Biosynthesis of Methanopterin†

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ABSTRACT: This paper establishes the pathway for the biosynthesis of methanopterin in *Methanosarcina thermophila* to proceed by the following series of reactions. First, 5-phospho-α-D-ribosyl diphosphate (PRPP) and 4-aminobenzoic acid condense together to produce 4-(β-D-ribofuranosyl)aminobenzene 5′-phosphate, which then reacts with 6-hydroxymethyl-7,8-H_2pterin pyrophosphate to produce 7,8-H_2pterin-6-ylmethyl-4-(β-D-ribofuranosyl)aminobenzene 5′-phosphate (**3′**). Compound **3′** is then reduced to 7,8-H_2pterin-6-ylmethyl-1-(4-aminophenyl)-1-deoxy-D-ribitol 5′-phosphate (**4′**) in a reaction stimulated by the addition of FMN or factor F_{420}. Dephosphorylation of compound **4′** leads to 7,8-H_2pterin-6-ylmethyl-1-(4-aminophenyl)-1-deoxy-D-ribitol (**5′**). Compound **5′** then condenses with another molecule of PRPP to form 7,8-H_2pterin-6-ylmethyl-1-(4-aminophenyl)-1-deoxy-5-[1-α-D-ribofuranosyl 5-phosphate]-D-ribitol (**9′**). Compound **9′**, in the presence of ATP, then condenses with (*S*)-2-hydroxyglutaric acid to form demethylated H_2methanopterin, a known precursor to methanopterin. The occurrence of this pathway was confirmed by (a) the chemical and/or biochemical synthesis of most of the proposed intermediates, (b) the detection of these intermediates in cell-free extracts, and (c) by the measurement of their conversion to demethylated methanopterin and/or other intermediates in the pathway.

Methanopterin (MPT),[1] (Figure 1) (Van Beelen et al., 1984a,b) was the first in a series of modified folates that have been identified in Archaea (Raemakers-Franken et al., 1989, 1991; White, 1991, 1993a,b; Zhou & White, 1992). The current evidence that cells containing modified folates do not contain folate (Leigh, 1983; Worrell & Nagle, 1988) indicates that each of these modified folates conducts all of the biochemical reactions normally carried out by folates present in other cells. A common structural unit present in all of these modified folates, with one exception (Raemakers-Franken et al., 1991), is 1-(4-aminophenyl)-1-deoxy-D-ribitol (**6**) known to be biosynthesized from 4-aminobenzoic acid (*p*AB), and ribose or ribose-P (White & Zhou, 1993). An understanding of the biochemical nature of this condensation is important because it represents one of the key biosynthetic steps that differentiates the biosynthesis of folate from that of the modified folates. This paper establishes the steps in the formation of this compound as well as how its formation is integrated in the complete biosynthesis of methanopterin. Also defined is the biosynthetic origin of the two unusual structural features of these molecules, the α-ribosidic bond and the phosphodiester linkage between the 5′-position of ribose and the hydroxyl group of (*S*)-2-hydroxyglutaric acid.

MATERIALS AND METHODS

Materials. PRPP, ATP, GTP, UTP, CTP, (*S*)-2-hydroxyglutaric acid, carboxypeptidase G (γ-glutamyl hydrolase, Sigma C9658), carboxypeptidase A (Sigma 0386), peptidase (from porcine intestinal mucosa, Sigma P7500), [*ring*-U-^{14}C]-4-aminobenzoic acid ([^{14}C]*p*AB) (6.8 mCi/mmol), chromatographically purified *Escherichia coli* alkaline phosphatase (type III: *E. coli*, Sigma P 4252), phosphodiesterase I (type VII: from *Crotalus atrox*, Sigma P6761), and bovine intestinal mucosa alkaline phosphatase (Sigma P7640) were obtained from Sigma Chemical Co. Proteinase K was obtained from Boehringer Mannheim GmbH. L-[2,3,3,4,4-$^2H_5$]-Glutamic acid was obtained from Cambridge Isotope Labs, Andover, MA. NaB^3H_4 (1 mCi/mmol) was obtained from Dupont NEN Research Products. [2,2′-$^2H_2$]-4-Aminobenzoic acid (89.0 atom % $^2H_2$) was prepared as previously described (White, 1985). Compound **2′** was prepared as previously described (White, 1990).

Bacterial Strains and Growth Conditions. Methanosarcina thermophila strain TM-1 (Zinder et al., 1985), used for the generation of cell extracts, was grown in the departmental laboratory of Dr. James G. Ferry as previously described (Sowers et al., 1984). Frozen cells of *Methano-*

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[1] Abbreviations and compound designations: MPT, methanopterin; SP, sarcinapterin; HG, (*S*)-2-hydroxyglutaric acid; F_{420}, factor F420; H_2MPT, 7,8-dihydromethanopterin; H_4MPT, 5,6,7,8-tetrahydromethanopterin; *p*AB, 4-aminobenzoic acid; PRPP, 5-phospho-α-D-ribosyl diphosphate; GC−MS, gas chromatography−mass spectrometry; TLC, thin-layer chromatography; CFE, cell-free extract; compound **1** is 4-(β-D-ribofuranosyl)aminobenzene 5′-phosphate; compound **2** is 6-hydroxymethylpterin pyrophosphate; compound **3** is pterin-6-ylmethyl-4-(β-D-ribofuranosyl)aminobenzene 5′-phosphate; compound **4** is pterin-6-ylmethyl-1-(4-aminophenyl)-1-deoxy-D-ribitol 5-phosphate; compound **5** is pterin-6-ylmethyl-1-(4-aminophenyl)-1-deoxy-D-ribitol; compound **6** is 1-(4-aminophenyl)-1-deoxy-D-ribitol; compound **7** is 1-(4-aminophenyl)-1-deoxy-D-ribitol 5-phosphate; compound **8** is 4-(β-D-ribofuranosyl)aminobenzene; compound **9** is pterin-6-ylmethyl-1-(4-aminophenyl)-1-deoxy-5-[1-α-D-ribofuranosyl 5′-phosphate]-D-ribitol; compound **10** is 4-(α-D-ribofuranosyl)aminobenzene 5′-phosphate; compound **11** is 1-(4-aminophenyl)-1-deoxy-5-[1-α-D-ribofuranosyl]-D-ribitol; compound **12** is pterin-6-ylmethyl-4-(α-D-ribofuranosyl)-aminobenzene 5′-phosphate; compound **13** is 1-(4-aminophenyl)-1-deoxy-5-[1-α-D-ribofuranosyl 5′-phosphate]-D-ribitol; compound **14** is pterin-6-ylmethyl-1-(4-aminophenyl)-1-deoxy-5-[1-α-D-ribofuranosyl]-D-ribitol; and compound **15** is 4-(α-D-ribofuranosyl)aminobenzene. 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.

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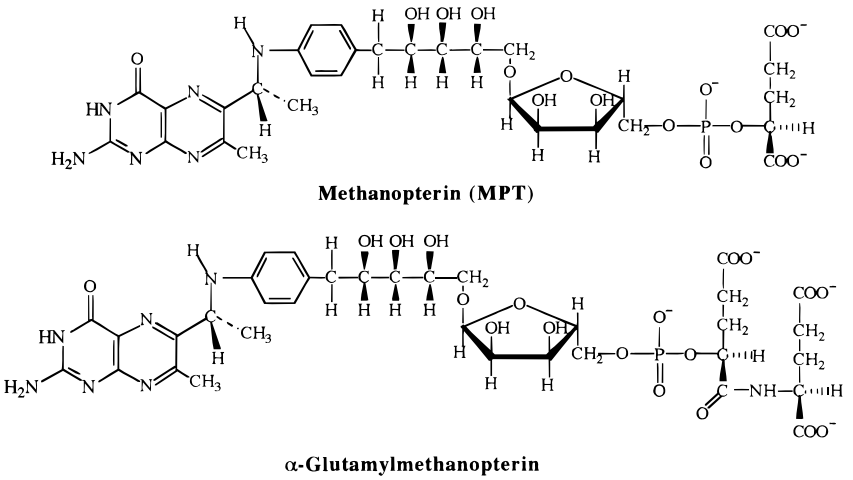

FIGURE 1: Chemical structures of methanopterin and its α-glutamyl derivative, sarcinapterin.

bacterium thermoautophicum were kindly supplied by Dr. Ralph S. Wolfe (Urbana, IL). *Methanococcus voltae* cells were supplied by Dr. David M. Ferber, Department of Microbiology, University of Illinois, Urbana, IL.

