REVIEW ARTICLE

Tetrahydrofolate and tetrahydromethanopterin compared: functionally distinct carriers in C_1 metabolism

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In most organisms, tetrahydrofolate (H_4folate) is the carrier of C_1 fragments between formyl and methyl oxidation levels. The C_1 fragments are utilized in several essential biosynthetic processes. In addition, C_1 flux through H_4folate is utilized for energy metabolism in some groups of anaerobic bacteria. In methanogens and several other Archaea, tetrahydromethanopterin (H_4MPT) carries C_1 fragments between formyl and methyl oxidation levels. At first sight H_4MPT appears to resemble H_4folate at the sites where C_1 fragments are carried. However, the two carriers are functionally distinct, as discussed in the present review. In energy metabolism, H_4MPT permits redox-flux features that are distinct from the pathway on H_4folate. In the reductive direction, ATP is consumed in the entry of carbon from CO_2 into the H_4folate pathway, but not in entry into the H_4MPT pathway. In the oxidative direction, methyl groups are much more readily oxidized on H_4MPT than on H_4folate. Moreover, the redox reactions on H_4MPT are coupled to more negative reductants than the pyridine nucleotides which are generally used in the H_4folate pathway. Thermodynamics of the reactions of C_1 reduction via the two carriers differ accordingly. A major underlying cause of the thermodynamic differences is in the chemical properties of the arylamine nitrogen N^{10} on the two carriers. In H_4folate, N^{10} is subject to electron withdrawal by the carbonyl group of *p*-aminobenzoate, but in H_4MPT an electron-donating methylene group occurs in the corresponding position. It is also proposed that the two structural methyl groups of H_4MPT tune the carrier's thermodynamic properties through an entropic contribution. H_4MPT appears to be unsuited to some of the biosynthetic functions of H_4folate, in particular the transfer of activated formyl groups, as in purine biosynthesis. Evidence bearing upon whether H_4MPT participates in thymidylate synthesis is discussed. Findings on the biosynthesis and phylogenetic distribution of the two carriers and their evolutionary implications are briefly reviewed. Evidence suggests that the biosynthetic pathways to the two carriers are largely distinct, suggesting the possibility of (ancient) separate origins rather than divergent evolution. It has recently been discovered that some eubacteria which gain energy by oxidation of C_1 compounds contain an H_4MPT-related carrier, which they are thought to use in energy metabolism, as well as H_4folate, which they are thought to use for biosynthetic reactions.

Key words: biosynthesis, evolution, methanogenesis, redox, thermodynamics.

INTRODUCTION

Tetrahydrofolate (H_4folate) is the biologically active form of the human vitamin folic acid. Its principal metabolic roles in man are in the provision of C_1 fragments for the biosynthesis of purines and thymidylate, and for the regeneration of methionine from homocysteine. The biochemistry of folic acid and its derivatives have been intensively studied for many years, particularly in relation to human health and disease. Folic acid is synthesized by many or most bacteria, plants and unicellular eukaryotes, and in such organisms H_4folate also mediates some additional functions (see below for bacteria).

In a landmark collaboration, analysis of rRNA gave evidence that methanogens are phylogenetically deeply separated from 'typical' bacteria [1–3]. (For an interesting personal recollection, see [4], pages 13 and 14.) The concept of the Archaea as a third domain of life, distinct from both the Bacteria and Eukarya, was born [5–7]. Methanogens were classified into various groups within the Archaea [8,9]. Several new cofactors of methanogenesis were revealed [10]. One of these became called methanopterin [11]. The biologically active form of this compound, tetrahydromethanopterin (H_4MPT), resembles H_4folate in that both compounds are pterin-containing carriers of C_1 fragments between formyl and methyl oxidation levels [12,13]. However, it has become clear that H_4folate and H_4MPT are not functionally equivalent.

The present review compares and contrasts H_4folate and H_4MPT. A qualitative outline of their structures and functions is followed by a summary of data indicating that the thermodynamics of C_1 flux on the two carriers are different. Distinctive structural features underly the thermodynamic differences, and the chemical differences in turn underlie distinct metabolic roles of the two carriers. Evolution of the two distinct C_1 pathways is obviously of interest and is discussed from the perspective of biosynthesis and phylogenetic distribution of the two carriers. An important recent finding is that some C_1-oxidizing members

Abbreviations used: H_4folate, tetrahydrofolate; H_4MPT, tetrahydromethanopterin; PABA, *p*-aminobenzoate; DHFR, dihydrofolate reductase; TS, thymidylate synthase; SHMT, serine hydroxymethyltransferase; MTHFR, methylenetetrahydrofolate reductase; MFR, methanofuran; H_4SPT, tetrahydrosarcinapterin; MCH, methenyl-H_4MPT cyclohydrolase; HMD, H_2-dependent CH_2-H_4MPT dehydrogenase; MER, methylene-H_4MPT reductase; CoM, coenzyme M; CoB, coenzyme B; MR, methyl-CoM reductase; RPG effect, R. P. Gunsalus effect; PRPP, phosphoribosyl diphosphate; β-RFA-P, 4-(β-D-ribofuranosyl)aminobenzene 5′-phosphate; ORF, open reading frame; PLP, pyridoxal phosphate; GAR, glycinamide ribonucleotide.

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of the domain Bacteria contain an H_4MPT-related carrier, which they use in energy metabolism, as well as containing H_4folate, which they are presumed to use for biosynthetic purposes [14].

STRUCTURAL AND CHEMICAL FEATURES

H_4folate

Figure 1(a) shows the structure of H_4folate and of several derivatives. The molecule consists of a reduced pterin linked to *p*-aminobenzoate (PABA) and thence to one or more glutamate residues. Polyglutamylation promotes intracellular retention, and different enzymes catalysing the various reactions of H_4folate biochemistry have preferences for different numbers of glutamate residues (for a review, see [15]).

Biological activity of the cofactor requires that the pyrazine ring be fully reduced. Full reduction introduces a chiral centre at C^6. In enzymically reduced H_4folate the absolute configuration is (6*S*) [15a], and only that isomer is biologically active. The C_1 substituent is carried at N^{10} or N^5 or bridged between both, as shown (see Table 1 for nomenclature). [Attachment of a C_1 fragment to N^{10}, or bridged between N^{10} and N^5, results in change of designation at C^6 from (*S*) to (*R*). This is solely a consequence of Cahn–Ingold–Prelog priority rules; bonding at C^6 remains unchanged.]

A crucial feature of H_4folate is that the chemical properties of N^5 and N^{10} are different. N^{10} has an extremely low pK_a of -1.25, whereas the pK_a of N^5 is 4.8 [16]. The very low pK_a of N^{10} is attributable to two interacting factors: the electron-withdrawing effect of the carbonyl group of PABA ([17]; discussed in detail below), and prior protonation of N^5 and N^1 as the pH is lowered [16].

H_4folate undergoes non-enzymic condensation *in vitro* with formaldehyde to give 5,10-CH_2-H_4folate. The 5-carbinolamine and cationic imine derivatives in Figure 1(a) are intermediates in the condensation [18]. The cationic imine is believed to be exceedingly short-lived, but is crucially important because it is a predicted intermediate in the pathways of several enzymic reactions of CH_2-H_4folate, as will be discussed.

H_4MPT

Figure 1(b) shows the structure of H_4MPT and derivatives. The structure of non-reduced methanopterin (MPT) was determined by two-dimensional NMR [19]. Fully reduced H_4MPT was

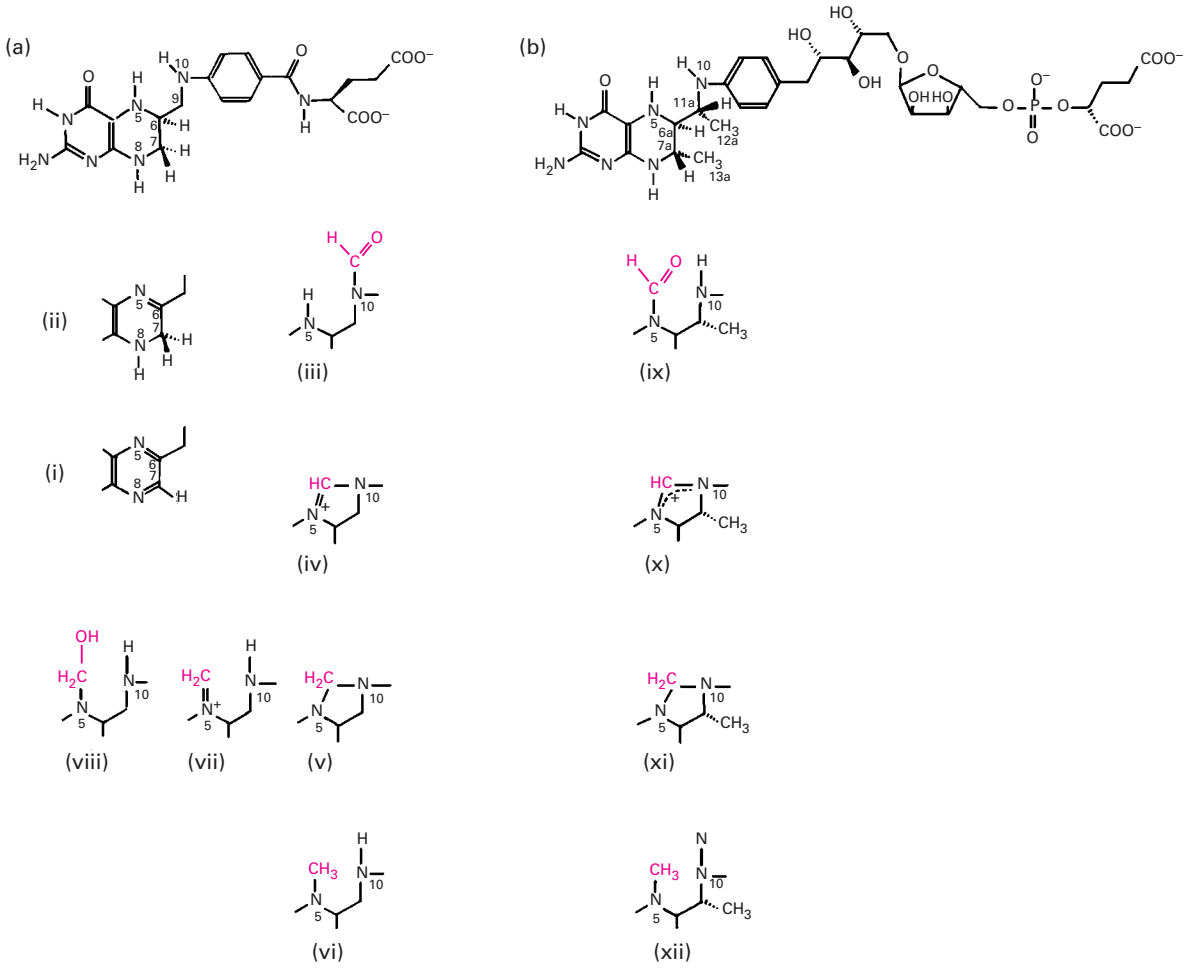


Figure 1 Structures of (a) H_4folate (monoglutamyl form) and (b) H_4MPT

(**a**) Partial structures (i)–(vi), clockwise: (i) pyrazine ring of folate (non-reduced); (ii) pyrazine ring of 7,8-H_2folate; (iii) 10-HCO-H_4folate; (iv) CH^+-H_4folate; (v) CH_2-H_4folate; (vi) 5-CH_3-H_4folate. Partial structures (vii), 5-cationic imine (also called iminium cation) and (viii) 5-carbinolamine. (**b**) Partial structures: (ix) 5-HCO-H_4MPT; (x) CH^+-H_4MPT, drawn to indicate greater delocalization than in CH^+-H_4folate; (xi) CH_2-H_4MPT; (xii) 5-CH_3-H_4MPT. (5-HCO-H_4folate, not shown, is structurally analogous to 5-HCO-H_4MPT.) Attached C_1 fragments are shown in red.

Table 1 Nomenclature of C_1 derivatives of H_4folate and H_4MPT

Names and abbreviations for H_4folate and its C_1 derivatives are generally as recommended in [182]. In particular, H_4folate is not further abbreviated (e.g. to H_4F), and since N^5 and N^{10} are uniquely numbered, the designations 5 and 10 suffice. For simplicity the configuration at C^6 is not specified; the biologically active configuration (see the text) is to be assumed throughout the present Review. Also, for simplicity, the number of glutamate residues attached to *p*-aminobenzoate is not specified. Some authors use $CH^+ \equiv H_4MPT$ and $CH_2 = H_4MPT$ for the methenyl and methylene derivatives of H_4MPT [78]. The formyl and methenyl compounds are at the level of oxidation of formate. The methylene and methyl compounds are at oxidation level of formaldehyde and methanol respectively. It should be borne in mind that the bound C_1 derivatives are related to the respective unbound compounds by loss of the elements of water as follows: formyl, −1 mol of water relative to formic acid and free carrier; methenyl, −2 mol of water + H^+ relative to formic acid and carrier; methylene, −1 mol of water relative to formaldehyde and carrier; methyl, −1 mol of water relative to methanol and carrier. These relationships can be seen from the structures in Figure 1.

	Bound to H_4folate		Bound to H_4MPT	
Unbound	Name	Abbreviation	Name	Abbreviation
Formate	Formyltetrahydrofolate (5 or 10, as specified)	HCO-H_4folate	Formyltetrahydromethanopterin (5 or 10, as specified)	HCO-H_4MPT
	5,10-Methenyltetrahydrofolate	CH^+-H_4folate	5,10-Methenyltetrahydromethanopterin	CH^+-H_4MPT
Formaldehyde	5,10-Methylenetetrahydrofolate	CH_2-H_4folate	5,10-methylenetetrahydromethanopterin	CH_2-H_4MPT
Methanol	5-Methyltetrahydrofolate	5-CH_3-H_4folate	5-Methyltetrahydromethanopterin	5-CH_3-H_4MPT

shown to utilize formaldehyde *in vitro* in the pathway to methanogenesis [12]. The C_1-H_4MPT derivatives in Figure 1(b) are intermediates in methanogensis from CO_2 and H_2 [10,13,20] (for pathway details, see below).

