PROGRESS IN
HAEMATOLOGY: 2

Edited by

Christopher J. Pallister
MSc PhD FIBMS CBiol MIBiol CHSM

and

Christopher D. R. Dunn
DSc PhD BPharm (Hons) MRPharmS CBiol FIBiol FRSH
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FOLATE METABOLISM

M. Lucock
THE DISCOVERY OF FOLIC ACID AND ITS DERIVATIVES

Various researchers independently contributed to the discovery and characterization of folic acid and its many biologically active forms. In 1931, Lucy Wills reported that injections of crude yeast or a liver autolysate were effective in the treatment of tropical macrocytic anaemia in the pregnant woman. It was subsequently demonstrated that when monkeys were provided with a diet similar to those associated with human tropical macrocytic anaemia, they also developed a blood condition that could be rectified upon administration of yeast or liver extract.

Concurrent research reported a factor in yeast, wheat bran and alfalfa that stimulated the growth of chicks kept on highly purified diets. Other workers isolated nutrients from spinach, liver and yeast that were essential for the growth of lactic acid bacteria. These nutritional haematopoietic factors were identified as N-[4-[(2-amino-4-hydroxy-6-pteridinyl)methyl]amino]benzoyl] glutamic acid and various glutamyl derivatives. Angier et al., who elucidated this structure, put forward the name pteroylmonoglutamic acid. However, Mitchell’s group had already proposed the alternative name ‘folic acid’ (from the Latin folium meaning ‘leaf’) for the nutritional factor they had isolated from four tons of spinach! Eventually all factors were identified as the same compound.
The bacterial growth-stimulating properties of ‘folate compounds’ were instrumental in their discovery. This phenomenon is still used today as the basis of microbiological assays which, by employing *Lactobacillus casei*, *Streptococcus faecalis* or *Pediococcus cerevisiae* (formerly *Leuconostoc citrovorum*), achieve differential analysis of the various folylco enzyme forms. This analytical technique, combined with paper chromatography, was utilized by Herbert in 1962 who first identified 5-methyltetrahydropteroylmonoglutamic acid (5CH$_3$-H$_4$PteGlu) in human serum. This compound is now known to be the most ubiquitous native extracellular folylcoenzyme derivative.

STRUCTURE OF PTEROYLMONOGLUTAMIC ACID AND ITS DERIVATIVES

Today we recognize pteroylmonoglutamic acid (PteGlu) as a relatively stable, synthetic substance that represents the parent molecule of a large family of chemically similar, highly labile, trace compounds. These native folates may differ in:

- the state of oxidation of the pteridine ring;
- the nature of the one-carbon substituent at the N5 and N10 positions; and
- the number of glutamic acid residues linked one to another via a g-glutamyl linkage to form an oligo-g-glutamyl chain.

Figure 1.1 shows the structure of PteGlu and of some of its reduced one-carbon substituted forms.

This multiplicity of form available to folylco enzyme derivatives, coupled to disadvantages associated with more classical analytical methods, has led to confusion over the biological occurrence and role of reduced native folates. Indeed, it has been calculated that with three known reduction states of the pyrazine ring, six potential one-carbon substituents on either N$_5$ or N$_{10}$, and a maximum of seven glutamyl residues, there are, in theory at least, 150 different forms of folic acid. Thus, ‘folate’ is a generic term used to describe all derivatives which exhibit vitamin activity. PteGlu (Figure 1.1) is a synthetic pteridine derivative consisting of a pteridine moiety linked by a methylene bridge to para-aminobenzoic acid which in turn is joined via a peptide-like bond to glutamic acid. In nature, reduced PteGlu derivatives may occur as folylpolyglutamates typically containing up to seven glutamic acid residues.

Because of the complexity of folate metabolism and since ‘folate’ and ‘folic acid’ are most often used in generic ways, it is important to be as specific as possible about individual forms of the vitamin. Compounds where pteroic acid is conjugated with one or more glutamic acid residues are termed pteroylglutamic acid, pteroyldiglutamic acid, pteroyltriglutamic acid, etc. Reduction of the pteridine ring is indicated
by the prefixes 'dihydro-' and 'tetrahydro-' placed directly before the stem names. One-carbon substituents that are covalently bonded to the N5 or N10 positions or bridged between both positions are indicated by prefixes taken from general Organic Nomenclature rules.

