Biosynthesis of Nucleotides, Flavins, and Deazaflavins in Methanobacterium thermoautotrophicum*

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The biosynthesis of deazaflavins, flavins, ribonucleotides, and selected amino acids was studied in Methanobacterium thermoautotrophicum by incorporation of $^{13}$C-labeled acetate and pyruvate. $^{13}$C enrichments were monitored by $^{13}$C and $^1$H NMR spectroscopy. The biosynthesis of ribonucleotides follows the standard pathway. The xylene ring of riboflavin is formed from two pentose moieties in agreement with studies in yeasts and eubacteria. The pyrimidine ring and the ribityl side chain of the deazaflavin chromophore of coenzyme F$_{420}$ are derived from the purine nucleotide pool. The phenolic ring and C-5 of the deazaflavin system are supplied by the shikimate pathway. A hypothetical mechanism for the assembly of the deazaflavin chromophore from 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidine-4-one-5-pyruvate is proposed.

Methanogenic bacteria generate energy and cell mass by the reduction of CO$_2$ with H$_2$ as electron donor. The pathway of CO$_2$ assimilation is well established (Fuchs and Stupperich, 1978, 1980). Briefly, the reduction of CO$_2$ yields active methyl groups that can be utilized for the formation of acetyl-CoA (Stupperich and Fuchs, 1984a, 1984b). Further reductive carboxylation yields pyruvate, which can serve as a precursor for a wide variety of metabolic pathways.

The biosynthesis of most amino acids has been studied in Methanospirillum hungatei and proceeds by known pathways (Eikeli et al., 1983). In Methanobacterium thermoautotrophicum the biosynthesis of glutamate, aspartate, alanine, lysine, and isoleucine was investigated in closer detail (Fuchs et al., 1978; Bakhiet et al., 1984; Kenely et al., 1982). Isoleucine is formed by an unusual biosynthetic pathway via citramalate (Eikemans et al., 1983; Eikeli et al., 1984).

A variety of unusual coenzymes was detected relatively recently in methanogenic bacteria (for review, see Wolfe, 1985). The green fluorescent coenzyme F$_{430}$ (Fig. 1) was detected by Wolfe and his co-workers in various species of methanogenic bacteria (Cheeseman et al., 1972), and its structure (1, Fig. 1) was elucidated by Eirich et al. (1978). The chromophoric moiety of the coenzyme was assigned the structure of 7,8-didemethyl-8-hydroxy-5-deazariboflavin (2, factor F$_{430}$). Whereas flavocoenzymes can participate in one- and two-electron redox steps, the deazaflavins are limited to two-electron reactions and can be considered as functional analogs of pyridine nucleotides (Jacobson and Walsh, 1984). In methanogenic bacteria, the deazaflavin coenzyme F$_{420}$ is involved in key steps of methanogenesis and carbon assimilation (Zeikus et al., 1977). Deazaflavin coenzymes have also been found in several other microbial species such as Streptomyces, mycobacteria, and nonmethanogenic Archaeabacteria, in which they serve special functions such as the biosynthesis of tetracycline and as cofactor of DNA photolysase (Daniels et al., 1985; Ecker et al., 1980; McCormick and Morton, 1982).

Jaenicke et al. (1984) observed the incorporation of [2,14$^C$]glutamine into coenzyme F$_{420}$ by M. thermoautotrophicum and concluded that the pyrimidine ring of the coenzyme is derived from a purine precursor. Scherer et al. (1984) observed the incorporation of label from [1,14$^C$]glycine into a carbon atom of coenzyme F$_{420}$ which is characterized by a $^{13}$C NMR signal at 162.7 ppm. The authors assumed that this signal represents C-10a of the deazaflavin chromophore and concluded that the incorporation should have occurred via a purine intermediate.

The biosynthesis of riboflavin has been studied extensively in fungi and eubacteria. Early studies documented that the pyrimidine ring of the flavin chromophore is derived from a purine precursor (for review see Bacher, 1990; Young, 1986). More recently, it was shown that the xylene ring is derived from two pentose moieties (Volk and Bacher, 1988, 1990). The biosynthesis of riboflavin in Archaeabacteria has not been studied up to now.

This paper reports on incorporation studies with $^{13}$C-labeled acetate and pyruvate which yield information on the biosynthetic pathways of deazaflavins, flavins, purines, pyrimidines, and amino acids in M. thermoautotrophicum.

EXPERIMENTAL PROCEDURES

Materials—The following materials were obtained commercially: [1,14$^C$]acetate, [2,14$^C$]acetate, and [1,2,14$^C$]acetate from Cambridge Isotope Laboratories, Woburn, MA; [1-14$^C$]pyruvate from Isotec Inc., Miamisburg, OH; [U-14$^C$]pyruvate, [1-14$^C$]acetate, [2-14$^C$]acetate, and [1-14$^C$]pyruvate from Amersham Corp. Other chemicals were reagent grade.

Microorganism—M. thermoautotrophicum Marburg (DSM no. 2133) was obtained from the Deutsche Sammlung von Mikroorganismen, Göttingen, Federal Republic of Germany. The strain was subcultured at weekly intervals in serum bottles under an atmosphere of H$_2$/CO$_2$ (4:1, v/v) which were incubated at 65 °C.