Preparation of Cell-Free Extracts. Cell-free extracts (CFE) of *M. thermophila* were prepared by French press lysis as described by Nelson and Ferry (1984) except that the breakage buffer contained 50 mM potassium TES, pH 6.8, 10 mM $MgCl_2$, 5% (v/v) glycerol, and 0.01 mg of DNase I per mL. Protein concentrations of the extracts typically ranged from 14 to 20 mg/mL, and the extracts were stored frozen at −78 °C until used.

Incubation of Cell-Free Extracts with Precursors. To 0.5 or 1 mL of CFE sealed in a bottle under the indicated atmosphere were added concentrated anaerobic solutions of the desired precursors. The solutions were then incubated at 50 °C for the described times.

Preparation and Purification of the Azo Dye Derivatives of the Arylamines. Cell-free extracts (1 mL), after incubation with substrates, were mixed with 0.2 mL of 6 M HCl and centrifuged to remove the precipitated proteins. This procedure resulted in the oxidative cleavage of the di- and tetrahydropterins present in the CFEs to their respective arylamines (Chippel & Scrimgeour, 1970; White, 1985). The arylamines contained in the resulting acid-treated samples were then converted to their Bratton−Marshall azo dye derivatives (Zhou & White, 1992), and separated and purified by adsorption chromatography using either a Bio-Gel P-4 column (1.0 × 48 cm) or a Bio-Gel P-6 column (1.5 × 26 cm) previously equilibrated with 50 mM HCl. The azo dye derivatives were eluted with 50 mM HCl and quantitated in the fractions (5.5 mL) by their absorbance at 562 nm, $\epsilon = 48\,000\ M^{-1}\ cm^{-1}$ (White, 1993a). On Bio-Gel P-4 columns, the following known samples eluted, relative to a retention volume of 1.0 for compound **6**, as follows: α-glutamylmethaniline, 0.71; methaniline, 0.74; compound **1**, 0.79; compound **11**, 0.89; compound **7**, 0.93; compound **15**, 0.97; compound **8**, 1.1; and *p*AB, 1.7. The samples were recovered from the fractions either by lyophilization or by evaporation with a stream of N_2 gas. Purified samples were then further identified by comparison to known compounds using TLC (visual inspection of the purple spots) and/or by HPLC (A_{556}) (White, 1993a).

Chemical and Enzymatic Analysis of the Azo Dye Products. Portions of each of the azo dye samples were subjected to acid hydrolysis or phosphatase cleavage, in order to obtain information about their chemical structures. Because of the sensitivity to acid hydrolysis of the α-ribosidic bond in MPT and related compounds (Keltjens et al., 1983), its presence in any given molecule was readily established by a brief acid hydrolysis of the sample. This was accomplished by heating the sample at 100 °C for 2 min in 1 M HCl, evaporating the acid, and determining the production of the azo dye derivative of compound **6** by HPLC or TLC.

Azo dye products were tested for the presence of phosphate monoesters by dissolving them in 0.1 M glycine buffer, pH 10.4, containing 1 mM $ZnCl_2$ and 1 mM $MgCl_2$, and treating them with either *E. coli* or bovine intestinal mucosa alkaline phosphatase for 2 h at 37 °C. The samples were then acidified, and the azo dyes were isolated by chromatography on small Bio-Gel P-4 columns (0.4 × 0.8 cm), eluted with 50 mM HCl, concentrated by evaporation, and examined by TLC or HPLC.

The azo dye derivatives of compounds **6** and **8** were also identified after their reductive cleavage to compounds **6** and **8** followed by GC−MS analysis of their trifluoroacetate derivatives. This was accomplished by dissolving the sample in 1 M HCl, adding Zn dust, and shaking for 2 min at room temperature. After removal of the Zn by filtration and evaporation of the HCl with a stream of nitrogen, the arylamines were isolated by absorption on Dowex 50W-8X H^+ and eluted with 3 M aqueous ammonia. The samples were reacted for 12 h at room temperature with an equal mixture of methylene chloride and trifluoroacetic anhydride, and after evaporation of the excess solvent, the trifluoroacetate derivatives were separated by GC−MS on a DB-1 column (30 m × 0.32 mm, J&W Scientific Co.) programmed from 75 to 250 °C at 10 °C/min. Compound **6** had a molecular ion at *m*/*z* 707, and compound **8** had a molecular ion at *m*/*z* 609.

TLC Analysis of Azo Dye Derivatives of Arylamines. The azo dye derivatives of the different arylamines were identified by TLC analysis on silica gel 60 F-254 TLC plates (E. Merck, Darmstadt, Germany) using acetonitrile/water/formic acid (88%) (40:10:5, v/v/v) as the developing solvent. With this solvent, the azo dye derivatives had the following indicated R_f values: aniline, 0.76; *p*AB, 0.65; compounds **8** and **15**, 0.63; compound **6**, 0.61; compound **11**, 0.25; α-glutamylmethaniline, 0.13; methaniline, 0.11; compound **7**, 0.11; compound **1**, ∼0.1; and compound **13**, 0.04. The

purple color of the azo dyes was most intense immediately after evaporation of the solvent from the TLC plate but completely disappeared from the plate after a few hours if exposed to the air. This fading of the TLC spots was prevented by placing the TLC plates in a closed chamber containing HCl vapors.

HPLC Analysis of the Azo Dye Derivatives. The azo dye derivatives of the arylamines were assayed by C_{18} reverse-phase chromatography, using acidic conditions, by monitoring absorbance at 556 nm, as previously described (White, 1993a). Under the conditions used, the azo dye derivatives of the following compounds eluted at the indicated times (min): α-glutamylmethaniline and methaniline, 27.57; compound **1**, 25.75; compound **7**, 28.30; compound **11**, 29.44; and compound **6**, 30.6.

*Chemical Synthesis of 4-(β-D-Ribofuranosyl)aminobenzene (**8**)*. The synthesis of compound **8** was accomplished by the coupling of 2,3,5-tri-*O*-benzoyl-D-ribosyl chloride with an organocuprate prepared from *N*,*N*-bis(trimethylsilyl)-4-bromoaniline (Pratt et al., 1975). The synthesis gave a low yield of a 60:40 mixture of the tribenzoylates of the α- and β-isomers, which were separated and purified by preparative TLC using the solvent system methyl acetate/hexane (1:1, v/v). In this solvent, the R_f value of the α-isomer was 0.28 and that of the β-isomer was 0.22.

The 4-(2,3,5-tribenzoyl-β-D-ribofuranosyl)aminobenzene had the following spectral properties: ^{1}H NMR ($CDCl_3$), δ 4.32 (1H, dd, $J_{2',3'} = 5.8$ Hz, $J_{2',1'} = 7.6$ Hz, H-2′), 4.58 (1H, m, H-4′), 4.63 (1H, dd, $J_{5',4'} = 3.7$ Hz, $J_{5',5''} = 12.0$ Hz, H-5′), 4.72 (1H, dd, $J_{5'',4'} = 3.7$ Hz, $J_{5'',5'} = 12$ Hz, H-5″), 4.80 (1H, bd, $J_{1',2'} = 7.6$ Hz, H-1′), 5.51 (1H, dd, $J_{3',2'} = 5.8$ Hz, $J_{3',4'} = 3.4$ Hz, H-3′), 6.65 (2H, d, $J_{2,3} = 8.4$ Hz, *ortho* aromatic hydrogens of the aniline), 7.23 (2H, d, $J_{3,2} = 8.4$ Hz, *meta* aromatic hydrogens of the aniline), 7.3−8.2 (15H, m, benzoate aromatic hydrogens). Mass spectrum (*m*/*z*): $M^+ - C_6H_5COOH$, 415; $M^+ - CHC_6H_4NH$, 433. The identification of the β-isomer was established by a series of nuclear Overhauser effect experiments as well as by comparison of chemical shifts to known compounds (unpublished results).