It has become accepted practice to represent pteroylglutamic acid and its derivatives by the symbols PteGlu, PteGlu₂, PteGlu₃, etc., with the subscripted numeral indicating the number of attached glutamic acid residues. Oxidation state is indicated by H₂ or H₄ in front of the main symbol which, if required, is preceded by the nature and position of the one-carbon substituent.

Table 1.1 gives the name and abbreviation for all folylmonoglutamates and some folylpolyglutamates. It also gives the oxidation level of the one-carbon substituent.

Figure 1.1 – Structure of pteroylmonoglutamic acid and its major reduced one-carbon substituted forms.
PHYSICO-CHEMICAL PROPERTIES

The various derivatives of folic acid exhibit differential stability and temperature, pH and the presence of metal cations can all influence the oxidative degradation of folate species.

The most stable folate is PteGlu, which is stable over a wide pH range (5–12) when boiled continuously for up to 10 h in the dark. However, its stability decreases below pH 5. Alkaline hydrolysis cleaves the PteGlu molecule to yield p-aminobenzoic acid (P-ABG) and pterin–6-carboxylic acid, while acid hydrolysis produces 6-methylpterin.

H4PteGlu is extremely unstable. Oxidative degradation yields H2PteGlu along with P-ABG and pterin–6-carboxylic acid, while acid hydrolysis produces 6-methylpterin.

H3PteGlu is extremely unstable. Oxidative degradation yields H3PteGlu along with P-ABG and 6-formylpterin at pH 10, but only a pterin and P-ABG at pH 4 and 7.

The addition of a methyl group at the N5 position to yield 5CH3-H4PteGlu greatly improves stability. At 25°C the oxidative degradation of 5CH3-H4PteGlu is greatest at pH 9.0 (t1/2 = 5.9 h) while the stability increases between pH 7.3 and 3.5 (t1/2 = 16.2 and 23.3 h respectively). Light does not influence this process although the thiol anti-oxidant dithiothreitol can protect 5CH3-H4PteGlu at neutral to alkali pH but not under mildly acidic conditions where ascorbic acid is a better anti-oxidant. Metal cations result in oxidative degradation of 5CH3-H4PteGlu with the following order of effect Zn2+ > Ca2+ = K+ > Mg2+ = Na+. Equimolar Zn2+ and Na+ enhance methylfolate decay 33.3- and 7.2-fold respectively when compared with decay in water alone.

5CH3-H4PteGlu is a ubiquitous food folate, the most important transport and storage form of the vitamin in most mammals, and the methyl donor for de novo methionine biosynthesis. As a result, it is particularly important that the factors that affect this folylco enzyme’s stability are fully understood.

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Table 1.1 – Folate co enzymes: abbreviations and oxidation levels of one-carbon unit.

<table>
<thead>
<tr>
<th>Congener</th>
<th>Abbreviation</th>
<th>Oxidation</th>
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<tr>
<td>Pteroylmonoglutamate</td>
<td>PteGlu</td>
<td></td>
</tr>
<tr>
<td>Pteroyltriglutamate</td>
<td>PteGlu3</td>
<td></td>
</tr>
<tr>
<td>5-Methyltetrahydropteroylmonoglutamate</td>
<td>5CH2H,PteGlu</td>
<td>methanol</td>
</tr>
<tr>
<td>5-Methyl–5,6-dihydropteroylmonoglutamate</td>
<td>5CH2H,PteGlu</td>
<td>methanol</td>
</tr>
<tr>
<td>Tetrahydropteroylmonoglutamate</td>
<td>H,PteGlu</td>
<td></td>
</tr>
<tr>
<td>Dihydropteroylmonoglutamate</td>
<td>H2,PteGlu</td>
<td></td>
</tr>
<tr>
<td>5-Formyltetrahydropteroylmonoglutamate</td>
<td>5CHOH,PteGlu</td>
<td>formic acid</td>
</tr>
<tr>
<td>10-Formyltetrahydropteroylmonoglutamate</td>
<td>10CHOH,PteGlu</td>
<td>formic acid</td>
</tr>
<tr>
<td>5,10-Methylenetetrahydropteroylmonoglutamate</td>
<td>5,10CHOH,PteGlu</td>
<td>formic acid</td>
</tr>
<tr>
<td>5,10-Methylenetetrahydropteroylmonoglutamate</td>
<td>5,10CHOH,PteGlu</td>
<td>formaldehyde</td>
</tr>
<tr>
<td>5-Formimino tetrahydropteroylmonoglutamate</td>
<td>5NHCHOH,PteGlu</td>
<td>formic acid</td>
</tr>
<tr>
<td>5-Methyltetrahydropteroylmonoglutamate</td>
<td>5CH2H,PteGlu</td>
<td>methanol</td>
</tr>
<tr>
<td>5-Methyltetrahydropteroylhexaglutamate</td>
<td>5CH2H,PteGlu</td>
<td>methanol</td>
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</table>
Food folate exists largely in the 5CH₃-H₄PteGlu and 10CHO-H₄PteGlu forms. The predominant folate is 5CH₃-H₄PteGlu which is readily oxidized to 5-methyl-5,6-dihydrofolate (5CH₃-5,6-H₂PteGlu) which may constitute as much as 50% of total food folate.

