# Biosynthesis of Methanopterin†

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ABSTRACT: The biosynthetic pathway for the generation of the methylated pterin in methanopterins was determined for the methanogenic bacteria *Methanococcus volta* and *Methanobacterium formicicum*. Extracts of *M. volta* were found to readily cleave L-7,8-dihydroneopterin to 7,8-dihydro-6-(hydroxymethyl)pterin, which was confirmed to be a precursor of the pterin portion of the methanopterin. [*methylene*-$^2$H]-6-(Hydroxymethyl)pterin was incorporated into methanopterin by growing cells of *M. volta* to an extent of 30%. Both the C-11 and C-12 methyl groups of methanopterin originate from [*methyl*-$^2H_3$]methionine, as confirmed by the incorporation of two $C^2H_3$ groups into 6-ethyl-7-methylpterin, a pterin-containing fragment derived from methanopterin. Cells grown in the presence of [*methylene*-$^2$H]-6-(hydroxymethyl)pterin, [*ethyl*-$^2H_4$]-6-[1(*RS*)-hydroxyethyl]pterin, [*methyl*-$^2H_3$]-6-(hydroxymethyl)-7-methylpterin, [*ethyl*-$^2H_4$, *methyl*-$^2H_3$]-6-[1(*RS*)-hydroxyethyl]-7-methylpterin, and [1-*ethyl*-$^3$H]-6-[1(*RS*)-hydroxyethyl]-7-methylpterin showed that only the non-7-methylated pterins were incorporated into methanopterin. Cells extracts of *M. formicicum* readily condensed synthetic [*methylene*-$^3$H]-7,8-$H_2$-6-(hydroxymethyl)-pterin-PP with methaniline to generate demethylated methanopterin, which is then methylated to methanopterin by the cell extract in the presence of *S*-adenosylmethionine. These observations indicate that the pterin portion of methanopterin is biosynthetically derived from 7,8-$H_2$-6-(hydroxymethyl)pterin, which is coupled to methaniline by a pathway analogous to the biosynthesis of folic acid. This pathway for the biosynthesis of methanopterin represents the first example of the modification of the specificity of a coenzyme through a methylation reaction.

Current biosynthetic evidence indicates that methanopterin (Figure 1), a coenzyme involved in $C_1$ metabolism in the methanogenic archaebacteria (Jones et al., 1987), is the first example of a naturally occurring, structurally modified folic acid (White, 1985a, 1986a,b) that functions as a coenzyme in cells. Despite these structural modifications, current evidence indicates that, biochemically, methanopterin functions in these bacteria in the same way folic acid does in other cells. In fact, since these cells appear to lack folic acid (Leigh, 1983; Worrell & Nagle, 1988), it is likely that methanopterin performs all the normal functions of folic acid in the methanogenic archaebacteria. Thus, methanopterin, not folic acid, serves as the $C_1$ carrier for serine hydroxymethyltransferase (Hoyt et al., 1986), 5,10-methylenetetrahydromethanopterin dehydrogenase (Hartzell et al., 1985), and 5,10-methenyltetrahydromethanopterin cyclohydrolase (Donnelly et al., 1985; DeMarco, 1986). In order to understand how this change in coenzyme structure arose, the details of the steps involved in the biosynthesis of the methylated pterin portion of methanopterin were explored. The results of this work are outlined herein.

## Materials and Methods

*Materials.* Folic acid, 6-(hydroxymethyl)pterin (**1**),[1] *S*-adenosylmethionine (**2**), and [$^2H_2$]water (99.8 atom % $^2$H) were purchased from Sigma Chemical Co. L-Neopterin (**3**) and pyrophosphoric acid were obtained from Fluka Chemical Co.

*Labeled Compounds.* [$^2H_4$]Sodium borohydride was obtained from Aldrich Chemical Co. [$^3H_4$]Sodium borohydride (>100 mCi/mmol) and [*methyl*-$^3H_3$]-*S*-adenosylmethionine (15 Ci/mmol) were supplied by Du Pont Co., NEN Research Products, Boston, MA.

*Chemical Syntheses.* (*A*) *Synthesis and Purification of 7,8-Dihydropterins.* Neopterin (**3**) and 6-(hydroxymethyl)-pterin (**1**) (~1 mg) were dissolved in 1 mL of water, reduced with sodium hydrosulfite, and purified by chromatography on Sephadex G-25 as described by Goto and Sugiura (1971), except that 0.05 mM mercaptoethanol was used instead of water as the eluting solvent. The 7,8-$H_2$-pterins were quantitated and identified by their UV absorbances at 257, 275, and 362 nm when dissolved in 0.1 M HCl.

(*B*) *Chemical Synthesis of Phosphorylated 7,8-Dihydropterins.* The mixture of phosphorylated and pyrophosphorylated 6-(hydroxymethyl)pterins were prepared by heating 6-(hydroxymethyl)pterin (**1**) with pyrophosphoric acid as described by Ho (1980). The phosphorylated pterins in the final synthetic product were assayed by separation on a DEAE-Sephadex column using an $NH_4HCO_3$ gradient as described below for the isolation of methanopterin. The pterins were quantitated by their absorbance at 344 nm, and the phosphorylated pterins were identified by their phosphate to pterin ratios after liberation of the phosphate by hydrolysis

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[1] The compounds described in this paper are identified by a number–letter code. Each oxidized pterin is assigned a different number. A single prime (′) after the number indicates that the pterin is in the form of a 7,8-dihydropterin, and a double prime (″) indicates that the pterin is in the form of a 5,6,7,8-tetrahydropterin. A "d" following the number indicates that the pterin is labeled with deuterium, "t" indicates that the pterin is labeled with tritium, "p" indicates that the pterin is in the form of a phosphate ester, and "pp" indicates that the pterin is in the form of a pyrophosphate ester. The numbers assigned to the different compounds are 6-(hydroxymethyl)pterin (**1**), *S*-adenosylmethionine (**2**), L-neopterin (**3**), 6-[1(*RS*)-hydroxyethyl]pterin (**4**), 6-(hydroxymethyl)-7-methylpterin (**5**), 6-[1(*RS*)-hydroxyethyl]-7-methylpterin (**6**), demethylated methanopterin (**7**), C-7 demethylated methanopterin (**8**), methanopterin (**9**), and methaniline (**10**).