The tribenzoylate compounds were debenzoylated by dissolving in 1 M sodium methoxide in methanol for 1 h at room temperature, isolated by absorption on Dowex 50W-8X H^+, and eluted with aqueous ammonia. Each isomer showed the expected molecular ion at *m*/*z* 609 for M^+ as the tetratrifluoroacetyl derivative and ran as a single spot on TLC with an $R_f = 0.48$.

*Synthesis of 4-(β-D-Ribofuranosyl)aminobenzene 5′-Phosphate (**1**) and 7-(4-Aminophenyl)-7-deoxy-D-ribitol 5-Phosphate (**7**)*. Compound **8** from the above synthesis and a synthetic sample of compound **6** were each phosphorylated with phosphoryl chloride in trimethyl phosphate as described by Yoshikawa et al. (1967). Chromatographic analysis of the azo dye derivatives of the products showed only the 5′-phosphorylated compound and the starting material. The starting compounds were separated from the phosphorylated compounds by chromatography on DEAE-Sephadex using a gradient (0−2 M) of aqueous NH_4HCO_3 as the eluting buffer. The yields of compounds **1** and **7** were 51% and 48%, respectively. ^{1}H NMR of compound 1 (D_2O): δ 3.88 (2H, m, H-5′,5″), 4.00 (1H, dd, $J_{2',3'} = 5.5$ Hz, $J_{2',1'} = 7.6$ Hz, H-2′), 4.02 (1H, m, H-4′), 4.12 (1H, dd, $J_{3',2'} = 5.5$ Hz, $J_{3',4'} = 7.6$ Hz, H-3′), 4.55 (1H, d, $J_{1',2'} = 7.6$ Hz, H-1′), 6.80 (2H, d, $J_{2,3} = 8.4$ Hz, *ortho* aromatic hydrogens of the aniline), 7.2 (2H, d, $J_{3,2} = 8.4$ Hz, *meta* aromatic hydrogens of the aniline). In this spectrum, a few of the hydrogen resonances for the α-isomer were also detected because of their separation from the resonances of compound **1**: δ 5.01 (1H, d, $J_{1',2'} = 2.8$ Hz, H-1′), 6.85 (2H, d, $J_{2,3} = 8.4$ Hz, *ortho* aromatic hydrogens of the aniline), 7.23 (2H, d, $J_{3,2} = 8.4$ Hz, *meta* aromatic hydrogens of the aniline). From the integration of the hydrogen signals from individual compounds, it was established that the sample was 87% β-isomer (**1**) and 13% α-isomer (**10**).

Generation of the Azo Dye Derivatives of Known Structures. Several different enzymes were tested for their ability to digest methanopterin and sarcinapterin, in order to produce biosynthetic reference compounds. These include carboxypeptidase G, carboxypeptidase A, peptidase, proteinase K, and phosphodiesterase I. Of all these enzymes, only the phosphodiesterase I was found to generate any cleavage of MPT or SP. This enzyme was found to slowly cleave methanopterin, to give an equal mixture of compounds **11** and **13**, or the azo dye derivative of α-glutamylmethaniline, to give the azo derivatives of compounds **11** and **13**.

*Chemical Synthesis of 1-(4-Aminophenyl)-1-deoxy-D-ribitol (**6**)*. The general outline of the synthesis of compound **6** has been presented in an earlier paper (White, 1986) and involves the condensation of 2,3:4,5-*O*-diisopropylidene-D-ribose with the organolithium compound prepared from *N*,*N*-bis(trimethylsilyl)-4-bromoaniline (Pratt et al., 1975). The 2,3:4,5-*O*-diisopropylidene-D-ribose used in the synthesis was prepared as described by Kumar and Dev (1987) and purified by column chromatography as described by Aslani-Shotorbani et al. (1985).

To a stirred solution of *N*,*N*-bis(trimethylsilyl)-4-bromoaniline (0.9 mL, 3.13 mmol) dissolved in 3.4 mL of diethyl ether and cooled to 0 °C under argon was slowly added 1.32 mL of 2.6 M *n*-butyllithium in hexane. After the solution was kept at 0 °C for 2 h, a solution of 2,3:4,5-*O*-diisopropylidene-D-ribose (0.6 g, 2.6 mmol) dissolved in 2 mL of diethyl ether was added and the resulting solution slowly warmed to room temperature overnight. After addition of water (2 mL) to the stirred reaction mixture and separation of the ether layer, the ether was evaporated and the residue was dissolved in 8 mL of methanol−water (90:10, v/v) and heated at 80 °C for 30 min. The solvents were then evaporated with a stream of nitrogen gas, leaving a red residue which was then dissolved in 5 mL of dioxane, mixed with 0.5 g of lithium aluminum hydride, and heated at 120 °C for 3 h in a sealed vial. After the addition of water (3 mL) to the cooled sample, the desired product was recovered by extraction with ether (3 × 5 mL). After evaporation of the ether, the resulting brown oil was purified by chromatography on silica gel using diethyl ether as the eluting solvent. The yield of the 2,3:4,5-*O*-diisopropylidene derivative of 5-(4-aminophenyl)-5-deoxy-D-ribitol (0.35 g) was 44% on the basis of the sugar derivative used. ^{1}H NMR ($CDCl_3$): δ 1.29, 1.37, 1.42, 1.45 (each 3H, s, isopropylidene CH_3), 2.73 (1H, dd, $J_{1,2} = 9.91$, $J_{1,1'} = 14.35$ Hz, H-1), 2.97 (1H, dd, $J_{1',2} = 3.2$, $J_{1',1} = 14.35$, H-1′), 3.57 (2H, bs, NH_2), 3.94 (1H, dd, $J_{5,4} = 6.0$, $J_{5,5'} = 8.3$, H-5), 4.01 (1H, dd, $J_{5',4} = 5.7$, $J_{5',5} = 8.8$, H-5′), 4.15 (1H, m, H-4), 4.20 (1H, m, H-3), 4.35 (1H, ddd, $J_{2,1'} = 3.35$, $J_{2,3} = 5.65$, $J_{2,1} = 9.91$, H-2), 6.63 (2H, d, $J = 8.4$, aromatic H-3 and H-3′), 7.09 (2H, d, $J = 8.5$ aromatic H-4 and H-4′). Mass spectrometry showed a molecular ion at *m*/*z* 307 for M^+ with characteristic fragment ions at *m*/*z* 292 for $M^+ - 15$ and *m*/*z* 106 for

$NH_2C_6H_4CH_2^{\bullet+}$. Deprotection of the sample by heating for 10 min at 100 °C in 0.1 M HCl followed by evaporation of the solvents gave 1-(4-aminophenyl)-1-deoxy-D-ribitol.

*Synthesis of Pterin-6-ylmethyl-1-(4-aminophenyl)-1-deoxy-D-ribitol (**5**).* To a solution of *N*-acetyl-6-formylpterin (23.3 mg, 0.1 mmol) (Bieri & Viscontini, 1973) and 2,3:4,5-*O*-diisopropylidene-1-(4-aminophenyl)-1-deoxy-D-ribitol (30 mg, 0.1 mmol) in 0.47 mL of DMSO, were added 7 (1−2 mm spheres, ∼60 mg) of 4 Å molecular sieves. Crystals of the Schiff base condensation product were apparent in 5 min, and the amount continued to increase over a 3 h period. After 12 h at room temperature, the yellow crystals were removed by filtration, washed with ether, and dissolved in 1 mL of DMF. Sodium borohydride (4.5 mg, 0.12 mmol) was then added, after 2 days at room temperature the DMF was removed in vacuo, and the residue, under argon, was dissolved in 25 mL of an anaerobic solution of 0.1 M NaOH. After 24 h, the solution was filtered and adjusted to pH 3−4 with HCl. The resulting yellow precipitate was isolated by centrifugation and washed with water. The material had UV absorbance maxima at 249, 279, and 322 nm in 0.1 M HCl and 250 and 358 nm in 0.1 M NaOH, indicating the presence of a pterin. FAB mass spectrometry gave the expected molecular ion of *m*/*z* 482. ^{1}H NMR (^2H_2O, $N^2H_4O^2H$): δ 1.32, 1.33, 1.36, 1.46 (each 3H, s, CH_3), 2.68 (1 H, dd, $J_{1',2}$ = 10.5 Hz, $J_{1',1}$ = 14.1 Hz, H-1′), 2.91 (1H, dd, $J_{1,2}$ = 3.65 Hz, $J_{1,1'}$ = 14.2 Hz, H-1), 3.95 (1H, dd, $J_{5,4}$ = 5.8 Hz, $J_{5,5'}$ = 8.7 Hz, H-5), 4.19 (1H, dd, $J_{5',4}$ = 6.4 Hz, $J_{5',5}$ = 8.4 Hz, H-5′), 4.25−4.40 (3H, m, H-2, H-3, and H-4), 4.53 (2H, s, CH_2), 6.83 (2H, d, J = 8.7 Hz, *ortho* aromatic hydrogens of the aniline), 7.14 (2H, d, *meta* aromatic hydrogens of the aniline), 8.59 (1H, s, C-7 hydrogen of the pterin).