H_4MPT resembles H_4folate in that it consists of a pterin linked to an arylamine, with C_1 binding at N^5 or both N^5 and N^{10}. However, to the right of the benzene ring the structures differ completely. Instead of the glutamate residue(s) in H_4folate, the side chain of H_4MPT consists of a ribitol residue linked to ribose 5-phosphate and thence to hydroxyglutarate. There are two potential consequences of this major structural difference. First, insofar as the 'right arms' of the respective molecules might interact with enzymes, the H_4MPT arm is completely different from the H_4folate arm. Secondly, and importantly, the benzene ring is linked directly to the methylene of ribitol, with no intervening carbonyl group. Thus the electron-withdrawing effect of the carbonyl on N^{10} of H_4folate, referred to above, is absent in H_4MPT ([17]; discussed later). A further difference between the two carriers is that H_4MPT contains two structural methyl groups on the carbon atoms designated C^{7a} and C^{11a}. Possible functions of these methyl groups are also discussed later. (For historical reasons the numbering system of H_4MPT differs from that of H_4folate [20].) The system shown in Figure 1(b) is used by most authors, e.g. [21], although some authors use the H_4folate numbering system [22].

H_4MPT, like H_4folate, is chiral at C^6. In addition, the two methyl groups introduce chiral centres at C^{7a} and C^{11a}. The configurations at these carbon atoms were deduced by NMR and optical methods to be (6*S*) (as in H_4folate), (7*S*,11*R*), the NMR data linking the assignments (see [21] and also Figure 4 in [17]). [22] provides independent verification of (*R*) stereochemistry at C^{11a} (termed C^9 in [22]).

REDUCTION OF THE PTERIN RINGS

Folate to H_4 folate

The enzyme dihydrofolate reductase (DHFR) catalyses NADPH-linked reduction of 7,8-H_2folate to H_4folate [reaction (1) in Table 2, below], and also, with slower kinetics, reduction of folate to 7,8-H_2folate. Folate can be a dietary source of the carrier (due to oxidation of some dietary H_4folate or to dietary supplementation), whereas 7,8-H_2folate is the product of the folate-biosynthetic pathway in organisms that synthesize the carrier. 7,8-H_2folate is also released from the thymidylate synthase reaction (see below).

The X-ray structures of DHFRs from bacterial [23] and vertebrate sources (for a review, see [24]), complexed with various substrates and inhibitors, have been determined, enabling deduction of the catalytic mechanism [23,24]. NADP(H) binds 'behind' the pterin ring in the pterin orientation shown in Figure 1(a), with transfer of hydride to the *si* face of C^6 (H_2folate reduction) or C^7 (folate reduction) and protonation at N^5 (or N^8) coming from bound water (discussed in [24]). Reduction of H_2folate to H_4folate generates the (6*S*) chirality referred to above.

DHFRs are generally very sensitive to the anticancer agent methotrexate, a competitive inhibitor which binds tightly to the enzyme (discussed in [23]). Bacterial DHFRs are also sensitive to trimethoprim [25] and protozoal DHFRs to cycloguanil and pyrimethamine ([26] and references cited therein). (For a discussion of DHFR and H_2folate reduction in *Escherichia coli*, see [27].)

Methanopterin to H_4MPT

The biochemistry of methanopterin reduction is less well characterized than that of folate reduction. Given the (6*S*) configuration of H_4MPT, hydride transfer to C6 must take place from 'behind' (Figure 1b) as in H_2folate reduction. However, the sterochemistry at C^{7a} (Figure 1b) [21] implies that the hydride for this reduction comes from 'in front'. Moreover, the cellular conditions for methanopterin reduction may be more restrictive than those for folate reduction. In an early study, reduction of methanopterin to H_4MPT by cell extracts of *Methanobacterium thermoautotrophicum* was observed to take place in the presence of methyl-coenzyme M (methyl-CoM) (an intermediate in methanogenesis, see below) together with formaldehyde, but not in their absence [12], suggesting that reduction to active carrier is linked in some way to C_1 flux.

The putative reductase does not seem to be closely related to DHFR, because no DHFR-related sequence has been identified in the complete genome sequences of three organisms that utilize H_4MPT: *Methanococcus jannaschii* [28], *M. thermoautotrophicum* [29] or *Archeoglobus fulgidis* [30]. Nor is the reduction cofactor known; possible candidates might be NAD(P)H or the deazaflavin $F_{420}H_2$ (see below). Characterization of the reductase system and comparison with DHFR would be of biochemical and evolutionary interest.

COMPARISON OF THE H_4FOLATE AND H_4MPT PATHWAYS

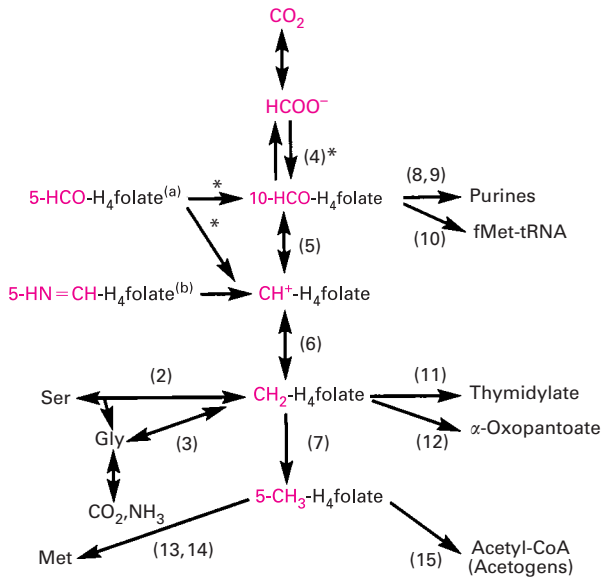
The H_4folate pathway

Reactions of H_4folate that are important for the present review are summarized in Scheme 1 and in Table 2. In the Table, the reactions are grouped into several functional categories. In the first category, generation of active cofactor, the DHFR reaction was discussed above. Reactions that determine the numbers of glutamic acid residues [see * in the legend to Table 2], though functionally important [15], are not directly relevant here and are not discussed further.

The next category comprises the loading of C_1 on to H_4folate. In man, the major loading route is from serine (reaction 2) yielding CH_2-H_4folate and glycine. Reaction (3) cleaves any glycine that is in excess of the organism's needs, yielding additional CH_2-H_4folate from C^2 of glycine (see [31] for both reactions). Processing of glycine in this manner is important in man and occurs in mitochondria. A severe genetic disorder, non-ketotic hyperglycinaemia, results from a defective enzyme system [32].

C_1 also enters the H_4folate pathway from formate, in an ATP-requiring reaction that generates 10-HCO-H_4folate (reaction 4) [33]. In man this is a quantitatively minor route [33], but it is the major route in some bacteria, especially acetogens (see below).

N^5 of H_4folate is an acceptor for some catabolically generated C_1 at the formyl oxidation level [34,35] (for brief details, see the legend to Scheme 1). Enzymic conversion of 5-HCO-H_4folate into 10-HCO-H_4folate requires energy input from coupled hydrolysis of ATP [36]. This affords evidence that 10-HCO-H_4folate is the more activated of the two formyl isomers. Generation of the methenyl derivative, CH^+-H_4folate, from 5-HCO-H_4folate also requires ATP (discussed in [36]).



Scheme 1 Map of C_1 flux through H_4folate

Single-headed arrows denote reactions that are normally unidirectional. Double-headed arrows denote reactions that flow in either direction, in different organisms or under different metabolic conditions. Numbers indicate the correspondingly numbered reactions in Table 2, in which redox and other cofactors and names of enzymes are given. (Reaction 1 of Table 2 is not included in the Figure.) Asterisks denote reactions in which ATP is consumed (liberating ADP and P_i). Reaction (4) consumes ATP, but the reverse reaction is most commonly catalysed by a separate hydrolase. However, reaction (4) as listed in Table 2 may run in reverse in purine fermenters [39]. Notes: [(a)] 5-HCO-H_4folate is metabolically derivable from formylglutamate [34] and is a minor source of H_4folate in human nutrition [183]; [(b)] 5-formimino-H_4folate is metabolically derived from formiminoglutamate (from histidine degradation in mammals [35]) or formiminoglycine (from purine degradation in some bacteria ([39] and references cited therein). The C_1 group of 5-formimino-H_4folate is sufficiently activated to be convertible into CH^+-H_4folate by a cyclodeaminase without hydrolysis of ATP [184], whereas enzymic conversion of 5-HCO-H_4folate into CH^+-H_4folate or 10-HCO-H_4folate involves consumption of ATP ([36]; see the text).

Reactions (2) and (3) are reversible, and operate in the reverse direction in some bacterial and plant metabolic settings (reviewed in [37,38]). Reaction (4) may run in reverse in the specialized, purine-fermenting *Clostridium cylindrosporum* [39], discussed in [40], again emphasizing the activated nature of 10-HCO-H_4folate.

The redox and associated interconversions of carrier-bound C_1, reactions (5)–(7), comprise the 'central superhighway' of C_1 metabolism on H_4folate. In mammals the redox cofactor for reactions (6) and (7) is NADPH. In various bacteria NADH is used by one or both enzymes. In reaction (7), enzyme-bound FAD participates as an intermediate in hydride transfer [41–43].

Reactions (5) and (6) are potentially reversible; they act together mainly in the oxidative direction in vertebrates to provide 10-HCO-H_4folate [44], and mainly in the reductive direction in some bacteria, including acetogens (see below). Reaction (7) is physiologically irreversible in mammals in the direction shown [45], a point to which reference will be made later.

In most bacteria the dehydrogenase and cyclohydrolase enzymes are on a single bifunctional polypeptide (see, e.g. [46, 46a,47]). Typically in eukaryotes a trifunctional polypeptide contains these two enzymes together with 10-HCO-H_4folate synthase (reaction 4) (reviewed in [44]). These arrangements promote substrate channelling [48]. However, in some fermentative anaerobic bacteria in which H_4folate concentrations are high, the enzymes are on separate polypeptides. This facilitated their purification and further analysis (see [49–51] for the dehydrogenase and [52] for the cyclohydrolase). It should also be mentioned that a monofunctional dehydrogenase has been isolated from yeast [53] and that eukaryotic bifunctional dehydrogenase/cyclohydrolases are known (see [53] for references).

Turning to biosynthetic C_1-transfer reactions, three major transfers occur at the formyl level. Reactions (8) and (9) occur in the classical purine-biosynthetic pathway, contributing C^8 and C^2 of the purine ring respectively (for a review, see [54]). Reaction (10) formylates initiator methionyl-tRNA of bacteria (for a review see [55]). These transfer reactions exploit the activated state of the formyl group of 10-HCO-H_4folate. A recent finding that is important in the context of the present review is that *Escherichia coli* contains two glycinamide ribotide (GAR) transformylases: PurN catalyses the classical reaction (8), but PurT utilizes free formate directly in an ATP-coupled reaction ([56] and references therein), instead of using 10-HCO-H_4folate. Thus H_4folate is not universally obligatory in provision of C^8 of the purine ring. Whether C^2 of the purine ring can be provided independently of H_4folate will be discussed later.

At the methylene level, the archetypal biosynthetic C_1 transfer is the thymidylate synthase (TS) reaction, whereby dUMP is reductively methylated to TMP (reaction 11) (for a review, see [57]). The source of reductant is the C^6–N^5 bond of the H_4folate carrier. 7,8-H_2folate is released, and this must be reduced again to H_4folate (reaction 1) before re-utilization. CH_2-H_4folate is also used in various other reductive methylations and non-reductive hydroxymethylations of nucleic acids and nucleotides; for examples, see footnote § of Table 2. Another important hydroxymethylation from CH_2-H_4folate is the formation of α-oxopantoate ('α-ketopantoate') (reaction 12), the first step in the biosynthesis of pantothenate and CoA ([58]; for a

Table 2 Reactions and enzymes of H_4folate metabolism

The Table lists the main reactions and enzymes of H_4folate metabolism that are mentioned in the text. Reactions are numbered as in Scheme 1 and the text. Commonly used abbreviations for some enzymes are given in parentheses. Any cofactors which function catalytically and which therefore do not feature in the reaction equations are shown with a plus sign in parentheses after the enzymes. Further abbreviations used: FGAR, formylglycinamide ribonucleotide; FAICAR, *N*-formylaminoimidazole-4-carboxamide ribonucleotide; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide.

	Reaction and equations	Enzyme
Generation of active carrier*		
(1)	7,8-H_2folate + NADPH + H^+ → H_4folate + $NADP^+$	Dihydrofolate reductase (DHFR)†
Loading/unloading of C_1‡		
(2)	Serine + H_4folate ↔ glycine + CH_2-H_4folate + H_2O	Serine hydroxymethyltransferase (SHMT) (+PLP)
(3)	Glycine + H_4folate + NAD^+ ↔ CO_2 + NH_4^+ + CH_2-H_4folate + NADH	Glycine-cleavage system (+PLP, lipoamide)
(4)	$HCOO^-$ + H_4folate + $MgATP^{2-}$ → 10-HCO-H_4folate + $MgADP^-$ + P_i^{2-}	10-Formyltetrahydrofolate synthase
C_1 redox pathway		
(5)	10-HCO-H_4folate + H^+ ↔ CH^+-H_4folate + H_2O	Methenyltetrahydrofolate cyclohydrolase
(6)	CH^+-H_4folate + NAD(P)H ↔ CH_2-H_4folate + $NAD(P)^+$	Methylenetetrahydrofolate dehydrogenase
(7)	CH_2-H_4folate + NAD(P)H + H^+ → 5-CH_3-H_4folate + $NAD(P)^+$	Methylenetetrahydrofolate reductase (MTHFR) (+bound FAD)
C_1 transfers		
Formyl		
(8)	10-HCO-H_4folate + GAR → FGAR + H_4folate	Glycinamide ribonucleotide (GAR) transformylase
(9)	10-HCO-H_4folate + AICAR → FAICAR + H_4folate	AICAR transformylase
(10)	10-HCO-H_4folate + Met-$tRNA_{fMet}$ → fMet-$tRNA_{fMet}$ + H_4folate	Formylmethionyl-tRNA synthase
Methylene§		
(11)	dUMP + CH_2-H_4folate → TMP + 7,8-H_2folate	Thymidylate synthase (TS)
(12)	α-Oxoisovalerate + CH_2-H_4folate ↔ α-oxopantoate + H_4folate	Oxopantoate hydroxymethyltransferase
Methyl		
(13)	5-CH_3-H_4folate + homocysteine → methionine + H_4folate	Methionine synthase (MetH) (+cobalamin)
(14)	5-CH_3-H_4folate + homocysteine → methionine + H_4folate	Methionine synthase (MetE)
(15)	5-CH_3-H_4folate + CO_2 + [2H] + CoA → acetyl-CoA + H_4folate + H_2O	Corrinoid protein, acetyl-CoA synthase (+Ni, Fe) (carbon monoxide dehydrogenase)

* Generation of active carrier: also in this category are γ-glutamylhydrolase and folylpolyglutamate synthase [15].
† DHFR also catalyses the reduction of folate to 7,8-H_2folate (see the text).
‡ Loading/unloading of C_1: also in this category are enzymes of formimiminoglutamate and formiminoglycine catabolism, and of conversion of 5-HCO-H_4folate into 10-HCO-H_4folate or to CH^+-H_4folate (see Scheme 1 and its legend).
§ C_1 transfers: methylene; also in this category are dCMP hydroxymethyltransferase of T-even bacteriophages [185] and m^5U^{54}-tRNA methyltransferase of Gram-positive bacteria [186,187].

review, see [59]) in organisms that do not require dietary pantothenate.