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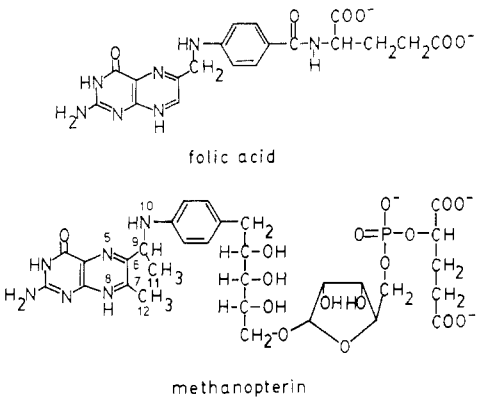


FIGURE 1: Chemical structures of folic acid and methanopterin.

in 3 M HCl. The phosphorylated pterins were reduced to dihydropterins by reduction with sodium hydrosulfite in ascorbic acid solution (Friedkin et al., 1962). [*methylene*-$^3$H]-7,8-$H_2$-6-(hydroxymethyl)pterin-PP (**1′tpp**) was prepared from [*methylene*-$^3$H]-6-(hydroxymethyl)pterin (**1t**) (see section C) as described by Ho (1980).

(*C*) *Synthesis of* [*methylene*-$^2$*H*]-6-(*Hydroxymethyl*)*pterin* (**1d**) *and* [*methylene*-$^3$*H*]-6-(*Hydroxymethyl*)*pterin* (**1t**). 6-Formylpterin (200 mg) [generated by cleavage of folic acid with bromine in HBr as described by Thijssen (1973)] was suspended in 10 mL of $^2H_2O$ (99.8 atom % $^2$H) and dissolved by the addition of 100 μL of 10 M NaO$^2$H (99+ atom % $^2$H). Sodium borohydride (300 mg) (98 atom % $^2$H) was then dissolved in the solution, and after 2 h at room temperature, the red, cloudy solution was filtered and adjusted to pH 4 by the addition of 3 M HCl. After the resulting suspension of pterin was stored overnight at 3 °C, the brown precipitate of [*methylene*-$^2$H]-6-(hydroxymethyl)pterin (**1d**) was removed by centrifugation, washed with water, and dissolved in ~2 mL of 1 M HCl. The HCl solution was decolorized by treatment with Norit, filtered to remove the Norit, and adjusted to pH 4 by the addition of 4 M ammonium hydroxide. The resulting precipitated pterin gave a single thin-layer chromatography (TLC) spot with an $R_f$ of 0.37 in solvent system 1 and had absorbance maxima at 363, 253, and 264 (s) nm in 0.1 N NaOH. The $(TMS)_2$ derivative of the pterin showed an $M^+$ ion at $m/z$ 410 and an $M^+ - 15$ ion at $m/z$ 395, thereby proving that the molecule contained one $^2$H. From the ratio of the intensities of the $M^+$ and $M^+ + 1$ ions, the pterin was determined to be labeled to an extent of >95% with a single $^2$H.

[*methylene*-$^3$H]-6-(Hydroxymethyl)pterin (**1t**) was prepared by the reduction of 6-formylpterin, prepared as described above and purified as described by Waller et al. (1950), with Na$B^3H_4$. Thus, to 8 mg of the 6-formylpterin dissolved in 0.6 mL of 1 M NaOH was added 1 mCi of Na$B^3H_4$ in 0.01 M NaOH. After 15 min at room temperature, 5 mg of $NaBH_4$ was added in 0.4 mL of 1 M NaOH, and after an additional 30 min, acetic acid was added to precipitate the pterin which was then washed with water and dried.

(*D*) *Synthesis of* [*ethyl*-$^2H_4$]-6-[*1*(*RS*)-*Hydroxyethyl*]*pterin* (**4d**). The synthesis and purification of **4** was as described by Sugimoto and Matsuura (1979) for the preparation of the *R* and *S* isomers of 6-(1-hydroxyethyl)pterin, except that racemic 2,3-dihydroxybutanal was added directly to the reaction mixture. The 2,3-dihydroxybutanal was prepared by the hydration of 2,3-epoxybutraldehyde, which was generated by the epoxidation of crotonaldehyde as described by Payne (1961).

An attempt to introduce deuterium into **4** by exchange of the C-1 proton of the ethyl side chain with 28% $N^2H_3$ in $^2H_2O$ at 100 °C showed the compound to be chemically stable to these conditions but to undergo no exchange at this position. In contrast, 6-acetylpterin, formed by the oxidation of **4** with chromium trioxide, was shown to be readily labeled under these conditions and to be reduced to the desired product with $NaBH_4$. Thus, 200 mg of **4** was dissolved in 6 mL of 1 M HCl, and after the addition of 0.55 g of $CrO_3$ in 5 mL of 1 M HCl, the resulting solution was heated at 80 °C for 5 min. (TLC of the products showed the pterin to be completely oxidized to 6-acetylpterin.) After an additional hour at room temperature, 0.5 mL of 2-propanol was added and the pH of the solution was adjusted to 3–4 by the addition of 1 M NaOH. The resulting precipitate of 6-acetylpterin was washed with water and dried to give 165 mg of product, which was then placed in 2 mL of 14% $N^2H_3$ in $^2H_2O$, and the solvent was evaporated to remove the rapidly exchangeable NH protons. The resulting product was dissolved in 7.5 mL of the same deuteriated ammonia solution and heated at 100 °C for 1 h in a sealed tube to exchange the methyl hydrogens for deuterium. After evaporation of the solvent, the sample was dissolved in 5 mL of $^2H_2O$ containing 64 μL of 40% NaO$^2$H, and 300 mg of Na$B^2H_4$ was added. After 6 h at room temperature, the pH of the solution was adjusted to 3–4 with 1 M HCl, and the precipitated pterin was washed with water and dissolved in ~2 mL of 1 M HCl. The resulting solution was decolorized with Norit, and the product (**4d**) was precipitated by adjusting the pH of the solution to 3–4 with 4 M ammonium hydroxide. The final pterin (**4d**) (37 mg) had absorbance maxima at 362, 254, and 274 (s) nm when dissolved in 0.1 M NaOH. The $(TMS)_3$ derivative gave $M^+$ $m/z$ 427 and $M^+ - 15$ $m/z$ 412 ions, indicating that the molecule had incorporated four deuteriums. The pterin was determined to be >95% $^2H_4$ from the intensities of the isotope ions of both the $M^+$ and the $M^+ - 15$ ions.

