Moreover, the redox reactions on H$_4$folate 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$_4$folate is utilized for energy metabolism in some groups of anaerobic bacteria. In methanogens and several other Archaea, tetrahydromethanopterin (H$_4$MPT) carries C$_1$ fragments between formyl and methyl oxidation levels. At first sight H$_4$MPT appears to resemble H$_4$folate 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$_4$MPT permits redox-flux features that are distinct from the pathway on H$_4$folate. In the reductive direction, ATP is consumed in the entry of carbon from CO$_2$ into the H$_4$folate pathway, but not in entry into the H$_4$MPT pathway. In the oxidative direction, methyl groups are much more readily oxidized on H$_4$MPT than on H$_4$folate. Moreover, the redox reactions on H$_4$MPT are coupled to more negative reductants than the pyridine nucleotides which are generally used in the H$_4$folate 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$_4$folate, N$^{10}$ is subject to electron withdrawal by the carbonyl group of p-aminobenzoate, but in H$_4$MPT an electron-donating methylene group occurs in the corresponding position. It is also proposed that the two structural methyl groups of H$_4$MPT tune the carrier’s thermodynamic properties through an entropic contribution. H$_4$MPT appears to be unsuited to some of the biosynthetic functions of H$_4$folate, in particular the transfer of activated formyl groups, as in purine biosynthesis. Evidence bearing upon whether H$_4$MPT 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$_4$MPT-related carrier, which they are thought to use in energy metabolism, as well as H$_4$folate, which they are thought to use for biosynthetic reactions.

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

INTRODUCTION

Tetrahydrofolate (H$_4$folate) 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$_4$folate 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$_4$MPT), resembles H$_4$folate 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$_4$folate and H$_4$MPT are not functionally equivalent.

The present review compares and contrasts H$_4$folate and H$_4$MPT. 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
of the domain Bacteria contain an H$_4$MPT-related carrier, which they use in energy metabolism, as well as containing H$_4$folate, which they are presumed to use for biosynthetic purposes [14].

**STRUCTURAL AND CHEMICAL FEATURES**

**H$_4$folate**

Figure 1(a) shows the structure of H$_4$folate 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$_4$folate 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'. In enzymically reduced H$_4$folate the absolute configuration is (6$^S$) [15a], and only that isomer is biologically active. The C' substituent is carried at N$^6$ or N$^{10}$ or bridged between both, as shown (see Table 1 for nomenclature). [Attachment of a C' fragment to N$^6$, or bridged between N$^6$ and N$^{10}$ as the pH is lowered [16].

H$_4$folate undergoes non-enzymic condensation *in vitro* with formaldehyde to give 5,10-CH$_2$-H$_4$folate. 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$_4$folate, as will be discussed.

**H$_4$MPT**

Figure 1(b) shows the structure of H$_4$MPT and derivatives. The structure of non-reduced methanopterin (MPT) was determined by two-dimensional NMR [19]. Fully reduced H$_4$MPT was
shown to utilize formaldehyde in vitro in the pathway to methanogenesis [12]. The C₇-H₄MPT derivatives in Figure 1(b) are intermediates in methanogenesis from CO₂ and H₂ [10,13,20] (for pathway details, see below).

H₄MPT resembles H₄folate in that it consists of a pterin linked to an arylamine, with C₇ binding to N⁵ or both N³ and N⁹. However, to the right of the benzene ring the structures differ completely. Instead of the glutamate residue(s) in H₄folate, the side chain of H₄MPT consists of a ribitol residue linked to ribose 5-phosphate and thence to hydroxylglutamate. 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 H₄MPT arm is completely different from the H₄folate 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 N¹⁰ of H₄folate, referred to above, is absent in H₄MPT (discussed later). A further difference between the two carriers is that H₄MPT contains two structural methyl groups on the carbon atoms designated C⁷a and C¹⁰a. Possible functions of these methyl groups are also discussed later. (For historical reasons the numbering system of H₄MPT differs from that of H₄folate [20].) The system shown in Figure 1(b) is used by most authors, e.g. [21], although some authors use the H₄folate numbering system [22].

H₄MPT, like H₄folate, is chiral at C⁶. In addition, the two methyl groups introduce chiral centres at C⁷a and C¹⁰a. The configurations at these carbon atoms were deduced by NMR and optical methods to be (6S) (as in H₄folate), (7S,11R), the NMR data linking the assignments (see [21] and also Figure 4 in [17]). [22] provides independent verification of (R) stereochemistry at C¹¹a (termed C⁶ in [22]).

Table 1 Nomenclature of C₁ derivatives of H₄folate and H₄MPT

<table>
<thead>
<tr>
<th>Unbound</th>
<th>Bound to H₄folate</th>
<th>Bound to H₄MPT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td>Abbreviation</td>
<td>Name</td>
</tr>
<tr>
<td>Formate</td>
<td>HCO-H₄folate</td>
<td>Formyltetrahydroxymethanopterin (5 or 10, as specified)</td>
</tr>
<tr>
<td>5,10-Methenyltetrahydrofolate</td>
<td>CH⁺-H₄folate</td>
<td>5,10-Methenyltetrahydroxymethanopterin (5 or 10, as specified)</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>CH₂-H₄folate</td>
<td>5,10-methenyltetrahydroxymethanopterin</td>
</tr>
<tr>
<td>Methanol</td>
<td>S-CH₃-H₄folate</td>
<td>5-Methyltetrahydroxymethanopterin</td>
</tr>
</tbody>
</table>

REDUCTION OF THE PTERIN RINGS

Folate to H₄folate

The enzyme dihydrofolate reductase (DHFR) catalyses NADPH-linked reduction of 7,8-H₄folate to H₄folate [reaction (1) in Table 2, below], and also, with slower kinetics, reduction of folate to 7,8-H₄folate. Folate can be a dietary source of the carrier (due to oxidation of some dietary H₄folate or to dietary supplementation), whereas 7,8-H₄folate is the product of the folate-biosynthetic pathway in organisms that synthesize the carrier. 7,8-H₄folate 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]. NAD(P)H binds ‘behind’ the pterin ring in the pterin orientation shown in Figure 1(a), with transfer of hydride to the si face of C⁶ (H₄folate reduction) or C⁷ (folate reduction) and protonation at N³ (or N⁹) coming from bound water (discussed in [24]). Reduction of H₄folate to H₄folate generates the (6S) 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 H₄folate reduction in Escherichia coli, see [27].)

Methanopterin to H₄MPT

The biochemistry of methanopterin reduction is less well characterized than that of folate reduction. Given the (6S) configuration of H₄MPT, hydride transfer to C₆ must take place from ‘behind’ (Figure 1b) as in H₄folate reduction. However, the stereochemistry at C⁷ (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 H₄MPT 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 C₁ 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 H₄MPT: Methanococcus jannaschii [28], M. thermoautotrophicum [29] or Archeoglobus fulgidus [30]. Nor is the reduction cofactor known; possible candidates might be NAD(P)H or the deazaflavin F₃⁺H₂ (see below). Characterization of the reductase system and comparison with DHFR would be of biochemical and evolutionary interest.
COMPARISON OF THE H\textsubscript{4}FOLATE AND H\textsubscript{4}MPT PATHWAYS

The H\textsubscript{4}folate pathway

Reactions of H\textsubscript{4}folate 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\textsubscript{1} on to H\textsubscript{4}folate. In man, the major loading route is from serine (reaction 2) yielding CH\textsubscript{2}-H\textsubscript{4}folate and glycine. Reaction (3) cleaves any glycine that is in excess of the organism’s needs, yielding additional CH\textsubscript{2}-H\textsubscript{4}folate from C\textsubscript{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 hyperglycinemia, results from a defective enzyme system [32].