Under mildly acidic conditions, typical of the post-prandial gastric environment, 5CH₃-5,6-H₂PteGlu is rapidly degraded, while 5CH₃-H₄PteGlu is relatively stable. Fortunately, ascorbate secreted into the gastric lumen can salvage labile 5CH₃-5,6-H₂PteGlu by reducing it back to acid-stable 5CH₃-H₄PteGlu in a process that may be critical for optimization of the bioavailability of food folate. Unlike 5CH₃-H₄PteGlu, 5CH₃-5,6-H₂PteGlu does not support the growth of *L. casei*, and in this oxidation state cannot enter the body’s active folate pool.

The mechanism of 5CH₃-H₄PteGlu oxidation has been the subject of considerable uncertainty and ambiguous nomenclature. In the following scheme only 5CH₃-H₄PteGlu and, given the appropriate conditions, 5CH₃-5,6-H₂PteGlu are biologically available, the other products no longer have metabolic activity (Figure 1.2).

10CHO-H₄PteGlu is the second major intracellular folate derivative, and is often referred to as ‘active formate’. It serves as a one-carbon donor in purine synthesis and in the formylation of met-tRNA. This co-enzyme is extremely unstable and readily oxidized to 10formylfolic acid (10CHOH₄PteGlu) which still exhibits full activity in certain bioassays. At neutral or mildly alkaline pH and in the absence of oxygen, 10CHO-H₄PteGlu undergoes isomerization to 5CHO-H₄PteGlu, the most stable one-carbon substituted tetrahydrofolate derivative. Under acidic conditions, 5CHO-H₄PteGlu loses a molecule of H₂O to form 5,10CHH₄PteGlu. This compound is stable to oxidation at acid pH but is hydrolysed to 10CHOH₄PteGlu at neutral or higher pHs (Figure 1.3).

![Degradation pathway of methylfolate.](image)

**Figure 1.2** – Degradation pathway of methylfolate.
5,10CH₂H₄PteGlu is sometimes referred to as active formaldehyde. The one-carbon moiety is carried as a CH₂ group bound via the N₅ and N₁₀ positions. This folylco-enzyme is responsible for the synthesis of thymidylate, and is the precursor of 5CH₃-H₄PteGlu. In our laboratory this compound appears to be marginally less stable than 5CH₃-H₄PteGlu.

H₂PteGlu is formed enzymatically from PteGlu and as a consequence of thymidylate synthesis, and is rapidly degraded on exposure to air. The principal oxidation product is an unstable quinoid dihydrofolate isomer although other pathways exist. Antioxidants prevent all degradation reactions. At 0°C in a pH 7.3 buffer containing 0.3 M mercaptoethanol, 2.3% of H₂PteGlu degraded in 10 h.

5-HCNH-H₄PteGlu is produced in the catabolism of histidine by a reaction between formiminoglutamic acid (FigGlu) and H₄PteGlu. It is stable to atmospheric oxygen.