(*E*) *Synthesis of* [*7-methyl*-$^2H_3$]-6-(*Hydroxymethyl*)-*7-methylpterin* (**5d**). 6-(Bromomethyl)-7-methylpterin was generated by the reduction of 6,7-bis(bromomethyl)pterin as described by Boothe et al. (1952). The 6-(bromomethyl)-7-methylpterin was hydrolyzed to **5** by heating with 1 M NaOH at 100 °C for 3 h. Pterin **5** was precipitated from the reaction by the addition of acetic acid and purified by dissolving in 6 M HCl, decolorizing with Norit, and reprecipitating by the addition of aqueous ammonia. The mass spectrum of the TMS derivative of **5** had the expected ions at $M^+$ $m/z$ 423 and $M^+ - 15$ $m/z$ 408. Pterin **5** was labeled by exchange of the 7-methyl protons with $^2H_2O$. This was accomplished by heating 35 mg of **5**, dissolved in 2.2 mL of 5% $N^2H_3$ in $^2H_2O$, at 100 °C for 5.5 h. After the solution was decolorized with Norit, evaporation of the solution gave 14.5 mg of [*7-methyl*-$^2H_3$]-6-(hydroxymethyl)-7-methylpterin (**5d**) with absorbance maxima at 360, 254, and 276 (s) nm when dissolved in 0.1 M NaOH. The mass spectrum of the TMS derivative of **5d** showed an $M^+$ ion at $m/z$ 426 and an $M^+ - 15$ ion at $m/z$ 411, indicating that three deuteriums were incorporated into the molecule. From the intensities of the isotope ions, the final product was calculated to be >95% $^2H_3$.

(*F*) *Synthesis of* [*ethyl*-$^2H_4$,*methyl*-$^2H_3$]-6-[*1*(*RS*)-*Hydroxyethyl*]-*7-methylpterin* (**6d**) *and* [*1-ethyl*-$^3$*H*]-6-(*1-Hydroxyethyl*)-*7-methylpterin* (**6t**). 6-Acetyl-7-methylpterin was prepared by the condensation of triketopentane (Dayer et al., 1974) with 2,4,5-triamino-6-hydroxypyrimidine as described by Sugiura and Goto (1969). The protons of both methyl groups of this pterin were labeled with deuterium by heating 100 mg of pterin with 2 mL of $^2H_2O$ and 3.5 mL of 28% $N^2H_3$ at 100 °C for 18 h. After evaporation of the

solvent, the resulting residue, as the $(TMS)_3$ derivative, had an $M^+$ at $m/z$ 369 and an $M^+ - 15$ at $m/z$ 354, indicating that the molecule contained six atoms of deuterium. The residue was suspended in 5 mL of $^2H_2O$ and dissolved by the addition of 20 μL of 40% $NaO^2H$. Reduction was effected by the addition of 154 mg of $NaB^2H_4$ (98 atom % $^2H$). After 1.5 h, the solution was filtered and adjusted to pH 4 by the addition of acetic acid. The resulting yellow precipitate was washed with water and dried to give 92.5 mg of material which showed absorbance maxima at 366, 262, and 284 (s) nm in 0.1 N NaOH. The $(TMS)_3$ derivative had ions at $M^+$ $m/z$ 444 and $M^+ - 15$ $m/z$ 429, indicating that most of the molecules had incorporated seven atoms of deuterium. From the isotope ion intensities for both the $M^+$ and $M^+ - 15$ ions it was calculated that >88% of the **6d** molecules contained seven deuteriums.

[1-*ethyl*-$^3$H]-6-(1-Hydroxyethyl)-7-methylpterin (**6t**) was prepared by the reduction of 6-acetyl-7-methylpterin by adding 70 μL of a 0.2 mM solution of $NaB^3H_4$ (500 mCi/mmol) to 8 mg of the pterin dissolved in 440 μL of 0.1 M NaOH. After 5 h at room temperature, the solution was acidified with acetic acid and the precipitated **6b** was washed with water.

*Isolation of Methanopterin from Cells. M. volta* cells (1–2 g wet wt) were heated at 100 °C in 5 mL of water for 30 min and centrifuged to remove the insoluble cellular material. Then, either the methanopterin in the resulting extract was cleaved directly to 6-ethyl-7-methylpterin (see below) after concentrating the extract to 0.5 mL or the methanopterin was isolated by column chromatography on a DEAE-Sephadex column (1 × 25 cm) using a linear gradient of $NH_4HCO_3$ as previously described (Leigh & Wolfe, 1983; White, 1988b). The methanopterin peak was identified by its absorbance at 342 nm and by the change in its fluorescence emission intensity at 440 nm with excitation at 360 nm with changing pH (Keltjens et al., 1983). [On the basis of its position of elution from the DEAE column and on TLC evidence, the methanopterin isolated from *M. volta* appears to be the monoglutamyl derivative of methanopterin (White, unpublished data). This compound was first isolated from *Methanosarcina barkeri* and assigned the name sarcinapterin (Van Beelen et al., 1984).]

*Cleavage of Methanopterin and Gas Chromatographic–Mass Spectrometric (GC–MS) Analysis of the Resulting 6-Ethyl-7-methylpterin.* The pterin portion of the methanopterin was isolated as 6-ethyl-7-methylpterin by Zn/HCl cleavage of either the crude hot water cell extract or the DEAE-Sephadex-purified methanopterin by a procedure similar to one developed for the cleavage of folic acid (Foo et al., 1980). In either case, the samples were dissolved in 0.5 mL of water, mixed with 0.5 mL of 1 M HCl and 0.1 mL of a suspension of Zn dust (0.5 g in 1 mL of 0.5% gelatin), and, after shaking for 8 min, centrifuged to remove the insoluble material. The pellet was washed with 0.5 mL of 0.5 M HCl, and 1 M NaOH was added to the combined clear aqueous layers until the first appearance of a $Zn(OH)_2$ precipitate. After the pH of the solution was adjusted to 4.5 by the addition of acetic acid, the precipitate was redissolved and the resulting clear solution was applied to a $C_{18}$ Sep-Pak cartridge. After the column was washed with water, the pterins were eluted with methanol. (The elution of the fluorescent pterins can easily be monitored by using a fluorescent lamp.) After evaporation of the methanol, the resulting residue was converted to the TMS derivative for GC–MS analysis. The TMS derivatives were separated on a 2 mm × 0.9 m glass column containing 3% OV-1 on 80/100 Supelcoport (Supelco Inc., Bellefonte, PA) programmed from 100 °C at 10 °C/min.

Reduction of a sample of **6d** by the above procedure produced 6-ethyl-7-methylpterin with retention of all of the deuteriums of the original pterin, indicating that no loss of deuterium occurred during the reduction. This experiment also shows the importance of separating all of the labeled **6** from the methopterin before the reductive cleavage step since this labeled pterin gives the same pterin product as methanopterin.

*Formation of TMS Derivatives of Pterins.* Pterins were converted to their TMS derivatives by reaction with 20 μL of an equal mixture of bis(trimethylsilyl)acetamide and pyridine for 15 min at 100 °C in a sealed vial.

*Thin-Layer Chromatographic Separation and Analysis of Pterins.* The pterins were both analyzed and purified by preparative TLC on precoated silica gel 60 F-254 TLC plates (E. Merck, Darmstadt, West Germany) with either solvent system 1 [acetonitrile–water–formic acid (88%) (40:10:5 v/v/v)] or solvent system 2 [1-butanol–acetic acid–water (12:3:5 v/v/v)]. The pterins were identified by their fluorescence on TLC plates when exposed to ultraviolet light.