Culture Medium—The culture medium (modified from Taylor and Pirt, 1977) contained, per liter: KHPO$_4$, 0.15 g; K$_2$HPO$_4$, 0.3 g; NH$_4$Cl, 1.5 g; Na$_2$CO$_3$, 0.6 g; MgSO$_4$·7H$_2$O, 0.18 g; CaCl$_2$·2H$_2$O, 80 mg; FeSO$_4$·7H$_2$O, 13 mg; MnCl$_2$·4H$_2$O, 1 mg; CoCl$_2$·6H$_2$O, 1.7 mg; ZnCl$_2$, 1 mg; H$_2$BO$_3$, 0.19 mg; Na$_2$MoO$_4$·2H$_2$O, 0.1 mg; NiCl$_2$·6H$_2$O, 0.48 mg; EDTA, tetrasodium salt, 52 mg; resazurin, 1 mg; Cysteine hydrochloride (1.5 g/liter) and Na$_2$S (1.5 g/liter) were added to the autoclaved medium as sterilized solutions. The pH was adjusted to 7.0 by the addition of NaOH.

Serum bottles (100 ml) equipped with butyl rubber septa and

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containing 20 ml of medium were gassed with a mixture of H₂/CO₂ (4:1, v/v) to a pressure of 2 bars and autoclaved at 121 °C. Culture medium in fermentor vessels (0.3–20 liters) was autoclaved and gassed immediately after removal from the autoclave with a mixture of H₂/CO₂ (4:1, v/v) at atmospheric pressure.

**Fermentation**—M. thermoautotrophicum was grown in a thermostatted vessel containing 300 ml of medium at 65 °C with stirring. The medium was gassed with a mixture of H₂/CO₂ (4:1, v/v, 150 ml/min). When the culture had reached late exponential growth phase, it was transferred into a 20-liter fermentor containing 18 liters of medium. The fermentor was equipped with a pH electrode and was gassed with the H₂/CO₂ mixture (4:1, v/v) at a rate of 3 liters/min. The cell suspension was stirred at 65 °C. A sterile solution of 5% sodium sulfite was added constantly at a rate of 2 ml/h. When the cell density had reached a value of 0.4 OD (660 nm, 1 cm), the pH was adjusted to 6.0 by the addition of 1 M HCl. Subsequently the pH was kept at this value by automated addition of 20% Na₂CO₃. ¹³C-Labeled sodium acetate was added as a sterile solution to a final concentration of 4 mM when the cell density had reached a value of 0.5 OD. ¹⁴C-Labeled sodium pyruvate was added to the culture in two portions at cell densities of 1.2 and 1.8 OD to a final concentration of 2 mM. The fermentor was kept in the dark to avoid photodecomposition of coenzymes. Fermentation was continued until early stationary phase. Cells were harvested aerobically by centrifugation and frozen at −20 °C. The medium was set aside for isolation of coenzymes.

**Isolation of Flavins and Deazaflavins from the Culture Medium**—The culture fluid was passed through a column of Dowex 1×8 (formate form, 20–50 mesh, 5 × 30 cm). The column was washed with water. The combined effluents were passed through a column of charcoal (35–50 mesh, 3 × 7 cm). The column was washed with water. Fluorescent material was eluted from the Dowex column by 10 liters of 2 M formic acid. The eluate was concentrated to dryness under reduced pressure. Fluorescent material was also eluted from the charcoal column by 3 liters of 0.8 M NH₄OH containing 50% ethanol. The eluate was concentrated to dryness under reduced pressure. Fluorescent material was eluted from both columns in water and combined. The solution was placed on a column of QAE Sephadex A-25 (HCO₃⁻ form, 3 × 41 cm). The column was developed with a linear gradient of 0–1.5 M NH₄HCO₃ (total volume, 2 liters). Fractions of 20 ml were collected. Elution of flavins and deazaflavins was monitored by photometry and by HPLC analysis. Riboflavin was eluted at 0.16 M NH₄HCO₃, FMN at 0.7 M NH₄HCO₃, and factor F₄₅₀ at 0.75 M NH₄HCO₃. The respective fractions were pooled and concentrated to dryness under reduced pressure.

**Isolation of Flavins and Deazaflavins from Bacterial Cells**—Cell paste of M. thermoautotrophicum (100 g) was suspended in 50% aqueous acetone (400 ml) at −10 °C. The suspension was stirred at 4 °C for 30 min. The suspension was centrifuged, and the residue was again extracted as described until the supernatant was colorless (approximately 10 times). The residual cell mass was suspended in 80% aqueous methanol (400 ml) and heated at 80 °C for 20 min in order to extract tightly bound FMN. The suspension was centrifuged, and the cells were again extracted as described (two to three times). The supernatants from the extraction procedures were combined and concentrated to dryness under reduced pressure. The residue was dissolved in 80 ml of water and placed on a column of QAE Sephadex A-25 (HCO₃⁻ form, 3 × 41 cm). The column was developed with a linear gradient of 0–1.5 M NH₄HCO₃ (total volume, 2 liters) followed by 1.5 M NH₄HCO₃ (1 liter). Fractions of 20 ml were collected. FMN was eluted at 0.7 M NH₄HCO₃, FAD at 0.75 M NH₄HCO₃, and coenzyme F₄₅₀ at 1.5 M NH₄HCO₃. The respective fractions were concentrated to dryness under reduced pressure. Corresponding fractions obtained from cell extract and cell culture medium were combined.

**Purification of Riboflavin**—Crude FAD was dissolved in 0.1 M phosphate buffer (pH 7) and treated with phosphodiesterase (Naja naja sp. venom, 100 µg) for 24 h at room temperature. The resulting FMN was combined with FMN obtained from culture fluid and cell extract and dissolved in 5 ml of 0.1 M NH₄HCO₃ containing 0.5 mM MgCl₂ and the pH of the solution was adjusted to 10.4. Alkaline phosphatase (7 units) was added, and the mixture was kept at 37 °C for 2 h. The resulting riboflavin was combined with crude riboflavin obtained from the culture fluid. Purification of riboflavin was achieved by preparative reversed phase HPLC using a column of Lichrosorb RP₈ (16 × 250 mm) and an eluent containing 30% methanol. Riboflavin was monitored by photometry at 365 nm. The retention volume was 260 ml. Fractions were collected and concentrated to dryness under reduced pressure.