Figure 1.4 shows the UV spectra of nine folate-related compounds at pH 3.5.

**SOURCES OF DIETARY FOLATE**

Humans are unable to synthesize folate and, therefore, depend on dietary sources of the vitamin. Since food folates are present in small amounts, efficient absorption is critical for maintaining the body’s folate status. Dietary folates occur in yeasts (i.e. extracts such as Marmite®), Bovril®, liver, kidney, leafy green vegetables and citrus fruits. Folates are also found in moderate amounts in bread, potatoes and dairy products. Since these latter foods are consumed in large quantities they actually provide a substantial contribution to the total folate intake.

Methylfolates as 5CH₃-H₄PteGlu and 5CH₃-H₂PteGlu are the most prevalent form of the vitamin found in the diet although the oxidized methyl form of the vitamin may account for as much as 50% of the total dietary folate.
FOLATE ABSORPTION

Most dietary folates exist as conjugated polyglutamate forms of 5CH$_3$H$_2$PteGlu or 10CHO-H$_2$PteGlu. Before they can be absorbed efficiently, these folylpolyglutamates must be hydrolysed (deconjugated) to their various monoglutamate congeners by the enzyme $\gamma$-glutamylcarboxy-peptidase (folate deconjugase, $\gamma$-glutamylhydrolase) which is present in saliva, juice of the small intestine and the mucosal brush border. It has been reported that many tissues contain both endo- and exo-peptidase activity while, in addition, both folylmono- and folyldiglutamate endproducts can be formed: PteGlu$_n$ $\rightarrow$ PteGlu$_1$ and $\gamma$-Glu$_{(n-2)}$ where $n \geq 5$ or PteGlu$_n$ $\rightarrow$ PteGlu$_1$ and $\gamma$-Glu$_{(n-1)}$. Synthetic folic acid found in vitamin supplements is present in the monoglutamate form as PteGlu and does not, therefore, require deconjugation before absorption.

Since it can salvage acid labile 5CH$_3$H$_2$PteGlu by reducing it back to 5CH$_3$H$_3$PteGlu, ascorbic acid actively secreted into the gastric lumen may play a crucial role.

Figure 1.4 – UV spectra of nine folate-related compounds obtained by photo-diode array detection following HPLC separation at pH 3.5.
role in optimizing the bioavailability of dietary methylfolate. In the absence of ascorbate at mildly acid pH, 5CH₃H₁PteGlu rapidly degrades via C₉-N₁₀ bond cleavage ($t_{1/2} \approx 16.9$ min) with complete loss of vitamin activity.

Absorption of folate is a pH-dependent, saturable process that occurs throughout the length of the small intestine although it is significantly more efficient proximally. PteGlu is reduced by dihydrofolate reductase (DHFR) and methylated via downstream enzymes to form 5CH₃-H₄PteGlu as it crosses the epithelial cells of the proximal jejunum.

The formation of plasma 5CH₃-H₄PteGlu from orally administered PteGlu proceeds with great efficiency at low doses where the overall process is essentially first order. A low apparent $K_m$ suggests that the system has a high substrate affinity well suited to the efficient and rapid production of 5CH₃-H₄PteGlu from normal dietary sources where folate compounds may be in short supply. While formation of 5CH₃-H₄PteGlu shows obvious saturation kinetics, at higher oral doses the amount of unmodified PteGlu transversing the intestine increases greatly relative to that portion which is reduced and methylated to form the transport and storage 5CH₃-H₄PteGlu form. Transport across the enterocyte brush border membrane is saturable and involves an anion-exchange mechanism driven by the transmembrane pH gradient. Anionic folate at intralumen pH is exchanged for a hydroxyl anion. It has been shown that H₂PteGlu, H₄PteGlu, 5CHOH₄PteGlu and 10CHOH₄PteGlu are all converted to 5CH₃-H₄PteGlu by the human intestine.