*Synthesis of Pterin-6-ylmethyl-4-(β-D-ribofuranosyl)aminobenzene 5′-Phosphate (**3**) and Pterin-6-ylmethyl-4-(α-D-ribofuranosyl)aminobenzene 5′-Phosphate (**12**).* The early steps in the synthesis of these materials were identical to those described above for the synthesis of compound **5**, except that the *N*-acetyl-6-formylpterin was condensed with the 2,5:3,4-diacetone derivative of 1-(4-aminophenyl)-D-ribitol, which was prepared as described above from 2,3:4,5-*O*-diisopropylidene-D-ribose but using the 2,5:3,5-*O*-diisopropylidene-D-ribose isomer. However, unlike the above reaction where the reaction product largely precipitated from the DMSO solution, this reaction produced much less precipitate. As a result, the entire reaction mixture was dissolved in the DMF and reduced with $NaBH_4$. Subsequent deprotection and precipitation of the product was as described above. The resulting isolated product was chromatographically pure as determined by TLC and had the following spectral properties: ^{1}H NMR (^2H_2O, $N^2H_4O^2H$); δ 1.11, 1.26, 1.31, 1.36 (each 3H, s, isopropylidene CH_3 groups), 3.75 (1H, dd, $J_{5',4}$ = 5.5 Hz, $J_{5',5}$ = 10.3 Hz, H-5′), 3.83 (1H, dd, $J_{2,3}$ = ∼2 Hz, $J_{2,1}$ = 15 Hz, H-2), 3.92 (1H, dd, $J_{5,4}$ = 4.1 Hz, $J_{5,5'}$ = 10.2 Hz, H-5), 3.95 (1H, d, $J_{1,2}$ = 15 Hz, H-1), 4.17 (1H, bd, $J_{3,2}$ = 5.6 Hz, H-3), 4.53 (2H, s, CH_2), 4.72 (1H, H-4, obscured by HOD), 6.85 (2H, d, J = 8.55 Hz, *ortho* aromatic hydrogens of the aniline), 7.22 (2H, d, J = 8.55 Hz, *meta* aromatic hydrogens of the aniline), 8.59 (1H, s, C-7 hydrogen of the pterin).

This material was then dissolved in deoxygenated 1 M HCl, heated under argon at 100 °C for 10 min and the solution lyophilized. This treatment caused the removal of the isopropylidene groups and subsequent cyclization of the sample to occur. The resulting mixture of isomers was then phosphorylated at the 5′-position in trimethyl phosphate with phosphoryl chloride as described by Yoshikawa et al. (1967). Separation of the resulting phosphorylated products was accomplished by chromatography on a DEAE-Sephadex column eluted with 0−2.0 M NH_4HCO_3 gradient. The α-isomer eluted at 1.2 M NH_4HCO_3 and the β-isomer eluted at 1.46 M NH_4HCO_3. The steps in the chemical synthesis of compound **3′** are outlined in Figure 2. The α-isomer had the following spectral properties: ^{1}H NMR (^2H_2O, $N^2H_4O^2H$): δ 3.57 (1H, t, $J_{5'',5'}$ = 11.0 Hz, $J_{5',4}$ = 11.0 Hz, H-5′), 3.66 (1H, dd, $J_{5',4'}$ = 5.0 Hz, $J_{5',5''}$ = 11 Hz, H-5), 3.73 (1H, dd, $J_{2',3'}$ = 2.8 Hz, $J_{2',1'}$ = 10.0 Hz, H-2), 4.14 (1H, bt, $J_{3',4'}$ = 2.8 Hz, $J_{3',2'}$ = 2.8 Hz, H-3), 4.25 (1H, d, $J_{1',2'}$ = 10.0 Hz, H-1′), 4.65 (2H, s, C-9, CH_2), 6.78 (2H, d, $J_{3,2}$ = 8.4 Hz, *ortho* aromatic hydrogens of the aniline), 7.16 (2H, d, $J_{2,3}$ = 8.5 Hz, *meta* aromatic hydrogens of the aniline), 8.51 (1H, s, C-7 hydrogen of the pterin). The ^{31}P NMR of this sample showed the presence of only one resonance at 4.73, indicating the sample contained a single monophosphate ester. Both synthetic isomers had absorbance maxima at 274 and 343 nm and contained 1 mol of organic phosphate per mol of pterin. Reductive cleavage of the α-isomer with Zn/HCl (White, 1990) gave 6-meth-

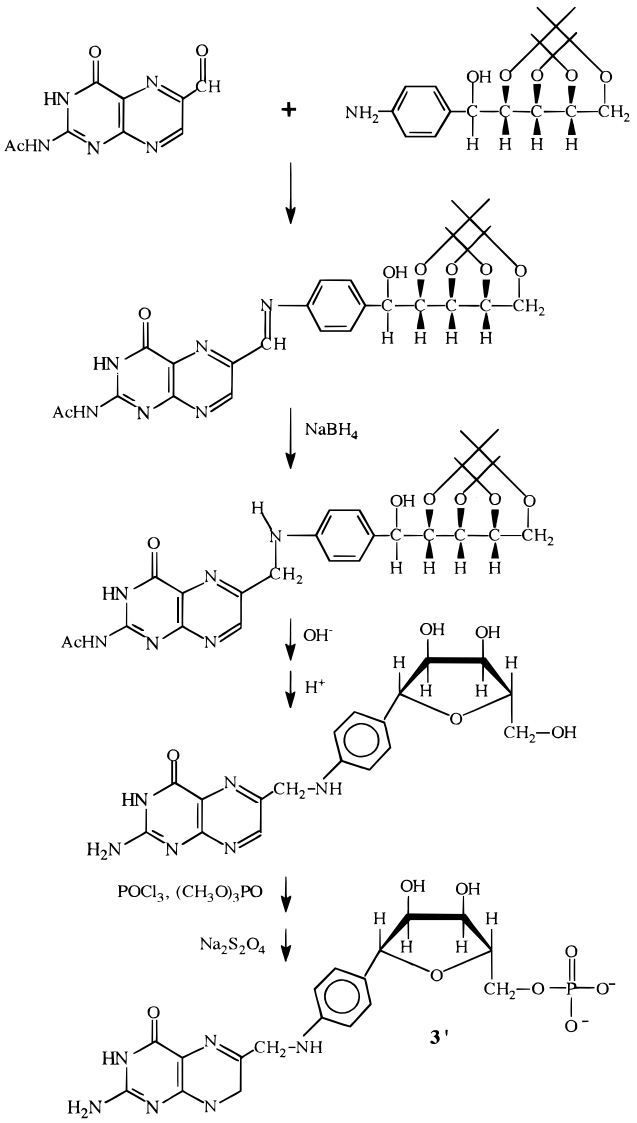

FIGURE 2: Chemical synthesis of compound **3′**.