**Purification of Deazaflavins**—Factor F₄₅₀ was purified by preparative HPLC on a column of Lichrosorb RP₈ (16 × 250 mm) with an eluent containing 27% MeOH and 30 mM formic acid. The effluent was monitored photometrically (405 nm). The retention volume was 200 ml. Fractions were combined and concentrated to dryness under reduced pressure.

Coenzyme F₄₅₀ was hydrolyzed by treatment with 1 M HCl at 110 °C for 1 h. The solution was adjusted to pH 9 with 3% NH₄OH and concentrated to dryness under reduced pressure. The resulting 5'-phosphate of factor F₄ was dissolved in 0.1 M ammonium bicarbonate buffer (pH 10.4) containing 0.5 mM MgCl₂ and treated with alkaline phosphatase (10 units) at 37 °C for 20 min. The solution was adjusted to pH 6 by the addition of formic acid and was concentrated under reduced pressure.

The samples of factor F₄ obtained as described above were combined and dissolved in 30 ml of 0.1 M phosphate buffer (pH 10). Insoluble material was removed by centrifugation. The pH of the yellow colored supernatant was adjusted to 6 by the addition of concentrated formic acid. The solution was kept overnight at 4 °C. The precipitate of factor F₄ was harvested by centrifugation and washed twice with ice water. The supernatant was placed on a preparative HPLC column of Lichrosorb RP₇ (16 × 250 mm) which was developed with 27% methanol. The retention volume of factor F₄ was 240 ml. Fractions were concentrated to dryness under reduced pressure. When necessary, factor F₄ was recrystallized from a water/dimethyl sulfoxide mixture (4:1, v/v).

**Isolation of Nucleosides**—The residue remaining after repeated acetone and methanol extraction of bacterial cell mass was suspended in a mixture of ethanol/ether (3:1, v/v, 200 ml) and boiled under reflux for 1 h. The suspension was filtered, and the residue was washed with ether and dried at room temperature. The residue was suspended in 100 ml of 1 M NaOH and was stirred for 24 h at room temperature. Concentrated hydrochloric acid (10 ml) and 25% trichloroacetic acid (10 ml) were added, and the suspension was centrifuged. The supernatant was adjusted to pH 8.0 by the addition of 25% NH₄OH. Barium acetate (50 mg) and ethanol (650 ml) were added. The mixture was kept at 4 °C for 12 h, and the precipitate of nucleotide barium salts was harvested by centrifugation. Barium acetate (200 mg) was added to the supernatant, which was again kept at 4 °C for 12 h and then centrifuged. The precipitates were combined and dissolved in 50 ml of 50 mM HCl. A concentrated solution of sodium sulfate (2 ml) was added, and the precipitate of BaSO₄ was removed by centrifugation. The supernatant was placed on a column.
of Dowex 50W-X8 (H+ form, 200–400 mesh, 2 × 34 cm). The column was developed with 160 ml of 50 mM HCl and subsequently with water. Fractions of 20 ml were collected and analyzed by HPLC. The retention volumes were as follows: UMP, 100 ml; GDP, 300 ml; CMP and AMP, 1,000 ml. CMP and AMP were separated by preparative HPLC using a column of Lichrosorb RP-18 (16 × 250 mm). An eluent containing 100 mM ammonium formate and 100 mM formic acid. The nucleotides were monitored at 254 nm. 2′-AMP and 3′-AMP had retention volumes of 260 and 560 ml, and 2′-CMP and 3′-CMP had a retention volume of 100 ml, respectively. Fractions were concentrated to dryness under reduced pressure. The isolated nucleotides were dissolved in 0.1 M ammonium formate and filtered through a 0.22-μm PES filter. The radioactivity was determined with a 2-Hz Gaussian in the t1 dimension and 90° shifted sine bell filter in the t2 dimension; (e) ROESY, 64 scans/t1, increment, 1.1-s relaxation delay, 200-ms continuous wave spin lock period; 90° pulse width, 60 μs; 450 × 1,024 raw data matrix size, zerofilled to 1 K in f1 and processed with 2 Hz Gaussian in the t1 dimension and 90° shifted sine bell filter in the t2 dimension; (f) INADEQUATE, 64 scans/t1, increment, 2.3-s relaxation delay, Ernst-type phase cycle; 3.6-s delay period for evolution of 1H–13C corresponding to a coupling constant of 145 Hz; 128 × 2,048 raw data matrix size, zerofilled to 512 words and processed with 90° shifted sine bell filter in t1; (g) 1H–13C HMBC, 192 scans/t1, increment, 1.0-s relaxation delay, 3.5-s delay period for suppression of 1H–13C, 62-ms and 125-ms delay periods for evolution of long range couplings, corresponding to coupling constants of 8 and 5 Hz, respectively; 128 × 2,048 raw data matrix size zerofilled to 512 words in f1 and processed with 90° shifted sine bell filter in t1; (f) INADEQUATE, 64 scans/t1, increment, 2.3-s relaxation delay, Ernst-type phase cycle; 3.6-s delay period for evolution of 1H–13C corresponding to a coupling constant of 145 Hz; 128 × 2,048 raw data matrix size, zerofilled to 2 K in f1 and processed with 60° sine bell filter in t1.