TRANSPORT OF BLOOD FOLATES

Following absorption of monoglutamyl folate into the portal circulation as 5CH₃-H₁PteGlu (with perhaps some 10CHOH₄PteGlu also present), a substantial amount is taken up by the liver where it is either metabolized to folylpolyglutamate derivatives and retained or released. Folates released into the bile are re-circulated by the enterohepatic cycle and reabsorbed in the small intestine. Plasma contains γ-glutamylhydrolase activity thus ensuring the exclusive presence in plasma of monoglutamyl folate species. Plasma levels of 5CH₃-H₁PteGlu are maintained by dietary intake and enterohepatic recycling: the normal plasma range varies according to which analytical technique is used (microbiological assay, radiometric binding assay or high-performance liquid chromatography (HPLC)), but it is reasonable to quote 3–30 ng/ml as typical.

During brief periods of dietary deprivation, folate supply is maintained by the monoglutamate pools of the enterohepatic cycle and cells: reduced cellular folylpolyglutamate synthesis combined with polyglutamate degradation to monoglutamate forms result from a decreased tissue uptake. The net result of these reactions is that the extracellular and, thus, available folate level (as 5CH₃-H₁PteGlu)
is increased. Re-absorption of $5\text{CH}_3\text{-H}_4\text{PteGlu}$ by the renal proximal tubule also helps maintain circulating levels. This process occurs by receptor-mediated endocytosis (see below).

Some confusion exists over the character of folate-binding proteins in human serum largely due to the varying reports and inconsistent terminology of different researchers. However, in a series of papers, Markkanen et al. showed that $30–40\%$ of endogenous serum folate was associated with $\alpha_2$ macroglobulin, albumin and transferrin, and that bound folate decreased during folate deficiency, while a shift in binding from $\alpha_2$ macroglobulin to transferrin occurred during pregnancy.

Although it is now recognized that endogenous circulating $5\text{CH}_3\text{-H}_4\text{PteGlu}$ is either free or non-specifically bound to various plasma proteins, a specific folate (PteGlu) binder has been identified during folate deficiency, uraemia, leukaemia, liver disease and pregnancy, and in serum from umbilical cord blood. If this specific binder is not saturated by PteGlu, it may weakly associate with $5\text{CH}_3\text{-H}_4\text{PteGlu}$. However, as PteGlu is not present in humans under normal circumstances, it is difficult to ascribe any physiological purpose to this specific folate-binding protein. It is not likely to act as a methylfolate transport protein since it has little affinity for $5\text{CH}_3\text{-H}_4\text{PteGlu}$. Nevertheless, its elevated levels during pregnancy, contraceptive therapy and disease does indicate a possible relationship to perturbed folate metabolism.

The low affinity complex formed between folylmonoglutamates and albumin ($K = 10^3 \text{ l/mol}$) is a result of electrostatic interaction between negative carboxyl groups on the folate molecule and positively charged residues on the protein. A similar, less extensively studied interaction occurs between folates (in predominantly polyglutamate form) and haemoglobin molecules within erythrocytes. One molecule of $5\text{CH}_3\text{-H}_4\text{PteGlu}$ binds one molecule of haemoglobin and, although this is a low-affinity, low-capacity relationship, an intra-erythrocyte molar ratio of haemoglobin to folate of about 10 000:1 actually renders it a ‘high-capacity’ system.

Erythrocyte folate is largely, but not entirely, $5\text{CH}_3\text{-H}_4\text{PteGlu}$: $60–70\%$ is in the form of folylpolyglutamates with penta- and hexa-glutamates predominating.

Figure 1.5 shows the distribution of polyglutamate chains within the erythrocyte determined by the current author using HPLC. The concentration range of erythrocyte folate using radiometric-binding assays varies from 140 to 450 ng/ml packed cells. Methylfolate is incorporated into the developing erythroblast during erythropoiesis in the marrow. Intra-erythrocyte folate has no metabolic role and is, therefore, presumably a storage reservoir and/or buffer for maintaining folate homeostasis. It is often used as a measure for long-term folate status and, unlike plasma levels, is unaffected by recent dietary intake. Folate is salvaged from
senescent erythrocytes by the reticuloendothelial system, transported to the liver, and appears in the bile for distribution to peripheral tissues.

CELLULAR TRANSPORT

Two basic systems of folate transport have been described: membrane carriers and folate-binding proteins.