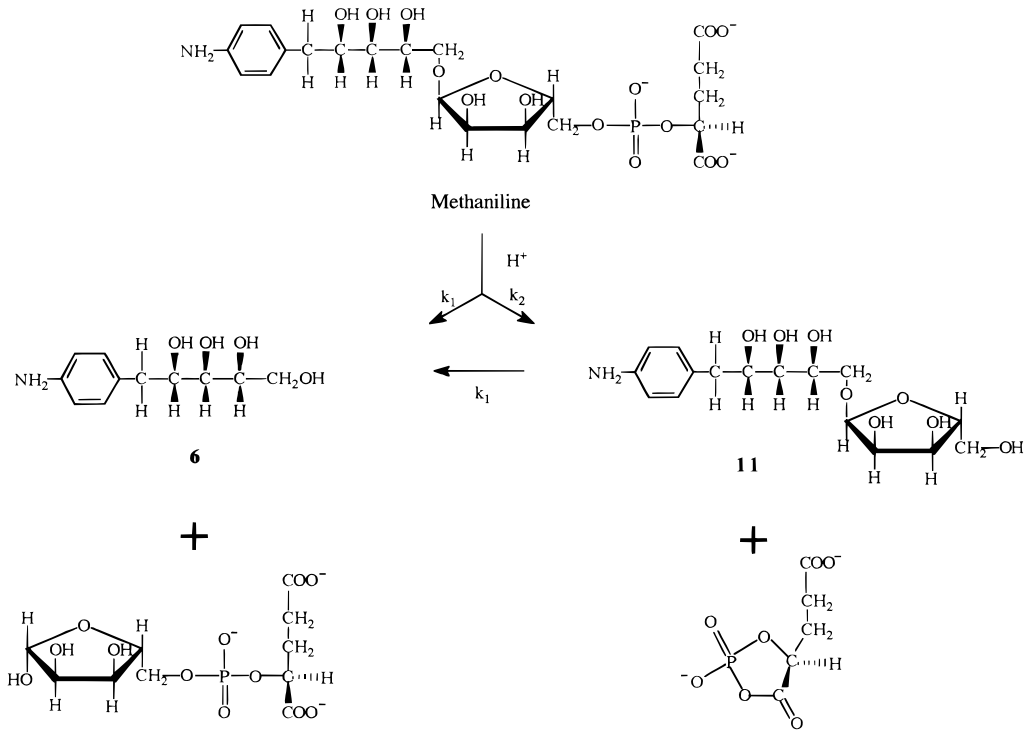

FIGURE 3: Routes for the acid-catalyzed hydrolysis of methanopterin and methaniline.

ylpterin and compound **10**, whereas reductive cleavage of the β-isomer gave 6-methylpterin and compound **1**.

Reduction of Pterin Substrates. Pterin substrates were reduced to the 7,8-dihydro stage with an excess of sodium dithionite (Kawai & Scrimgeour, 1972) at room temperature for 30 min before being added to the incubation mixtures. Reductions were also effected with Zn·NaOH as described by Whiteley and Huennekens (1967). Reduction of the pterins to the 5,6,7,8-tetrahydro stage was accomplished with sodium dithionite at 75 °C as described by Zakrzewski and Sansone (1971) for the reduction of folic acid. In each case, the reduction was followed by changes in the ultraviolet absorption spectrum, and were greater than 90% complete.

Synthesis of [2-^{3}H]-2-Hydroxyglutaric Acid. To 50 μL of a 0.5 M solution of 2-ketoglutaric acid was added 14.3 mg of sodium carbonate decahydrate to give a solution with a pH of 10; to this solution was then added 2.5 μmol of solid NaB^3H_4 (1 Ci/mmol). After 2 h at room temperature, 2.1 mg of unlabeled $NaBH_4$ was added to ensure complete reduction. After an additional 12 h at room temperature, the reaction was acidified with 1 M HCl and evaporated to dryness with a stream of nitrogen gas. The resulting residue was redissolved in methanol and evaporated 3 times in order to remove the boric acid reaction product as well as any tritiated water. The final residue was dissolved in water and adjusted to pH 8.0 with NaOH. The resulting sample was 99% radiochemically pure on the basis of TLC analysis using acetonitrile/water/formic acid (88%) (40/10/5, v/v/v) as the eluting solvent; it had an R_f value of 0.57.

Synthesis of (S)-[2,3,3,4,4-$^2H_5$]-2-Hydroxyglutaric Acid and Isotopic Dilution Analysis of 2-Hydroxyglutaric Acid in the Cell-Free Extract. (*S*)-(+)-5-Oxo-2-tetrahydrofurancarboxylic acid was formed by the nitrous acid deamination of (*S*)-[2,3,3,4,4-$^2H_5$]glutamic acid as described by Ravid et al. (1978). The resulting lactone was saponified with sodium hydroxide to produce the disodium salt, and then dissolved in a specific volume of water to generate a solution of known concentration. A portion of this solution was then added to a given volume of cell-free extract, and the resulting solution was heated 14 min at 100 °C, centrifuged to remove the precipitated proteins, and passed through a bed of Dowex-50W-8X H^+. The resulting solution was then evaporated to dryness and extracted with 3 M HCl in *n*-butanol. The resulting extract was heated at 100 °C for 3 h, concentrated with a stream of nitrogen gas, and extracted with methylene chloride. GC−MS of the resulting extract showed the presence of butyl 5-oxo-2-tetrahydrofurancarboxylate: M^+, *m*/*z* 187; $M^+ - 101$, *m*/*z* 86. From the ratios of the molecules containing zero and five deuteriums, it was calculated that the CFE contained 0.213 mM (*S*)-2-hydroxyglutaric acid. Gas chromatography of the diastereomeric ester of the isolated (*S*)-2-hydroxyglutaric acid with (*S*)-(+)-2-butanol was used to establish the stereochemistry of the HG (unpublished results).

RESULTS AND DISCUSSION

Chemical and Biochemical Preparation and Analysis of Proposed Biosynthetic Intermediates. In order to establish the intermediates involved in methanopterin biosynthesis, compounds of known structure had to be obtained and characterized so that they could be used to identify the intermediates involved in MPT biosynthesis. In order to accomplish this goal, the azo dye derivatives of the following compounds were prepared and characterized: compounds **1**, **6**−**8**, **10**, **11**, **13**, methaniline, and α-glutamylmethaniline.

The azo dyes of compounds **1** and **6**−**8** were prepared from the synthetic compounds. The azo dyes of methaniline and α-glutamylmethaniline were prepared from the arylamines produced from the oxidative cleavage of H_4MPT and tetrahydrosarcinapterin, respectively. Compound **6** could also be prepared by the mild acid hydrolysis of any of the compounds that contained compound **6** α-linked to a ribose. Compound **11** was generated by the mild acid hydrolysis of

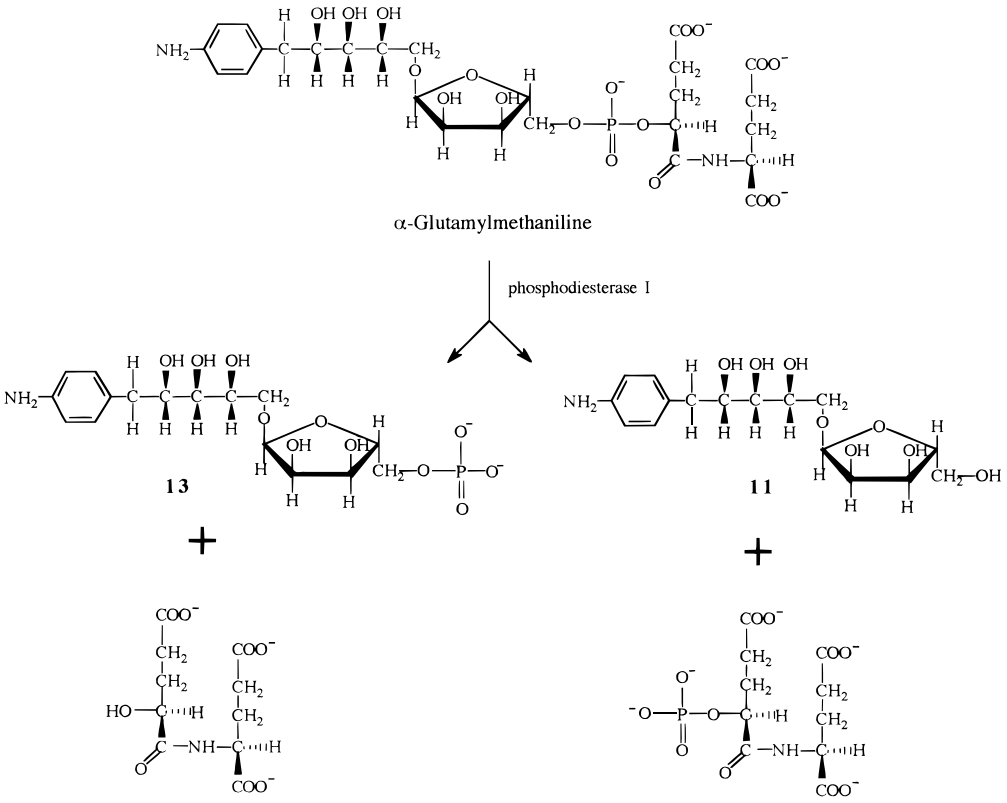

FIGURE 4: Phosphodiesterase cleavage of α-glutamylmethaniline.