*Incubation of Cell Extract with [methyl-$^3H_3$]-S-Adenosylmethionine (**2t**) and a Mixture of 7,8-$H_2$-6-(hydroxymethyl)pterin-P (**1'p**) and 7,8-$H_2$-6-(hydroxymethyl)-pterin-PP (**1'pp**).* A cell-free extract of *M. formicicum* was generated as described below but with a buffer containing 0.1 M tris instead of 50 mM 2-[[tris(hydroxymethyl)methyl]-amino]ethanesulfonic acid (TES). To 2.2 mL of the extract (~15 mg of protein/mL) were added 22 μL of 10 mM SAM, 10 μL of [*methyl*-$^3H_3$]SAM (**2t**) (0.55 mCi/mL, 15 Ci/mmol), and 180 μL of a solution 10 mM in 7,8-$H_2$-6-(hydroxymethyl)pterin-P (**1'p**) and 25 mM in 7,8-$H_2$-6-(hydroxymethyl)pterin-PP (**1'pp**). After incubation at 37 °C for 1 h, an equal volume of 10% trichloroacetic acid was added, and after removal of the precipitated proteins by centrifugation, the remaining trichloroacetic acid was removed by extraction (3×) with an equal volume of diethyl ether. The resulting aqueous solution was applied to a DEAE-Sephadex column which was eluted with a linear gradient of sodium chloride as previously described (White, 1988b). This chromatographic procedure readily separated all of the desired compounds, including noncharged 6-(hydroxymethyl)pterin, 6-(hydroxymethyl)pterin-P, 6-(hydroxymethyl)pterin-PP, SAM, and methanopterin, from each other. The fractions containing each of the two phosphorylated pterins were combined, and the pterins were adsorbed onto Norit (Ho, 1980). After the Norit was washed with water, the pterins were eluted with 3 M ammonia and purified by TLC.

*Incubation of Cell Extract with [methyl-$^3H_3$]-S-Adenosylmethionine (**2t**) and 7,8-$H_2$-6-(hydroxymethyl)pterin (**1'**).* To a cell extract of *M. volta* (0.9 mL, ~20 mg of protein/mL), prepared as described below, was added 50 μL of a 1.43 mM solution of 7,8-$H_2$-6-(hydroxymethyl)pterin (**1'**), 10 μL of 50 mM SAM, and 20 μL of [*methyl*-$^3H_3$]SAM (0.55 mCi/mL, 15 Ci/mmol). The reaction mixture was incubated for 3 h at 37 °C, after which 0.24 mL of 2 M TCA was added, and after centrifugation to remove the precipitated protein, 150 μL of a 0.2 M TCA solution (0.5% in $I_2$, 1% in KI) was added (Fukushima & Nixon, 1980). After 1 h at room temperature, 100 μL of 1 M sodium acetate was added and the pH was adjusted to 5.0. The resulting oxidized pterins were adsorbed onto a $C_{18}$ Sep-Pack cartridge (Waters Associates, Milford, MA), which was washed with water, and the pterins were eluted with methanol. The methanol-eluted pterin was mixed with carrier **6** dissolved in 0.1 M phosphate buffer (pH

Table I: Incorporation of Deuteriated Pterins into the 6-Ethyl-7-methylpterin Fragment Derived from the Methanopterin Biosynthesized by *M. volta*[a]

| pterin fed | level (mg/500 mL) | distribution of $^2H$[b] | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | 7 |
| [*methylene*-$^2$H]-6-(hydroxymethyl)pterin (**1d**) | 3.8 | 70.7 | 29.3[c] | 0.0 | | | |
| [*ethyl*-$^2H_4$]-6-[1(*RS*)-hydroxyethyl]pterin (**4d**) | 30 | 91.0 | 0.0 | 0.0 | 1.8 | 7.2 | |
| [*methyl*-$^2H_3$]-6-(hydroxymethyl)-7-methylpterin (**5d**) | 14.4 | 100 | 0.0 | 0.0 | 0.0 | | |
| [*ethyl*-$^2H_4$,*methyl*-$^2H_3$]-6-[1(*RS*)-hydroxyethyl]-7-methylpterin (**6d**) | 30 | 100 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

[a] Samples were measured by GC–MS of the $(TMS)_2$ derivative of 6-ethyl-7-methylpterin. The $(TMS)_2$ derivative of an unlabeled sample of 6-ethyl-7-methylpterin had intensities of 100, 28.8, 11.3, and 2.1 for the $M^+$, $M^+ + 1$, $M^+ + 2$, and $M^+ + 3$ and 100, 28.3, 13.7, and 2.7 for the $(M^+ - 15)$, $(M^+ - 15) + 1$, $(M^+ - 15) + 2$, and $(M^+ - 15) + 3$, respectively. [b] Expressed as the percent of the total molecules containing the indicated number of $^2H$. The average values for the $M^+$ $m/z$ 349 and $M^+ - 15$ $m/z$ 334 are reported. The measured normalized intensities for the $M^+$, $M^+ + 1$, $M^+ + 2$, $(M^+ - 15)$, $(M^+ - 15) + 1$, and $(M^+ - 15) + 2$ from the experiment using **1d** were 100, 71.4, 22.4, 100, 68.5, and 21.2, respectively. The measured normalized intensities for the $M^+$, $M^+ + 3$, $M^+ + 4$, $(M^+ - 15)$, $(M^+ - 15) + 3$, and $(M^+ - 15) + 4$ for the experiment using **4d** were 100, 4.8, 8.2, 100, 4.0, and 9.0, respectively. The measured normalized intensities for the $M^+$, $M^+ + 1$, $M^+ + 2$, $M^+ + 3$, $(M^+ - 15)$, $(M^+ - 15) + 1$, $(M^+ - 15) + 2$, and $(M^+ - 15) + 3$ for the experiment using **5d** were 100, 30.4, 11.5, 2.7, 100, 28.8, 11.3, and 2.6, respectively. No ion intensities were recorded for the $M^+ + 7$ and $(M^+ - 15) + 7$ in the experiment using **6d**. [c] It should be noted that the true incorporation is actually twice this value since the single deuterium is randomly incorporated at the two asymmetric proton sites on the hydroxymethyl side chain and only one of the isomers would be expected to be incorporated with the retention of the deuterium.

5.8), and **6** was isolated by column chromatography on Sephadex G-25 with water as the eluting solvent (Dewey & Kidder, 1967). (This column procedure did not separate the different methylated pterins, but it did cleanly separate the SAM and the salts from the pterins.) The fractions containing **6** were combined, and the pterins were purified by two separate preparative TLC separations prior to counting. The first separation was effected by using solvent system 1 and the second by using solvent system 2. Areas of the plates containing **4**–**6** were removed and counted to establish if any monomethylated or dimethylated pterins were produced.