C\textsubscript{1} also enters the H\textsubscript{4}folate pathway from formate, in an ATP-requiring reaction that generates 10-HCO-H\textsubscript{4}folate (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\textsuperscript{0} of H\textsubscript{4}folate is an acceptor for some catabolically generated C\textsubscript{1} at the formyl oxidation level [34,35] (for brief details, see the legend to Scheme 1). Enzymic conversion of 5-HCO-H\textsubscript{4}folate into 10-HCO-H\textsubscript{4}folate requires energy input from coupled hydrolysis of ATP [36]. This affords evidence that 10-HCO-H\textsubscript{4}folate is the more activated of the two formyl isomers. Generation of the methenyl derivative, CH\textsuperscript{2+}-H\textsubscript{4}folate, from 5-HCO-H\textsubscript{4}folate also requires ATP (discussed in [36]).

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\textsubscript{4}folate.

The redox and associated interconversions of carrier-bound C\textsubscript{1}, reactions (5)–(7), comprise the ‘central superhighway’ of C\textsubscript{1} metabolism on H\textsubscript{4}folate. 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\textsubscript{4}folate [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 cyclodihydrolase 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\textsubscript{4}folate synthase (reaction 4) (reviewed in [44]). These arrangements promote substrate channelling [48]. However, in some fermentative anaerobic bacteria in which H\textsubscript{4}folate 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 cyclodihydrolase). It should also be mentioned that a monofunctional dehydrogenase has been isolated from yeast [53] and that eukaryotic bifunctional dehydrogenase/cyclodihydrolases are known (see [53] for references).

Turning to biosynthetic C\textsubscript{1}-transfer reactions, three major transfers occur at the formyl level. Reactions (8) and (9) occur in the classical purine-biosynthetic pathway, contributing C\textsuperscript{6} and C\textsuperscript{5} 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\textsubscript{4}folate. 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\textsubscript{4}folate. Thus H\textsubscript{4}folate is not universally obligatory in provision of C\textsuperscript{6} of the purine ring. Whether C\textsuperscript{5} of the purine ring can be provided independently of H\textsubscript{4}folate will be discussed later.

At the methylene level, the archetypal biosynthetic C\textsubscript{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\textsuperscript{6}–N\textsuperscript{5} bond of the H\textsubscript{4}folate carrier. 7,8-H\textsubscript{4}folate is released, and this must be reduced again to H\textsubscript{4}folate (reaction 1) before re-utilization. CH\textsubscript{3}-H\textsubscript{4}folate 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 hydroxymethylated from CH\textsubscript{3}-H\textsubscript{4}folate is the formation of-z-oxopantoate (‘z-ketopantoate’) (reaction 12), the first step in the biosynthesis of pantothenate and CoA (58); for a

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Scheme 1 Map of C\textsubscript{1} flux through H\textsubscript{4}folate

Single-headed arrows denote reactions that are normally unidirectional. Double-headed arrows denote reactions that flow in different directions, or 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\textsubscript{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: \(\text{5-HCO-H}_{4}\text{folate}\) is metabolically derivable from formylglutamate [34] and is a minor source of H\textsubscript{4}folate in human nutrition [183]; \(\text{5-formimino-H}_{4}\text{folate}\) 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 \textsubscript{C}_{1} group of 5-formimino-H\textsubscript{4}folate is sufficiently activated to be convertible into CH\textsuperscript{2+}-H\textsubscript{4}folate by a cyclodihydrolase without hydrolysis of ATP [184], whereas enzymic conversion of 5-HCO-H\textsubscript{4}folate into CH\textsuperscript{2+}-H\textsubscript{4}folate or 10-HCO-H\textsubscript{4}folate involves consumption of ATP [36]; see the text).
Table 2  Reactions and enzymes of H₄folate metabolism

The Table lists the main reactions and enzymes of H₄folate 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 collectors which function cataclysmically 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-formylaminomimidazole-4-carboxamide ribonucleotide; AICAR, 5-aminomimidazole-4-carboxamide ribonucleotide.

<table>
<thead>
<tr>
<th>Reaction and equations</th>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Generation of active carrier</strong></td>
<td></td>
</tr>
<tr>
<td>(1) 7,8-H₂folate + NADPH + H⁺ → H₄folate + NADP⁺</td>
<td>Dihydrofolate reductase (DHFR)†</td>
</tr>
<tr>
<td>(2) Serine + H₄folate → glycine + CH₃H₂folate + H₂O</td>
<td>Serine hydroxymethyltransferase (SHMT) (+ PLP)</td>
</tr>
<tr>
<td>(3) Glycine + H₄folate + NAD⁺ → CO₂ + NH₃ + CH₃H₂folate + NADH</td>
<td>Glycine-cleavage system (+ PLP, lipoamide)</td>
</tr>
<tr>
<td>(4) HCOO⁻ + H₂folate + MgATP²⁻ → 10-HCO-H₂folate + MgADP + P²⁻</td>
<td>10-Formyltetrahydrofolate synthase</td>
</tr>
<tr>
<td><strong>C₅ redox pathway</strong></td>
<td></td>
</tr>
<tr>
<td>(5) 10-HCO-H₂folate + H⁺ → CH⁺⁺H₂folate + H₂O</td>
<td>Methylenetetrahydrofolate cyclohydrolase</td>
</tr>
<tr>
<td>(6) CH⁺⁺H₂folate + NAD(P)⁺ → CH₃H₂folate + NAD(P)⁺</td>
<td>Methylenetetrahydrofolate dehydrogenase</td>
</tr>
<tr>
<td>(7) CH₃H₂folate + NAD(P)⁺ → H⁺ + 5-CH₃H₂folate + NAD(P)⁺</td>
<td>Methylenetetrahydrofolate reductase (MTHFR) (+ bound FAD)</td>
</tr>
<tr>
<td><strong>C₅ transfers</strong></td>
<td></td>
</tr>
<tr>
<td>Formyl</td>
<td></td>
</tr>
<tr>
<td>(8) 10-HCO-H₂folate + GARP → FGAR + H₄folate</td>
<td>Glycinamide ribonucleotide (GAR) transformylase</td>
</tr>
<tr>
<td>(9) 10-HCO-H₂folate + AICAR → FAICAR + H₂folate</td>
<td>AICAR transformylase</td>
</tr>
<tr>
<td>(10) 10-HCO-H₂folate + Met-tRNAfMet → IMet-tRNAfMet + H₂folate</td>
<td>Formylmethionyl-RNA synthase</td>
</tr>
<tr>
<td>Methylene</td>
<td></td>
</tr>
<tr>
<td>(11) dUMP + CH₃H₂folate → TMP + 7,8-H₂folate</td>
<td>Thymidylate synthase (TS)</td>
</tr>
<tr>
<td>(12) α-Oxosovalerate + CH₃H₂folate → α-oxopantoate + H₂folate</td>
<td>Oxopantoate hydroxymethyltransfase</td>
</tr>
<tr>
<td>Methyl</td>
<td></td>
</tr>
<tr>
<td>(13) 5-CH₃H₂folate + homocysteine → methionine + H₂folate</td>
<td>Methionine synthase (MetH) (+ cobalamin)</td>
</tr>
<tr>
<td>(14) 5-CH₃H₂folate + homocysteine + H₂O → methionine + H₂O</td>
<td>Methionine synthase (MetE)</td>
</tr>
<tr>
<td>(15) 5-CH₃H₂folate + CO₂ + [Ni] → CoA → acetyl-CoA + H₂O</td>
<td>Cobalamin protein, acetyl-CoA synthase (+ Ni, Fe) (carbon monoxide dehydrogenase)</td>
</tr>
</tbody>
</table>