methaniline as shown in Figure 3. The very facile hydrolysis of the phosphodiester in methaniline is the result of the generation of the cyclic phosphate intermediate shown in Figure 3, and has been observed in other analogously substituted phosphodiesters (Steffens et al., 1975). This mechanism of hydrolysis was clearly responsible for the decomposition of a derivative of methanopterin observed during the preparation of methenyl-H_4MPT (Donnelly et al., 1985). Since a free carboxylic acid α to a phosphodiester bond in a molecule is required for this type of hydrolysis to proceed, methanopterin derivatives in which this carboxylic acid is involved in a peptide bond will not undergo this type of hydrolysis. Thus, hydrolysis of the azo dye of α-glutamylmethaniline under the same conditions was found to produce only the azo dye of compound **6**. These observations can be taken as strong evidence that the glutamyl moiety in sarcinapterin is α-linked.

The azo dye derivatives of compounds **11** and **13** were prepared by phosphodiesterase I cleavage of the azo dye derivative of α-glutamylmethaniline (Figure 4). The identification of compound **11** from the acid hydrolysis of MPT, as well as the phosphodiesterase cleavage of α-glutamylmethaniline, confirmed the chemical connection between these two compounds.

With these reference compounds in hand, the following analytical problems were confronted. First, the azo dyes of α-glutamylmethaniline, methaniline, and compound **13** could not be completely resolved on the Bio-Gel P-4 column used for the initial separation of the azo dyes, and, as a result, these compounds could not be individually measured after such a separation. The azo dye of compound **13**, however, in such a mixture could be assayed by HPLC where it easily separates from the other two compounds. The remaining two compounds could not be eluted separately by the HPLC methods used. These two compounds, however, could be differentiated in a mixture, since upon subjecting the mixture to mild acid hydrolysis, only methaniline would give compound **11**, and this was easily separated from the other product by HPLC or a Bio-Gel P-4 column.

From the above procedures, it was established that *M. thermophila*, *M. voltae*, and *Methanococcus barkerii* all contained sarcinapterin (α-glutamylmethaniline) and *Methanobacterium thermoautotrophicum* contained methanopterin. The presence of glutamic acid in the sarcinapterin samples was confirmed by its direct detection by TLC after acid hydrolysis of the purified sample.

*Incorporation of [2,2′-$^2H_2$]-4-Aminobenzoic Acid into Compound **1***. Incubation of 0.5 mL of a cell-free extract of *M. thermophila* with 6.4 mM [2,2′-$^2H_2$]-4-aminobenzoic acid and 7.7 mM PRPP, under argon for 3 h at 50 °C, led to the formation of 11.7 μmol of compound **1** as assayed by the amount of azo dye formed. GC−MS analysis of the trifluoroacetyl derivative of compound **8** prepared from this biosynthesized compound **1** showed that all of the molecules were labeled with two deuteriums. These results established that PRPP and *p*AB condensed together to give compound **1**, and that *p*AB is the only biosynthetic precursor to the aromatic portion of compound **1**. To the author's knowledge a condensation of this type has never been described in a biochemical reaction. The closest analogy appears to be the proposed involvement of PRPP in the biosynthesis of several C-ribosides (Buchanan, 1993).

Direct Identification of Intermediates in Cell-Free Extracts. One approach to establishing the intermediates in a biosynthetic pathway is to chemically identify the compounds involved by analysis of the tissues or cells supporting the pathway. In the specific case of the analysis of intermediates in MPT biosynthesis in methanogenic *Archaea*, this analysis procedure is expedited by the oxidative cleavage of the reduced pterin intermediates to arylamines (White, 1985).

Table 1: Concentrations of the Intermediates of Methanopterin Biosynthesis in CFE of *M. thermophila* after Incubation with Different Precursors

		concentration of intermediate found[b] (μM)			
expt[a]	added substrates	**1**	**4′**	**5′**	**9′**
1	none	nd[c]	nd	28	6.9
2	PRPP, ATP	nd	nd	10	15[d]
3	H_2pteroic acid, PRPP, ATP	nd	nd	9.4	14
4	compound **2′**, PRPP, *p*AB	152	1.1	52	28

[a] Experiments were performed with 0.5 mL of CFE, incubated under H_2 for 3 h at 50 °C, containing the indicated concentrations of substrates: *p*AB, 4 mM; PRPP, 15.4 mM; ATP, 14.5 mM; and compound **2′**, 0.26 mM. All samples were found to contain 220 μM α-glutamylmethaniline, resulting from the oxidative cleavage of the H_4sarcinapterin present in the CFE. [b] The values reported are based on the amount of the arylamine formed and recovered as the azo dye derivative. [c] nd, not detected. [d] An increase in the amount of compound **11** was also observed in this experiment (from 0.6 μmol to 1.6 μmol); this presumably resulted from cleavage of compound **9′** by a phosphatase, or by acid hydrolysis of demethylated MPT during the reaction work up.

Table 2: Incorporation of [2-^{3}H]-2-Hydroxyglutaric Acid into Demethylated H_2Methanopterin by CFE of *M. thermophila*

expt[a]	added substrate(s)	nM methaniline produced
1	none	76
2	compound **6**	83
3	compound **6**, ATP, UTP, ribose, ribose-P, NAD	83
4	PRPP, ATP	200
5	compound **6**, PRPP, ATP	200
6	*p*AB, PRPP, ATP	280
7	compound **5′**, PRPP, ATP	270

[a] Experiments were performed with 0.5 mL of CFE, incubated under H_2 at 50 °C for 3 h, containing 20 μL of carrier-free [2-^{3}H]-2-hydroxyglutaric acid and the indicated substrates at the following concentrations: compound **6**, 0.27 mM; PRPP, 15.4 mM; ATP, 14.5 mM; compound **5′**, 0.05 mM; and *p*AB, 4 mM. The CFE contained 213 μM HG and 220 μM H_4sarcinapterin measured from the amount α-glutamylmethaniline produced upon its oxidative cleavage.

These arylamines can then be purified and selectively assayed after conversion to their azo dye derivatives (White, 1993a). Application of these analytical methods to the analysis of the oxidatively-cleaved reduced pterin intermediates present in CFE of *M. thermophila*, identified (1) the azo dye of α-glutamylmethaniline, resulting from the oxidative cleavage of H_4sarcinapterin present in the cells, and (2) the azo dye derivatives of compounds **6** and **13**, resulting from the oxidative cleavage of the parent compounds **5′** and **9′** (Table 1, experiment 1), which are in the proposed biosynthetic pathway (Figure 6).

Considerations of Possible Pathways. At first it was thought, since compound **6** was found in the CFE, that this compound was, in fact, a pathway intermediate which would then react with PRPP to generate compound **13**. However, as can be seen from data presented in Table 2, incubation of CFE with compound **6** did not lead to an increase in the incorporation of HG to MPT (compare experiment 1 with 2 and experiment 4 with 5). It was concluded that the true intermediate was not compound **6**, but its dihydropterin derivative, compound **5′**, which produced compound **6** only upon oxidative cleavage. The question then arises as to how compound **5′** was formed.