Membrane carriers

Several membrane carriers that transport folate have been characterized in mammalian tissue. One of the most extensively studied transporter systems occurs in some tumour cells and foetal tissue and is distinct from similar systems found in normal adult tissue. It is saturable with a low avidity for reduced folates, although this affinity is much greater than for PteGlu and slightly greater than for the anti-folate chemotherapeutic agent methotrexate.

A wide range of different major folate transporters occur in adult tissues and exhibit differential affinity for various folyko enzymes. Transport in hepatocytes is energy-dependent and has saturable and low affinity non-saturable components. Basolateral membrane preparations from rat and human liver have an electroneutral folate H⁺ co-transporter, while the basolateral membrane of the small intestine has an anion exchange folate transporter. Mitochondria also possess folate specific transporters.
Folate-binding proteins

A specific folate-binding protein exists that binds a variety of folylco enzymes with high affinity, and which is attached to the plasma membrane by a glycosylphosphatidylinositol anchor. In normal tissue, this protein is confined to the apical membrane of certain epithelial cells. The folate–protein complex internalizes folate by a non-clathrin-mediated endocytotic pathway which does not involve lysosomes. The phrase ‘potocytosis’ has been coined for the recycling of binding protein via vesicular structures known as caveolae. Furthermore, acidification may release anionic folate from the carrier before liberation from the vesicle into the cytosol. The binding protein subsequently cycles back to the plasma membrane.

CELLULAR ONE-CARBON TRANSFER REACTIONS

The major metabolic function of folylco enzymes is the transfer of single carbon units within cells. Five major reactions occur: conversion of serine to glycine; catabolism of histidine; and synthesis of thymidylate, methionine and purine. The vitamin interconversions responsible for these reactions take place through various electron transfer steps facilitated by specific enzyme systems and co-enzymes such as FADH₂ and NADPH.

In general, folypolyglutamates are better substrates for enzymes than their monoglutamyl counterparts: \( K_m \) decreases with increasing glutamate chain. Many of these folate-dependent enzymes are multifunctional and channel one-carbon units from reaction-to-reaction without reaching equilibrium with the intracellular medium.

Liver, in which the enzyme sarcosine synthase is the main binding protein, harbours the body’s main store of folates, largely in folylpolyglutamate form although polyglutamylation occurs in many cells. Folate enters the cell largely as \( 5\text{CH}_3\text{H}_4\text{PteGlu} \), thus vitamin \( 	ext{B}_12 \)-dependent methionine synthase (MS), being the only enzyme capable of demethylating \( 5\text{CH}_3\text{H}_4\text{PteGlu} \), is rate-limiting for intracellular accumulation of folates.

It has been shown that differential polyglutamate specificity for MS is required for incorporation of plasma methyl folate into the cellular folate pool. That is, \( 5\text{CH}_3\text{H}_4\text{PteGlu} \) metabolism is strongly inhibited by the presence of intracellular \( 5\text{CH}_3\text{H}_4\text{PteGlu} \); therefore, intracellular incorporation of plasma \( 5\text{CH}_3\text{H}_4\text{PteGlu} \) via MS only occurs when cellular \( 5\text{CH}_3\text{H}_4\text{PteGlu} \) is low. MS may, therefore, have two classes of binding site for methylfolate polyglutamates consistent with negative cooperativity of substrate binding providing a regulatory mechanism for intracellular one-carbon metabolism.

Internalized \( 5\text{CH}_3\text{H}_4\text{PteGlu} \) is a poor substrate for polyglutamylation via folylpolyglutamate synthase. MS-dependant demethylation to \( \text{H}_4\text{PteGlu} \) must
occur before polyglutamylation can take place. Formation of polyglutamates or larger chain compounds ensures cellular retention.

Figure 1.6 depicts the metabolic pathways of one-carbon metabolism.

**Sources of one-carbon units**

The β-carbon of serine is the principal source of one-carbon units in a reaction
catalysed by serine hydroxymethyltransferase (SHMT). In this reaction glycine is formed and H$_4$PteGlu is converted to 5,10CH$_2$-H$_4$PteGlu.

Other sources of one-carbon units include formiminoglutamic acid (FiGlu), the mitochondrial glycine cleavage pathway and choline catabolism. In addition, a minor source in mammalian tissue is the formation of 10CHOH$_4$PteGlu from formate, H$_4$PteGlu and ATP.