*Enzymatic Conversion of 7,8-$H_2$-neopterin (**3'**) to 7,8-$H_2$-6-(hydroxymethyl)pterin (**1'**).* 7,8-$H_2$-L-neopterin (20 μL of a 3.94 mM solution) was added to 1 mL of a cell extract of *M. volta*, and the resulting solution was incubated at 39 °C for 3 h under a hydrogen atmosphere. At the end of incubation, 0.27 mL of 2 M trichloroacetic acid was added to the reaction, and after centrifugation to remove the precipitated protein, 175 μL of a 0.2 M TCA solution (0.5% in $I_2$, 1% in KI) was added. After 1 h at room temperature, 50 μL of 1 M phosphate buffer (pH 5.8) was added, the pH was adjusted to 4.9, and the oxidized pterins were isolated and analyzed as described above.

*Incubation of Cell Extract with [methylene-$^3H$]-7,8-$H_2$-6-(hydroxymethyl)pterin-PP (**1'tpp**) and Methaniline (**10**).* To 2 mL of a cell extract of *M. formicicum* (12.6 mg of protein/mL) were added 50 μL of a solution 2.5 mM in [*methylene*-$^3$H]-7,8-$H_2$-6-(hydroxymethyl)pterin-PP (**1'tpp**) (0.4 Ci/mmol) and 50 μL of a 6.2 mM solution of methaniline (**10**).[2]

The resulting solution was shaken at 39 °C for 3 h under a $H_2/CO_2$ atmosphere and then heated for 6 min at 100 °C. After cooling, the $H_2/CO_2$ atmosphere was replaced with oxygen and the sample was shaken for an additional 8 h in order to effect the oxidation of the dihydropterins. After centrifugation to remove the denatured proteins, the sample was separated on the DEAE-Sephadex column using the same conditions described above for the isolation methanopterin.

*Bacterial Strains and Growth Conditions.* *M. volta* strain P.S. (DSM 1537) and *M. formicicum* JF-1 were used for most of the work described herein. Each bacterial strain was grown at 39 °C in 2-L bottles pressurized to 30 psi with $H_2/CO_2$ (80/20) on 500 mL of medium. The medium described by Whitman et al. (1982) was used for the growth of *M. volta*, and the medium described by Schauer and Ferry (1980) was used for the growth of *M. formicicum* JF-1. For those experiments where *M. volta* and *M. formicicum* cells were grown in the presence of different pterins, the pterins were added to the anaerobic medium prior to autoclaving. After autoclaving, the medium was allowed to cool to 37 °C before inoculating with a 10–20% volume of cells actively growing on a medium of the same composition but without the pterin. The cells were isolated from the medium by centrifugation; if the extraction of the methanopterin or the preparation of a cell extract was not carried out within a few minutes, the cell pellets were stored at −20 °C under nitrogen.

*Preparation of Cell Extracts.* These procedures involved sonication of cell suspensions under nitrogen in an anaerobic buffer (pH 7.5) consisting of 50 mM TES, 10 mM $MgCl_2$, and 2 mM mercaptoethanol as previously described (White, 1988a).

## Results

*Growth of M. volta with [methyl-$^2H_3$]Methionine.* That both methyl groups of methanopterin are derived from methionine was established by growing *M. volta* with [*methyl*-$^2H_3$]methionine (0.3 mg/mL) and measuring the extent of deuterium incorporation into the methanopterin-derived 6-ethyl-7-methylpterin. The $M^+ - 15$ $m/z$ 349 fragment in the mass spectrum of the $(TMS)_2$ derivative of this methylated pterin showed 40.6% of the molecules with no label, 39.0% with one $C^2H_3$, and 20.4% with 2 $C^2H_3$. On the basis of the observed distribution of deuteriated methyl groups and on the assumption that the [*methyl*-$^2H_3$]-methionine is incorporated into both the methyl groups to the same extent, it was calculated that 83% of the methanopterin was biosynthesized by the cells during the incubation period and that each methyl group incorporated the labeled methionine to an extent of 51%. The remaining 17% of the molecules, which would have been produced by the cells when grown in the absence of the labeled methionine, can be accounted for by the presence of unlabeled methanopterin in the ~20% inoculum used to start the culture.

*Growth of M. volta with Deuteriated Pterins.* The C-7 and C-9 methylations can occur either with **1'** or at an intermediate

[2] Methaniline [4-[5'-*O*-(5''-phosphono-α-D-ribofuranosyl)-2',3',4',5'-tetrahydroxypent-1'-yl]aniline in which the phosphate group is esterified to α-hydroxyglutaric acid] (**10**), the side-chain structure of methanopterin, is produced by the oxidative cleavage of tetrahydromethanopterin. It was purified from hot water extracts of *M. formicicum* by DEAE-Sephadex chromatography as described in the text for the isolation of methanopterin. The fractions containing the methaniline were identified by using the fluorescamine assay for primary arylamines as described by White (1988b).

that can be derived from **1'**, since **1d** is incorporated into methanopterin to the extent of ~29% when fed to growing cells of *M. volta* (Table I). The C-7 (**5d**) and C-9 (**4d**) monomethylated 6-(hydroxymethyl)pterins and the C-7 and C-9 dimethylated pterin (**6d**) were also fed to *M. volta*. The C-9 methylated pterin (**4d**) was incorporated into the methanopterin to an extent of 7%, whereas the C-7 methylated pterins (**5d** and **6d**) were not incorporated to an extent of greater than 0.02%, the detection limit of the GC–MS procedure used in this analysis (Table I).[3]

*Growth of M. volta and M. formicicum with [1-ethyl-$^3H$]-6-[1(RS)-Hydroxyethyl]-7-methylpterin* (**6t**). The incorporation of **6t** into methanopterin by growing cells of *M. volta* and *M. formicicum* was found to be <0.0004%.