* Generation of active carrier: also in this category are γ-glutamylhydrolase and folylpolyglytarnate synthase [15].
† DHFR also catalyses the reduction of folate to 7,8-H₂folate (see the text).
‡ Loading/unloading of C₅: also in this category are enzymes of formimimoglutamate and formimimoglycine catabolism, and of conversion of 5-HCO-H₄folate into 10-HCO-H₂folate or to CH⁺⁺H₂folate (see Scheme 1 and its legend).
§ C₅ transfers: methylene; also in this category are dCMP hydroxymethyltransferase of T-even bacteriophages [185] and m⁰U₅₄-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 N⁹⁰ or that to N⁵? In the TS reaction, the internal source of reductant (C⁶–N⁵, see above) and crystallographic data on the enzyme [57] support a mechanism whereby the CH₆–N⁵ bond breaks first, yielding the N⁵ cationic imine (Figure 1a) as reactive intermediate [57]. This mechanistic prediction is from that of the reversible condensation of formaldehyde with H₂folate [18], and is very likely the pattern of C₅ transfer reactions at the methyl level generally, including the reversible serine hydroxymethyltransferase (SHMT) reaction (2) [60]. Furthermore, the cationic imine is a presumed intermediate in the methylene-tetrahydrofolate 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 C₅ 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]. MethH utilizes cobalamin as intermediate in methyl transfer (reaction 13) [42,62,63], whereas MethE is cobalamin-independent (reaction 14) [64,65]. Mammalian methionine synthase, like MethH, is cobalamin-dependent [61,66]. Thus, in Man, reaction (13) cannot take place in the absence of vitamin B₁₂. Because reaction (7) is functionally irreversible in Man, 5-CH₃H₂folate has ‘no way out’ in the absence of the cobalamin vitamin B₁₂. 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, CH₃H₂folate 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 CO₂. Most of the C₂ flux through the pathway is used for energy generation; the remaining acetyl-CoA generates carbon. In the ‘methyl’ branch of the pathway one molecule of CO₂ is reduced to formate by formate dehydrogenase [69], and the formate is activated to 10-HCO-H₂folate at the expense of ATP (reaction 4), and reduced to 5-CH₃H₂folate (reactions 5–7). The methyl group of 5-CH₃H₂folate 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 CO₂, and CoA (reaction 15) [70]. Energy that is consumed in reaction (4) is...
recovered at the end of the pathway by conversion of acetyl-CoA into acetate 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-CH₃-H₄MPT to acetyl-CoA and/or the MTHFR reaction (reaction 7) [71,72]. It should be mentioned that acetogens are a phyllogenetically diverse group, comprising heterotrophs and autotrophs (as a general reference, see [68]), and also that, in a single member of a fifth order, the hyperthermophile Methanopyrus kandleri, the primary reductant in the MTHFR reaction is reduced ferredoxin [73] and in others NADH [72].

### The H₄MPT Pathway

Before focusing on H₄MPT, 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, Methanomicrobiales and Methanosarcinales, and also a single member of a fifth order, the hyperthermophile Methanopyrus kandleri. The main modes of methanogenesis are from CO₂ and H₂ (hydrogenotrophic), from methanol or other C₁ methyl compounds (methylotrophic), or from acetate. Each mode is separately reviewed in [74], and in a recent compilation of the reactions and enzymes of all three modes is in [75].

The reactions in Table 3 (to be discussed shortly), several novel cofactors appear. The structures, discoveries, 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. F₄³⁰ is a 5-deazaflavin which carries hydride on C₈ (see below). Coenzyme M (CoM, HS-CoM) and coenzyme B (CoB, HS-CoB, also called HS-HTTP) are thiol cofactors. F₄₃₅ is a nickel tetapyrrole. The functions of these cofactors are described below. F₄₃₅ and CoB are unique to methanogens. H₄MPT, F₄₃₀ and MFR are more widely distributed (see below), and this has recently also been found to be true for CoM [77a].

Methanofuran is reduced ferredoxin [139] and in others NADH [72].

### Table 3 Reactions and enzymes of methanogenesis

The Table lists the main reactions and enzymes of methanogenesis from CO₂ plus H₂. Reactions are numbered as in Figure 3 and the text. Commonly used abbreviations for the enzymes are given in parentheses. Collectors 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 H₄SPT instead of H₄MPT (see the text).

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Equation</th>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>(16)</td>
<td>CO₂ + MFR⁺⁺ + 2[H] ↔ HCO-MFR + H₂O + H⁺</td>
<td>Formylmethanofuran dehydrogenase* (+ Mo or W, and Fe)</td>
</tr>
<tr>
<td>(17)</td>
<td>HCO-MFR + H₂MPT + H⁺ ↔ 5-HCO-H₄MPT + MFR⁺⁺</td>
<td>Formylmethanofuran: H₄MPT formyltransferase</td>
</tr>
<tr>
<td>(18)</td>
<td>5-HCO-H₄MPT + H⁺ ↔ CH⁺⁺-H₄MPT + H₂O</td>
<td>Methenyl-H₄MPT cyclohydrolase (MCH)</td>
</tr>
<tr>
<td>(19)</td>
<td>CH⁻⁻-H₄MPT + F₄₃₀H₂ ↔ CH₂⁺⁺-H₄MPT + F₄₃₀</td>
<td>F₄₃₀-dependent methylene-H₄MPT dehydrogenase</td>
</tr>
<tr>
<td>(20)</td>
<td>CH⁺⁺-H₄MPT + H₂ ↔ CH₂⁺⁺-H₄MPT + H⁺</td>
<td>H₂-forming methylene-H₄MPT dehydrogenase (HMD)</td>
</tr>
<tr>
<td>(21)</td>
<td>CH₂⁺⁺-H₄MPT + F₄₃₀H₂ ↔ 5-CH₃-H₄MPT + F₄₃₀</td>
<td>Methylene-H₄MPT reductase (MER)</td>
</tr>
<tr>
<td>(22)</td>
<td>CH₂⁺⁺-H₄MPT + HS-CoM ↔ CH₂⁺⁺-S-CoM</td>
<td>Methyl-H₄MPT:CoM methyltransferase (+ corrinoid protein)</td>
</tr>
<tr>
<td>(23)</td>
<td>CH₂⁺⁺-S-CoM + HS-CoB ↔ CH₂⁺⁺ + CoM+S-CoB</td>
<td>(MR) Methyl-CoM reductase (+ bound F₄₃₀)</td>
</tr>
<tr>
<td>(24)</td>
<td>CoM+S-CoB + 2[H] ↔ HS-CoM + HS-CoB</td>
<td>Heterodisulphide reductase†</td>
</tr>
</tbody>
</table>

* The immediate electron donor for reaction (16) is not known, but may be a polyferredoxin [139].† Different heterodisulphide reductases are linked to H₂ as reductant in the hydrogenotrophic pathway [116] or to F₄₃₀H₂ as reductant in the methylotrophic pathway [123].

CO₂ + 4H₂ ↔ CH₄ + 2H₂O

The energy yield under standard conditions is −131 kJ mol⁻¹ (see under ‘Thermodynamic aspects’). Under ecological conditions of 1–10 Pa H₂, the energy yield is only about 15–35 kJ 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. CO₂ 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: H₄MPT formyltransferase [79,88–90] to N₂ of H₄MPT, yielding 5-HCO-H₄MPT (reaction 17). In contrast with the H₂MPT pathway, no free formate is formed as an intermediate in these reactions, and no ATP is expended.

5-HCO-H₄MPT is reduced to 5-CH₃-H₄MPT in a reaction sequence that formally resembles reactions (5–7) of the H₂MPT pathway, in that water is released and two consecutive two-electron reductions occur. However, the reactions on H₄MPT differ in major respects from those on H₂MPT. First, a cyclohydrolase (methenyl-H₄MPT cyclohydrolase, MCH) [80,91,92] generates CH⁻⁻-H₄MPT directly from 5-HCO-H₄MPT without ATP (reaction 18). (Recall that, in the H₂MPT pathway, 10-
The methyl group is transferred from $H_2$MPT 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 $H_2$folate pathway (reaction 13). However, the $CH_2$-H$\text{MPT}$-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 $CH_2$-S-CoM (reaction 23). The enzyme methyl-CoM reductase (MR) contains the unique nickel tetrapyrrole cofactor F$_{\text{inv}}$ [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 $H_2$MPT occurs (in methanogens) only under conditions of methanogenesis.