Which bond breaks first in methylene transfers: the bond to N^{10} or that to N^5? In the TS reaction, the internal source of reductant (C^6–N^5, see above) and crystallographic data on the enzyme [57] support a mechanism whereby the CH_2–N^{10} bond breaks first, yielding the N^5 cationic imine (Figure 1a) as reactive intermediate [57]. This mechanistic pattern is predicted from that of the reversible condensation of formaldehyde with H_4folate [18], and is very likely the pattern of C_1 transfer reactions at the methylene level generally, including the reversible serine hydroxymethyltransferase (SHMT) reaction (2) [60]. Furthermore, the cationic imine is a presumed intermediate in the methylenetetrahydrofolate reductase (MTHFR) reaction (7) [42]. Crystallographic data are available on this enzyme [43], and further mechanistic details may emerge soon.

At the methyl level, the main or exclusive C_1 transfer in most organisms is in the biosynthesis or regeneration of methionine from homocysteine (for a review, see [61]). *E. coli* produces either of two methionine synthases, depending on the growth conditions [37]. MetH utilizes cobalamin as intermediate in methyl transfer (reaction 13) [42,62,63], whereas MetE is cobalamin-independent (reaction 14) [64,65]. Mammalian methionine synthase, like MetH, is cobalamin-dependent [61,66]. Thus, in Man, reaction (13) cannot take place in the absence of vitamin B_{12}. Because reaction (7) is functionally irreversible in Man, 5-CH_3-H_4folate has 'no way out' in the absence of the cobalamin vitamin B_{12}. This state of affairs is called the 'methyl trap', and underlies much of the clinical picture of pernicious anaemia. Detailed accounts appear elsewhere [61,67].

In acetogenic bacteria, CH_3-H_4folate has a separate major role, in the 'acetyl-CoA' pathway. This topic is briefly discussed here because it provides a conceptual link with methanogenesis. In the pathway (see, as a general reference, [68]) acetyl groups are generated from two molecules of CO_2. Most of the C_1 flux through the pathway is used for energy generation; the remaining acetyl-CoA generates cell carbon. In the 'methyl' branch of the pathway one molecule of CO_2 is reduced to formate by formate dehydrogenase [69], and the formate is activated to 10-HCO-H_4folate at the expense of ATP (reaction 4), and reduced to 5-CH_3-H_4folate (reactions 5–7). The methyl group of 5-CH_3-H_4folate is then transferred by a corrinoid protein to acetyl-CoA synthase (also called carbon monoxide dehydrogenase), a metalloenzyme that assembles acetyl-CoA from the methyl group, a carbonyl group derived from the second CO_2, and CoA (reaction 15) [70]. Energy that is consumed in reaction (4) is

Table 3 Reactions and enzymes of methanogenesis

The Table lists the main reactions and enzymes of methanogenesis from CO_2 plus H_2. Reactions are numbered as in Figure 3 and the text. Commonly used abbreviations for the enzymes are given in parentheses. Cofactors which do not feature in the reaction equations are shown with a plus sign in parentheses after the enzymes. Enzymes of methanol oxidation in the oxidative branch of the methylotrophic pathway utilize H_4SPT instead of H_4MPT (see the text).

Reaction	Equation	Enzyme
(16)	$CO_2 + MFR^+ + 2[H] \leftrightarrow HCO\text{-}MFR + H_2O + H^+$	Formylmethanofuran dehydrogenase* (+Mo or W, and Fe)
(17)	$HCO\text{-}MFR + H_4MPT + H^+ \leftrightarrow 5\text{-}HCO\text{-}H_4MPT + MFR^+$	Formylmethanofuran:H_4MPT formyltransferase
(18)	$5\text{-}HCO\text{-}H_4MPT + H^+ \leftrightarrow CH^+\text{-}H_4MPT + H_2O$	Methenyl-H_4MPT cyclohydrolase (MCH)
(19)	$CH^+\text{-}H_4MPT + F_{420}H_2 \leftrightarrow CH_2\text{-}H_4MPT + F_{420} + H^+$	F_{420}-dependent methylene-H_4MPT dehydrogenase
(20)	$CH^+\text{-}H_4MPT + H_2 \rightarrow CH_2\text{-}H_4MPT + H^+$	H_2-forming methylene-H_4MPT dehydogenase (HMD)
(21)	$CH_2\text{-}H_4MPT + F_{420}H_2 \leftrightarrow 5\text{-}CH_3\text{-}H_4MPT + F_{420}$	Methylene-H_4MPT reductase (MER)
(22)	$CH_3\text{-}H_4MPT + HS\text{-}CoM \leftrightarrow CH_3\text{-}S\text{-}CoM + H_4MPT$	Methyl-H_4MPT:CoM methyltransferase (+corrinoid protein)
(23)	$CH_3\text{-}S\text{-}CoM + HS\text{-}CoB \rightarrow CH_4 + CoM\text{-}S\text{-}S\text{-}CoB$	(MR) Methyl-CoM reductase (+bound F_{430})
(24)	$CoM\text{-}S\text{-}S\text{-}CoB + 2[H] \rightarrow HS\text{-}CoM + HS\text{-}CoB$	Heterodisulphide reductase†

* The immediate electron donor for reaction (16) is not known, but may be a polyferredoxin [139].

† Different heterodisulphide reductases are linked to H_2 as reductant in the hydrogenotrophic pathway [116] or to $F_{420}H_2$ as reductant in the methylotrophic pathway [123].

recovered at the end of the pathway by conversion of acetyl-CoA into acetyl phosphate, followed by substrate-level phosphorylation, releasing acetate. However, this only regenerates ATP already consumed and does not amount to net synthesis. Instead, net ATP synthesis is chemiosmotically coupled to the pathway [71]. Likely energy-yielding steps are methyl transfer from 5-CH_3-H_4folate to acetyl-CoA and/or the MTHFR reaction (reaction 7) [71,72]. It should be mentioned that acetogens are a phylogenetically diverse group, comprising heterotrophs and autotrophs (as a general reference, see [68]), and also that, in some acetogens, the primary reductant in the MTHFR reaction is reduced ferredoxin [73] and in others NADH [72].

The H_4MPT pathway

Before focusing on H_4MPT, some comments on methanogenesis may be helpful. The reader is also referred to a multiauthor volume [74], as a general reference, and to two recent reviews which treat methanogenesis from biochemical [75] and molecular-genetic [76] viewpoints.

All methanogens are anaerobic Archaea, comprising a diverse array among the Euryarchaeota. Phylogenetic analysis [8,9] has distinguished four major orders: the Methanobacteriales, Methanococcales, Methanomicrobiaceae and Methanosarcinales, and also a single member of a fifth order, the hyperthermophile *Methanopyrus kandleri*. The main modes of methanogenesis are from CO_2 and H_2 (hydrogenotrophic), from methanol or other C_1 methyl compounds (methylotrophic), or from acetate. Each mode is separately reviewed in [74], and a recent compilation of the reactions and enzymes of all three modes is in [75].

In the reactions in Table 3 (to be discussed shortly), several novel cofactors appear. The discoveries, structures and functions of these cofactors are reviewed, e.g. in [10,77]. Brief notes may be helpful here. Methanofuran (MFR) is a substituted furan with an aminomethylene group on which a formyl group can be carried. F_{420} is a 5-deazaflavin which carries hydride on C^5 (see below). Coenzyme M (CoM, HS-CoM) and coenzyme B (CoB, HS-CoB, also called HS-HTP) are thiol cofactors. F_{430} is a nickel tetrapyrrole. The functions of these cofactors are described below. F_{430} and CoB are unique to methanogens. H_4MPT, F_{420} and MFR are more widely distributed (see below), and this has recently also been found to be true for CoM [77a].

It should also be mentioned that the Methanosarcinales contain tetrahydrosarcinapterin (H_4SPT) [78], which differs from H_4MPT only in the presence of a terminal glutamate residue linked to the hydroxyglutarate. Other variations on the H_4MPT structure are described later.

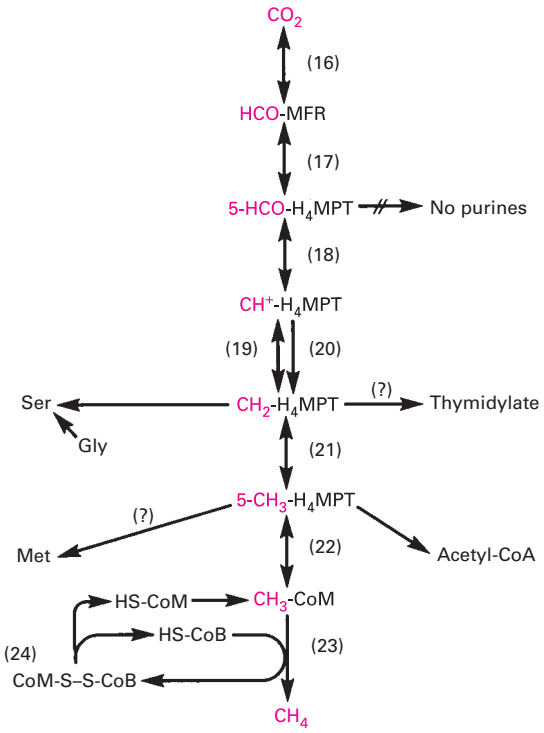
Hydrogenotrophic methanogenesis was elucidated largely by studies on *M. thermoautotrophicum* (for a review, see [77]); also the hyperthermophile *M. kandleri* has afforded a source of highly thermostable enzymes (see, e.g. [79–81a]). The net pathway can be written:

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$$

The energy yield under standard conditions is $-131\ kJ \cdot mol^{-1}$ (see under 'Thermodynamic aspects'). Under ecological conditions of 1–10 Pa H_2, the energy yield is only about $15–35\ kJ \cdot mol^{-1}$ ([82], pp. 142–147). This is insufficient for synthesis of 1 mol of ATP per methane molecule produced [75,77]. Therefore net ATP synthesis would not be possible by substrate-level phosphorylation. Instead, phosphorylation of ADP is chemiosmotically coupled to methane formation [83,84].

The reactions of the pathway are summarized in Scheme 2 and Table 3. CO_2 is reduced to the formyl level upon the carrier MFR (reaction 16). Catalysis of reaction (16) is by formylmethanofuran dehydrogenase, a multisubunit, oxygen-labile metalloenzyme with Mo or W at the active site ([85–87]; further references in [75]). The formyl group is then transferred by formylmethanofuran:H_4MPT formyltransferase [79,88–90] to N^5 of H_4MPT, yielding 5-HCO-H_4MPT (reaction 17). In contrast with the H_4folate pathway, no free formate is formed as an intermediate in these reactions, and no ATP is expended.

5-HCO-H_4MPT is reduced to 5-CH_3-H_4MPT in a reaction sequence that formally resembles reactions (5–7) of the H_4folate pathway, in that water is released and two consecutive two-electron reductions occur. However, the reactions on H_4MPT differ in major respects from those on H_4folate. First, a cyclohydrolase (methenyl-H_4MPT cyclohydrolase, MCH) [80,91,92] generates CH^+-H_4MPT directly from 5-HCO-H_4MPT without ATP (reaction 18). {Recall that, in the H_4folate pathway,10-



Scheme 2 Map of C_1 flux through H_4MPT

Conventions for arrows are as in Scheme 1. Numbers indicate the correspondingly numbered reactions in Table 3, in which the redox and other co-reactants and names of enzymes are given. The crossed arrow indicates that this pathway is thought not to occur, and the queries denote uncertainties concerning aspects of the indicated reactions; see under the section entitled 'Biosynthetic roles for H_4MPT?'.

HCO-H_4folate is the substrate for cyclohydrolase (reaction 5), whereas 5-HCO-H_4folate can only be converted into the methenyl derivative at the expense of ATP, either in a direct reaction or indirectly via 10-HCO-H_4folate; see above and [36].}

Then either of two alternative enzymes reduce CH^+-H_4MPT to CH_2-H_4MPT. In reaction (19), F_{420}-dependent CH_2-H_4MPT dehydrogenase catalyses a reversible reduction using the low-redox-potential deazaflavin $F_{420}H_2$ as cofactor [93–96,81]. The $F_{420}/F_{420}H_2$ midpoint potential (E_0') is about -350 mV [97–99], well below that of $NAD(P)^+/NAD(P)H$ (-320 mV) used in the H_4folate pathway. In hydrogenotrophic methanogenesis, H_2 and an F_{420}-dependent nickel–iron hydrogenase maintain F_{420} in the reduced state. Alternatively (reaction 20), an H_2-dependent CH_2-H_4MPT dehydrogenase (HMD) catalyses reduction of the methenyl derivative directly by H_2 [17,21,100]. Unlike most hydrogenases, the enzyme contains no redox-active metal centre [17]. Notwithstanding its name, the enzyme operates predominantly in the reductive direction. It is expressed and functions under conditions of moderately high H_2 pressure [77,101] or low Ni concentration [102].