*Incubation of Cell Extract with [methyl-$^3H_3$]-S-Adenosylmethionine* (**2t**) *and a Mixture of 7,8-$H_2$-6-(hydroxymethyl)pterin-P* (**1'p**) *and 7,8-$H_2$-6-(hydroxymethyl)-pterin-PP* (**1'pp**). The methylated pterins which may have been produced in this incubation would consist of the monomethylated derivatives of 7,8-$H_2$-6-(hydroxymethyl)pterin-P or 7,8-$H_2$-6-(hydroxymethyl)pterin-PP containing a methyl group at either C-7 or C-9 or the dimethylated derivatives of 7,8-$H_2$-6-(hydroxymethyl)pterin-P or 7,8-$H_2$-6-(hydroxymethyl)pterin-PP containing methyl groups at both C-7 and C-9. Synthetic samples of these methylated pterin phosphate and pyrophosphate esters were found to elute from the DEAE-Sephadex column at the same positions as the corresponding nonmethylated pterin phosphate and pyrophosphate esters. Thus, the **1'p** and **1'pp** added to the incubation mixture not only served as potential substrates for the reaction but also served as markers to identify where the methylated pterins elute from the column. Their recovery from the incubation mixture as the oxidized pterins also demonstrates that the procedure used to isolate the oxidized pterins worked and that the pterin phosphates were stable under the incubation conditions. Analysis of the DEAE-Sephadex fractions from this incubation showed the presence of **1p** and **1pp** and that these fractions contain no detectable tritium associated with the pterins. On the basis of the specific activity of the [*methyl*-$^3H_3$]SAM used in the reaction and on the basis of the amount of the phosphorylated pterins present in the reaction, and assuming that at least 50% of the pterins were isolated from the incubation mixture, then less than $(14.2 \times 10^{-5})$% of **1'p** and less than $(5.6 \times 10^{-5})$% of **1'pp** could have been converted to a methylated pterin by the cell extract.

*Incubation of Cell Extract with [methyl-$^3H_3$]-S-Adenosylmethionine* (**2t**) *and 7,8-$H_2$-6-(hydroxymethyl)pterin* (**1'**). The expected methylated dihydropterins generated in this incubation were oxidized with $I_2$, and carrier 6-(1-hydroxyethyl)-7-methylpterin (**6**) was added. The **6** was then isolated from the reaction by four separate steps of purification to assure the purity of the product prior to determining its radioactivity. No detectable radioactivity was found in the final purified product. Assuming that 50% of the pterins were recovered from the incubation mixture, then <0.026% of **1'** would have been converted to **6** by the cell extract. Fractions containing the monomethylated pterins **4** and **5** were also found to contain no detectable radioactivity.

*Enzymatic Conversion of 7,8-$H_2$-neopterin* (**3'**) *to 7,8-$H_2$-6-(hydroxymethyl)pterin* (**1'**). Analysis (TLC) of the isolated pterins generated by the incubation of an *M. volta* extract with **3'** using solvent systems 1 and 2 showed the complete conversion of **3'** to **1'**. The identity of the **1** formed by the oxidation of the product of the reaction, **1'**, was confirmed by GC–MS of the $(TMS)_3$ derivative, which gave a peak with the same retention time and mass spectrum as a known sample of **1** showing an $M^+$ ion at *m/z* 409 and an $M^+ - 15$ ion at *m/z* 394. A control experiment, conducted without cell extract, showed only the recovery of neopterin.

*Incubation of Cell Extract with [methylene-$^3H$]-7,8-$H_2$-6-(hydroxymethyl)pterin-PP* (**1'tpp**) *and Methaniline* (**10**). Incubation of a cell extract of *M. formicicum* with **1'tpp** and **10** was found to readily lead to the production of demethylated methanopterin (**7't**) with 17% of the substrate being converted to this product. Measurement of the absorbance at 353 nm in the fractions eluting from the DEAE-Sephadex column used to separate the reaction products showed three major peaks which, on the basis of their fluorescent spectra and their position of elution, were determined to be **1t**, **1tpp**, and **9t**. These compounds eluted in the order given with an increasing concentration of $NH_4HCO_3$. Three major radioactive peaks were observed, the first coinciding with the elution position of **1** and the second coinciding with the elution position of **1pp**. The third radioactive peak, eluting at a slightly higher $NH_4HCO_3$ concentration than the methanopterin peak, was determined to be demethylated methanopterin (**7**) on the basis of the following observations. It eluted from the DEAE-Sephadex column at almost the same position as methanopterin, which is consistent with the total charge on the molecule and, therefore, the structure of the side chain being the same as that of methanopterin. Also, cleavage with mild acid (5 min, 1 M HCl) gave a single radioactive fragment with almost the same $R_f$ on TLC as the HP-1 fragment derived from methanopterin under the same hydrolytic conditions (Van Beelen et al., 1984). Finally, reductive cleavage of the material with Zn/HCl generated 6-methylpterin as the major pterin fragment (95% of the total pterin produced). A small amount of 6-ethyl-7-methylpterin (5%) was also observed, apparently resulting from the production of a small amount of methanopterin during incubation. No indication of a monomethylated methanopterin, which would have generated either 6-ethylpterin or 6,7-dimethylpterin during the reductive cleavage, was found.

*Conversion of Demethylated [$^3H$]Methanopterin* (**7t**) *to Methanopterin* (**9'**) *by Cell Extracts*. The **7t** generated in the above experiment and purified by DEAE-Sephadex column chromatography was further purified by preparative TLC and incubated with 1 mL of the same cell extract of *M. formicicum* as used above but containing 5 mM SAM. After incubation for 3 h at 39 °C, the reaction was fractionated as described above and the isolated methanopterin subjected to the Zn/HCl cleavage procedure used for the cleavage of methanopterin. (The production of a reduced form of methanopterin during this incubation was inferred by the isolation of methanopterin after the air oxidation of the reaction products.) TLC analysis of the pterin products, generated by a reductive cleavage, showed 6-ethyl-7-methylpterin to be 65% of the product and 6-methylpterin to be 35% of the product. No indication of the occurrence of 6,7-dimethylpterin or 6-ethylpterin, the cleavage products from monomethylated methanopterins, was found.

A summary of the incubations of cell extracts with the different labeled precursors is presented in Table II.

## Discussion

The pathway for the biosynthesis of folic acid, first established in *Escherichia coli* by Brown and co-workers and in

[3] It should be noted that, since the C-9 methylated pterins are a racemic mixture of the *R* and *S* isomers and since only one isomer would be expected to be the precursor, the amount of the correct isomer fed is only half of that indicated in Table I.