Under conditions of autotrophic growth, some $C_3$ is diverted into cell carbon via a modified version of the acetyl-CoA pathway; 5-CH$_3$-H$\text{MPT}$ rather than 5-CH$_2$H$\text{MPT}$ folate 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 $H_2$folate pathway [92,96,121]. Moreover, MCH and the two methylene-H$\text{MPT}$ dehydrogenases are physically distinct enzymes, in contrast with the corresponding $H_2$folate enzymes, which are physically linked in most bacteria and in eukaryotes (see above). Generally, $H_2$folate derivatives cannot substitute for H$\text{MPT}$ derivatives, although 5-CH$_2$H$\text{MPT}$ folate can substitute to some extent for 5-CH$_2$H$\text{MPT}$ 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 Methanosaarcina barkeri (for a review, see [122]). The pathway may be written:

$$3CH_3OH + 6 \cdot H^- \rightarrow 3CH_4 + 3H_2O$$
$$CH_3OH + H_2O \rightarrow CO_2 + 6 \cdot H^-$$

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·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$\text{SPT}$ (a close structural relative of H$\text{MPT}$; see above). The 5-CH$_2$H$\text{MPT}$ is oxidized to 5-HCOH$\text{MPT}$ by reversal of reactions (21), (19) and (18). The formyl group of 5-HCO-H$\text{MPT}$ is transferred to FMR and oxidized to

HCO-H$_2$folate is the substrate for cyclohydrolase (reaction 5), whereas 5-HCO-H$_2$folate can only be converted into the methenyl derivative at the expense of ATP, either in a direct reaction or indirectly via 10-HCO-H$_2$folate; see above and [36].

Then either of two alternative enzymes reduce CH$_3$-H$\text{MPT}$ to CH$_2$-H$\text{MPT}$. In reaction (19), $F_{\text{inv}}$-dependent CH$_2$-H$\text{MPT}$ dehydrogenase catalyses a reversible reduction using the low-redox-potential deazaflavin $F_{\text{inv}}$, as cofactor [93–96,81]. The $F_{\text{inv}}$/F$_{\text{inv}}$midpoint potential ($E_\text{m}$) is about $-350$ mV [97–99], well below that of NAD(P)$^+$/NAD(P)H ($-320$ mV) used in the $H_2$folate pathway. In hydrogenotrophic methanogenesis, $H_2$ and an $F_{\text{inv}}$-dependent nickel-iron hydrogenase maintain $F_{\text{inv}}$ in the reduced state. Alternatively, (reaction 20), an $H_2$-dependent CH$_2$-H$\text{MPT}$ 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$\text{MPT}$ reductase (MER; reaction 21) also uses $F_{\text{inv}}$ as cofactor. Unlike the $H_2$folate enzyme, MER is not a flavoprotein [103,104]. Hydride is delivered directly to the CH$_2$-H$\text{MPT}$ substrate by the reduced deazaflavin [105]. (In the $H_2$folate reaction, a synthetic deazaflavin can substitute in vitro for FAD [106].) Deazaflavins are obligate two-electron oxido-reductants [97,98], implying a two-electron mechanism for both reactions [105,106]. In contrast with the $H_2$folate reaction, the CH$_2$-H$\text{MPT}$ reductase reaction is reversible (see below).
CO₂ 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 $F_{420}H₂$-linked heterodisulphide reductase [123].

The non-methanogenic sulphate reductor *A. fulgidis* also carries out oxidation of methyl groups, using H₂MPT 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 H₂MPT as well as H₂SPT 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 activated to acetyl-CoA at the expense of ATP. Acetyl-CoA is sulphate reduction [124].

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### THERMODYNAMIC ASPECTS

The above, qualitative differences between the C₄ pathways on H₂folate and H₂MPT are now examined from the viewpoint of thermodynamic data, namely, free energies and midpoint redox potentials of the C₄ reduction steps. The important conclusion will emerge that the two carriers ‘tune’ the energetics of the intermediate steps in C₄ reduction differently. The chemical basis of the differences will be discussed below.

Thermodynamic tabulations have been presented previously for the H₂folate pathway (see, e.g. [130,131]), the H₂MPT 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 C₄ reduction pathway by H₂ without pterin carrier is given for reference. Then, literature values for the thermodynamics of C₄ reduction on H₂folate and H₂MPT are given, with reference both to the immediate redox cofactors that were listed in Tables 2 and 3, and to H₂—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 C₄ 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. $K_r$ is the overall equilibrium constant and may include hydrogen ions in the equilibrium expression. For such reactions, $K_r$ is the apparent equilibrium constant at pH 7. Unless otherwise stated, reaction free energies are for 298 K, pH 7, and are designated $ΔG_r$ and $E_r$ respectively. The following values were used for $E_r$ at 298 K: $2H^+ + 2e^− / H₂ = −414 mV$ [40]; NAD(P)⁺/NAD(P)H $H^+ / H^− = −320 mV$ [135] (values differing from this by plus or minus a few millivolts are found in various sources); $F_{420}/F_{420}H₂ = −350 mV$ (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.

**CO₂ to formyl**

In the H₂folate pathway, CO₂ is first reduced to formate. For the present comparative purposes, $ΔG_r$ with H₂ as reductant under standard conditions is the relevant value. This value is slightly endergonic: $+3.5 kJ \cdot mol^{−1}$ [40] and Table 4a).

As already noted, formate activation to 10-HCO-H₂folate is coupled to ATP hydrolysis. $K_{eq}$ for the reaction was determined in [137] to be approx. 41 in the direction of 10-HCO-H₂folate synthesis. This value is used here for calculating $ΔG_r$ (reaction 4a in Table 4b). (The $ΔG_r$ is probably accurate to within $±2 kJ \cdot mol^{−1}$ 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 $ΔG_r$ for the hypothetical reaction (4) without ATP. Taking $−31.8 kJ \cdot mol^{−1}$ for $ΔG_r$ of hydrolysis of the terminal phosphate of ATP [40], one can estimate that formate activation without ATP would be endergonic by about $+22 kJ \cdot mol^{−1}$ with respect to free formate. This value is included as reaction (4a) in Table 4(b).

Turning to the H₂MPT pathway, for the conversion of CO₂ into HCO-MFR (reaction 16), a theoretical estimate of $ΔG_r$ was based on the sum of the free energies of CO₂ reduction to formate ($+3.5 kJ \cdot mol^{−1}$, see above) and of condensation of a model amide, N-formylglutamate ($+12.5 kJ \cdot mol^{−1}$ [138]), giving $+16 kJ \cdot mol^{−1}$ 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 conjuction with CO₂ 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 C₃ reduction with no carrier (a), H₄folate (b) and H₄MPT (c)