Methylene-H_4MPT reductase (MER; reaction 21) also uses $F_{420}H_2$ as cofactor. Unlike the H_4folate enzyme, MER is not a flavoprotein [103,104]. Hydride is delivered directly to the CH_2-H_4MPT substrate by the reduced deazaflavin [105]. (In the H_4folate reaction, a synthetic deazaflavin can substitute *in vitro* for FAD [106]. Deazaflavins are obligate two-electron oxidoreductants [97,98], implying a two-electron mechanism for both reactions [105,106].) In contrast with the H_4folate reaction, the CH_2-H_4MPT reductase reaction is reversible (see below).

The methyl group is transferred from H_4MPT to the SH group of CoM (reaction 22). The transfer proceeds via a corrinoid [107–109] and is thus formally analogous to the MetH methionine synthase reaction of the H_4folate pathway (reaction 13). However, the CH_3-H_4MPT:CoM methyltransferase is a complex membrane-bound enzyme [110,111], in contrast with methionine synthase, and is a primary sodium pump [112] that contributes to energy conservation [83,84].

The end product, methane, is generated by reductive demethylation of CH_3-S-CoM (reaction 23). The enzyme methyl-CoM reductase (MR) contains the unique nickel tetrapyrrole cofactor F_{430} [113]. The co-reductant is the thiol cofactor CoB. The X-ray structure of MR has been determined to high resolution, permitting deduction of the spatial arrangement of the substrates at the active site [114]. In the reaction the cofactors are oxidized to yield the heterodisulphide CoM-S–S-CoB. This is subsequently reduced by heterodisulphide reductase [115,116] to regenerate the two thiol cofactors (reaction 24).

Much of the net free-energy change of the pathway is made available from reactions (22–24) and is harnessed for chemiosmotic ATP synthesis [83,84]. Also a portion of the energy from the late steps drives the first reductive step of the pathway (reaction 16). This coupling was first observed by R. P. Gunsalus and is called the 'RPG effect' [117–119]. It may be related to the fact mentioned above that reduction of MPT to H_4MPT occurs (in methanogens) only under conditions of methanogenesis.

Under conditions of autotrophic growth, some C_1 is diverted into cell carbon via a modified version of the acetyl-CoA pathway; 5-CH_3-H_4MPT rather than 5-CH_3-H_4folate provides the methyl group of acetyl-CoA. As in the acetogenic pathway, a corrinoid intermediate is involved in the methyl transfer, and the assembly of acetyl-CoA takes place upon acetyl-CoA synthase (for a review, see [120]).

Enzymes that catalyse reactions (18–21) show no significant sequence similarity to enzymes that catalyse reactions (5–7) of the H_4folate pathway [92,96,121]. Moreover, MCH and the two methylene-H_4MPT dehydrogenases are physically distinct enzymes, in contrast with the corresponding H_4folate enzymes, which are physically linked in most bacteria and in eukaryotes (see above). Generally, H_4folate derivatives cannot substitute for H_4MPT derivatives, although 5-CH_3-H_4folate can substitute to some extent for 5-CH_3-H_4MPT in one reported instance, namely the methyltransferase reaction (22) catalysed by the enzyme from Methanosarcinales [112].

Methanogenesis from methanol, in contrast with the hydrogenotrophic pathway, involves no net consumption (or production) of H_2. The process occurs mainly in Methanosarcinales, and has been studied in detail in *Methanosarcina barkeri* (for a review, see [122]). The pathway may be written:

$$3CH_3OH + 6\,\text{'H'} \rightarrow 3CH_4 + 3H_2O$$

$$CH_3OH + H_2O \rightarrow CO_2 + 6\,\text{'H'}$$

$$\text{Net: } 4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$$

Thus reduction of three molecules of methanol to methane is made possible by coupling to oxidation of one molecule of methanol to CO_2. Under standard conditions the pathway yields 106 $kJ \cdot mol^{-1}$ CH_4 [122].

In the reductive branch of the pathway the methyl group of methanol is transferred to CoM and is thence reduced to methane by reaction (23). In the oxidative branch a methyl group is transferred via CoM to H_4SPT (a close structural relative of H_4MPT; see above). The 5-CH_3-H_4SPT is oxidized to 5-HCO-H_4SPT by reversal of reactions (21), (19) and (18). The formyl group of 5-HCO-H_4SPT is transferred to MFR and oxidized to

CO_2 by reversal of reactions (17) and (16). Reducing equivalents from the three redox reactions are channelled to methane production in the reductive part of the pathway via an $F_{420}H_2$-linked heterodisulphide reductase [123].

The non-methanogenic sulphate reducer *A. fulgidis* also carries out oxidation of methyl groups, using H_4MPT as carrier. The same reactions are used as those just described for the oxidative branch of the methanogenesis pathway from methanol: reactions (21) and (19–16) [105,124–126]. Thus H_4MPT as well as H_4SPT are used for methyl group oxidation by various Archaea. In *A. fulgidis* the methyl groups originate from lactate, and the reductant generated by methyl-group oxidation is consumed in sulphate reduction [124].

For accounts of methanogenesis from acetate the reader is referred to [127–129]. In brief, the pathway differs in a number of ways from those described above. In particular, acetate is first activated to acetyl-CoA at the expense of ATP. Acetyl-CoA is split by reversal of the acetyl-CoA synthase (or carbon monoxide dehydrogenase) reaction. The carbonyl group of the acetyl moiety is thereby oxidized to CO_2. The methyl group proceeds via H_4SPT to CoM and thence to methane. As in other methanogenic pathways, there is no generation of ATP by substrate-level phosphorylation. Therefore more than the ATP that is consumed in acetate activation must be regenerated chemiosmotically (for discussion, see [127]).

THERMODYNAMIC ASPECTS

The above, qualitative differences between the C_1 pathways on H_4folate and H_4MPT are now examined from the viewpoint of thermodynamic data, namely, free energies and midpoint redox potentials of the C_1 reduction steps. The important conclusion will emerge that the two carriers 'tune' the energetics of the intermediate steps in C_1 reduction differently. The chemical basis of the differences will be discussed below.

Thermodynamic tabulations have been presented previously for the H_4folate pathway (see, e.g. [130,131]), the H_4MPT pathway [75,77,83,132] and both pathways together [133,134]. The various primary data originated from many laboratories over many years. I attempt here to give a unified overview in order to support the conclusions and inferences to be presented.

Topics are treated in the following sequence. First, the hypothetical C_1 reduction pathway by H_2 without pterin carrier is given for reference. Then, literature values for the thermodynamics of C_1 reduction on H_4folate and H_4MPT are given, with reference both to the immediate redox cofactors that were listed in Tables 2 and 3, and to H_2 – the latter to permit direct comparison between the different pathways. All values are for the reactants under hypothetical standard conditions – again to enable comparisons. The bearing of the standard values on the directions of C_1 flux under real conditions is briefly mentioned.

Appropriate thermodynamic equations (as, for example, in [72]) were used for calculating relationships between equilibrium constants, reaction standard free energies and midpoint redox potentials. K_{eq} is the overall equilibrium constant and may include hydrogen ions in the equilibrium expression. For such reactions, K'_{eq} is the apparent equilibrium constant at pH 7. Unless otherwise stated, reaction free energies are for 298 K, pH 7, and are designated $\Delta G'_0$ and E'_0 respectively. The following values were used for E'_0 at 298 K: $2H^+ + 2e/H_2 = -414$ mV [40]; $NAD(P)^+/NAD(P)H + H^+ = -320$ mV [135] (values differing from this by plus or minus a few millivolts are found in various sources); $F_{420}/F_{420}H_2 = -350$ mV (based on data in [97]; a review [98] citing [97] gives -360 mV). The data are compiled in Table 4 and are depicted in summary form in Figure 2.

C_1 reduction, no carrier

For the (theoretical) stepwise reduction of CO_2 by H_2 in the absence of pterin carrier, $\Delta G'_0$ and E'_0 values can be calculated from the free energies of formation ($\Delta_f G_0$) of the compounds. Previous calculations [40] have been slightly revised here (Table 4a) owing to minor revisions in some $\Delta_f G_0$ values, particularly that of formaldehyde ([136]; see notes to Table 4a). The total $\Delta G'_0$ of reduction of CO_2 to CH_4 is -130.6 kJ·mol^{-1}, in close agreement with the value [40] of -131 kJ·mol^{-1}.

The C_1 pathways on carriers

The above approach, which relies on primary thermochemical data [enthalpies and absolute (Third Law) entropies for obtaining $\Delta_f G_0$ values] is not readily applicable to compounds as large as pterin derivatives. Therefore experimentally derived equilibrium constants have been used in most instances for calculating $\Delta G'_0$ and E'_0 for the C_1 pathways on the carriers. The currently 'best available' thermodynamic values for the C_1 reduction in pathways on the two carriers are collected in Tables 4(b) and 4(c).

CO_2 to formyl

In the H_4folate pathway, CO_2 is first reduced to formate. For the present comparative purposes, $\Delta G'_0$ with H_2 as reductant under standard conditions is the relevant value. This value is slightly endergonic: $+3.5$ kJ·mol^{-1} ([40] and Table 4a).

As already noted, formate activation to 10-HCO-H_4folate is coupled to ATP hydrolysis. K_{eq} for the reaction was determined in [137] to be approx. 41 in the direction of 10-HCO-H_4folate synthesis. This value is used here for calculating $\Delta G'_0$ (reaction 4a in Table 4b). (The $\Delta G'_0$ is probably accurate to within ± 2 kJ·mol^{-1} based also on two later determinations of K_{eq} cited in [33].) Because ATP is not consumed in the pathways in Tables 4(a) and 4(c), it is also necessary, for comparison, to estimate $\Delta G'_0$ for the hypothetical reaction (4) without ATP. Taking -31.8 kJ·mol^{-1} for $\Delta G'_0$ of hydrolysis of the terminal phosphate of ATP [40], one can estimate that formate activation without ATP would be endergonic by about $+22$ kJ·mol^{-1} with respect to free formate. This value is included as reaction (4a) in Table 4(b).

Turning to the H_4MPT pathway, for the conversion of CO_2 into HCO-MFR (reaction 16), a theoretical estimate of $\Delta G'_0$ was based on the sum of the free energies of CO_2 reduction to formate ($+3.5$ kJ·mol^{-1}, see above) and of condensation of a model amide, *N*-formylglutamate ($+12.5$ kJ·mol^{-1} [138]), giving $+16$ kJ·mol^{-1} for the overall reaction [133]. Experimental data with various artificial electron acceptors and donors enabled estimation of about the same value [85]. This value is cited in recent literature [75,84] and is used here (Table 4c). Thus the reaction is substantially endergonic. No ATP is consumed, but the reaction is coupled chemiosmotically to a later exergonic step of methanogenesis, probably the methyl-transfer reaction (reaction 22), as there is evidence that some Na^+ extruded in that reaction re-enters in conjunction with CO_2 reduction to HCO-MFR; discussed in [84]. This coupling affords an explanation of the RPG effect [117–119] mentioned above. The immediate electron donor for the reduction is probably a polyferredoxin

Table 4 Thermodynamics of C_1 reduction with no carrier (a), H_4folate (b) and H_4MPT (c)

(a) No carrier. All values are for standard conditions (298 K, pH 7). $\Delta G'_0$ values were derived from [$\Delta_f G_0$ (products) − $\Delta_f G_0$ (reactants)]. The following $\Delta_f G_0$ values, in kJ · mol^{-1}, were used [$_{(g)}$ is gaseous and $_{(aq)}$ is aqueous]: $CO_{2(g)} = -394.36$ [40,136]; $H_{2(g)} = 0$; $HCOO^- = -351.04$ [40]; $H^+ = -39.87$ [40]; $HCHO_{(aq)} = -121.5$ (see below); $CH_3OH_{(aq)} = -175.3$ [136]; $CH_{4(g)} = -50.72$ [136]; $H_2O_{(l)} = -237.13$ [136]. Values in [136] are almost identical with those cited previously in [40], except for HCHO. The previous values [40] for $HCHO_{(g)}$ and $HCHO_{(aq)}$ were −112.97 and −130.54 kJ · mol^{-1} respectively; the $_{(aq)}$ values were used for calculations in [40]. The revised value [136] for $HCHO_{(g)}$ is −102.53 kJ · mol^{-1}. On the basis of two sets of literature values for the *difference* between $HCHO_{(g)}$ and $HCHO_{(aq)}$ (17.6 kJ · mol^{-1} more negative for $HCHO_{(aq)}$ in [40], see the above values, and 20 kJ · mol^{-1} more negative for $HCHO_{(aq)}$ in [188]), a value for $HCHO_{(aq)}$ of 19 kJ · mol^{-1} more negative than the revised value for $HCHO_{(g)}$ was used for calculations here, i.e. −121.5 kJ · mol^{-1}. The consequences of the revised value for $HCHO_{(aq)}$ are that reaction (26) is approx. 9 kJ · mol^{-1} more endergonic herein than in [40] and reaction (27) is approx. 9 kJ · mol^{-1} more exergonic. The revised value for reaction (26) is important in relation to data in part (b), as discussed in the text. (b) H_4folate. The reactions of the C_1 pathway are (25) of part (a) and (4–7) of Table 2. $\Delta G'_0$ values for reactions (4–7) were calculated firstly with ATP (reaction 4) and with NAD(P)H as reductant (reactions 6 and 7). Then, in order to enable direct comparison with data in parts (a) and (c), reaction (4) was recalculated without ATP and is listed as reaction (4a), and reactions (6) and (7) were recalculated for H_2 as reductant. Cumulative free-energy values ($\Sigma\Delta G'_0$) are given in separate columns, starting both from formate and from CO_2. The sums are based on reaction (4a) (i.e. without ATP) and H_2 as reductant. Values in **bold** in these two columns are specifically mentioned in the text. Reaction (29) is the hydrolysis of CH_2-H_4folate to formaldehyde and H_4folate; the associated cumulative value is for reactions (4a), (5), (6) and (29). Reaction (26) is from part (a). See the text for the reasons for including reactions (29) and (26). Also see the text for the two sets of values for reaction (7). Most of the $\Delta G'_0$ values are rounded to the nearest 0.5 kJ · mol^{-1}. (c) H_4MPT. The reactions are (16)–(21) of Table 3. $\Delta G'_0$ values are calculated for 298 K to enable comparison with parts (a) and (b). It is recognized that K_{eq} was determined at about 338 K in most instances; it is provisionally assumed that any variation of K_{eq} with temperature is likely to be small in the temperature range of interest. An outline of the derivations of the values is given in the text. For reactions (19) or (20) plus (21), most sources are in agreement that the combined $\Delta G'_0$ is approximately zero with $F_{420}H_2$ as reluctant and approx. −24 kJ · mol^{-1} with H_2 as reductant, although some sources [75,77,130,131] differ from others [94,104] in the apportionment of $\Delta G'_0$ between the two successive reductions (see the text). Because the total $\Delta G'_0$ for reduction of CO_2 to CH_4 by H_2 is approx. −131 kJ · mol^{-1} (part a), and $\Sigma G'_0$ from CO_2 through to reaction (21) is −16 kJ · mol^{-1}, the $\Delta G_0{}'$ available from the final reactions of methanogenesis (reactions 22–24) is approx. −115 kJ · mol^{-1}; these exergonic reactions are coupled to chemiosmotic energy conservation [83,84].