**Histidine catabolism**

FiGlu arises from the breakdown of histidine. Further metabolism involves transfer of the formimino group to H$_4$PteGlu and removal of the =NH group by the bifunctional enzyme 5-formiminotetrahydrofolate cyclodeaminase/transferase to yield 5,10CH$_2$H$_4$PteGlu. The excretion of urinary FiGlu following a histidine load was an early test for folate deficiency.

**Purine and pyrimidine synthesis**

The importance of folic acid derivatives lies particularly with their role as coenzymes in the intracellular synthesis of the purine ring and pyrimidine nucleotides.

Through the actions of the enzymes GAR transformylase and AICAR transformylase, glycinamide ribonucleotide (GAR) and aminimidazole–4-carboxamide ribonucleotide (AICAR) both receive a one-carbon moiety from 10CHOH$_4$PteGlu. This moiety becomes carbon atom 8 and 2 respectively of the developing purine ring.

In the pathway leading to pyrimidine nucleotides, 5,10CH$_2$-H$_4$PteGlu is responsible for the methylation of deoxyuridylate monophosphate (DUMP) to form thymidylate monophosphate (TMP). Catalysed by thymidylate synthase, this is the rate-limiting step in the elaboration of DNA. A major advance in medical science, and particularly in cancer chemotherapy, has been the development of antagonists to the enzymes that play a crucial role in mammalian folate metabolism. Folate anti metabolites such as methotrexate and aminopterin structurally resemble H$_2$PteGlu, and, therefore, inhibit the enzyme dihydrofolate reductase (DHFR) which converts H$_4$PteGlu to H$_4$PteGlu. As the formation of TMP is sensitive to depressed levels of H$_4$PteGlu, DNA synthesis in proliferating malignant cells is inhibited.

In mammalian tissues the level of thymidylate synthase is related to replication rate with expression of the enzyme being highest during the S-phase of the cell cycle. A protein complex containing thymidylate synthase, dihydrofolate reductase, DNA polymerase, thymidine kinase, deoxycytidine monophosphate kinase, nucleoside diphosphate kinase and ribonucleotide reductase has been
described. This multi-enzyme complex has been termed replicase, and forms during the S-phase of the cellular cycle.

H₂PteGlu inhibits the enzyme 5,10 methylenetetrahydrofolate reductase (5,10MTHFR) and provides a possible regulatory mechanism for ensuring priority is given to nucleic acid synthesis: Increased thymidylate synthesis in proliferating cells elevates H₂PteGlu levels (Figure 1.6) and, through inhibition of 5,10MTHFR, decreases the one-carbon flux into methionine formation. This may be a mechanism to conserve one-carbon units for thymidylate and purine biosynthesis.

**Glycine–serine interconversions**

The reversible interconversion of serine and glycine is catalysed by SHMT in a reaction requiring pyridoxal phosphate (PLP). This step allows the β carbon of serine to enter the one carbon pool at the formaldehyde oxidation level:

\[
(\text{PLP}) \quad \text{Serine} + \text{H}_2\text{PteGlu} + \text{NAD}^+ \leftrightarrow 5,10\text{CH}_2\text{H}_4\text{PteGlu} + \text{Glycine} + \text{H}_2\text{O}.
\]

The product 5,10CH₂H₄PteGlu has a pivotal role in one-carbon metabolism not the least because it is involved in the formation of DNA-thymine and 5CH₃-H₄PteGlu, the precursor of de novo methionine biosynthesis. Its importance is best emphasized by the fact that SHMT, thymidylate synthase, 5,10MTHFR and methylenetetrahydrofolate dehydrogenase all either produce or utilize 5,10CH₂H₄PteGlu in mammalian tissue.

Another entry into the formaldehyde oxidation level involves the glycine cleavage reaction – a complex four-stage process occurring exclusively in the mitochondria:

\[
\text{Glycine} + \text{H}_2\text{PteGlu} + \text{NAD}^+ \leftrightarrow \text{CO}_2 + \text{NH}_4^+ + 5,10\text{CH}_2\text{H}_4\text{PteGlu} + \text{NADH}.
\]

Carbon 2 of glycine is transferred to H₂PteGlu forming 5,10CH₂H₄PteGlu and releasing NH₃. Carbon 1 is oxidized to CO₂.