(a) No carrier. All values are for standard conditions (298 K, pH 7). ΔG° values were derived from ΔGₚ (products) → ΔGₚ (reactants). The following ΔGₚ values, in kJ·mol⁻¹, were used [31]: CO₂(g) = −394.36 [40,136]; H₂(g) = 0; HCOO⁻ = −351.04 [40]; H⁺ = −39.87 [40]; HCHO(aq) = −121.5 (see below); CH₂DH₂ = −175.3 [136]; CH₃OH = −50.72 [136]; H₂O = −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; these 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(g) 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) H4folate. The reactions of the C₃ pathway are (25) of part (a) and (4–7) of Table 3. ΔΔG° 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 H₂ as reductant. Cumulative free-energy values (ΣΔG°) are given in separate columns, starting both from formate and from CO₂. The sums are based on reaction (4a) (i.e. without ATP) and H₂ as reductant. Values in bold in these two columns are specifically mentioned in the text. Reaction (29) is the hydrolysis of CH₂H₂folate to formaldehyde and H₂folate; 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 reasons for using H₂(g) as a reductant. The reaction can proceed non-enzymically in either direction according to pH. Multiple de-oxidations of H₂(g) are possible, thus 1 mol of H₂(g) liberates 237.13 mol of energy. All other values are calculated for 298 K to enable comparison with parts (a) and (b). It is recognized that Kₚ was determined at about 338 K in most instances; it is provisionally assumed that any variation of Kₚ, 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 ΔG° is approximately zero with F₄20H₂ as reductant and approx. −24 kJ·mol⁻¹ with H₂ as reductant, although some sources [75,77,130,131] differ from others [94,104] in the apportionment of ΔΔG° between the two successive reductions (see the text). Because the total ΔΔG° for reduction of CO₂ to CH₄ by H₂ is approx. −131 kJ·mol⁻¹ (part a), and ΔΔG° from CO₂ through to reaction (21) is −16 kJ·mol⁻¹, the ΔΔG° 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].

(b) H₄folate

<table>
<thead>
<tr>
<th>Reaction</th>
<th>ΔG° (kJ·mol⁻¹)</th>
<th>ΣΔG° (kJ·mol⁻¹)</th>
<th>E° (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(25) CO₂ + H₂ → HCOO⁻ + H⁺</td>
<td>+3.5</td>
<td></td>
<td>−432</td>
</tr>
<tr>
<td>(26) HCOO⁻ + H₂ → HCHO + H₂O</td>
<td>+32.3</td>
<td>+35.8</td>
<td>−581</td>
</tr>
<tr>
<td>(27) HCHO + H₂ → CH₂DH₂</td>
<td>−53.8</td>
<td>−18.0</td>
<td>−135</td>
</tr>
<tr>
<td>(28) CH₂DH₂ → H₂ + CH₄</td>
<td>−112.6</td>
<td>−130.6</td>
<td>+169</td>
</tr>
<tr>
<td>(net) CO₂ + 4H₂ → CH₄ + 2H₂O</td>
<td>−130.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(c) H₄MPT

<table>
<thead>
<tr>
<th>Reaction</th>
<th>ΔG° (kJ·mol⁻¹)</th>
<th>ΣΔG° (kJ·mol⁻¹)</th>
<th>E° (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(16) F₄₂₀H₂ + H₂ → CH₂H₂folate</td>
<td>+28</td>
<td>+12.5</td>
<td>+16</td>
</tr>
<tr>
<td>(17)</td>
<td>+3.5</td>
<td>−3.5</td>
<td>+12.5</td>
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<tr>
<td>(18)</td>
<td>−4.2</td>
<td>−4.2</td>
<td>+8.3</td>
</tr>
<tr>
<td>(19)</td>
<td>+2</td>
<td>−2</td>
<td>+12.5</td>
</tr>
<tr>
<td>(20)</td>
<td>−10</td>
<td>−5.2</td>
<td>−2</td>
</tr>
<tr>
<td>(21)</td>
<td>−14</td>
<td>−19.2</td>
<td>−16</td>
</tr>
</tbody>
</table>

[139]. Formyl transfer from MFR to N₃ of H₄MPT (reaction 17) is slightly exergonic [90] (Table 4c).

Although free formate is not an intermediate in the H₄MPT pathway, it affords a reference point for the energy levels of the actual intermediates. Summation of the appropriate ΔG° values indicates that 5-HCO-H₄MPT is endergonic with reference to free formate, but only by about +9 kJ·mol⁻¹, in contrast with 10-HCO-H₄folate, which, as stated above, is endergonic with reference to free formate by about +22 kJ·mol⁻¹. As mentioned above, Figure 2 depicts the C₃ free-energy relationships during progression through the respective pathways.

Formyl to methenyl

In the H₄folate pathway, cyclization from 10-HCO-H₄folate is also endergonic at pH 7. ΔG° 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 C1 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 H4folate pathway CO2 is first reduced to formate, whereas in the H4MPT pathway CO2 is first reduced to HCO-MPR. 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). ΔG° values for the methyl-transfer reactions (13) and (22) have been reported. In a detailed study of reaction (22), a ΔG° of −10 kJ·mol⁻¹ was found for methyl transfer from H4MPT to cobamide, and −20 kJ·mol⁻¹ from cobamide to CH3-S-CoM, i.e. −30 kJ overall [108]. The only study on the equilibrium of reaction (13) [189] gave a value for KE of 7 × 10⁻⁶ in the direction of homocysteine and CH3-H4folate formation, which converts to −29 kJ·mol⁻¹ in the direction of methionine synthesis. The potential of the S-methyl group in methionine and CH3-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.

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In the H4MPT pathway, cyclization from 5-HCO-H4MPT is exergonic at pH 7. The reaction is pH-dependent for the same reason as that given above. An experimental value of KE of 5.4 × 10⁻⁶ M⁻¹ was obtained for the direction of cyclization [91], corresponding to a KE of 5.4 at pH 7 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⁺⁻H4folate is endergonic by approx. 28 kJ·mol⁻¹ with reference to free formate, whereas CH⁺⁻H4MPT 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 H4folate pathway were complicated by the physical linkage of the dehydrogenase and cyclohydrase enzymes from most organisms (discussed above), and also by the tendency of CH⁺⁻H4folate to undergo spontaneous hydrolysis to 10-HCO-H4folate 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 ΔG°ᵦ ≈ approx. −4.9 kJ·mol⁻¹ in the direction of reduction from CH⁺⁻H4folate to CH₃-H4folate. This value is generally cited [130, 131], and corresponds to E°ₚ = 200 mV for the CH⁺⁻/CH₃-H4folate couple. ΔG°ₚ for H₃ 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 H4folate has been studied in detail [18]. An overall equilibrium constant of 3.2 × 10¹⁸ 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 ΔG° values in Table 4(b) for reactions (4a) (i.e. without ATP), (5) and (6) (H₃ as reductant) are approximately correct, then the sum of these ΔG° values plus that for reaction (29) should be equal to ΔG°ₚ 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 C¹ to H4folate modifies the E°ₚ values of the C¹ redox reactions. The overall reaction (reaction 26) is split into smaller steps in the H4folate 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 H4folate also contributes to the magnitude of the redox step.

Thermodynamic values for interconversion between CH⁺⁻H4MPT and CH₃-H4MPT 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 F₁₂₅₈ known. A value for ΔG°ₚ of −4.85 kJ·mol⁻¹ was obtained for the H₃-coupled reaction, giving E°ₚ = −390 mV for the CH⁺⁻/CH₃-H4MPT couple [140, 133]. Subsequent purification of the F₁₂₅₈-utilizing enzyme allowed determination of KE of 2.24 × 10⁻⁷ M⁻¹ in the direction of CH₃-H4MPT oxidation [94] (reaction 19). In the reductive direction and at pH 7 this yields the ΔG° values shown in Table 4(c) for reaction (19) and, indirectly, reaction (20). E°ₚ of the CH⁺⁻/CH₃⁻H₄MPT couple is −362 mV from this study [94].