(a) No carrier

Reaction	$\Delta G'_0$ (kJ · mol^{-1})	$\Sigma\Delta G'_0$	E'_0 (mV)
(25) $CO_2 + H_2 \rightarrow HCOO^- + H^+$	+3.5		−432
(26) $HCOO^- + H_2 + H^+ \rightarrow HCHO + H_2O$	+32.3	+35.8	−581
(27) $HCHO + H_2 \rightarrow CH_3OH$	−53.8	−18.0	−135
(28) $CH_3OH + H_2 \rightarrow CH_4 + H_2O$	−112.6	−130.6	+169
(net) $CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$	−130.6		

(b) H_4 folate

Reaction	Reductant...	$\Delta G'_0$ (kJ · mol^{-1})		$\Sigma\Delta G'_0$ (H_2 as reductant)		E'_0 (mV)
		NAD(P)H	H_2	From formate	From CO_2	
(25)		(+21.5)	+3.5			−432
(4)		−10	−10			
(4a)		+22	+22	+**22**	+25.5	
(5)		+6	+6	+**28**	+31.5	
(6)		−5	−23	+**5**	+8.5	−295
(29) (see the text)		+25.7	+25.7	+**30.7**		
(26)			+32.3	+**32.3**		
(7a) (see the text)		−39	−57	−52	−48.5	−120
(7b) (see the text)		−23	−41	−36	−**32.5**	−200

(c) H_4MPT

Reaction	Reductant...	$\Delta G'_0$ (kJ · mol^{-1})		$\Sigma G'_0$ (H_2 as reductant)		E'_0 (mV)
		$F_{420}H_2$	H_2	From formate (hypothetical)	From CO_2	
(16)		+28	+16	+12.5	+16	−497
(17)		−3.5	−3.5	+**9**	+12.5	
(18)		−4.2	−4.2	+**4.8**	+8.3	
(19)		+2				−360
(20)			−10	−5.2	−2	
(21)		−1.7	−14	−19.2	−**16**	−330

[139]. Formyl transfer from MFR to N^5 of H_4MPT (reaction 17) is slightly exergonic [90] (Table 4c).

Although free formate is not an intermediate in the H_4MPT pathway, it affords a reference point for the energy levels of the actual intermediates. Summation of the appropriate $\Delta G'_0$ values indicates that 5-HCO-H_4MPT is endergonic with reference to free formate, but only by about +9 kJ · mol^{-1}, in contrast with 10-HCO-H_4folate, which, as stated above, is endergonic with reference to free formate by about +22 kJ · mol^{-1}. As mentioned above, Figure 2 depicts the C_1 free-energy relationships during progression through the respective pathways.

Formyl to methenyl

In the H_4folate pathway, cyclization from 10-HCO-H_4folate is also endergonic at pH 7. ΔG_0 for cyclization is pH-dependent because H^+ is a reactant. The reaction can proceed non-enzymically in either direction according to pH. Multiple de-

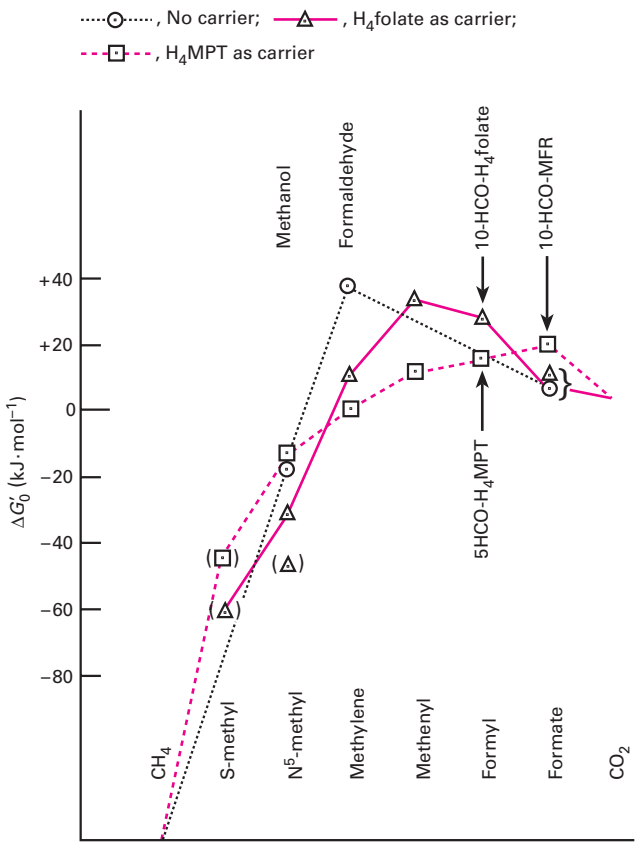


Figure 2 Free-energy profiles of C_1 reduction pathways, based on 'best available' thermodynamic values as discussed in the text

Reduction is from right to left. The data are those in Tables 4(a)–(c) for hydrogen under standard conditions as reductant, and without ATP for reaction (4) (i.e. reaction 4a in Table 4b). These values allow direct comparability between the pathways; see the text. The relationships between compounds in the different pathways are defined in the text and in Tables 1–3. (For example, in the H_4folate pathway CO_2 is first reduced to formate, whereas in the H_4MPT pathway CO_2 is first reducted to HCO-MFR.) At the methyl-oxidation level the triangle below the continuous line is derived from the early value for the MTHFR reaction (reaction 7) [41], whereas the triangle on the line is from the recent value [72] (see the text and Table 4b). $\Delta G'_0$ values for the methyl-transfer reactions (13) and (22) have been reported. In a detailed study of reaction (22), a $\Delta G'_0$ of -10 kJ·mol^{-1} was found for methyl transfer from H_4MPT to cobamide, and -20 kJ·mol^{-1} from cobamide to CH_3-S-CoM, i.e. -30 kJ overall [108]. The only study on the equilibrium of reaction (13) [189] gave a value for K_{eq} of 7×10^{-6} in the direction of homocysteine and CH_3-H_4folate formation, which converts to -29 kJ·mol^{-1} in the direction of methionine synthesis. The potential of the S-methyl group in methionine and CH_3-S-CoM might be expected to be fairly similar, but not necessarily identical, because CoM also contains a strongly negative sulphate group [10] which might conceivably influence the overall reactivity of the compound. Given that the equilibrium points of the S-methyltransferase reactions (13) and (22) are far to the right in the directions written, the reported free-energy values may be viewed as being somewhat approximate.

terminations of the non-enzymic reaction at different pH values gave $K_{eq} = 0.9 \times 10^6$ M^{-1} [36], which converts at pH 7 into $K'_{eq} = 9 \times 10^{-2}$ M^{-1} and $\Delta G'_0 = +5.9$ kJ·mol^{-1} in the direction of cyclization, as cited in [134].

In the H_4MPT pathway, cyclization from 5-HCO-H_4MPT is exergonic at pH 7. The reaction is pH-dependent for the same reason as that given above. An experimental value of K_{eq} of 5.4×10^7 M^{-1} was obtained for the enzymic reaction in the direction of cyclization [91], corresponding to a K'_{eq} of 5.4 at pH7 and -4.2 kJ·mol^{-1} for cyclization.

Note that there is a substantial difference between the energy levels of the methenyl derivatives of the two carriers. At pH 7 CH^+-H_4folate is endergonic by approx. 28 kJ·mol^{-1} with reference to free formate, whereas CH^+-H_4MPT is endergonic by only about 5 kJ·mol^{-1} with reference to free formate (Tables 4b and 4c; Figure 2).

Methenyl to methylene

Early attempts to determine the equilibrium of reaction (6) in the H_4folate pathway were complicated by the physical linkage of the dehydrogenase and cyclohydrolase enzymes from most organisms (discussed above), and also by the tendency of CH^+-H_4folate to undergo spontaneous hydrolysis to 10-HCO-H_4folate at neutral pH. These difficulties were overcome with the purification of a physically separate dehydrogenase from *C. cylindrosporum* and development of a rapid assay [49]. The enzyme utilizes NADP(H). An equilibrium constant of 0.14 was reported in the direction of cyclization [49], corresponding to $\Delta G'_0 =$ approx. -4.9 kJ·mol^{-1} in the direction of reduction from CH^+-H_4folate to CH_2-H_4folate. This value is generally cited [130,131], and corresponds to $E'_0 = -295$ mV for the CH^+/CH_2-H_4folate couple. $\Delta G'_0$ for H_2 as reductant is -23 kJ·mol^{-1} (Table 4b).

It would be useful to have a means of checking the cumulative accuracy of the values so far presented in Table 4(b). This may be achieved as follows. As noted above, the non-enzymic condensation of formaldehyde with H_4folate has been studied in detail [18]. An overall equilibrium constant of 3.2×10^4 M^{-1} in the direction of condensation was reported [18]. This corresponds to $+25.7$ kJ·mol^{-1} in the direction of hydrolysis, reaction (29) in Table 4. Reactions (4), (5), (6) and (29) represent, overall, the conversion of formic acid into formaldehyde, the same overall change as reaction (26) in Table 4(a). If the $\Delta G'_0$ values in Table 4(b) for reactions (4a) (i.e. without ATP), (5) and (6) (H_2 as reductant) are approximately correct, then the sum of these $\Delta G'_0$ values plus that for reaction (29) should be equal to $\Delta G'_0$ for reaction (26). The sum of the reactions in Table 4(b) is approx. 31 kJ·mol^{-1}, which compares well with the value 32.3 kJ·mol^{-1} for reaction (26), affording some confidence that the cumulative values so far discussed in Table 4(b) are reliable.

In an earlier review [130] it was pointed out that binding of C_1 to H_4folate modifies the E'_0 values of the C_1 redox reactions. The overall reaction (reaction 26) is split into smaller steps in the H_4folate
pathway, including the activation reaction (4). The magnitude of the redox reaction (6) is thereby diminished relative to (26) and is rendered suitable for coupling with pyridine nucleotides. As will be discussed, the chemistry of H_4folate also contributes to the magnitude of the redox step.

Thermodynamic values for interconversion between CH^+-H_4MPT and CH_2-H_4MPT were first derived [140] from early data [13] on interconversions in cell extracts under different partial hydrogen pressures, before the enzymes had been characterized or the role of F_{420} was known. A value for $\Delta G'_0$ of -4.85 kJ·mol^{-1} was obtained for the H_2-coupled reaction, giving $E'_0 = -390$ mV for the CH^+/CH_2-H_4MPT couple [140,133]. Subsequent purification of the F_{420}-utilizing enzyme allowed determination of $K_{eq} = 2.24 \times 10^7$ M^{-1} in the direction of CH_2-H_4MPT oxidation [94] (reaction 19). In the reductive direction and at pH 7 this yields the $\Delta G'_0$ values shown in Table 4(c) for reaction (19) and, indirectly, reaction (20). E'_0 of the CH^+/CH_2-H_4MPT couple is -362 mV from this study [94].

Thus, on H_4MPT, E'_0 for CH^+/CH_2 couple is more negative by about 65 mV than on H_4folate. Consequently, the free-energy change for the CH^+/CH_2 reduction, expressed relative to H_2 as reductant, is less steeply 'downhill' on H_4MPT than on H_4folate (see Figure 2).

Methylene to methyl

There is general agreement that the equilibrium point for the MTHFR reaction (reaction 7) is far in the direction of CH_3-H_4folate formation. However, for this very reason, quantitatively accurate values of K_{eq} and related thermodynamic values for the overall reaction are technically not easy to ascertain. There have been two sets of literature values, designated (7a) and (7b) in Table 4(b). The matter is discussed here because it bears upon topics to be addressed shortly.

As already mentioned, MTHFR is a flavoprotein. FAD is non-covalently bound and is an intermediate in hydride transfer from NAD(P)H to CH_2-H_4folate [41–43]. Thus $\Delta G'_0$ for the overall reaction is the sum of the $\Delta G'_0$ values for two partial reactions: (i) hydride transfer from NAD(P)H to enzyme-bound FAD; (ii) hydride transfer from $FADH_2$ to CH_2-H_4folate. Vanoni and Matthews [41] determined that reaction (ii) is reversible (-0.5 kcal, i.e. -2 kJ·mol^{-1}), and calculated that reaction (i) is irreversible (-9 kcal, i.e. -38 kJ·mol^{-1}), giving about -40 kJ·mol^{-1} for the overall reaction.

The derivation in [41] drew in part from an earlier study [141]. In the first reported purification of the *E. coli* enzyme, enzyme-bound FAD became dissociated [141]. It was therefore necessary to add FAD and an $FADH_2$-generating system in the form of NADH and 'diaphorase' in order to bring about enzymic reduction of CH_2-H_4folate. Under these experimental conditions the equilibrium point for the reaction between $FADH_2$ and CH_2-H_4folate was found to be far in the direction of CH_3-H_4folate formation: K_{eq} was reported as approx. 3×10^3 [141]. Assuming E'_0 for free FAD/$FADH_2$ $= -220$ mV, then E'_0 for CH_2/CH_3-H_4folate can be calculated to be approx. -120 mV; values of -133 mV [41] and -117 mV [130] were given in the literature. Accordingly, from the value -133 mV for CH_2/CH_3-H_4folate, and from the *reversible* equilibrium of reaction (ii) when FAD is enzyme-bound, E'_0 for *enzyme-bound* FAD was calculated as -143 mV [41]. The remaining free-energy parameters for the overall reduction were thence derived [41], as listed above (Table 4b, reaction 7a). Although the derivation was somewhat indirect, the steps were clear and the $\Delta G'_0$ value was accepted for several years, e.g. [45,130].