RESULTS

M. thermoautotrophicum has a highly specialized metabolism which enables it to grow autotrophically on CO2 and H2. Complex organic nutrients are utilized rather inefficiently, probably for lack of appropriate uptake systems. Simple organic nutrients such as acetate and pyruvate are at least metabolized to some extent and can be used for 13C incorporation studies. Predictably, such simple precursors will contribute 13C label to many different positions in virtually all complex metabolic products. These complex labeling patterns can be assessed with high accuracy by 13C NMR spectroscopy. A rigorous interpretation of the 13C labeling obtained with simple two- or three-atomic precursors can be achieved by comparison among the labeling patterns of a variety of metabolic products from the same fermentation. With this aim, we have analyzed quantitatively the labeling patterns of purine and pyrimidine nucleotides as well as amino acids. These data subsequently served as the background for the interpretation of the 13C labeling pattern in the coenzymes, deazaflavin and riboflavin.

Hüster and Thauer (1983) observed that the utilization of pyruvate in M. thermoautotrophicum is optimal at pH 6. The same is true for acetate as shown by preliminary experiments with [13C]acetate (data not shown). Based on these data, the microorganism was grown at a constant pH of 6 in a mineral salts medium under an atmosphere of H2/CO2 (4:1, v/v). Labeled acetate (1-13C, 2-13C, or 1,2-13C2) was added to a final concentration of 4 mM.

Pyruvate was unstable in long time fermentations. After the addition of sodium [1-13C]pyruvate to a culture of M. thermoautotrophicum, 50% of the radioactivity was lost within 15 h, probably as 13CO2. [1-13C]Pyruvate was therefore added sequentially in two portions to a rapidly growing culture.

In all feeding experiments, incubation was continued until early stationary phase. The cells were then separated from the culture medium. Riboflavin, FMN, and factor F0 were isolated from the culture fluid. Coenzyme F420, FAD, and FMN were isolated from the cell mass. The flavoenzymes were converted to riboflavin, and coenzyme F420 was converted to factor F0, by hydrolysis. Purine and pyrimidine nucleosides...
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FIG. 2. Biosynthesis of purines in M. thermoautotrophicum. Carbon atoms labeled from [1-13C]acetate (O), [2-13C]acetate (●), and [1-13C]pyruvate (*) are indicated. The joint transfer of both carbon atoms from [1,2-13C2]acetate as shown in 13C-13C coupling is indicated by bold lines. The labeling pattern of compounds shown in boxes has been determined by NMR spectroscopy. Other labeling patterns shown were deduced on the basis of known biosynthetic pathways.

TABLE I
Relative 13C enrichments of ribonucleosides from M. thermoautotrophicum grown with 13C-labeled acetates and 13C-labeled pyruvate.

| Position | Chemical shift | % Coupling | Relative 13C enrichment |
|----------|----------------|------------|-------------------------|
|          | ppm            | Hz         | [1-13C]-Acetate | [2-13C]-Acetate | [1,2-13C2]-Acetate | [1-13C]-Pyruvate |
| Cytidine |                |            | 1.0'            | ND             | 1.1              | 1.0'            |
| 4        | 168.4          | 1.0'       | 1.0'            | ND             | 1.1              | 1.0'            |
| 2        | 159.7          | 1.4        | ND              | 1.1            | 1.0              |
| 6        | 144.4          | 3.4        | ND              | 4.3            | 1.3              |
| 5        | 98.8           | 1.0        | ND              | 4.6            | 1.3              |
| 1'       | 92.9           | 3.2        | ND              | 3.9            | 1.3              |
| 4'       | 86.4           | 3.4        | ND              | 4.1            | 1.4              |
| 2'       | 76.6           | 1.1        | ND              | 1.0            | 4.4              |
| 3'       | 71.9           | 1.0        | ND              | 1.0            | 4.5              |
| 5'       | 63.3           | 1.1        | ND              | 4.5            | 1.4              |
| Guanosine|                |            |                 |                |                  |
| 2        | 156.4          | 1.0        | 1.0'            | ND             | 1.1              |
| 6        | 153.8          | 1.1        | 1.1             | 1.3            | 1.2              |
| 4        | 151.1          | 1.0'       | 1.0             | 3.5            |
| 8        | 135.6          | 1.1        | 1.1             | 1.3            |
| 5        | 115.9          | 3.9        | 1.0             | 4.3            | 1.0'            |
| 1'       | 86.5           | 3.9        | 1.1             | 3.8            | 1.2              |
| 4'       | 85.2           | 1.1        | 1.1             | 4.2            | 1.2              |
| 2'       | 73.7           | 3.9        | 1.1             | 4.2            | 1.2              |
| 3'       | 70.2           | 1.2        | 1.2             | 4.4            |
| 5'       | 61.4           | 1.3        | 5.2             | 4.7            | 1.1              |

a 13C-13C couplings observed in the spectrum of the biosynthetic sample from the [1,2-13C2]acetate fermentation indicating the incorporation of intact acetate units.

b Percent coupling calculated as percentage of total signal for a given carbon atom involved in 13C-13C coupling.

c Carbon atom arbitrarily assigned a relative 13C enrichment of 1.0.

TABLE I

ND, not determined.

FIG. 3. Biosynthesis of pyrimidines in M. thermoautotrophicum.

For details see Fig. 2.

were isolated after hydrolysis of RNA by alkali treatment of cell mass, and amino acids were isolated after acid hydrolysis of cellular proteins.

13C enrichments of the isolated metabolites were determined by 13C NMR spectroscopy. Integrals of individual signals were calibrated using the spectra of samples with natural 13C abundance which were measured under identical experimental conditions. In each compound under study, the carbon atom with the lowest relative enrichment was assigned a relative enrichment value of 1.0.

When possible, 13C enrichments of selected carbon atoms were also determined by evaluation of the 13C satellites in 1H NMR spectra. This approach was limited to carbon atoms attached to protons with simple NMR signatures, preferably singlets. The enrichment values derived for a given carbon atom from 13C and 1H NMR spectroscopy were generally in good agreement.