Thus, on H₄MPT, E°ₚ for CH⁺⁻/CH₃⁻ couple is more negative by about 65 mV than on H₄folate. Consequently, the free-energy change for the CH⁺⁻/CH₃⁻ reduction, expressed relative to H₃ as reductant, is less steeply ‘downhill’ on H₄MPT than on H₄folate (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$_2$-folate formation. However, for this very reason, quantitatively accurate values of $K_e$ 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$_2$-folate [41-43]. Thus $\Delta G_f$ for the overall reaction is the sum of the $\Delta G_f$ values for two partial reactions: (i) hydride transfer from NAD(P)H to enzyme-bound FAD; (ii) hydride transfer from FADH$_2$ to CH$_2$-folate. Vanoni and Matthews [41] determined that reaction (ii) is irreversible ($-0.5$ 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$_2$-folate. Under these experimental conditions the equilibrium point for the reaction between FADH$_2$ and CH$_2$-folate was found to be far in the direction of CH$_2$-folate formation: $K_e$ was reported as approx. 3 x 10$^9$ [141]. Assuming $E_0'$ for free FAD/FADH$_2$ = $-220$ mV, then $E_0'$ for CH$_2$/CH$_2$-H$_2$folate 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$_2$-H$_2$folate, 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 $\Delta G_f$ 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$_2$-H$_2$folate couple than in (7a). It should be mentioned, 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$_2$-H$_2$folate, suggesting that $E_0'$ for the latter couple may not be quite as negative as $-200$ mV.

Turning to the H$_2$MPT 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$_{red}$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$_2$-H$_2$MPT couple is considerably more negative than $E_0'$ for the CH$_2$/CH$_2$-H$_2$folate 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$_2$-H$_2$folate retaining a lower potential energy than CH$_2$-H$_2$MPT by about $-16$ kJ mol$^{-1}$. If a less negative value than in (7b) is taken for the CH$_2$/CH$_2$-H$_2$folate couple (see above discussion), then the energy difference is even greater.

C$_5$ flux

The thermodynamic values in Tables 4(b) and 4(c), determined in vitro, are broadly concordant with patterns of C$_5$ flux in vivo. As outlined in the previous section, C$_5$ 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$_2$folate pathway to pyridine nucleotides, in most if not all organisms the NADP$^-$ pool is maintained in a much more reduced state than the NADP$^+$ pool (e.g. [143,144]). Therefore, whether a given enzyme uses NADP(H) or NAD(H) may be important in determining direction of C$_5$ 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$_2$-H$_2$MPT 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$_2$-H$_2$folate is $-200$ mV, as in (7b) in Table 4(b), then $\Delta G_f'$ 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$_2$folate 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$_2$folate pathway, given a supply of methyl groups, although this process would be thermodynamically less facile than by the H$_2$MPT pathway. (iii) In the H$_2$MPT pathway the CH$^+/\text{CH}_2$ and CH$_2$/CH$_2$ couples are substantially more negative than in the H$_2$folate pathway. This enables reversible coupling to the low-redox-potential cofactor F$_{red}$C$_5$ which can readily flow in either direction through the entire H$_2$MPT pathway, including the methylene reductase reaction, the actual direction being determined in different organisms by their overall patterns of C$_5$ metabolism; examples were given above. Additionally, C$_5$ can enter the H$_2$MPT pathway in the reductive direction from CO$_2$ without expenditure of ATP. This is largely due to the low energy level of CH$^-$/H$_2$MPT as compared...
with CH\textsuperscript{−}-H\textsubscript{4}folate (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\textsubscript{4}MPT pathway.

**Chemical basis of the differences**

Thermodynamic differences between the C\textsubscript{1} pathways on H\textsubscript{4}folate and H\textsubscript{4}MPT can be attributed to differences between the chemistries at N\textsuperscript{10} and, possibly, the presence of the two structural methyl groups in H\textsubscript{4}MPT. These chemical differences are discussed in turn.

N\textsuperscript{10} and N\textsuperscript{5}

A detailed early study on model chemical compounds for H\textsubscript{4}folate established that the nature of the group para to N\textsuperscript{10} of the arylamine is crucial in tuning the reactivity of N\textsuperscript{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\textsuperscript{10} in the methyl-substituted compound [145]. That study was carried out before H\textsubscript{4}MPT had been discovered, but can now be seen as a model for differences between H\textsubscript{4}folate and H\textsubscript{4}MPT.

The effect of the group para to N\textsuperscript{10} on thermodynamic differences between H\textsubscript{4}folate and H\textsubscript{4}MPT 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 increased the rate of condensation of the model compound with formaldehyde, whereas that for CH\textsubscript{2}-H\textsubscript{4}MPT was estimated to be about +2.4 ([146], cited in [17]). (ii) The pK\textsubscript{a} values (basicities) at N\textsuperscript{10} confer different properties to the bond C\textsubscript{1} units, with more negative E\textsubscript{c} values of the C\textsubscript{1} redox transitions upon H\textsubscript{4}MPT than on H\textsubscript{4}folate. (iv) The methylene of CH\textsubscript{2}-H\textsubscript{4}MPT epimerizes more slowly than that of CH\textsubscript{2}-H\textsubscript{4}folate (t\textsubscript{1/2} = 1 h and < 15 min respectively [21]). Likewise, the methylene of CH\textsubscript{2}-H\textsubscript{4}MPT epimerizes more slowly than that of H\textsubscript{4}folate (0.01 s\textsuperscript{−1} and 0.1 s\textsuperscript{−1} [21].) (v) The chemical environments and reactivities of N\textsuperscript{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\textsuperscript{5} are felt throughout the C\textsubscript{1} redox pathways on the pterins. See also [146] for further discussion.

**Formyl to methenyl**

N\textsuperscript{10}, being a better nucleophile in H\textsubscript{4}MPT than in H\textsubscript{4}folate, is thermodynamically able to promote spontaneous cyclization from 5-HCO-H\textsubscript{4}MPT to the methenyl derivative at pH 7. In contrast, cyclization from 5-HCO-H\textsubscript{4}folate requires energy input from ATP. (For discussion of cyclohydrolase reactions and N\textsuperscript{5}, N\textsuperscript{10} equivalibra on H\textsubscript{4}folate, see [36,138], and on H\textsubscript{4}MPT, see [146]).

**Methenyl to methyl**

Because the electron density at N\textsuperscript{10} is greater in H\textsubscript{4}MPT than in H\textsubscript{4}folate, the bridged N\textsuperscript{5},N\textsuperscript{10} C\textsubscript{1} compounds are more strongly bound to H\textsubscript{4}MPT than to H\textsubscript{4}folate; 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\textsuperscript{−}-H\textsubscript{4}MPT than in CH\textsuperscript{−}-H\textsubscript{4}folate, but in both cases more reducing power is required to bring about the C\textsubscript{1} reductions on H\textsubscript{4}MPT than on H\textsubscript{4}folate. (This is equivalent to saying that E\textsubscript{c} for the redox transitions is more negative on H\textsubscript{4}MPT than on H\textsubscript{4}folate.) In addition to the thermodynamic profile data, independent evidence that C\textsubscript{1} at the methylene level is more strongly bound to H\textsubscript{4}MPT than to H\textsubscript{4}folate is given by the smaller exchange of CH\textsubscript{2}-H\textsubscript{4}MPT with formaldehyde and the slower epimerization than for CH\textsubscript{2}-H\textsubscript{4}folate (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\textsuperscript{10} more basic [145]. Extending this reasoning to H\textsubscript{4}MPT and H\textsubscript{4}folate, ring (re)-closuore will be more favoured for H\textsubscript{4}MPT than for H\textsubscript{4}folate; hence the steady-state level of the cationic imine will be lower for H\textsubscript{4}MPT, and the overall reduction will be thermodynamically less favourable on H\textsubscript{4}MPT than on H\textsubscript{4}folate, as is found. (The lower steady-state level of the cationic imine also underlies the slower exchange of formaldehyde with CH\textsubscript{2}-H\textsubscript{4}MPT.)

