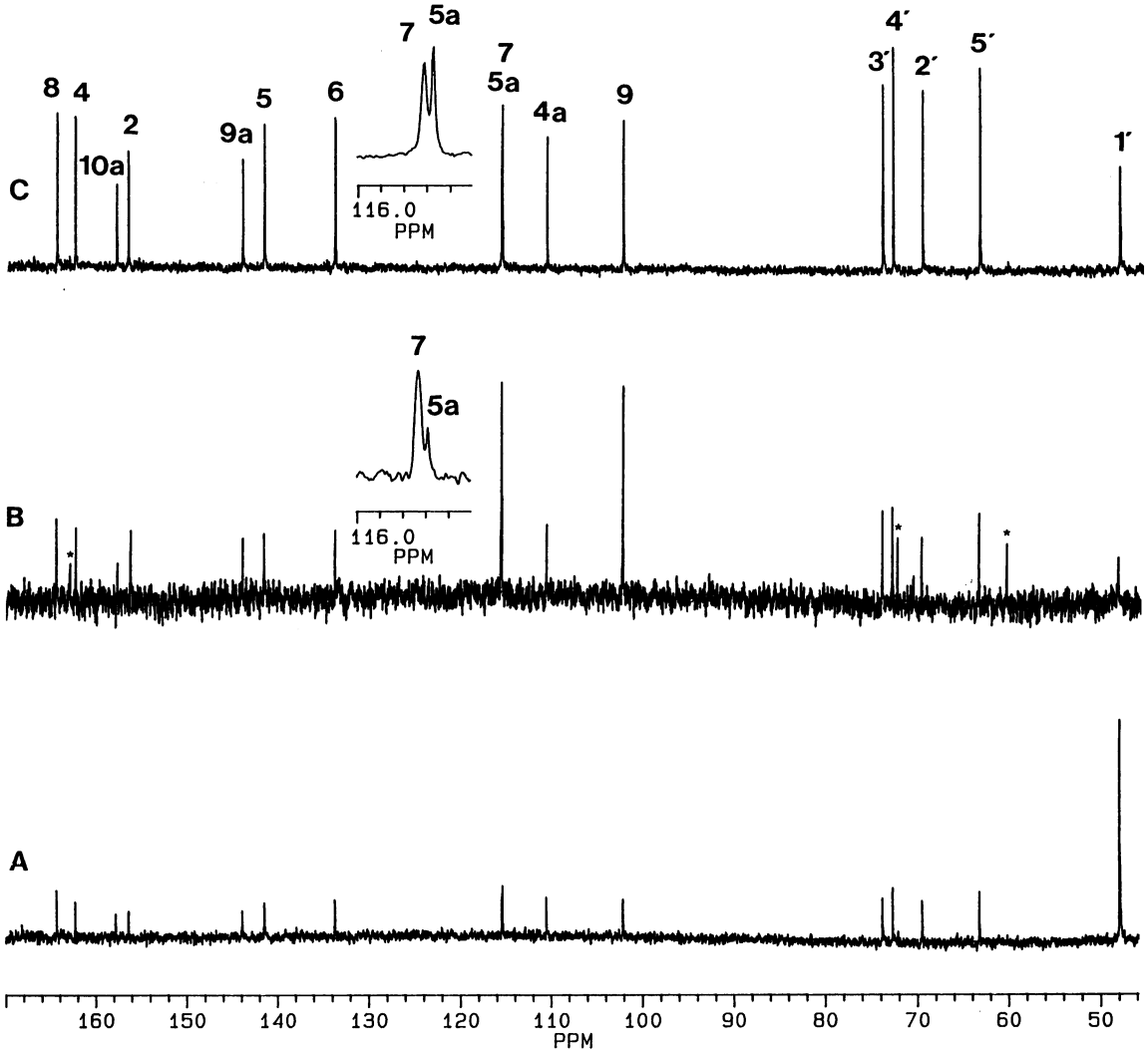


FIG. 3. ^{13}C NMR spectra of factor F_0. Samples from [1′-^{13}C]5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (A) and [6,8-$^{13}C_2$] tyrosine (B) and with natural ^{13}C abundance (C). Asterisks indicate impurities.