The labeling patterns of guanosine (5, Fig. 2) and cytidine (7, Fig. 3) isolated from RNA are summarized in Table I. The labeling pattern of adenosine was identical to that of guano-
sine, and the labeling pattern of uridine was identical to that of cytidine (data not shown). The labeling pattern of the ribose moiety was the same in all four nucleosides studied and can be easily rationalized on the basis of known metabolic pathways in *M. thermoautotrophicum* (Fig. 4). It is well established that pyruvate (8) is formed by reductive carboxylation of acetate (Fuchs and Stupperich, 1980). The labeling pattern of pyruvate is directly reflected by alanine (9) formed from pyruvate by transamination (Table II).

Starting from pyruvate, gluconeogenesis leads to a symmetrical duplication of the labeling pattern. Pentose formation by the oxidative branch of the pentose phosphate cycle then yields the observed labeling pattern of ribose (Fig. 4). The incorporation of intact acetate units into the positions 4 and 5 of the ribose moiety was clearly shown by 13C-13C coupling observed in the samples from the fermentation with double labeled acetate.

The pathway of purine biosynthesis in bacteria and fungi is well documented and has been reinvestigated recently in yeast by Kozluk and Spenser (1987). The observed labeling pattern of purines in *M. thermoautotrophicum* is well in line with the standard pathway as shown in Fig. 2. Similarly, the labeling pattern of pyrimidine nucleotides (Fig. 3) is in line with the respective standard pathway.

The labeling patterns of phenylalanine and tyrosine were identical within the experimental limits (Table II). Ekkel *et al.* (1983) studied the biosynthesis of these amino acids with 13C-labeled acetates, and their data were consistent with the shikimate pathway. The same is true for our data. However, it should be noted that only the ring atom 7 is enriched in the fermentation with [1-13C]pyruvate. As shown in Fig. 5, this

### Table II

| Position | Chemical shift | 13C coupling | Relative 13C enrichment | Relative 13C enrichment |
|----------|----------------|--------------|-------------------------|-------------------------|
|          | ppm            | Hz           | [1,13C]Acetate | [2,13C]Acetate | [1,2,13C]Acetate | [1-13C]Pyruvate |
| Aspartate|                |              |              |              |              |                |
| 1        | 185.0          |              | 1.9          | 1.9          | 1.9          | 3.1            |
| 2        | 56.6           | 74           | 3.5          | 1.0          | 5.4          | 1.0            |
| 3        | 45.9           | 68           | 1.1          | 5.6          | 6.5          | 1.0            |
| Tyrosine |                |              | 1.6          | 1.0          | 1.0          | 2.0            |
| 1        | 185.3          |              | 1.6          | 1.0          | 1.0          | 2.0            |
| 2        | 166.3          |              | 1.2          | 1.1          | 1.1          | 3.8            |
| 3        | 133.4          | 65           | 1.7          | 7.1          | 7.4          | 1.0            |
| 4        | 126.9          | 64           | 5.3          | 1.1          | 5.6          | 1.0            |
| 5/6      | 121.0          | 62           | 3.8          | 1.4          | 4.0          | 1.1            |
| Glycine  |                |              | 5.0          | 1.2          | 6.4          | 1.0            |
| 1        | 172.5          |              | 1.3          | 7.0          | 6.5          | 1.0            |
| 2        | 42.7           |              | 3.5          | ND          | ND          | 4.5            |
| Alanine  |                |              | 1.0          | ND          | ND          | 1.0            |
| 1        | 175.5          |              | 1.0          | 1.1          | 1.1          | 4.9            |
| 2        | 51.8           | 70           | 4.5          | 1.0          | 3.6          | 1.0            |
| 3        | 18.4           | 65           | 1.2          | 4.6          | 4.6          | 1.0            |
| Phenylalanine |         |              | 1.5          | 1.8          | 1.0          | 5.8            |
| 1        | 174.0          |              | 1.5          | 1.8          | 1.0          | 5.8            |
| 4        | 136.6          | 69           | 2.9          | 1.2          | 4.7          | 1.6            |
| 5/6      | 132.1          | ND           | 1.2          | 5.6          | 5.6          | 1.1            |
| 7        | 130.7          | ND           | 2.7          | 1.0          | 3.5          | 1.1            |
| 2        | 56.7           | 77           | 1.0          | 1.0          | 3.4          | 1.2            |
| 3        | 38.2           | 67           | 1.0          | 5.3          | 4.0          | 1.0            |
| Serine   |                |              | 1.0          | 1.0          | 1.0          | 4.1            |
| 1        | 173.9          |              | ND           | 1.0          | 1.0          | 4.1            |
| 2        | 57.9           | 75           | ND           | 1.0          | 4.2          | 1.0            |

* a 13C-13C couplings observed in the spectrum of the biosynthetic sample from the [1,2-13C2]acetate fermentation indicating the incorporation of intact acetate units.
* b Percent coupling calculated as percentage of total signal for a given carbon atom involved in 13C-13C coupling.
* c Carbon atom arbitrarily assigned a relative 13C enrichment of 1.0.
* d ND, not determined.
* e Average due to multiplet overlapping.
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![Diagram of biosynthesis of tyrosine](image)

**Fig. 5.** Biosynthesis of tyrosine (15) in *M. thermoautotrophicum*.

For details see Fig. 2.