It was stated (v, above) that N\textsuperscript{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\textsuperscript{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\textsuperscript{5}-CH\textsubscript{2} bond should be similar or identical between 5-CH\textsubscript{2}-H\textsubscript{4}folate and 5-CH\textsubscript{2}-H\textsubscript{4}MPT. It further follows from Hess’s Law that the overall enthalpy change for reduction of CO\textsubscript{2} to 5-CH\textsubscript{2}-H\textsubscript{4}folate and to 5-CH\textsubscript{2}-H\textsubscript{4}MPT (expressed relative to H\textsubscript{2}, as ‘reference’ reductant) should be approximately equal, notwithstanding differences in the intermediate steps in which N\textsuperscript{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\textsubscript{2} to 5-CH\textsubscript{2}-H\textsubscript{4}MPT is about −16 kJ·mol\textsuperscript{−1}, whereas that from CO\textsubscript{2} to CH\textsubscript{2}-H\textsubscript{4}folate is about −32 kJ·mol\textsuperscript{−1} (or possibly even greater, as discussed above). Are these different ‘end points’ real or ‘artefactual’? In other words, do the many individual AG\textsubscript{c} 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\textsubscript{1} flux patterns, as discussed above. By any reckoning there appears to be an irreducible difference of at least 12 kJ·mol\textsuperscript{−1}, and more probably about 16 kJ·mol\textsuperscript{−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\textsubscript{4}MPT, C\textsuperscript{12s} and C\textsuperscript{13s} in Figure 1.

**Structural methyl groups of H\textsubscript{4}MPT**

The methyl groups are each separated by two carbon atoms from N\textsuperscript{5}, with no intervening conjugation. It seems unlikely that they could affect the electron density at N\textsuperscript{5}. Thus the assumption that N\textsuperscript{5} is chemically similar in the two carriers remains sound, as does in turn the expectation that the overall enthalpy changes for C\textsubscript{1} reduction to the respective 5-methyl pterins should be similar. Might the structural methyl groups contribute an entropy effect?
The following considerations suggest that this possibility is plausible.

In the condensation of formaldehyde with $\text{H}_4\text{folate}$ to $\text{H}_2\text{MPT}$ there is a large decrease in entropy [147] (63 entropy units/mol by the notation in [147], or about 264 J K$^{-1}$ mol$^{-1}$). This is largely attributed [147] to constraint in the methylene compound of rotation around the bonds $\text{C}_6\text{a-C}_9$ and $\text{C}_6\text{a-N}^{9a}$, both of which can rotate freely in $\text{H}_4\text{folate}$. Conversely, in the TS–DHFR reaction cycle, in which free $\text{H}_4\text{folate}$ 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, $\text{C}_1$ remaining attached to $\text{N}^a$. Thus after reaction (7) free rotation also becomes possible around $\text{C}_9\text{a-C}_9$ and $\text{C}_9\text{a-N}^{9a}$. 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-$\text{H}_2\text{MPT}$ 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 $\text{H}_2\text{MPT}$ are bulkier than the hydrogen atoms that occur at the same positions in $\text{H}_4\text{folate}$. There is now potential for steric hindrance to free rotation around $\text{C}_9\text{a-C}_9$; when the methyl $\text{C}^{12a}$ rotates in one direction, it can swing into cyclopentane-like apposition with $\text{C}^{12a}$; in the other direction it can swing into the $\text{C}_1$ methyl group on $\text{N}^9$. 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 $\text{C}^{12a}$ and $\text{N}^9$ methyl groups. Due to limitation in free rotation around $\text{C}_9\text{a-C}_9$, 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 K$^{-1}$ mol$^{-1}$ for interconversion between $\text{H}_4\text{folate}$ and $\text{CH}_2\text{H}_4\text{folate}$ (above) corresponds to 79 kJ mol$^{-1}$ at 298 K. Only about 20 % of that value is required to explain the discrepancy between the overall $\Delta G^\circ$ of CO$_2$ reduction to 5-CH$_2$H$_4$MPT (at least $-32 \text{ kJ}$ with $\text{H}_4$ as reductant) and $\text{CO}_2$ reduction to 5-CH$_2$H$_2$MPT (about $-16 \text{ kJ}$ with $\text{H}_4$ as reductant). Hindrance of free rotation around $\text{C}^{9a}\text{C}^{11a}$ might afford such a difference in the form of an entropy difference between reactions (7) and (21).

A difference between $\Delta G^\circ$ values from CO$_2$ to 5-CH$_2$H$_4$MPT and 5-CH$_2$H$_2$MPT respectively may confer advantage upon $\text{H}_2\text{MPT}$ for its biological roles. In the reductive direction, if about 16 kJ of energy are ‘saved’ in the pathway to $\text{CH}_2\text{H}_2\text{MPT}$, this extra energy is available for the final reduction steps to CH$_3$, where energy is transformed chemiosmotically. In the oxidative direction $\text{CH}_2\text{H}_2\text{MPT}$ is at an energy level allowing ready oxidation to $\text{CH}_2\text{H}_4\text{MPT}$ by $\text{F}_{\text{ox}}$.

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$_2$H$_4$MPT and to CH$_2$H$_2$MPT 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 $\text{H}_2\text{MPT}$ 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 $\text{H}_2\text{MPT}$-like by the criterion of possessing a methylene $\text{para}$ to $\text{N}^{9a}$. Such carriers exist in some Archaea (see below). Last, the energetics of methyl transfer from $\text{N}^a$ of the
pteryns to homocysteine and CoM respectively are potentially relevant, as noted in the legend to Figure 2.

**BIOSYNTHETIC ROLES FOR H\textsubscript{MPT}?**

Given the many differences between the H\textsubscript{folate} and H\textsubscript{MPT} C\textsubscript{1} pathways, the question arises whether H\textsubscript{MPT} performs any of the biosynthetic roles that are played by H\textsubscript{folate} in most organisms. The question is important because, in general, H\textsubscript{MPT}-containing Archaea are thought to lack H\textsubscript{folate}, 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\textsubscript{folate}.

As discussed above, formyl groups are carried by H\textsubscript{MPT} on N\textsubscript{3}, where they are only slightly activated with reference to free formate, and are therefore unsuited to biosynthetic formyl transfers. 10-HCO-H\textsubscript{MPT} is not a known biochemical intermediate. Moreover, because N\textsuperscript{10} of H\textsubscript{MPT} is not electron-deficient, it would be unlikely to be as good a leaving group as N\textsuperscript{10} of H\textsubscript{folate}, and therefore 10-HCO-H\textsubscript{MPT}, even if it were metabolically available, would not be expected to be as good a formyl donor as 10-HCO-H\textsubscript{folate}. Interestingly, there is currently no evidence for biosynthetic formyl transfers via H\textsubscript{MPT}.

In the purine-biosynthetic pathway, PurT but not PurN is identifiable in the genome of *M. jannaschii* [28], indicating that any C\textsuperscript{1} transfer from CH\textsubscript{3}-H\textsubscript{MPT} would be less strongly driven than from CH\textsubscript{3}-H\textsubscript{folate}. Any entropic difference, as discussed in the previous section, might also favour H\textsubscript{folate} rather than H\textsubscript{MPT} for this class of C\textsuperscript{1}-donor reactions. With these considerations in mind, the following well-characterized H\textsubscript{folate} reactions, SHMT (reaction 2), TS (reaction 11) and 2-oxopantoate synthase (reaction 12) are now considered with regard to known or possible H\textsubscript{MPT} counterparts.