More recently, and arising primarily from considerations of literature on microbial metabolism, the thermodynamics of the overall MTHFR reaction were reinvestigated, using the enzyme from *Peptostreptococcus productus* and, for some of the experiments, the artificial redox cofactor acetylpyridine–adenine dinucleotide [72]. The latter was chosen because its E'_0 (-248 mV) was more suitable than that of NAD(P)H (E'_0 about -320 mV) for determining the equilibrium of the overall reaction. The experiments [72] yielded the approximate values in Table 4(b) (7b). These values indicate a significantly less exergonic reaction than the (7a) values, and a correspondingly more negative E'_0 for the CH_2/CH_3-H_4folate couple than in (7a). It should be mentioned that, in the initial purification of the *P. productus* enzyme [142], Methylene Blue ($E'_0 = +11$ mV) was found to react reversibly with CH_2/CH_3-H_4folate, suggesting that E'_0 for the latter couple may not be quite as negative as -200 mV.

Bearing all of the above in mind, -200 mV will be taken as the current value of E'_0 for the CH_2/CH_3-H_4folate couple, with the possibility that the true value may be somewhat less negative, and -23 kJ·mol^{-1} will be taken as the value of $\Delta G'_0$ for the overall reaction with NAD(P)H under standard conditions, corresponding to a value of -41 kJ·mol^{-1} with H_2 as reference reductant, with the possibility that the true values may be somewhat more exergonic.

Turning to the H_4MPT pathway, thermodynamic values for reaction (21) were first inferred indirectly on the basis of differences between values for other related reactions (for details see [133,134]). More recently the thermodynamics were investigated with purified reductase [104]. There is fairly good agreement between values in the literature [75,104,134]; those in Table 4(c) (reaction 21) are representative to within about 4 kJ·mol^{-1} and about 20 mV respectively. Importantly, the sum for reactions (19) or (20) plus (21) is the same whether the individual values in [75] or in Table 4(c) [94,104] are taken: 0 to -1 kJ·mol^{-1} relative to $F_{420}H_2$ and -24 kJ·mol^{-1} relative to H_2; see the note to Table 4(c).

It is evident that E'_0 for the CH_2/CH_3-H_4MPT couple is considerably more negative than E'_0 for the CH_2/CH_3-H_4folate couple. If the values in Table 4(b) (reaction 7b) and Table 4(c) (reaction 21) are taken, then the difference between the redox potentials is about 140 mV, and reaction (7) is 27 kJ·mol^{-1} more exergonic than reaction (21) with reference to H_2 for both reactions. As a consequence, the free-energy relationships depicted in Figure 2 become reversed in the methylene reductase reactions, CH_3-H_4folate attaining a lower potential energy than CH_3-H_4MPT by about -16 kJ mol^{-1}. If a less negative value than in (7b) is taken for the CH_2/CH_3-H_4folate couple (see above discussion), then the energy difference is even greater.

C_1 flux

The thermodynamic values in Tables 4(b) and 4(c), determined *in vitro*, are broadly concordant with patterns of C_1 flux *in vivo*. As outlined in the previous section, C_1 flux may be predominantly reductive or oxidative in different organisms and metabolic circumstances. It is not intended here to survey all of these circumstances, but rather to note a few key points. (i) Concerning the coupling of the H_4folate pathway to pyridine nucleotides, in most if not all organisms the $NADP^+$ pool is maintained in a much more reduced state than the NAD^+ pool (e.g. [143,144]). Therefore, whether a given enzyme uses NAD(H) or NADP(H) may be important in determining direction of C_1 flux. (ii) This choice may be especially important in the MTHFR reaction. As discussed above, it was formerly thought that E'_0 for CH_2/CH_3-H_4folate was about -120 mV (value 7a in Table 4b). That value would be sufficiently different from that of pyridine nucleotides, $E'_0 = -320$ mV, to drive the reaction irreversibly under practically any physiological circumstances. However, if E'_0 for CH_2/CH_3-H_4folate is -200 mV, as in (7b) in Table 4(b), then $\Delta E'_0$ is smaller and resembles (for example) that for lactate dehydrogenase, which runs in opposite directions in different mammalian tissues. Therefore the use of NADPH rather than NADH could be a key factor in driving the reaction in the direction of CH_2-H_4folate reduction, and hence in the causation of the methyl trap of pernicious anaemia mentioned above. Organisms that couple MTHFR to NAD^+ could in principle be capable of methyl-group oxidation via the H_4folate pathway, given a supply of methyl groups, although this process would be thermodynamically less facile than by the H_4MPT pathway. (iii) In the H_4MPT pathway the CH^+/CH_2 and CH_2/CH_3 couples are substantially more negative than in the H_4folate pathway. This enables reversible coupling to the low-redox-potential cofactor F_{420}. C_1 can readily flow in either direction through the entire H_4MPT pathway, including the methylene reductase reaction, the actual direction being determined in different organisms by their overall patterns of C_1 metabolism; examples were given above. Additionally, C_1 can enter the H_4MPT pathway in the reductive direction from CO_2 without expenditure of ATP. This is largely due to the low energy level of CH^+-H_4MPT as compared

with CH^+-H_4folate (Figure 2), although, as mentioned above, chemiosmotic energy coupling is required [84] to surmount the initial 'hill' of HCO-MFR formation (reaction 16) in the H_4MPT pathway.

Chemical basis of the differences

Thermodynamic differences between the C_1 pathways on H_4folate and H_4MPT can be attributed to differences between the chemistries at N^{10} and, possibly, the presence of the two structural methyl groups in H_4MPT. These chemical differences are discussed in turn.

N^{10} and N^5

A detailed early study on model chemical compounds for H_4folate established that the nature of the group *para* to N^{10} of the arylamine is crucial in tuning the reactivity of N^{10} [145]. Replacement of the electron-withdrawing carbonyl by a methyl group increased the rate of condensation of the model compound with formaldehyde; this was attributed to an increase in the rate constant for ring closure from the cationic imine intermediate, due in turn to greater basicity (electron density) at N^{10} in the methyl-substituted compound [145]. That study was carried out before H_4MPT had been discovered, but can now be seen as a model for differences between H_4folate and H_4MPT.

The effect of the group *para* to N^{10} on thermodynamic differences between H_4folate and H_4MPT was discussed in [146] and [17]. Several points that are collected in [17] (see page 3035 and Table 1 therein) are now summarized. (i) The functionally most important difference between the two carriers is that the electron-withdrawing carbonyl group in conjugation with N^{10} in H_4folate is replaced by a (slightly) electron-donating methylene group in H_4MPT. (ii) Relatedly, the pK_a of N^{10} in H_4folate is -1.2 (from [16], see above), whereas that for N^{10} in H_4MPT was estimated to be about $+2.4$ ([146], cited in [17]). (iii) The different pK_a values (basicities) at N^{10} confer different properties to the bound C_1 units, with more negative E'_0 values of the C_1 redox transitions upon H_4MPT than on H_4folate. (iv) The methylene of CH_2-H_4MPT exchanges more slowly with formaldehyde than does that of CH_2-H_4folate ($t_{\frac{1}{2}} = 1$ h and < 15 min respectively [21]). Likewise, the methylene of CH_2-H_4MPT epimerizes more slowly than that of H_4folate (0.01 s^{-1} and 0.1 s^{-1}) [21]. (v) The chemical environments and reactivities of N^5 are essentially indistinguishable between the two carrier molecules.

[17] addresses principally the HMD reaction of methanogenesis (reaction 20). The discussion is extended here to indicate in outline how the different chemical properties of N^{10} are felt throughout the C_1 redox pathways on the pterins. See also [146] for further discussion.

Formyl to methenyl

N^{10}, being a better nucleophile in H_4MPT than in H_4folate, is thermodynamically able to promote spontaneous cyclization from 5-HCO-H_4MPT to the methenyl derivative at pH 7. In contrast, cyclization from 5-HCO-H_4folate requires energy input from ATP. (For discussion of cyclohydrolase reactions and N^5, N^{10} equilibria on H_4folate, see [36,138], and on H_4MPT, see [146]).

Methenyl to methyl

Because the electron density at N^{10} is greater in H_4MPT than in H_4folate, the bridged N^5,N^{10} C_1 compounds are more strongly bound to H_4MPT than to H_4folate; this is seen as lower values in the free-energy profiles in Figure 2. The difference is greater for the methenyl than for the methylene compounds, with greater delocalization in CH^+-H_4MPT than in CH^+-H_4folate, but in both cases more reducing power is required to bring about the C_1 reductions on H_4MPT than on H_4folate. (This is equivalent to saying that E'_0 for the redox transitions is more negative on H_4MPT than on H_4folate.) In addition to the thermodynamic profile data, independent evidence that C_1 at the methylene level is more strongly bound to H_4MPT than to H_4folate is given by the slower exchange of CH_2-H_4MPT with formaldehyde and the slower epimerization than for CH_2-H_4folate (iv, above).

In the methylene reductase reactions the first step is assumed to be ring-opening to form the cationic imine (discussed for the MTHFR reaction in [42,106]; see above). This ephemeral species is then reduced to the 5-methyl compound, or recloses to the methylene compound. As discussed above, the rate constant for ring closure is enhanced in model compounds in which the *para* substituent renders N^{10} more basic [145]. Extending this reasoning to H_4MPT and H_4folate, ring (re-)closure will be more favoured for H_4MPT than for H_4folate; hence the steady-state level of the cationic imine will be lower for H_4MPT, and the overall reduction will be thermodynamically less favourable on H_4MPT than on H_4folate, as is found. (The lower steady-state level of the cationic imine also underlies the slower exchange of formaldehyde with CH_2-H_4MPT.)

It was stated (v, above) that N^5 is chemically similar in the two carriers. This is based on the reasonable argument (see also below) that there are no structural differences near N^5 that might affect the electron density differently at this N atom in the two carriers. It follows that the bond energy, or enthalpy, of the N^5–CH_3 bond should be similar or identical between 5-CH_3-H_4folate and 5-CH_3-H_4MPT. It further follows from Hess's Law that the overall enthalpy change for reduction of CO_2 to 5-CH_3-H_4folate and to 5-CH_3-H_4MPT (expressed relative to H_2 as 'reference' reductant) should be approximately equal, notwithstanding differences in the intermediate steps in which N^{10} is involved. However, this expectation is for enthalpies, whereas the data in Tables 4(b) and 4(c) and Figure 2 are free energies.

The apparent free-energy change from CO_2 to CH_3-H_4MPT is about -16 kJ·mol^{-1}, whereas that from CO_2 to CH_3-H_4folate is about -32 kJ·mol^{-1} (or possibly even greater, as discussed above). Are these different 'end points' real or 'artefactual'? In other words, do the many individual $\Delta G'_0$ values contain sufficient errors to generate, collectively, the observed discrepancy? My attempts to find sufficient 'leeway' in the data to 'close the energy gap' have generated other inconsistencies, either internally in various cross-checks or overall with C_1 flux patterns, as discussed above. By any reckoning there appears to be an irreducible difference of at least 12 kJ·mol^{-1}, and more probably about 16 kJ·mol^{-1}, between the overall free energies of the two pathways. It should therefore be considered whether some other chemical factor(s), not yet entertained, might contribute to the overall free-energy difference. So far, little has been said about the two structural methyl groups of H_4MPT, C^{12a} and C^{13a} in Figure 1.

Structural methyl groups of H_4MPT

The methyl groups are each separated by two carbon atoms from N^5, with no intervening conjugation. It seems unlikely that they could affect the electron density at N^5. Thus the assumption that N^5 is chemically similar in the two carriers remains sound, as does in turn the expectation that the overall enthalpy changes for C_1 reduction to the respective 5-methyl pterins should be similar. Might the structural methyl groups contribute an entropy effect?

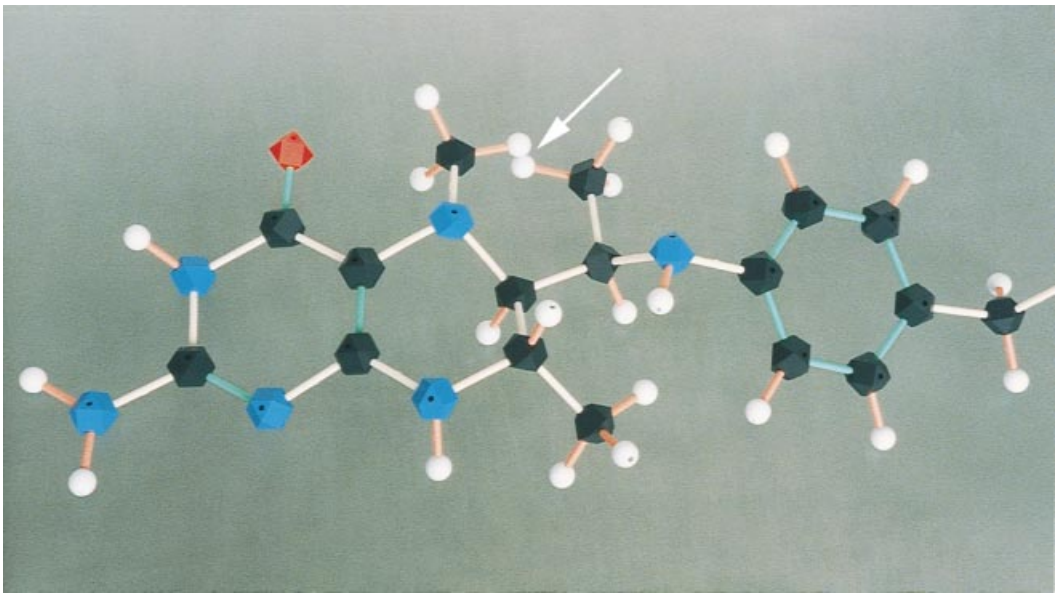


Figure 3 Colliding methyl groups in 5-CH_3-H_4MPT

Colour key to atoms: black, carbon; white, hydrogen; blue, nitrogen; red, oxygen. The model shows the pteridine and benzene-ring parts of the structure, and is in the extended conformation, with C^{6a} below the plane of the pteridine and the C^{6a}–H bond axial as in the minimum-energy conformation depicted in [190] for CH^+-H_4MPT (see Figure 1 for numbering of atoms). Since reduction to 5-CH_3-H_4MPT entails breakage of the bond linking the C_1 unit to N^{10}, the C^{6a}–C^{11a} bond has greater freedom to rotate than in the bridged compounds. However, the structural methyl group C^{12a} limits the extent of rotation by contacting the N^5 methyl group, as shown, or the C^{13a} structural methyl. The possibility for the contacts exists notwithstanding nitrogen inversion [191] at N^5, given some degree of flexibility of the pyrazine ring. 5-CH_3-H_4folate is not sterically hindered in this manner.