![Diagram of biosynthesis of riboflavin](image)

**TABLE III**

Relative $^{13}$C enrichments of riboflavin from *M. thermoautotrophicum* grown with $^{13}$C-labeled acetates and pyruvate

| Position | Chemical shift (ppm) | $\Delta_{oc}$* $^{\hbox{a}}$ | % Coupling $^{\hbox{a}}$ | Relative $^{13}$C enrichment |
|----------|----------------------|-----------------|-----------------|--------------------------|
|          | H$^{\hbox{a}}$       |                 |                 | [1-$^{13}$C]-Acetate | [2-$^{13}$C]-Acetate | [1,2-$^{13}$C]$^{2}$-Acetate | [1-$^{13}$C]-Pyruvate |
| 4        | 159.8                | 1.6             | 1.4             | 1.3                      | 1.5                      |
| 2        | 155.3                | 1.6             | 1.5             | 1.0                      | 1.0                      |
| 10$\alpha$ | 150.7          | 1.4             | 2.1             | 1.0                      | 2.8                      |
| 8        | 145.9                | 1.6             | 1.4             | 1.0                      | 3.9                      |
| 4$\alpha$ | 136.4          | 4.5             | 1.2             | 7.8                      | 1.0$^{d}$               |
| 7        | 136.7                | 1.0$^{d}$       | 1.8             | 1.1                      | 4.1                      |
| 5$\alpha$ | 133.8          | 1.3             | 1.2             | 1.1                      | 3.2                      |
| 9$\alpha$ | 131.9          | 1.3             | 1.5             | 1.1                      | 4.8                      |
| 6        | 130.6                | 4.5             | 1.5             | 10.0                     | 1.3                      |
| 9        | 117.3                | 1.4             | 8.9             | 7.6                      | 1.0                      |
| 3$'$     | 73.6                 | 1.1             | 1.2             | 1.0$^{d}$               | 3.7                      |
| 4$'$     | 72.7                 | 3.5             | 1.1             | 6.0                      | 1.4                      |
| 2$'$     | 68.8                 | 1.2             | 1.5             | 1.0                      | 4.3                      |
| 5$'$     | 63.4                 | 1.1             | 8.7             | 6.8                      | 1.0                      |
| 1$'$     | 47.3                 | 4.5             | 1.5             | 4.7                      | 1.0                      |
| 8$\alpha$ | 20.7             | 3.7             | 1.0$^{d}$       | 5.0                      | 1.0                      |
| 7$\alpha$ | 18.7             | 1.8             | 0.8             | 7.5                      | 1.1                      |

$^{a}$ $^{13}$C-$^{13}$C couplings observed in the spectrum of the biosynthetic sample from the [1,2-$^{13}$C]$^{2}$acetate fermentation indicating the incorporation of intact acetate units.

$^{d}$ Percent coupling calculated as percentage of total signal for a given carbon atom involved in $^{13}$C-$^{13}$C coupling.

Carbon atom arbitrarily assigned a relative $^{13}$C enrichment of 1.0.

implies that erythrose 4-phosphate (11) formed from [1-$^{13}$C] pyruvate should only be labeled in position 2. On the other hand, the formation of erythrose 4-phosphate by the pentose phosphate cycle should contribute label to both C-1 and C-2 from [1-$^{13}$C]pyruvate. Thus, the mechanism of erythrose 4-phosphate formation in *M. thermoautotrophicum* needs further study.

The $^{13}$C labeling patterns of biosynthetic riboflavins (21)
Table IV
Assignment of the ^1H NMR signals of factor F_o measured in MeSO-d_6

| Position     | Chemical shift | Observed NMR connectivities | 2QF-COSY | ROESY |
|--------------|----------------|-----------------------------|-----------|--------|
| OH (phenolic)| 11.20 (s)      |                             |           |        |
| NH           | 10.93 (s)      |                             |           |        |
| 5            | 8.86 (s)       | 6                           | 7         |        |
| 6            | 8.00 (d)       | 5                           | 2'        |        |
| 9            | 7.37 (d)       | 1'                          | 1", 1', 2' |    |
| 7            | 7.01 (dd)      | 5                           | 1", 1', 2' |    |
| OH (3')      | 5.08 (d)       | 3'                          | 3", 4'    |        |
| OH (2')      | 4.92 (d)       | 2'                          |           |        |
| OH (4', 1')  | 4.76 (m)       | 4', 2', 1"                  | 4', 5', 7, 9 |    |
| 1"           | 4.65 (dd)      | 2', 1"                      | 7, 9      |        |
| OH (5')      | 4.44 (tr)      | 5', 5"                      |           |        |
| 2'           | 4.22 (m)       | 1', 1", OH (2'), 3'         | 7, 9      |        |
| 4', 5'       | 3.65 (m)       | OH (4'), 3'                 |           |        |
| 3'           | 3.57 (m)       | 4', 2'                      |           |        |
| 5"           | 3.45 (m)       | 5', 5'                      |           |        |

*Internal standard MeSO-d_6. ^1H signal at 2.49 ppm relative to tetramethylsilane.

Table V
Assignment of the ^13C NMR signals of factor F_o by DEPT, inverse ^1H-^13C coherence transfer experiments (HMQC and HMBC) and by INADEQUATE of the biosynthetic sample from [1,2-^13C_jacetate

| Position     | Chemical shift | DEPT | HMQC | HMBC transfer delay | INADEQUATE |
|--------------|----------------|------|------|---------------------|------------|
| ppm          |                |      | 8 Hz | 4 Hz                |            |
| 8            | 164.2 C        | 6, 9, 7 | 6, 9 |                    |            |
| 4            | 162.6 C        | 5    |      |                     |            |
| 10a          | 158.0 C        | 5, 1" |      |                     |            |
| 2            | 156.8 C        |      |      |                     |            |
| 9a           | 144.3 C        | 5, 6, 9, 1" | 5, 9 | 9, 5a              |            |
| 5            | 141.2 CH       | 5    | 6    | 6                   | 4a, 5a     |
| 6            | 133.7 CH       | 6    | 5    | 5                   | 5a, 7      |
| 7            | 115.3 CH       | 7    | 9    | 9                   | 6          |
| 5a           | 115.1 C        | 7, 9, 6, 5 | 5, 6 | 6, 5, 9a           |            |
| 4a           | 109.8 C        |      |      | NH                  | 5          |
| 9            | 102.2 CH       | 9    | 7    | 9a                  |            |
| 3'           | 74.6 CH        | 3'   | 4'   |                     |            |
| 4'           | 72.8 CH        | 4'   | 5'   |                     |            |
| 2'           | 69.2 CH        | 5'   |      |                     |            |
| 5'           | 63.3 CH        | 5', 5" | 4'  |                     |            |
| 1'           | 47.8 CH        | 1', 1", 2' |    |                     |            |