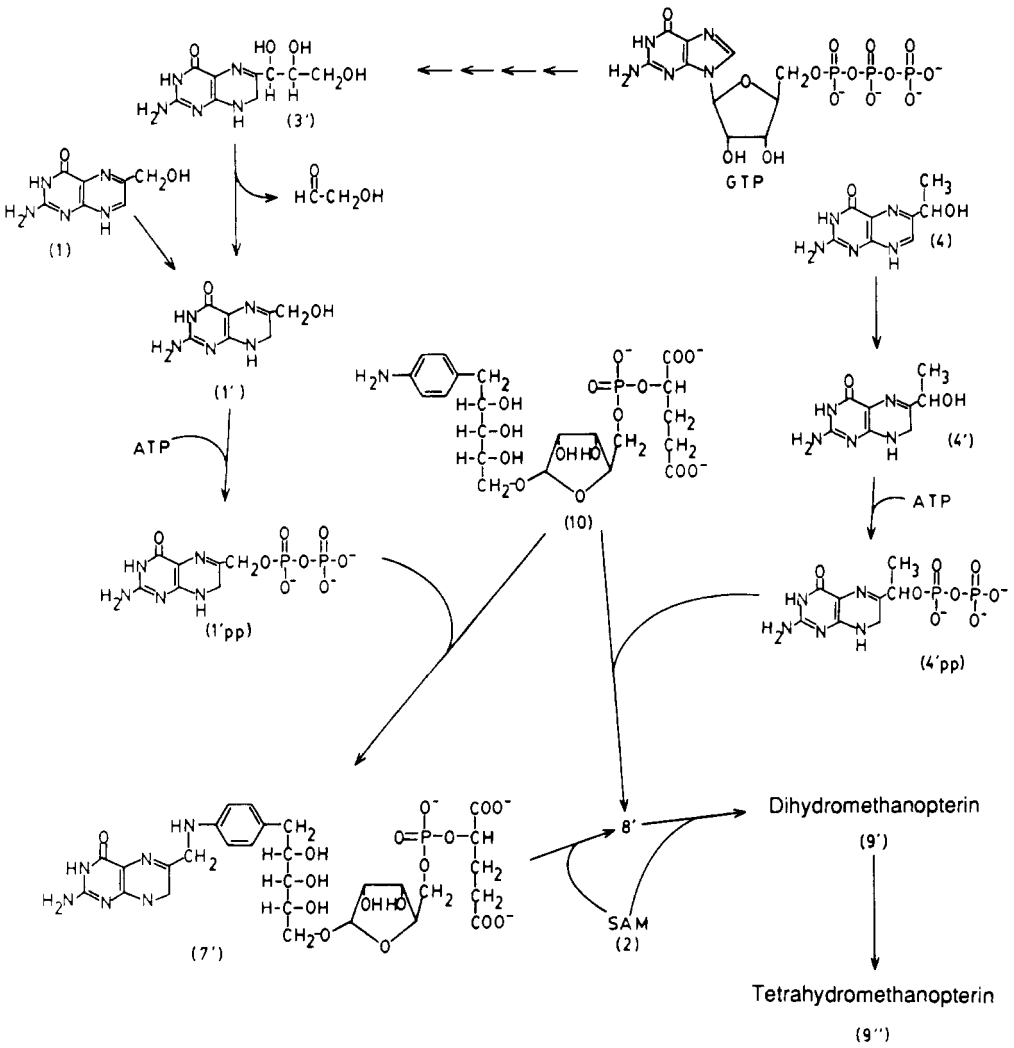


FIGURE 2: Proposed pathway for the biosynthesis of the pterin portion of methanopterin.

*Lactobacillus plantarum* by Shiota and collaborators, has now been confirmed in other microorganisms and in plants (Brown & Williamson, 1987). The close structural similarities between folic acid and methanopterin raise the question as to whether portions of the pathway for folic acid biosynthesis are utilized in methanopterin biosynthesis as well. Recent work on the biosynthesis of the individual units of methanopterin has shown that the pterin ring incorporates $^{15}N$ from glycine (White, 1986a) and $^{13}C$ from [1-$^{13}$C]acetate (Keller et al., 1986) to the same extent and at the same position as the guanine in the cells, indicating that, as in folic acid biosynthesis, GTP is a likely precursor of the pterin. The cleavage of GTP by GTP cyclohydrase, the first committed step in pterin biosynthesis, generates 2,5-diamino-6-(ribosylamino)-4(3*H*)-pyrimidinone 5'-triphosphate, an important intermediate in the biosyntheses of riboflavin and $F_{420}$ as well as in pterin biosynthesis. The presence of this activity in methanogenic bacteria was recently confirmed when label from [2-$^{14}$C]-guanine, but not from [8-$^{14}$C]guanine, was incorporated into the deazaflavin of $F_{420}$ by *Methanobacterium thermoautotrophicum* (Jaenchen et al., 1984). The C-7 methyl group of the pterin was shown to arise from the methyl group of methionine (White, 1986a), and the aniline portion of the 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane was shown to arise from *p*-aminobenzoic acid (White, 1985a). The five-carbon side chain was shown to arise from a pentose (Kelley et al., 1986) which was determined to be ribose on the basis of the stereochemistry of the side chain (White, 1986b).

Thus, from current information, it appears that the pathway for methanopterin biosynthesis is, in many ways, analogous to that of folic acid biosynthesis. If this is true, then part of the problem of defining the biosynthesis of methanopterin is determining exactly where the two pathways diverge. Considering the chemical differences in the structures of folic acid and methanopterin, this would require the determination of the step or steps at which the methylation of the pterin portion of the methopterin occurs and the step at which the modification to the *p*-aminobenzoic acid side chain is introduced.

The methylations are most likely to occur at the carbons $\alpha$ to C-6 of a 7,8-dihydropterin. Methylation at these positions is consistent with both the anionic nature of these carbons and the cationic nature of the expected methylating agent, SAM. That both methyl groups of methanopterin are derived from methionine was established by growing *M. volta* cells with [*methyl*-$^2$H$_3$]methionine (0.3 mg/mL) and determining the incorporation of trideuteriated methyl groups into both the methyl groups of methanopterin to an extent of 51%.

The quantitative conversion of **3'** to **1'** by methanogenic bacteria indicates that the methylations are not likely to occur at any of the intermediate steps between GTP and 7,8-H$_2$-6-(hydroxymethyl)pterin (Figure 2). The C-7 methylation can occur at **1'** or at an intermediate that can be derived from it, since 6-(hydroxymethyl)pterin is incorporated into methanopterin to the extent of ~29% when fed to growing cells of *M. volta*. The observation that this oxidized pterin is incorporated into methanopterin, which exists in the cell as