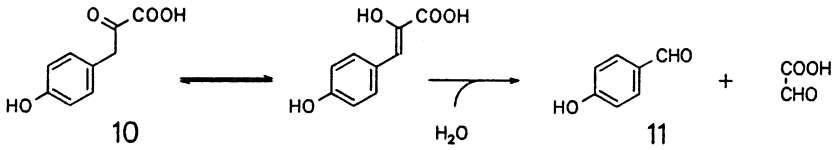


FIG. 4. Formation of 4-hydroxybenzaldehyde (compound 11) from 4-hydroxyphenylpyruvate (compound 10) (9).

in the deazaflavin factor F_0 from a shikimate derivative has been demonstrated, but the structure of the committed precursor remains unknown (11). Tyrosine (compound 14), 4-hydroxyphenylpyruvate (compound 10), and 4-hydroxybenzaldehyde (compound 11, which can be formed from compound 10 via the loss of two carbon atoms [9]) appeared as possible precursors. Therefore we decided to perform isotope incorporation experiments with each of these candidates.

[U-^{14}C]tyrosine was added to a growing culture of *M. thermoautotrophicum* (300 ml) at a concentration of 0.23 mM. Factor F_0 was isolated from the culture medium, and tyrosine was isolated after hydrolysis of cell protein. The radioactivity of the reisolated tyrosine was diluted to a value of about 3% by endogenous synthesis (Tables 1 and 2). Factor F_0 and reisolated tyrosine had the same specific activities within the experimental limits, thus indicating that exogenous tyrosine can be incorporated into compound 2.

In a subsequent experiment, [6,8-$^{13}C_2$]tyrosine (99% ^{13}C enrichment) was added to a culture of *M. thermoautotrophicum* (1.9 liters) at a concentration of 0.23 mM. After termination of bacterial growth, factor F_0 was isolated from the cell mass and the culture fluid. The ^{13}C NMR signals of C-7 and C-9 of deazaflavin were significantly increased in the fermentation product (Fig. 3). These signals were also slightly broadened as a consequence of two-bond ^{13}C-^{13}C coupling. By using quantitative analysis of the relative integrals of the NMR signals, a relative ^{13}C enrichment of 7.1% was calculated for C-7 and C-9 of the deazaflavin chromophore (Table 2). The ^{13}C NMR spectrum of tyrosine isolated after acid hydrolysis of cell protein showed 8.8% ^{13}C enrichment at C-6 and C-8 (data not shown), in close similarity with the enrichment of biosynthetic factor F_0. As expected, the proffered amino acid had been diluted substantially by endogenous synthesis. The results show that the proffered tyrosine had been incorporated into the deazaflavin chromophore and into protein with comparable efficiencies (Table 2).

[U-^{14}C]4-hydroxyphenylpyruvate, prepared by enzymatic deamination of [U-^{14}C]tyrosine, was added to a growing culture of *M. thermoautotrophicum* (300 ml) at a concentration of 0.25 mM. At the end of the growth experiment, 10% of the proffered 4-hydroxyphenylpyruvate had been converted to 4-hydroxybenzaldehyde as shown by HPLC analysis. Factor F_0 isolated from the culture medium and tyrosine obtained by acid hydrolysis of cell mass showed comparable radioactive labeling (Tables 1 and 2). The incorporation rates were similar to those observed with tyrosine (Table 2).