*Internal standard MeSO-d_6. ^13C signal at 39.5 ppm relative to tetramethylsilane.

are summarized in Table III and Fig. 6. The label distribution of the pyrimidine moieties in the purine nucleosides and in riboflavin are virtually identical. The ribitol side chain of riboflavin has the same ^13C labeling signature as the ribose moieties of the nucleosides.

The biosynthetic origin of the xylene ring of riboflavin has been established recently by studies with yeast and Bacillus subtilis (Bacher et al., 1983, 1985; Le Van et al., 1985; Volk and Bacher, 1988, 1990). The loss of C-4 from ribulose 5-phosphate (16, Fig. 6) by an intramolecular rearrangement yields the novel carbohydrate, Z-3,4-dihydroxy-2-butanone 4-phosphate (I7). Condensation with 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (18) yields 6,7-dimethyl-8-ribityllumazine (20). Subsequent dismutation of the lumazine yields riboflavin (21) as shown earlier by Plaut (1960, 1963). Thus, the xylene ring of the vitamin is ultimately

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**Fig. 7.** ^1H-decoupled ^13C NMR spectra of factor F_o (2, 7 mg in 0.4 ml of MeSO-d_6) isolated from M. thermoautotrophicum. a, natural ^13C abundance; b, from [2-^13C_jacetate; c, from [1,2-^13C_jacetate; d, from [1-^13C]pyruvate.**

**Fig. 8.** Part of the two-dimensional INADEQUATE spectrum of factor F_o (2, 7 mg in 0.4 ml of MeSO-d_6) obtained from the fermentation with [1,2-^13C_jacetate. For details see "Experimental Procedures." Inspection of the two-dimensional matrix at lower contour levels shows additional weak correlation patterns indicated in Table V. The presence of these signals with low intensity resulted from statistical recombination of ^13C. The one-dimensional ^13C NMR spectrum of factor F_o from the fermentation with [1,2-^13C_jacetate is shown at the top of the figure.
experiment, all proton signals could be assigned unambiguously. The NOE data obtained from the ROESY combination with the COSY data which revealed the directly correlated experiments optimized for this purpose (Table IV). The NMR spectrum of factor F0 was subsequently assigned by DEPT spectroscopy and inverse 'H-'C correlation experiments (Table V). Quaternary carbon atoms were assigned by multiple quantum multiple bond correlation experiments optimized for JCH and JCH.

13C NMR spectra of factor F0 samples from different fermentations are shown in Fig. 7. The presence of strong satellites in the sample from [1,2-13C2]acetate is due to 13C-13C coupling resulting from the incorporation of several intact acetate units. These are best observed in the INADEQUATE experiment shown in Fig. 8. Moreover, this experiment gave additional confirmation to the 13C NMR signal assignments.

A portion of the 'H NMR spectrum obtained with factor F0 from [1,2-13C2]acetate is shown in Fig. 9. The protons at C-5 and C-6 give rise to a singlet and a doublet, respectively. The 13C enrichments can be calculated from the 13C-coupled satellites of the proton signals. As shown in Table VI for selected examples, the enrichment values derived from 13C and 'H NMR measurements are in close agreement. The combined results of 13C incorporation into factor F0 are summarized in Table VII and Fig. 10.

A comparison of the labeling patterns of factor F0, riboflavin, and guanosine confirms immediately the common origin of the pyrimidine ring in all of these compounds. Not surprisingly, the ribityl chain of the deazaflavin cofactor shows the same labeling pattern as the ribityl chain of riboflavin and the ribose moiety of nucleosides. On the other hand, it is immediately obvious that the carbocyclic rings of riboflavin and deazaflavin have entirely different origins. The labeling pattern of the phenolic ring in the deazaflavin is characterized by a mirror symmetry that is indicated in Fig. 10 by a dashed line. This labeling pattern virtually duplicates that of tyrosine, thus indicating that the deazaflavin chromophore is derived from the shikimate pathway.

The question arises as to whether we can identify the specific shikimate derivative serving as the committed precursor. The labeling of the phenolic ring in factor F0 would be consistent with tyrosine (15), 4-hydroxyphenylpyruvate (14), or 4-hydroxybenzoate (23) as precursor. Prephenate (13, Fig. 5) is less likely as a precursor; as a consequence of its prochiral character, the labeling of factor F0 resulting from this precursor should not show symmetric label distribution in the carbocyclic ring.

Some additional information can be obtained from the analysis of the labeling pattern of C-5 of the deazaflavin chromophore. C-5 is labeled from [2-13C2]acetate and from [1,2-13C2]acetate but not from the other precursors studied. Although the labeling pattern of 4-hydroxybenzoate (23) could not be observed directly, it is obvious from the shikimate pathway that its carboxyl group should reflect the labeling of the carboxyl group of shikimate (12, Fig. 5) and not C-3 of pyruvate. It follows that C-5 of the deazaflavin chromophore cannot be derived from the carboxylic group of shikimate via 4-hydroxybenzoate. However, C-5 could well be derived from the aliphatic side chain of 4-hydroxyphenylpyruvate or tyrosine.