Considering the fact that *M. thermophila* cells readily form compound **1** from *p*AB and PRPP, as well as knowledge about folate biosynthesis, two possible pathways seem plausible. In the first pathway, 7,8-dihydro-6-hydroxymethylpterin pyrophosphate (compound **2′**, Figure 6) would react with compound **1** to form compound **3′**, which would then be reduced to compound **4′** and dephosphorylated to compound **5′** (Figure 6). In an alternate pathway, compound **2′** could first combine with *p*AB to form pteroic acid, as occurs in folate biosynthesis, and the pteroic acid could then combine with PRPP to form Compound **3′**. Compound **3′** would then be converted to compound **5′** as described in the first pathway. To test which was the operational pathway, CFE were incubated either with H_2pteroic acid and PRPP, or with compound **2′**, *p*AB and PRPP. The production of compounds **4′** and **5′** were established by measuring their oxidative cleavage products, compounds **7** and **6**. As can be seen from the data shown in Table 1, experiment 3,

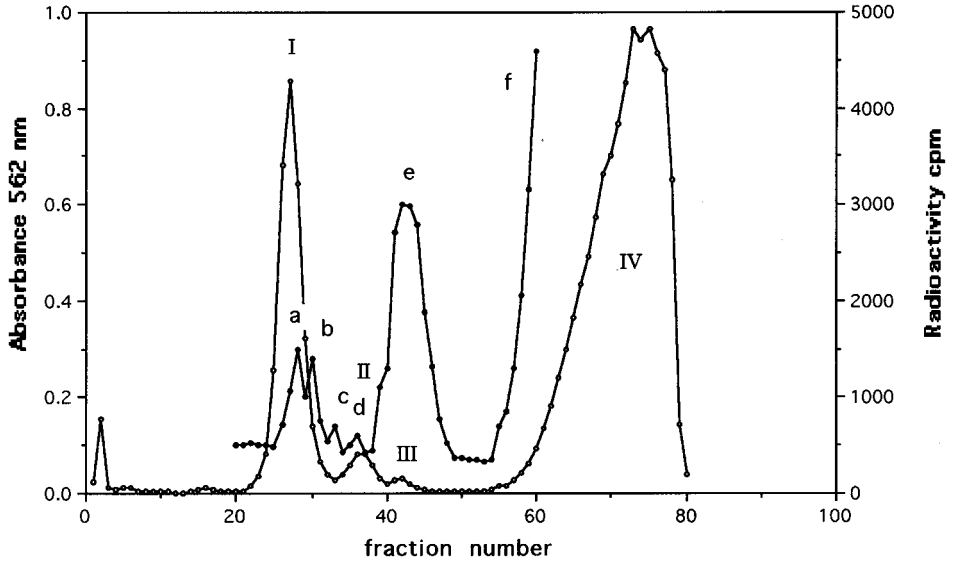

FIGURE 5: Elution profiles from a Bio-Gel P-6 column of the azo dyes generated from the arylamines produced by incubation of CFE of *M. thermophila* with [^{14}C]*p*AB, ribose-P, and ATP. Peaks I−IV are the optically-detected azo dye-containing compounds, and peaks a−f are the radioactive product peaks detected.

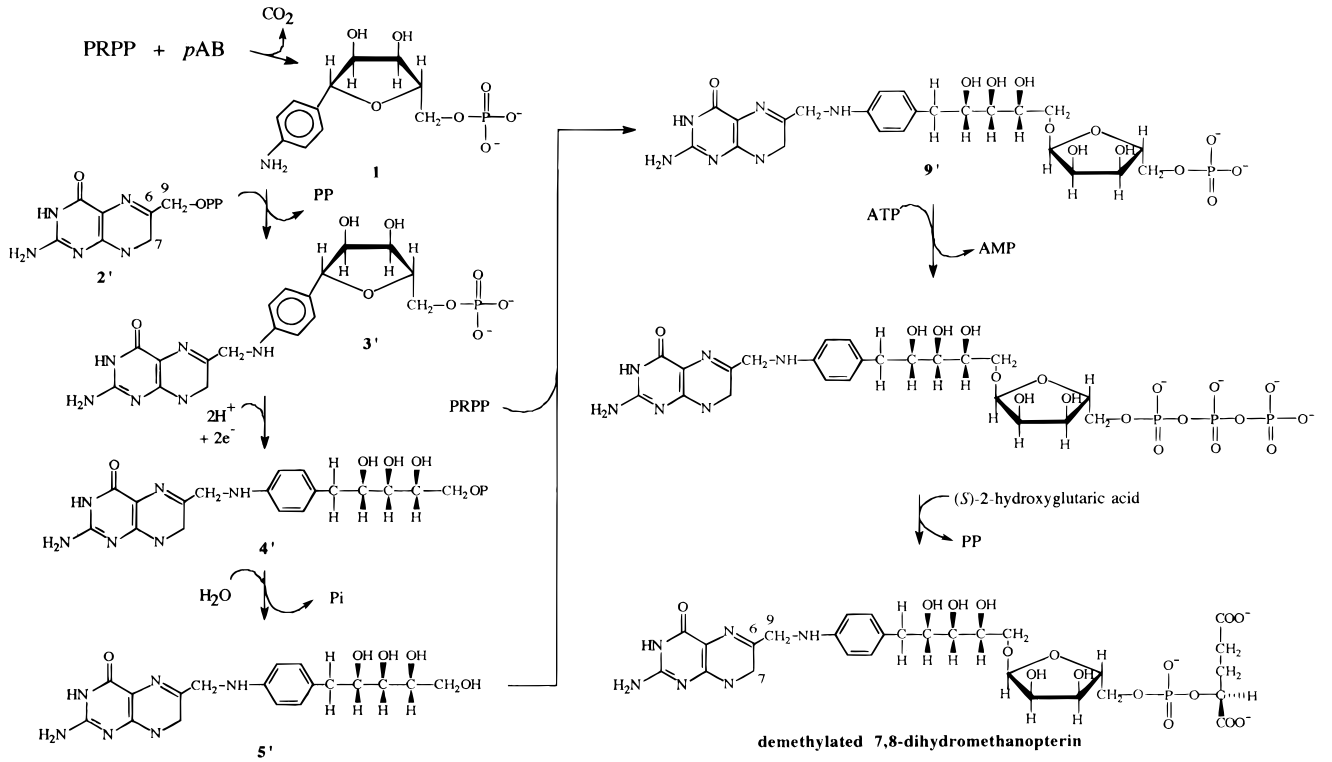

FIGURE 6: Proposed pathway for the biosynthesis of demethylated methanopterin.

Table 3: Conversion of 7,8-H_2Pterin-6-ylmethyl-4-(β-D-ribofuranosyl)aminobenzene 5′-Phosphate (**3′**) to 7,8-H_2Pterin-6-ylmethyl-1-(4-aminophenyl)-1-deoxy-D-ribitol 5-Phosphate (**5′**) by CFE of *M. thermophila*

				concentration of products[b] (μM)		
expt	compd(s)[a]	reductant	cofactor	**1**	**1** + **10**	**7**
1	**3′** (38 μM)	H_2		4.1		4.3
2	**3′** (230 μM)	H_2		14		17
3	**3″** (76 μM)[c]	H_2		12		2.8
4	**3′** + **12′** (440 μM)[d]	H_2			38	8.1
5	**3′** + **12′** (220 μM)[d,e]	CO			11	5
6	**3′** + **12′** (440 μM)[d]	H_2	NADH, NADPH		17	2.9
7	**3′** + **12′** (360 μM)[f]	H_2			24	3.7
8	**3′** + **12′** (360 μM)[f]	H_2	FMN, F_{420},[g] FAD[h]		11.2	10.8
9	**3′** + **12′** (360 μM)[f]	H_2	FMN (0.87 mM)		13.4	16.0
10	**3′** + **12′** (360 μM)[f]	H_2	F_{420} (0.34 μM)			7.4
11	**3′** + **12′** (360 μM)[f]	H_2	FAD (4.0 mM)			4.5

[a] This concentration of substrate in the reaction is calculated from the absorbance of the sample before dithionite reduction and represents the total concentration of the compounds **3′** and/or **12′** in the incubation mixture. [b] The values reported are those measured from the amount of azo dye derivative recovered from the mixture after incubation with the substrate(s). [c] The observed conversion of compound **3″** (the tetrahydro form of compound **1**) reported in experiment 3, is believed to result from the presence of compound **3′** present in the substrate preparation. This is expected, because the reduction conditions used are known to also produce some dihydropterins (Zakrzewski & Sansone, 1971). [d] The ratio of compounds **3′** to **12′** were 0.26. [e] The substrate pterin was reduced with Zn/NaOH for this experiment. [f] The ratios of compounds **3′** to **12′** was 0.53. [g] F_{420} is a 5-deazaflavin-containing cofactor present in methanogens (Eirich et al., 1978). [h] The concentrations of FMN, F_{420}, and FAD were 2 mM, 170 μM, and 1.7 mM, respectively.

incubation of CFE with H_2pteroic acid and PRPP gave no detectable indication of compounds **1** or **4′**, whereas incubation of CFE with compound **2**, *p*AB, and PRPP produced a 152-μM solution of compound **1** (Table 1, experiment 4). Furthermore, only in experiment 4 was there observed any increase in the amounts of compounds **5′** and **9′**, which are proposed to be intermediates in MPT biosynthesis. These data are thus consistent with compound **5′** being formed by the first pathway and compound **5′** being converted to compound **9′** by its reaction with PRPP.