4-Hydroxybenzaldehyde (compound 11) can be formed by spontaneous cleavage of 4-hydroxyphenylpyruvate (compound 10) in alkaline aqueous solutions (9) (Fig. 4). The same reaction could proceed under catalysis by an appropriate enzyme. For this reason, we performed an incorporation experiment with [U-^{14}C]4-hydroxybenzaldehyde. The incorporation into factor F_0 was very low, in the range of the noise level (Tables 1 and 2).

Enzyme studies. It has been shown that both 4-hydroxyphenylpyruvate and tyrosine can be incorporated into the coenzyme and into cell protein. However, to determine the committed precursor of the coenzyme, it was of interest to determine whether the label from tyrosine can be contributed to the coenzyme via 4-hydroxyphenylpyruvate. In vitro experiments with cell extracts of *M. thermoautotrophicum* showed that the cells contain a significant level of tyrosine transaminase. The specific activities of tyrosine transaminase were 0.29 μmol mg^{-1} min^{-1} at 37°C and 0.79 μmol mg^{-1} min^{-1} at 65°C.

DISCUSSION

The biosynthesis of riboflavin has been studied in some detail in eubacteria and fungi (1, 31). The release of formate and pyrophosphate from GTP (compound 3) yields the pyrimidine compound 4 (Fig. 2) (15). Hydrolytic cleavage of the 2-amino group and reduction of the ribose side chain yield 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione 5′-phosphate (compound 5) (2, 18, 22). Removal of the phosphate group yields 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (compound 6), which is condensed with 3,4-dihydroxy-2-butanone 4-phosphate (compound 7) under formation of 6,7-dimethyl-8-ribityllumazine (compound 8). The lumazine derivative is converted to riboflavin (compound 9) by an unusual dismutation reaction (24, 29, 30). The labeling pattern of riboflavin biosynthesized from ^{13}C-labeled precursors in *M. thermoautotrophicum* was in line with this sequence of reactions (11). The present study confirms that the pyrimidine compound 6 is incorporated into riboflavin by *M. thermoautotrophicum* without apparent dilution.

Compound 6 is also incorporated into the deazaflavin chromophore compound 2 without apparent dilution, and the yield of factor F_0 in cultures of *M. thermoautotrophicum* is increased about 10-fold by the pyrimidine supplement. This indicates that compound 6 is a direct precursor of the deazaflavin.

In eubacteria and *S. cerevisiae*, it has been shown that the pyrimidine compound 6 is formed by dephosphorylation of the 5′-phosphate compound 5, which is biosynthesized de novo from GTP (compound 3). The dephosphorylation step has not been studied by direct enzymatic analysis. However, it has been shown that the subsequent reaction steps, i.e., formation of 6,7-dimethyl-8-ribityllumazine (compound 8) and riboflavin (compound 9), can only proceed with unphosphorylated compounds. 5′-Phosphates cannot act as substrates for lumazine synthase and riboflavin synthase.

Since the existence of a 5′-kinase for compound 6 is not likely, our data suggest that compound 6 is the branching point of the riboflavin and deazaflavin biosynthetic pathways. The data also suggest that the formation of the deazaflavin chromophore proceeds at the level of unphosphorylated intermediates.

The de novo biosynthesis of the pyrimidine precursor appears to be rate limiting in the biosynthesis of the dea-

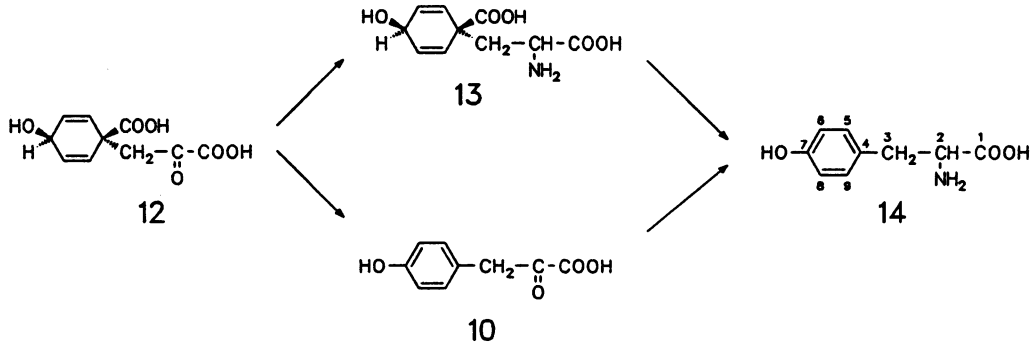


FIG. 5. Pathways of tyrosine biosynthesis.