The following considerations suggest that this possibility is plausible.

In the condensation of formaldehyde with H_4folate to CH_2-H_4folate there is a large decrease in entropy [147] (63 entropy units/mol by the notation in [147], or about 264 $J \cdot K^{-1} \cdot mol^{-1}$). This is largely attributed [147] to constraint in the methylene compound of rotation around the bonds C^6–C^9 and C^9–N^{10}, both of which can rotate freely in H_4folate. Conversely, in the TS–DHFR reaction cycle, in which free H_4folate is regenerated, there is a large entropy gain which contributes to driving the cycle [147]. In the MTHFR reaction (7) the constraining methylene bridge is also broken, C_1 remaining attached to N^5. Thus after reaction (7) free rotation also becomes possible around C^6–C^9 and C^9–N^{10}. The enthalpy and entropy components of reaction (7) are currently unresolved experimentally, but a substantial entropy increase seems likely from the above [147] considerations.

In the methylene-H_4MPT reductase reaction (reaction 21) the methylene bridge is also broken. However, there is a subtle difference in the end result, because the structural methyl groups of H_4MPT are bulkier than the hydrogen atoms that occur at the same positions in H_4folate. There is now potential for steric hindrance to free rotation around C^{6a}–C^{11a}: when the methyl C^{12a} rotates in one direction, it can swing into cyclopentane-like apposition with C^{13a}; in the other direction it can swing into the C_1 methyl group on N^5. An analogy may be drawn between a freely swinging door compared with one which swings between doorstoppers. The potential contacts are readily demonstrable, as shown in Figure 3 for the C^{12a} and N^5 methyl groups. Due to limitation in free rotation around C^{6a}–C^{11a}, it is reasonable to infer that any gain in entropy in reaction (21) is less than in reaction (7).

An entropy change of 264 $J \cdot K^{-1} \cdot mol^{-1}$ for interconversion between H_4folate and CH_2-H_4folate (above) corresponds to 79 $kJ \cdot mol^{-1}$ at 298 K. Only about 20 % of that value is required to explain the discrepancy between the overall $\Delta G'_0$ of CO_2 reduction to 5-CH_3-H_4folate (at least -32 kJ with H_2 as reductant) and CO_2 reduction to 5-CH_3-H_4MPT (about -16 kJ with H_2 as reductant). Hindrance of free rotation around C^6–C^{11a} might afford such a difference in the form of an entropy difference between reactions (7) and (21).

A difference between $\Delta G'_0$ values from CO_2 to 5-CH_3-H_4folate and 5-CH_3-H_4MPT respectively may confer advantage upon H_4MPT for its biological roles. In the reductive direction, if about 16 kJ of energy are 'saved' in the pathway to CH_3-H_4MPT, this extra energy is available for the final reduction steps to CH_4, where energy is transformed chemiosmotically. In the oxidative direction CH_3-H_4MPT is at an energy level allowing ready oxidation to CH_2-H_4MPT by F_{420}.

It would be useful to obtain further evidence as to the existence (or otherwise) of an entropy difference between the two pathways. First, an independent check of whether the overall free-energy changes are indeed as deduced from the above sums (Tables 4b and 4c) might be obtainable. Determination of equilibria and free energies of methyl-group transfer from a common C_1 source such as methanol to CH_3-H_4folate and to CH_3-H_4MPT would provide an independent, and direct, check, if appropriate enzymes were to become available. Secondly, calorimetric analysis, such as that carried out in connection with the TS reaction ([147] above), might be applicable to the methylene reductase reactions, in an attempt to distinguish between enthalpy and entropy contributions. Thirdly, to test the idea that the structural methyl groups of H_4MPT contribute to entropy in the manner suggested, it would be of interest to examine the energetics of C_1 pathways on carriers which lack one or both structural methyl groups, but are otherwise H_4MPT-like by the criterion of possessing a methylene *para* to N^{10}. Such carriers exist in some Archaea (see below). Last, the energetics of methyl transfer from N^5 of the

pterins to homocysteine and CoM respectively are potentially relevant, as noted in the legend to Figure 2.

BIOSYNTHETIC ROLES FOR H_4MPT?

Given the many differences between the H_4folate and H_4MPT C_1 pathways, the question arises whether H_4MPT performs any of the biosynthetic roles that are played by H_4folate in most organisms. The question is important because, in general, H_4MPT-containing Archaea are thought to lack H_4folate, judged by indicator microbial growth assays [148,149] and, more recently, by genome analyses [28–30], which reveal a lack of known enzymes that either utilize or synthesize H_4folate.

As discussed above, formyl groups are carried by H_4MPT on N^5, where they are only slightly activated with reference to free formate, and are therefore unsuited to biosynthetic formyl transfers. 10-HCO-H_4MPT is not a known biochemical intermediate. Moreover, because N^{10} of H_4MPT is not electron-deficient, it would be unlikely to be as good a leaving group as N^{10} of H_4folate, and therefore 10-HCO-H_4MPT, even if it were metabolically available, would not be expected to be as good a formyl donor as 10-HCO-H_4folate. Interestingly, there is currently no evidence for biosynthetic formyl transfers via H_4MPT. In the purine-biosynthetic pathway, PurT but not PurN is identifiable in the genome of *M. jannaschii* [28], indicating that ATP-dependent insertion of purine C^8 occurs from free formate. Insertion of C^2 from free formate has also been demonstrated in an ATP-linked reaction in three Archaea that contain H_4MPT or related pterins (two species of *M. thermoautotrophicum* and the sulphur-dependent *Sulfolobus solfataricus* [150]). Thus the purine-biosynthetic pathway in H_4MPT-utilizing Archaea appears to be independent of H_4MPT (or H_4folate) [150]). It is important in relation to biochemical evolution that purines can thus be made without pterins, which themselves are made in part from the purine GTP (see below).

The other major role of 10-HCO-H_4folate in bacteria is formylation of initiator tRNA. It is believed that Archaea generally, like eukaryotes, use non-formylated initiator tRNA [151]. Direct evidence for use of non-formylated initiator tRNA was first obtained from *Halobacterium cutirubrum* ([152], reviewed in [153]). Halobacteria are generally H_4folate-utilizing Archaea [149]. More general evidence for eukaryotic-like translation initiation machinery in Archaea is afforded by the presence of an unusual amino acid, hypusine, that is characteristic of eukaryotic initiation factor 4D (reviewed in [154]) and from genomic evidence for eukaryotically related initiation factors [28–30], and is consistent with the general use of non-formylated methionyl-tRNA in initiation of protein synthesis. From these considerations and those on purine biosynthesis above, it can be inferred that H_4MPT-utilizing Archaea have no requirement for an activated formyl donor such as 10-HCO-H_4folate. Further work on a range of Archaea might be useful to establish this point.

At the methyl level, 5-CH_3-H_4MPT and 5-CH_3-H_4folate are less different than the respective formyl derivatives. However, the two methyl derivatives may not be energetically equivalent, as discussed in the preceding section. The methionine synthase reaction in H_4MPT-containing Archaea has thus far only been partially characterized. An open reading frame (ORF) corresponding to the C-terminal half of the *E. coli* cobalamin-independent MetE was identified in the complete genome of *M. thermoautotrophicum*, whereas no homologue of the cobalamin-dependent MetH was found [29]. Purification of the respective *M. thermoautotrophicum* enzyme led to the suprising finding that it catalyses methyl transfer to L-homocysteine, using methylcobalamin as methyl donor, but not 5-CH_3-H_4MPT (nor 5-CH_3-H_4folate) [155]. It was proposed that, *in vivo*, a so-far-unidentified enzyme catalyses methyl transfer from 5-CH_3-H_4MPT to a corrinoid protein, and that the MetE gene product catalyses the further transfer to homocysteine [155]. The other known transfer reactions from 5-CH_3-H_4MPT are also corrinoid-mediated: to CoM and into acetyl-CoA (the latter via acetyl-CoA synthase) as mentioned above. Acetyl-CoA is a major biosynthetic precursor in methanogens [120].

Perhaps the biggest challenges in elucidating putative biosynthetic functions of H_4MPT are at the methylene level. It is expected that any transfers at this level would be initiated by breakage of the methylene–N^{10} bond, generating a cationic imine, as already described for CH_2-H_4folate. As emphasized in early literature, a suitable ΔpK_a between N^{10} and N^5 facilitates methylene C_1 transfers by appropriately tuning the availability of the cationic imine [145,156]. As discussed above, ΔpK_a between N^{10} and N^5 is much smaller in H_4MPT (pK_a values of about 2.4 and 4.8) than in H_4folate (-1.2 and 4.8). This would suggest that any C_1 transfer from CH_2-H_4MPT would be less strongly driven than from CH_2-H_4folate. Any entropic difference, as discussed in the previous section, might also favour H_4folate rather than H_4MPT for this class of C_1-donor reactions. With these considerations in mind, the following well-characterized H_4folate reactions, SHMT (reaction 2), TS (reaction 11) and α-oxopantoate synthase (reaction 12) are now considered with regard to known or possible H_4MPT counterparts.

An SHMT has been purified from *M. thermoautotrophicum*. The enzyme utilizes pyridoxal phosphate (PLP) (like the H_4folate enzyme), but is specific for H_4MPT [157]. The enzyme was proposed to function *in vivo* in the direction of serine biosynthesis [157]. K_{eq} was not reported for the reaction. For reference, K_{eq} for the H_4folate reaction is approx. 8–12 in the direction of CH_2-H_4folate synthesis [158], permitting ready reversibility. From the considerations in the above paragraph, one might expect K_{eq} for the H_4MPT reaction to be more strongly in the direction of methylene–pterin formation than in the case of the H_4folate reaction. Nevertheless, the reverse reaction of serine biosynthesis might be driven by high C_1 flux through the H_4MPT pathway if K_{eq} for the SHMT reaction were not too unfavourable, say 10^2–10^3, in the CH_2-H_4MPT direction. Determination of K_{eq} for the H_4MPT reaction would clearly be of interest in providing further experimental information on the relative affinity of the methylene group for H_4MPT as compared with H_4folate, and on the likely direction of the reaction *in vivo*.

Early attempts to identify TS in *M. thermoautotrophicum* revealed a candidate enzyme which catalysed some partial reactions of thymidylate synthesis (hydrogen exchange, dehalogenation of bromodeoxyuridine), but not the complete reaction [159]. Subsequent sequencing of the gene (together with that of the cyclohydrolase MCH) [92] indicated a relationship to a dUMP hydroxymethyltransferase [160]. No clear evidence for TS was found at the level of primary structure in the three sequenced genomes already mentioned [28–30], despite the fact that known TS enzymes are highly conserved at the level of primary structure [161]. Biochemical evidence was meanwhile obtained for dTMP synthesis in cell extracts of *M. thermophila* and *S. solfatoricus*, which contain H_4MPT-related pterins [162]. Recently, a sequence that is consistent with a TS-like secondary structure was identified in the genome of *M. jannaschii* by means of a novel computer program [163]. The gene was cloned and expressed in *E. coli*. *In vitro* activity was obtained, but, surprisingly, CH_2-H_4folate rather than CH_2-H_4MPT served as substrate [163]. Moreover, in the non-methanogen *S. solfataricus*,

the otherwise H_4MPT-like pterin lacks the two structural methyl groups ([164]; see below). A fragment of this pterin carrying the methylene group was a donor for thymidylate synthesis in [162]. These lines of evidence were taken to suggest that the methylene donor in H_4MPT-containing cells may be the unmethylated biosynthetic precursor to H_4MPT [163]. If this proves to be correct, it will also be in accord with the above proposal that CH_2-H_4folate (lacking the structural methyl groups) can give methylene transfer or reduction reactions a greater 'entropic boost' than can CH_2-H_4MPT (containing the structural methyl groups). However, the problem of the smaller ΔpK_a between N^{10} and N^5 in H_4MPT than in H_4folate remains.

Yet another unresolved aspect of thymidylate synthesis in organisms that utilize H_4MPT is that the putative H_2MPT reductase, which would be expected to be needed to complete the thymidylate synthase reaction cycle, remains unidentified (see 'Reduction of the pterin rings' section above). Overall the conclusion seems inescapable that the provision of dTMP in H_4MPT-utilizing Archaea is currently incompletely understood.

So far, no enzymes (or genes) corresponding to α-oxopantoate synthase or the glycine-cleavage system have been identified in methanogens. As already discussed, CoA is a major player in methanogenic metabolism. Therefore an α-oxopantoate synthase would be expected to be an essential enzyme. Its identification might shed further light on C_1-transfer processes at the methylene level in methanogens and related organisms.

BIOSYNTHESIS OF THE CARRIERS, AND PHYLOGENETIC ASPECTS

It follows from the above discussion that the steps in the biosynthetic pathway to H_4MPT that determine the carriers' distinctive functional properties are those that generate the methylene group *para* to N^{10} and also probably the addition of the structural methyl groups. Therefore the biochemistry of these steps, and the phylogeny of their distribution, are of considerable ecological and evolutionary interest.

In both carriers the pterin originates from GTP, and the arylamine from PABA. The pathway to H_4folate in *E. coli* is well known and has recently been reviewed [27]. Key steps are briefly outlined here so as to enable comparison with the pathway to H_4MPT.