An SHMT has been purified from *M. thermoautotrophicum*. The enzyme utilizes pyridoxal phosphate (PLP) (like the H\textsubscript{folate} enzyme), but is specific for H\textsubscript{MPT} [157]. The enzyme was proposed to function *in vivo* in the direction of serine biosynthesis [157]. K\textsubscript{eq} for the H\textsubscript{folate} reaction is approx. 8–12 in the direction of CH\textsubscript{3}-H\textsubscript{folate} synthesis [158], permitting ready reversibility. From the considerations in the above paragraph, one might expect K\textsubscript{eq} for the H\textsubscript{MPT} reaction to be more strongly in the direction of methylene-pterin formation than in the case of the H\textsubscript{folate} reaction. Nevertheless, the reverse reaction of serine biosynthesis might be driven by high C\textsuperscript{1} flux through the H\textsubscript{MPT} pathway if K\textsubscript{eq} for the SHMT reaction were not too unfavourable, say 10\textsuperscript{10}–10\textsuperscript{12}, in the CH\textsubscript{3}-H\textsubscript{MPT} direction. Determination of K\textsubscript{eq} for the H\textsubscript{MPT} reaction would clearly be of interest in providing further experimental information on the relative affinity of the methylene group for H\textsubscript{MPT} as compared with H\textsubscript{folate}, 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 cyclohydrase MCH) [92] indicated a relationship to 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. solfataricus*, which contain H\textsubscript{MPT}-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\textsubscript{3}-H\textsubscript{folate} rather than CH\textsubscript{3}-H\textsubscript{MPT} served as substrate [163]. Moreover, in the non-methanogen *S. solfataricus*,
the otherwise H,MPT-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,MPT-containing cells may be the unmethylated biosynthetic precursor to H,MPT [163]. If this proves to be correct, it will also be in accord with the above proposal that CH, -H,MPT (lacking the structural methyl groups) can give methylene transfer or reduction reactions a greater 'entropic boost' than can CH, -H,MPT (containing the structural methyl groups). However, the problem of the smaller &Delta;G, between N10 and N8 in H,MPT than in H,MPT remains.

Yet another unresolved aspect of thymidylate synthesis in organisms that utilize H,MPT is that the putative H,MPT 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,MPT-utilizing Archaea is currently incompletely understood.

So far, no enzymes (or genes) corresponding to a-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 a-oxopantoate synthase would be expected to be an essential enzyme. Its identification might shed further light on C, -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,MPT that determine the carriers' distinctive functional properties are those that generate the methylene group para to N10 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,MPT 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,MPT.

**Biosynthesis of H,MPT**

The pathway is initiated by GTP cyclohydrolase I, which converts GTP into the compound 7,8-H,MPT-neopterin triphosphate in a remarkable multistep reaction. In the reaction, C8 of GTP is eliminated as formate, C1 and C4 of the original ribose triphosphate become C4 and C8 of the pterin, and the remainder of the ribose triphosphate becomes the side chain of 7,8-H,MPT-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,OH-7,8-H,MPT.

PABA is synthesized from chorismate and is condensed with the pterin by dihydropteroate synthase with elimination of dihydropteroate. The first glutamate residue is then added, and the resulting 7,8-H,MPT is reduced by DHFR (reaction 1, above) to generate active carrier. The timing of addition of further glutamate residues and their relation to H,MPT 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 H4MPT**

From the structures (Figure 1), one might expect intuitively that the pathway to H4MPT would be similar to the pathway to H,MPT in the early steps, and that only the later steps that generate the distinguishing features of H4MPT 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,MPT, even in the early part of the pathway.

GTP cyclohydrolase I is absent in H,MPT-utilizing Archaea. Instead, ring restructuring proceeds by multiple steps, catalysed by two or more enzymes. The intermediate following expulsion of C4 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, H3-neopterin 2,3-cyclic phosphate, is an intermediate in dephosphorylation of the pterin [167]. The GTP cyclohydrolase of the Archaeal H,MPT 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 H3-neopterin, subsequent steps, which remain to be fully characterized enzymologically, generate the diphosphate of 6-CH,OH-7,8-H,perin [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-(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 anomic carbon atom of β-RFA-P is reduced to the functionally important methylene that tunes the reactivity of N10 (see above), and the 'right-hand end' of the structure (Figure 1) is completed in several further steps [168].

The methyl groups at C7s and C11s are donated by S-adenosylmethionine. For these C methylations it is proposed that an enzyme thiol combines with C11s of the pteridine [170] in similar manner to pyrimidine C6 methylations [171–173]. The methylation reactions 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 sequenced by reduction and might function in cellular biosynthetic C, -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 C6s [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 H₄SPT in Methanococci is based on analysis of one member of the group, M. voltae [193]. (For the difference between HₛSPT and H₄MPT, see the text.) Notes: (a) several Methanomicrobiaceae [193] contain one or more variants of the taliotepin/thermopterin structure shown; taliotepin O: R₁ = O; R₂ = H; thermopterin I: R₁ = glutamate, R₂ = H; thermopterin: R₁ = O; R₂ = 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 H₄MPT structure and their phylogenetic distribution

H₄MPT-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 known instance, in hydroxylation on the benzene ring.

As already mentioned, Methanosarcinales contain HₛSPT, which differs from H₄MPT in containing a terminal glutamate residue linked to the α-hydroxyglutarate of H₄MPT [78]. The following are further examples of variants, of generally increasing magnitude (Figure 4).

Taliotepin O, from Methanococcus thermophilius, has an aspartate residue linked to the α-hydroxyglutarate, and lacks the methyl C₁₂a; taliotepin I is the same, except that a glutamate residue is attached to the aspartate; thermopterin, from the same organism, resembles taliotepin O, except that the benzene ring is doubly para-hydroxylated [175,176]. Among non-methano-genic Euryarchaeae, Pyrococcus furiosus contains a pterin which resembles H₄MPT 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 H₄MPT-like in containing β-RFA. However, as mentioned above, the pterin lacks the structural methyl groups of H₄MPT [164].

Except for the sulphate-reducing A. fulgidis mentioned above, the metabolic functions of H₄MPT-like pterins in non-methano-genic Archaea are not well characterized. A synthetic fragment of sulfopterin was active with a recently isolated SHMT from S. solfataricus [177]. H₄folate did not support serine synthesis by this enzyme [177]. Further elucidating the functions of H₄MPT-like pterins in C₁ metabolism in non-methano-genic Archaea is likely to be of considerable metabolic interest. The occurrence of pterins which differ from H₄MPT 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 H₄MPT-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 H₄folate (Figure 4). As suggested above, the idea at first seemed intuitively likely that the pathway to H₄MPT 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 H₄MPT-biosynthetic pathway arose de novo in Archaea.

Among the present day Archaea, it is notable that halophiles contain H₄folate [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 point 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$_4$MPT 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 pyrroloquinolinequinoxine-dependent dehydrogenase [178]. A possible route of formaldehyde oxidation was thought to be entry into the H$_4$folate pathway at the methane level and oxidation to 10-HCO-H$_4$folate, formate and CO$_2$[179]. However, recently several ORFs showing significant homology to genes or ORFs known previously only in methanogens and *A. fulgidus* 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$_4$MPT cyclohydrolase (reaction 18 above), HCO-MFR: HCO-H$_4$MPT 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$_4$MPT substrates. The H$_4$MPT-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 z-hydroxyglutylate [14]. Further work revealed a novel, NADP$^+$-dependent CH$_2$-H$_4$MPT 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$_4$MPT path [14, 180]. Coupling of CH$_2$-H$_4$MPT dehydrogenase with NADP$^+$ would ensure oxidative flux at this step because of the much more negative $\Delta G^\circ$ of the CH$^+$/CH$_2$-H$_4$MPT couple than of the NADP$^+$/NADPH couple [180].

The evolutionary interpretation is that the H$_4$folate and H$_4$MPT pathways have co-existed in the methylotrophic lineage from early during Bacterial evolution, the H$_4$MPT 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$_4$folate, the H$_4$MPT class of compounds as C$_1$ carriers. The functions of H$_4$MPT in methanogenesis are now well characterized. However, there remain unanswered and intrinsically interesting questions concerning the bioenergetic roles of H$_4$MPT 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$_4$folate and H$_4$MPT pathways.

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