zaflavin chromophore, since the biosynthetic formation of the cofactor was increased about 10-fold by adding the precursor at a high concentration to the culture medium. In contrast, the level of flavin formation was not increased by feeding of the pyrimidine intermediate, although the proffered precursor was effectively incorporated into the vitamin. Thus, the de novo biosynthesis of compound 6 appears not to be rate limiting in the biosynthesis of riboflavin. It should be noted that relatively small amounts of riboflavin relative to the amounts of deazaflavin are biosynthesized in *M. thermoautotrophicum*. The amount of compound 6 biosynthesized de novo appears to saturate the enzyme machinery for the final steps in the biosynthesis of riboflavin but not of deazaflavin. It is possible that the biosynthesis of the 4-carbon precursor compound 7 limits the rate of biosynthesis of riboflavin.

In in vivo studies with simple precursors, the labeling pattern of the benzenoid ring of factor F_0 has shown the signature of a shikimate derivative (11). Moreover, these studies indicated that C-5 of the pyridine ring of the deazaflavin chromophore was derived from the β carbon of the tyrosine side chain. In connection with the symmetric isotope label distribution in the phenolic ring of factor F_0, this suggested that a metabolite posterior to prephenate (compound 12; Fig. 5) could serve as the specific biosynthetic precursor of deazaflavins. In line with this hypothesis, we have now found that both 4-hydroxyphenylpyruvate (compound 10) and tyrosine (compound 14) are efficiently incorporated into factor F_0.

An earlier incorporation experiment had failed to show incorporation of [^{14}C]tyrosine into coenzyme F_{420} in a culture of *M. thermoautotrophicum* (16). The reason for this discrepancy remains unknown.

It is unknown whether the biosynthesis of tyrosine proceeds via 4-hydroxyphenylpyruvate (compound 10) or arogenate (compound 13) in methanogenic bacteria (Fig. 5). The observed conversion of the keto acid to tyrosine is no definitive proof for its obligatory involvement in the tyrosine pathway under physiologic conditions, since some aminotransferases can use both prephenate and 4-hydroxyphenylpyruvate as substrates (3, 17).

The incorporation of tyrosine into the deazaflavin chromophore could imply that tyrosine is the committed precursor. However, the presence of tyrosine transaminase in cell extracts of *M. thermoautotrophicum* suggests that the tyrosine pool may actually be in equilibrium with the 4-hydroxyphenylpyruvate pool via reversible deamination. On the other hand, arogenate, prephenate, and earlier shikimate precursors cannot be the committed precursors of factor F_0, since the aromatization energy of the benzenoid metabolites precludes their equilibration with the pools of earlier nonaromatic intermediates.

It should be noted that 4-hydroxyphenylpyruvate (compound 10) is easily converted to 4-hydroxybenzaldehyde (compound 11) (9) (Fig. 4). About 10% of the proffered keto acid had been converted to the aldehyde at the end of the growth experiment. Thus, it is worth considering that the keto acid could be enzymatically converted to the aldehyde as the committed deazaflavin precursor. The aldehyde could then add to the 5 position of the pyrimidine precursor in an electrophilic reaction. An incorporation experiment with [U-^{14}C]4-hydroxybenzaldehyde gave no evidence for incorporation into the deazaflavin chromophore. However, this negative result does not provide definitive evidence against a biosynthetic involvement of the aldehyde, since we have no proof that the proffered compound was metabolized by the cells.