Table II: Summary of Incubations of Cell Extracts with Labeled Precursors

| experiment | expected products | % conversion |
|---|---|---|
| [*methyl*-$^3$H]SAM (**2t**) + 7,8-$H_2$-6-(hydroxymethyl)pterin-P (**1'p**) + 7,8-$H_2$-6-(hydroxymethyl)pterin-PP (**1'pp**) | 7,8-$H_2$-6-(hydroxymethyl)pterin-P or -PP methylated at C-7 and/or C-9 (**4'tp**, **4'tpp**, **5'tp**, **5'tpp**, **6'tp**, and/or **6'tpp**) | $<10^{-5}$ |
| [*methylene*-$^3$H]-7,8-$H_2$-6-(1-hydroxyethyl)-7-methylpterin (**6'tpp**) + ATP + methaniline (**10**) | 7,8-$H_2$-methanopterin (**10't**) | $<10^{-4}$ |
| [*methyl*-$^3$H]SAM (**2t**) + 7,8-$H_2$-6-(hydroxymethyl)pterin (**1'**) | 7,8-$H_2$-6-(hydroxymethyl)pterin methylated at C-7 and/or C-9 (**4't**, **5't**, and/or **6't**) | <0.03 |
| 7,8-$H_2$-neopterin (**3'**) | 7,8-$H_2$-6-(hydroxymethyl)pterin (**1'**) | 100 |
| 7,8-$H_2$-6-(hydroxymethyl)pterin (**1'**) + ATP | 7,8-$H_2$-6-(hydroxymethyl)pterin-PP (**1'pp**) | *a* |
| [*methylene*-$^3$H]-7,8-$H_2$-6-(hydroxymethyl)pterin-PP (**1'tpp**) + methaniline (**10**) | demethylated 7,8-$H_2$-methanopterin (**7't**) | 17 |
| demethylated methanopterin (**7t**) + SAM (**2**) | 7,8-$H_2$-methanopterin (**10'**) | 65 |

[a] The compound could not be trapped in the incubation mixture and is presumed to have been formed and converted to **7'**.

tetrahydromethanopterin (Escalante-Semerena et al., 1984a,b), indicates that the pterin must be reduced by the cells. Since dihydrofolate reductases are nonspecific and can reduce pterins to dihydropterins (Blakley, 1969), it is assumed that the first step in the incorporation of **1** into **9''** is its reduction to **1'**.

The next question to be considered is the sequence of the methylations at C-7 and C-9. When the C-7 (**5d**) and C-9 (**4d**) monomethylated 6-(hydroxymethyl)pterins and the C-9 and C-7 dimethylated 6-(hydroxymethyl)pterin (**6'**) were fed to *M. volta*, only the C-9 methylated 6-(hydroxymethyl)pterin (**4d**) was incorporated into the methanopterin (Table I). These data suggest that either **1'** or a phosphorylated derivative of **1'** such as **1'pp** is first methylated at C-9, with methylation at C-7 occurring only after the resulting 7,8-$H_2$-6-[1-(*R* or *S*)hydroxyethyl]pterin (**4'**) is converted to another compound. To test this idea, a mixture of **1'p** and **1'pp** was incubated with a cell extract supplemented with [*methyl*-$^3H_3$]SAM. The methylated **1'p** and **1'pp** were isolated from the reaction mixture as the oxidized pterin phosphates and were found to contain no detectable tritium.

Since the phosphorylated pterins were not found to be labeled, **1'** was incubated with a cell extract of *M. volta* and [*methyl*-$^3H_3$]SAM. As with the phosphorylated pterins, no detectable radioactivity was found in the final purified methylated pterin products. If a 50% recovery of the pterins from the incubation mixture is assumed, then <0.026% of **1'** could have been converted to either of the stereoisomers of **7** by the cell extract. The lack of any observed methylation of **1'** or its monophosphate or pyrophosphate esters by [*methyl*-$^3H_3$]SAM indicated that both methylations must occur after the condensation of **1'** with the arylamine. That this arylamine is methaniline (Van Beelen et al., 1984) is supported by the following observations. First, methanogenic archaebacteria contain no pteroic acid or folic acid (Leigh, 1983; Worrell & Nagle, 1988), and since all archaebacteria are completely resistant to sulfonamides (White, 1988b), except for the halobacteria which contain large amounts of folic acid, one could argue that the normal condensation of **1'pp** with *p*-aminobenzoic acid, a step that is central to folic acid biosynthesis and is blocked by sulfa drugs (Brown, 1962; Roland et al., 1979), is not operative in methanogenic bacteria. Second, several strains of methanogenic bacteria contain relatively large amounts of methaniline which could serve as a biosynthetic precursor for methanopterin (White, 1985b). Finally, it has recently been demonstrated that methaniline can be generated by cell extracts incubated with *p*AB, ribose-P, ATP, and $\alpha$-hydroxyglutaric acid (White, unpublished results).

In analogy with folic acid biosynthesis, **1'pp** was considered the logical activated substrate for the reaction with methaniline. Several attempts to trap **1'pp** in different cell extracts incubated with **1'** and ATP gave inconclusive results (data not shown). Incubation of cell extracts of *M. formicicum* with **1'pp** and **10**, however, resulted in 17% of the substrate being converted to **7'**. A small amount of methanopterin was also produced during the incubation. No indication of monomethylated **7**, which would have generated 6-ethylpterin and 6,7-dimethypterin during the reductive cleavage, was found.

The **7t** generated in the above incubation was isolated and incubated with the same cell extract of *M. formicicum* but supplemented with 5 mM SAM. The production of methanopterin, readily observed during this incubation, was confirmed by TLC analysis of the pterins generated by the reductive cleavage of the methanopterin, which showed 6-ethyl-7-methylpterin to be 65% of the product and 6-methylpterin to be 35% of the product. Pterins produced from **7'** with only one methyl group were not observed, indicating that when one site in **7'** is methylated, methylation of the other site is very rapid.

The order in which the methylations occur can be inferred from other experimental data. Since it was determined that **4** is readily incorporated into methanopterin, it would appear that the enzymes used for the activation and coupling of **1'** to the methaniline can also activate and couple **4'** with methaniline to form the C-7 demethylated 7,8-$H_2$-methanopterin (**8'**). This product must then be methylated at C-7 to generate 7,8-$H_2$-methanopterin. This observation and the recent isolation and characterization of a C-7 demethylated analogue of methanpterin, tatiopterin, from *Methanogerium tationis* (Raemakers-Franken et al., 1989), strongly suggest that the C-9 methyl is added before the C-7.

From the above discussion, it appears that all of the steps for the generation and subsequent condensation of **1'** with an arylamine appear to be analogous to the reactions that have been described for folic acid biosynthesis.

Carbon atoms C-6, C-7, and C-9 of the product of the condensation reaction, **7'**, only become asymmetric in the final product, tetrahydromethanopterin. The stereochemistry of C-7 and C-9 is determined by the stereoselectivity of each individual methylation reaction and has no analogy in folic acid biochemistry. The stereochemistry of the C-6 would, presumably, be controlled by dihydromethanopterin reductase as is the stereochemistry of C-6 in the reduction of dihydrofolate to tetrahydrofolate by dihydrofolate reductase. If the resulting tetrahydromethanopterin functions by the same mechanism and by the same stereochemical constraints as does tetrahydrofolate in the dTMP synthase reaction, then the C-6 proton transferred during the oxidation of tetrahydromethanopterin must originate from the C-6 with the same *R* absolute configuration as tetrahydrofolate (Fontecilla-Camps et al., 1979). It would be interesting to learn if the stereochemistry of this center is the same as or different from that found in folate biochemistry.

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