Biosynthesis of H_4folate

The pathway is initiated by GTP cyclohydrolase I, which converts GTP into the compound 7,8-H_2-neopterin triphosphate in a remarkable multistep reaction. In the reaction, C^8 of GTP is eliminated as formate, $C^{1'}$ and $C^{2'}$ of the original ribose triphosphate become C^7 and C^6 of the pterin, and the remainder of the ribose triphosphate becomes the side chain of 7,8-H_2-neopterin triphosphate. For a proposed mechanism based on the crystal structure, see [165]. Genes encoding homologous sequences to the enzyme have been identified from many Bacteria and Eukarya ([165] and references cited therein). In subsequent reactions the side chain is dephosphorylated, trimmed by neopterin aldolase and rephosphorylated to yield the diphosphate of 6-CH_2OH-7,8-H_2pterin.

PABA is synthesized from chorismate and is condensed with the pterin by dihydropteroate synthase with elimination of diphosphate, yielding dihydropteroate. The first glutamate residue is then added, and the resulting 7,8-H_2folate is reduced by DHFR (reaction 1, above) to generate active carrier. The timing of addition of further glutamate residues and their relation to H_4folate functions in *E. coli* are described in [27]. Also see [27] and references therein for details of other enzymes of the biosynthetic pathway and their genes.

Biosynthesis of H_4MPT

From the structures (Figure 1), one might expect intuitively that the pathway to H_4MPT would be similar to the pathway to H_4folate in the early steps, and that only the later steps that generate the distinguishing features of H_4MPT would involve different enzymes. Biosynthetic labelling studies with stable isotopes were initially consistent with this expectation [166]. However, it is emerging from genomic analyses [28–30] and from enzymological studies (see below) that, surprisingly, there are few, if any, close homologues to enzymes of folate biosynthesis among Archaea that utilize H_4MPT, even in the early part of the pathway.

GTP cyclohydrolase I is absent in H_4MPT-utilizing Archaea. Instead, ring restructuring proceeds by multiple steps, catalysed by two or more enzymes. The intermediate following expulsion of C^8 and before reclosure to form the pterin is separately identifiable *in vitro* in the Archaeal reaction sequence [167], but not in the classical cyclohydrolase I reaction sequence. A novel compound, H_2-neopterin 2′,3′-cyclic phosphate, is an intermediate in dephosphorylation of the pterin [167]. The GTP cyclohydrolase of the Archaeal H_4MPT biosynthetic pathway appears to be distinct not only from GTP cyclohydrolase I, but also from GTP cyclohydrolase II of the riboflavin pathway, and has been tentatively named 'GTP cyclohydrolase III'; for a discussion, see [167]. After conversion of the 2′,3′-cyclic phosphate into H_2-neopterin, subsequent steps, which remain to be fully characterized enzymologically, generate the diphosphate of 6-CH_2OH-7,8-H_2pterin [167].

In the arylamine part of the pathway, PABA is first condensed with phosphoribosyl diphosphate (PRPP) in a reaction in which the diphosphate of PRPP and the carboxyl group of PABA are both eliminated [168]. The product, 4-(β-D-ribofuranosyl)-aminobenzene 5′-phosphate (β-RFA-P), is the defining intermediate of the pathway, since the carboxyl group of the original PABA has now been removed. The enzyme that catalyses this crucial reaction has been partly purified, and a mechanism for the reaction has been proposed [169].

β-RFA-P is then condensed with the pterin diphosphate. The dihydropteroate synthase that catalyses the condensation shows little resemblance in primary structure to the folate-pathway enzyme. The synthase was identified [163] by a procedure similar to that described above for the putative TS of methanogens. Then the anomeric carbon atom of β-RFA-P is reduced to the functionally important methylene that tunes the reactivity of N^{10} (see above), and the 'right-hand end' of the structure (Figure 1) is completed in several further steps [168].

The methyl groups at C^{7a} and C^{11a} are donated by *S*-adenosylmethionine. For these C methylations it is proposed that an enzyme thiol combines with C^{6a} of the pteridine [170] in similar manner to pyrimidine C^5 methylations [171–173]. The methylations may precede the final steps of assembly of the right-hand end of the molecule in some organisms [170]. Alternatively, some unmethylated molecules might be sequestered by reduction and might function in cellular biosynthetic C_1-transfer reactions [170] such as the TS reaction [163] as mentioned above. (The proposed mechanism for C methylations would not operate on fully reduced carrier, because initiation of the mechanism requires a double bond at C^{6a} [170].)

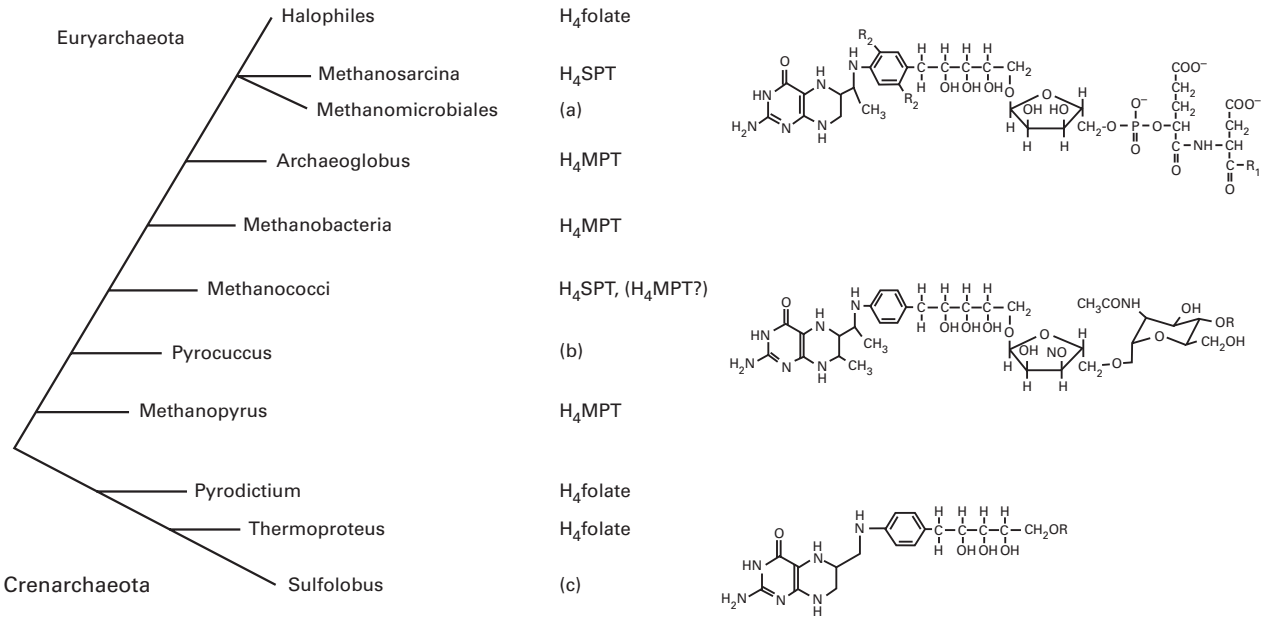


Figure 4 Pterins in Archaea

The partial phylogenetic tree is based on [7,9]. The distribution of pterins is based on survey data in [149,192,193]. It should be noted that these data, while extensive, are not exhaustive. For example, identification of H_4SPT in Methanococci is based on analysis of one member of the group, *M. voltae* [193]. (For the difference between H_4SPT and H_4MPT, see the text.) Notes: (a) several Methanomicrobiaceae [193] contain one or more variants of the tatiopterin/thermopterin structure shown; tatiopterin O: $R_1 = O^-$, $R_2 = H$; tatiopterin I: R_1 = glutamate, $R_2 = H$; thermopterin: $R_1 = O^-$, $R_2 = OH$ [175,176]; (b) *Pyrococcus furiosus*, a member of the Thermococci, contains the pterin shown, where R = H or one or two *N*-acetylglucosamine residues linked by β1–4 bonds [176]; *Thermococcus celer* contains a related pterin also with two structural methyl groups and β-RFA; the side chain was not fully characterized [176,192]; (c) Partial structure of sulfopterin; R = incompletely characterized side chain [164,192].

Variants on the H_4MPT structure and their phylogenetic distribution

H_4MPT-like pterins based on β-RFA (see above) are deeply rooted in Archaea, occurring in Crenarchaeota as well as Euryarchaeota. Among various Archaeal lineages there are numerous variations upon the core structure, generally arising from alternative terminal extensions of the biosynthetic pathway. (See [174], especially pp. 122–124, for classification of evolutionary processes in biochemical pathways.) Variations occur in the distal part of the 'right-hand' side chain, and in the presence or absence of structural methyl groups, and, in one known instance, in hydroxylation on the benzene ring.

As already mentioned, Methanosarcinales contain H_4SPT, which differs from H_4MPT in containing a terminal glutamate residue linked to the α-hydroxyglutarate of H_4MPT [78]. The following are further examples of variants, of generally increasing magnitude (Figure 4).

Tatiopterin O, from *Methanoculleus thermophilicum*, has an aspartate residue linked to the α-hydroxyglutarate, and lacks the methyl C^{12a}; tatiopterin I is the same, except that a glutamate residue is attached to the aspartate; thermopterin, from the same organism, resembles tatiopterin O, except that the benzene ring is doubly *para*-hydroxylated [175,176]. Among non-methanogenic Euryarchaeota, *Pyrococcus furiosus* contains a pterin which resembles H_4MPT in the presence of both structural methyl groups, and in the side chain up to and including the ribofuranose of Figure 1(b). However, there is no phosphate; instead the side chain is extended by one, two or three *N*-acetylglucosamine residues [176]. *S. solfataricus*, a Crenarchaeote, contains sulfopterin, which is H_4MPT-like in containing β-RFA. However, as mentioned above, the pterin lacks the structural methyl groups of H_4MPT [164].

Except for the sulphate-reducing *A. fulgidis* mentioned above, the metabolic functions of H_4MPT-like pterins in non-methanogenic Archaea are not well characterized. A synthetic fragment of sulfopterin was active with a recently isolated SHMT from *S. solfataricus* [177]. H_4folate did not support serine synthesis by this enzyme [177]. Further elucidating the functions of H_4MPT-like pterins in C_1 metabolism in non-methanogenic Archaea is likely to be of considerable metabolic interest. The occurrence of pterins which differ from H_4MPT in the absence of one or both structural methyl groups may enable investigation of possible roles that were discussed above for the methyl groups.

The presence of H_4MPT-like molecules in a wide range of Archaea suggests that the β-RFA part of the methanopterin-biosynthetic pathway became established early in the Archaeal lineage. However, some deeply rooted Crenarchaeota contain H_4folate (Figure 4). As suggested above, the idea at first seemed intuitively likely that the pathway to H_4MPT biosynthesis arose from modifications to a pre-existing folate-biosynthetic pathway. The discovery of major differences between the GTP sections of the two pathways [167] put this intuitive expectation into question, and the possibility should be considered that the H_4MPT-biosynthetic pathway arose *de novo* in Archaea.

Among the present day Archaea, it is notable that halophiles contain H_4folate [149]. Given the very different metabolic activities of the (aerobic) halophiles [153] and their closest relatives the (anaerobic) methanogens [6,74], it seems likely that acquisition of metabolic genes by lateral transfer may have occurred at some period during evolution of the halophile lineage. In this context it would be of interest to know whether the pathway to

folate biosynthesis in halophilic Archaea is initiated by a GTP cyclohydrolase I (Bacteria-like) or cyclodrolase III (Archaeal [167], see above).

Dephospho-H_4MPT in methylotrophic Bacteria

Methylobacterium extorquens AM1 is one of a group of aerobic methylotrophic bacteria that obtain energy by oxidizing methanol. Methanol is first oxidized to formaldehyde by a pyrroloquinolinequinone-dependent dehydrogenase [178]. A possible route of formaldehyde oxidation was thought to be entry into the H_4folate pathway at the methylene level and oxidation to 10-HCO-H_4folate, formate and CO_2 [179]. However, recently several ORFs showing significant homology to genes or ORFs known previously only in methanogens and *A. fulgidis* were identified adjacent to a cluster of genes for C_1 metabolism in the *M. extorquens* genome [14]. Among the ORFs were those for CH^+-H_4MPT cyclohydrolase (reaction 18 above), HCO-MFR: HCO-H_4MPT formyltransferase (reaction 17) and three of the subunits of HCO-MFR dehydrogenase (reaction 16). Cloning and expression of the first two in *E. coli* yielded enzymes that were functional with the H_4MPT substrates. The H_4MPT-like substrate in *M. extorquens* was identified as the dephospho form of the carrier, that is, the structure up to and including the ribofuranosyl residue (Figure 1b), but excluding the phosphate and α-hydroxyglutarate [14]. Further work revealed a novel, $NADP^+$-dependent CH_2-H_4MPT dehydrogenase [180].

These remarkable findings beg both a functional and an evolutionary interpretation. The proposed functional interpretation is that C_1 in *M. extorquens* (and related organisms) is oxidized by the H_4MPT path [14,180]. Coupling of CH_2-H_4MPT dehydrogenase with $NADP^+$ would ensure oxidative flux at this step because of the much more negative E_0' of the CH^+/CH_2-H_4MPT couple than of the $NADP^+$/NADPH couple [180]. The H_4folate pathway, which co-exists in this organism, would be used for biosynthetic purposes [180].

The evolutionary interpretation is that the H_4folate and H_4MPT pathways have co-existed in the methylotrophic lineage from early during Bacterial evolution, the H_4MPT pathway having probably entered either from an Archaeal/Bacterial fusion event or (perhaps more likely) from lateral gene transfer, and having been retained in C_1-oxidizing bacteria (see [181] for discussion).

CONCLUDING COMMENTS

Nature has invented, in addition to H_4folate, the H_4MPT class of compounds as C_1 carriers. The functions of H_4MPT in methanogenesis are now well characterized. However, there remain unanswered and intrinsically interesting questions concerning the bioenergetic roles of H_4MPT or related carriers in non-methanogenic Archaea, their possible roles in biosynthetic C_1 transfers, their own biosynthesis, and their evolutionary origins. Those origins appear to be deeply rooted in the origins of the Archaea themselves, but the picture is obscured by the mists of time and by the possibility of lateral gene transfers, as exemplified by the methylotrophic Bacteria [14]. Approaches based on comparative genomics and enzymology should continue to illuminate these fundamental questions on the early evolutionary history of C_1 metabolism and its consequences in the biosphere. Last, but not least, a comparative approach may continue to enhance understanding of specific chemical details of both the H_4folate and H_4MPT pathways.

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