On the basis of the available evidence, it is obvious that the formation of the deazaflavin system from the pyrimidine precursor involves the replacement of nitrogen by carbon at the 5 position of compound 6. This type of reaction has precedents in the biosynthetic formation of various deazapurines, such as tubercidin, toyocamycin, and sangivamycin (13).

We propose tentatively that the formation of factor F_0 could begin with the formation of a C-C bond between the β carbon of the committed aromatic precursor and C-5 of the pyrimidine (Fig. 6). On the basis of the present results, it remains open whether the committed precursor is tyrosine or 4-hydroxyphenylpyruvate. Whereas the keto acid could act directly as a nucleophile, tyrosine could be activated by an appropriate cofactor, such as pyridoxal phosphate.

Hypothetically, the reaction could start by the dehydrogenation of the pyrimidine compound 6, yielding the quinoid form (compound 15), which could then perform an electrophilic attack on the enolate of the keto acid or an activated form of tyrosine (Fig. 6). Elimination of ammonia and a two-carbon fragment could yield compound 16. Ring closure could then be achieved by oxidative condensation.

In summary, we propose that the unphosphorylated pyrimidine compound 6 is the direct biosynthetic precursor of the deazaflavin series. This suggests that factor F_0, rather than a phosphorylated intermediate, is the initial deazaflavin intermediate, which is subsequently converted to a phosphoric acid ester. This situation is analogous to the biosynthesis of flavins, which proceeds from 5′-phosphorylated to 5′-

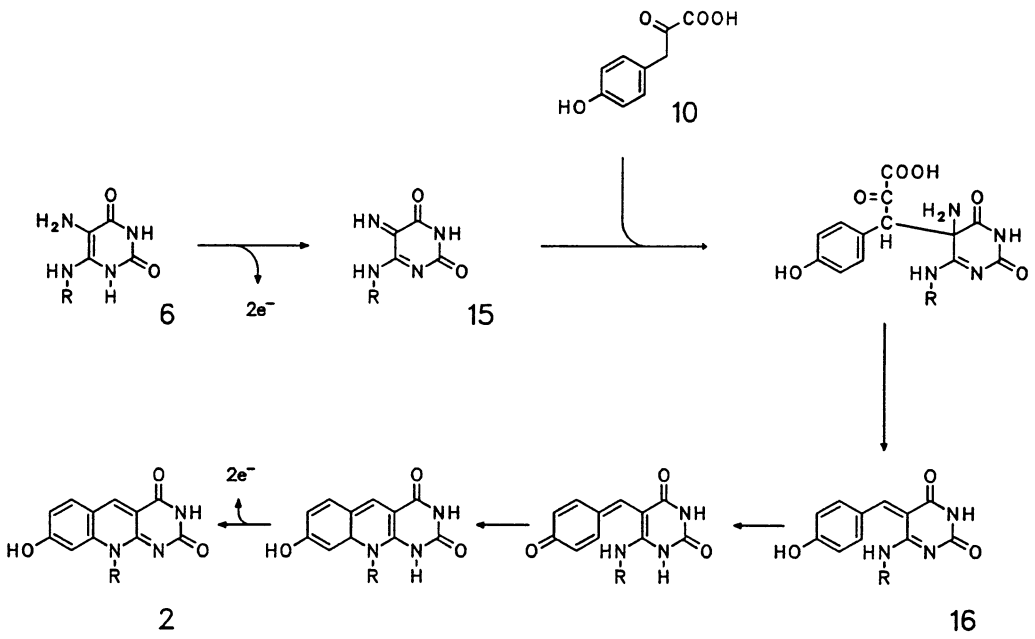


FIG. 6. Hypothetical mechanism for the biosynthesis of the deazaflavin chromophore (R, ribityl).

unphosphorylated products, although the 5'-phosphate residue is ultimately required for coenzyme activity.

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