Conversions of Intermediates in the Pathway. In order to verify this proposed involvement of compound **3′** in the pathway (Figure 6), this compound was synthesized and incubated with CFE and its conversion to compounds **4′** and **5′** was established by measuring the amounts of compounds **7** and **6** produced by their oxidative cleavage. In all incubations conducted with compound **3′**, the production of compound **4′** was established by measuring the generation of compound **7** (Table 3). Compound **6**, resulting from the dephosphorylation of compound **7**, was also detected in each experiment. However, since compound **6** was also produced from compound **5′**, which was present in the CFE, quantitation of its total production was difficult. Only in experiment 2 was the production of compound **5′** high enough above the background level that its concentration (21 μM) could be accurately measured.

Biochemical Reduction of Compound 3′ to Compound 4′. To gain an understanding of the process involved in the

conversion of compound **3′** and **4′**, the extent of this conversion was measured in CFE supplemented with different cofactors. For convenience some of these experiments were carried out with a mixture of the synthetic α and β compounds (compounds **3′** and **12′**). From experiments 4 and 5 (Table 3), we see that the reducing power for the reaction can come from either H_2 or CO; consistent with the presence of a hydrogenase and a CO dehydrogenase in these cells (Terlesky et al., 1986). Incubation of the CFE with a mixture of NADH and NADPH resulted in a lowering of the total product produced (experiment 6). On the other hand, incubation of the CFE with a mixture of FMN, FAD, and F_{420} led to a stimulation of the reduction observed under H_2 (compare experiment 7 with experiment 8). This stimulation was mostly a result of the added FMN (experiment 9), but F_{420} was also found to stimulate the reaction (experiment 10). These data are consistent with an FMN and/or an F_{420}-dependent enzyme(s) being involved in the conversion of compound 3′ to compound 4′. The details of the reaction will have to await the isolation of the enzyme(s) involved.

Tests for Substrates Involved in the Formation of the α-Ribosidic Bond. Possible precursors of the ribose present in MPT include PRPP, nucleotides, and NAD. In order to test if any of these compounds were involved in the biosynthesis, cell-free extracts of *M. thermophila* were incubated with compound **6** and PRPP and ATP, or individually with NAD^+, ATP, CTP, or GTP, and the generation of new arylamine-containing compounds was assayed by chromatography of the azo dye derivatives of the arylamines on Bio-Gel P-4 columns. Only when the incubations were done in the presence of PRPP was any new arylamine observed. This new arylamine consisted of a single peak which amounted to less than 5% of the compound **6** peak. The new arylamine peak was observed midway between the α-glutamylmethaniline and the compound **6** peaks and eluted at the same position as a known sample of compound **11**. The presence of compound **11** in this peak was confirmed by HPLC analysis using a known sample of compound **11** obtained by the mild acid hydrolysis of the azo dye derivative of methaniline. (These experiments were done before it was established that compound **6** was not an intermediate in MPT biosynthesis.)

These experiments suggested that PRPP is involved in the addition of the ribose moiety of MPT and SP. This proposal was verified by incubation of a CFE, which contained both compound **5′** and compound **9′**, with PRPP and ATP (Table 1, experiment 2). A decrease in the concentration of compound **5′** and an increase in the concentration of compound **9′** were observed, consistent with PRPP and compound **5′** reacting together to form compound **9′**.

These data indicated that compound **5′** could combine with PRPP to generate compound **9′**, which was then converted, in the presence of ATP and HG, to demethylated MPT. This reaction sequence was confirmed by the stimulation of demethylated MPT biosynthesis in the incubation of CFE with compound **5′**, PRPP, and ATP (Table 2, experiment 7).

Formation of the Phosphodiester Bond in MPT. A possible route for the conversion of compound **9′** to demethylated H_2MPT would be its reaction with ATP to form the triphosphate (compound **9′**-PP, Figure 6) which would then react with HG, with displacement of pyrophosphate, to form demethylated H_2MPT. This reaction sequence would be analogous to that used by nature for the formation of the phosphodiester bonds in DNA and RNA from nucleotide triphosphates. Incubation of CFE, which contains ATP, with [2-^{3}H]HG leads to the formation of 76 nM demethylated H_2-MPT as measured by the oxidatively released methaniline (experiment 1, Table 2). The observed incorporation of radioactivity was found to be diminished 3-fold when the incubation was performed with a CFE that had been desalted on a column of G-25 Sephadex. This indicated that precursors, such as the triphosphate of compound **14′**, were present in the extract that could combine with [2-^{3}H]HG to produce demethylated H_2MPT. Incubation of CFE with PRPP and ATP along with a series of different substrates expected to increase the concentration of compound **9′** (see experiments 4, 6, and 7 in Table 2) all showed a 2.5−3-fold increase in the amount of demethylated H_2MPT formed.

Table 4: Compounds Characterized from the Incubation of Cell-Free Extracts of *M. thermophila* with [U-^{14}C]-4-Aminobenzoic Acid, Ribose 5-P, and ATP

compd identified[a]	visible peak	radioactive peak[b]
α-glutamylmethaniline	I[c]	
1		a
7		b
11		c
6	II[c]	d
8		shoulder of peak e
aniline	III[d]	e
*p*AB	IV[d]	f

[a] All compounds were separated and identified as their azo dye derivatives. [b] The peak designations refer to Figure 5. [c] These peaks had a purple color because of strong absorbance at 562 nm. [d] These peaks had a pink color because of strong absorbance at 553 nm.

Incubation of a Cell-Free Extract with [^{14}C]pAB. If the above pathway is operational in *M. thermophila*, then incubation of CFE with [^{14}C]*p*AB should label the intermediates discussed above. Thus, 1 mL of a cell-free extract of *M. thermophila* was incubated with 0.33 mM [^{14}C]*p*AB (5 mCi/mmol), 2 mM ribose 5-phosphate, and 5 mM ATP under hydrogen for 3 h at 37 °C. The azo dye adducts of the oxidatively-derived arylamine-containing products were formed and separated on a Bio-Gel P-6 column, giving the elution profiles shown in Figure 5. Four visible azo dye-containing peaks, designated I−IV, and six peaks containing radioactivity, designated a−f, were observed. These peaks were established to be the compounds listed in Table 4 on the basis of comparison to the known compounds. These comparisons were made using a combination of different chromatographic methods that include Bio-Gel column chromatography, TLC, and HPLC, as well as other tests including both acid and phosphatase cleavages to the expected products.

Compounds **6** and **7** and possibly some of compound **1** would have arisen by the oxidative cleavage of intermediate compounds **5′**, **4′**, and **3′**, respectively. Compounds **8** and **11** would have been derived in the same manner from compounds **1** and **11**, respectively, after phosphatase cleavage. The aniline would have been derived by the decarboxylation of *p*AB and may have been produced as a side product of the condensation of *p*AB with PRPP.

Concluding Remarks. The above data are consistent with the formation of demethylated H_2MPT proceeding as outlined in Figure 6. Previous work has shown that demethylated

H_2MPT is methylated to H_2MPT by *S*-adenosylmethionine-dependent methylations (White, 1990). The H_2MPT is then reduced to the active form of the coenzyme H_4MPT. It is most likely that this reduction is carried out by a dihydromethanopterin reductase, analogous to the dihydrofolate reductases found in other organisms, although this has yet to be established.

Finally, the complete biosynthesis of sarcinapterin will require the addition of an α-linked glutamic acid to H_2MPT. Although this reaction would, at first sight, appear to be analogous to the addition of glutamic acid to folate, catalyzed by pteroylpolyglutamate synthetase (Shane, 1980), the fact that the glutamyl group is α-linked, as shown in this paper, makes one strongly doubt this idea. It is possible that the synthesis is more closely related to the biosynthesis of the α-glutamyl-linked folates found in *E. coli* (Ferone, 1986).

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