DISCUSSION

Complex labeling patterns result from feeding experiments with simple two- and three-carbon compounds as a consequence of the considerable number of reaction steps between the precursor and the product. Progress in NMR techniques and instrumentation enables the accurate experimental assessment of these complex labeling patterns. As shown in this paper, a rigorous interpretation is possible on the basis of comparison with the labeling patterns of a sufficiently large number of different metabolites.

The pyrimidine moieties of both riboflavin and deazaflavin are derived from a purine precursor. Moreover, we could show by studies with [1,2-13C2]adenine that the pyrimidine carbon atoms of the purine nucleotide precursor are transferred to-
TABLE VII
Relative 13C enrichments of factor F6 samples from cultures of M. thermoautotrophicum supplemented with 13C-labeled precursors

| Position | Chemical shift | 1Jx* Hz | % Coupling | Relative 13C enrichment |
|----------|----------------|---------|------------|-------------------------|
|          | ppm            |         |            | [1,2-13C2] Acetate | [2-13C2] Acetate | [1,2-13C2] Phosphate | [1-13C2] Pyruvate |
| 8        | 164.2          |         | 1.6        | 1.0                     | 1.1                     | 3.9                     |
| 4        | 162.6          |         | 1.0        | 1.0                     | 1.0                     | 1.2                     |
| 10        | 158.0          |         | 1.0        | 1.2                     | 1.1                     | 2.8                     |
| 2        | 156.8          |         | 1.0        | 1.2                     | 1.5                     | 1.1                     |
| a        | 144.3          | 69.0, 57.8 | 70        | 1.0                     | 3.3                     | 8.4                     | 1.2                     |
| 5        | 141.2          |         | 1.0        | 5.6                     | 7.9                     | 1.2                     |
| 6        | 133.7          | 58.3    | 9          | 1.0                     | 6.2                     | 8.0                     | 1.2                     |
| 7        | 115.3          | 59.9    | 68         | 2.9                     | 1.1                     | 5.5                     | 1.2                     |
| 5a       | 115.1          | 57.9    | 68         | 2.9                     | 1.1                     | 5.5                     | 1.2                     |
| 4a       | 109.8          |         | 3.3        | 1.2                     | 7.2                     | 1.2                     |
| 9        | 102.2          | 69.0    | 70         | 1.9                     | 1.0                     | 3.9                     | 1.1                     |
| 3'       | 74.0           |         | 1.0        | 1.0                     | 1.0                     | 2.9                     |
| 4'       | 72.8           | 41.6    | 78         | 2.3                     | 1.0                     | 6.5                     | 1.1                     |
| 2'       | 69.7           |         | 1.0        | 1.0                     | 1.0                     | 3.1                     |
| 5'       | 63.3           | 41.6    | 70         | 1.0                     | 5.4                     | 7.1                     | 1.0                     |
| 1'       | 47.8           |         | 2.4        | 1.0                     | 6.3                     | 1.2                     |

* *13C-13C couplings observed in the spectrum of the biosynthetic sample from the [1,2-13C2]acetate fermentation indicating the incorporation of intact acetate units.

* Percent coupling calculated as percentage of total signal for a given carbon atom involved in 13C-13C coupling.

* Carbon atom arbitrarily assigned a relative 13C enrichment of 1.0.

* Average due to multiplet overlapping.

It is well established that the biosynthesis of riboflavin starts from GTP (19, Fig. 6). Opening of the imidazole ring (Foor and Brown, 1975), reduction of the ribityl side chain, and deamination of the pyrimidine ring lead to 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione 5'-phosphate. Whereas the dephosphorylation of 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione 5'-phosphate is an obligatory step in the biosynthesis of flavins, it is conceivable that the condensation with a shikimate intermediate could proceed at the level of the ribitylaminopyrimidine phosphate. In this case, the biosynthetic pathway would lead directly to the FMN analog of the deazaflavin series.

**Fig. 10. Biosynthesis of the deazaflavin chromophore (2) in M. thermoautotrophicum.** For details see Fig. 2.
The benzenoid ring of the deazaflavin chromophore is derived from a shikimate derivative, 4-Hydroxybenzoate (23, Fig. 10) can be ruled out on the basis of the labeling pattern of C-5 of the chromophore. Prephenate appears unlikely as a precursor since one would expect nonsymmetric label distribution in the product. Thus, 4-hydroxyphenylpyruvate (14) and tyrosine (15) must be considered as potential precursors. It should be noted that 4-hydroxyphenylpyruvate in aqueous solution can undergo a spontaneous cleavage reaction yielding 4-hydroxybenzaldehyde (Doy, 1960). Conceivably, this reaction could be catalyzed enzymatically, and the aldehyde could serve as an intermediate in deazaflavin biosynthesis. However, 4-hydroxyphenylpyruvate could also serve directly as precursor.

The pyrimidine 18 (Fig. 11) is easily dehydrogenated. Wood and co-workers have observed the formation of a pyrimido[5,4-g]pteridine derivative (24, Scheme 1) from 18 in the presence of oxygen (Cresswell et al., 1960). This dimerization could proceed via oxidative formation of a quinoid product 25. We propose that such a quinoid species could react with the thermodynamically favorable enol of 4-hydroxyphenylpyruvate (26), resulting in the formation of the adduct 27. The subsequent elimination of NH₂ and oxalate could generate the mesomeric system 28. This intermediate could be cyclized to the deazaflavin chromophore by a two-electron oxidation step. This proposed sequence of events is as yet hypothetical and requires further confirmation.

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