Sarcosine dehydrogenase and dimethylglycine dehydrogenase are mitochondrial enzymes which further incorporate one-carbon units at the level of formaldehyde. Dimethylglycine arises from the catabolism of choline in the liver. One of its methylgroups is transferred to H₂PteGlu by dimethylglycine dehydrogenase and is oxidized to 5CH₂H₄PteGlu. The other product is sarcosine which is oxidized with the remaining methyl group being transferred to H₂PteGlu by sarcosine dehydrogenase. The products are 5CH₂H₄PteGlu and glycine.

**Role of 5 formyltetrahydrofolate**

Until recently, the biological role of 5CHOH₂PteGlu, if it had one at all, was
unclear. This folate is often referred to as leucovorin and is used as a rescue therapy following methotrexate treatment. Although 5CHOH,PteGlu occurs in mammalian systems, it does not serve as a one-carbon donor in biosynthetic reactions leading to methionine, thymidylate, or purine. A single unidirectional enzyme, methenyltetrahydrofolate synthetase, can salvage the one-carbon unit of 5CHOH,PteGlu by producing 5,10CHH,PteGlu, while the same co enzyme can be recycled to 5CHOH,PteGlu via SHMT. However, it is thought that the actual substrate for SHMT may be hydrated 5,10CHH,PteGlu (5,10CHOH-H,PteGlu). This cycle shown in Figure 1.7 is referred to as the futile cycle. The presence of an enzyme – 5,10 methenyltetrahydrofolate synthetase – which can utilize 5CHOH,PteGlu suggests this is in fact a native metabolically functional co-enzyme.

Three other biosynthetic pathways may be regulated through the futile cycle and the level of 5CHOH,PteGlu of which 5CHOH,PteGlu inhibition of aminimidazolecarboxamide formyltransferase (which is required for purine biosynthesis) may be particularly important.

Figure 1.7 – 5-Formyltetrahydrofolate and the futile cycle.
**De novo** methionine biosynthesis and regulatory control of homocysteine re methylation and trans-sulphuration

Homocysteine (Hcy) occupies a metabolic site at the intersection of the re methylation and trans-sulphuration pathways, with its biochemical fate being linked to vitamin B₁₂ and the various reduced folate co enzymes.

In the re methylation cycle, a methyl group from either 5CH₃-H⁴PteGlu or betaine is used to convert Hcy to methionine. The 5CH₃-H⁴PteGlu one-carbon unit is produced *de novo* via incorporation of the β carbon of serine into H⁴PteGlu thus converting it to 5,10CH₂-H⁴PteGlu, which is subsequently reduced to 5CH₃-H⁴PteGlu by the flavoprotein 5,10MTHFR. This folate co-enzyme donates its methyl group to Hcy through the action of MS – a vitamin B₁₂ (cyanocobalamin)-dependent enzyme (see above). In this step, 5CH₃-H⁴PteGlu transfers its methyl group to cyanocobalamin which, as methylcyanocobalamin, transfers it to Hcy yielding H₃PteGlu and methionine. Utilization of the betaine methyl group for conversion of Hcy to methionine involves the vitamin B₁₂-independent enzyme betaine homocysteine methyltransferase.

Methionine thus formed can be activated by ATP to yield the methyl donor S-adenosylmethionine (SAM) that methylates a variety of important biomolecules such as adrenaline, phosphatidylcholine and carnitine. In the process, SAM is converted to S-adenosylhomocysteine (SAH) which is subsequently hydrolysed back to Hcy to commence a new methyl transfer cycle. This is the only known route of Hcy formation in vertebrates.

The trans-sulphuration pathway involves condensation of Hcy with serine to form cystathionine – a vitamin B₆-dependent step catalysed by cystathionine β-synthase (CβS). Beyond this step, Hcy can no longer serve as a methionine precursor: indeed, increased cystathionine synthesis may be a metabolic adaptation to methionine excess. Cystathionine is hydrolysed to cysteine and α-ketobutyrate by a second B₆-dependent enzyme γ-cystathionase.

Metabolic coordination of re methylation and trans-sulphuration is under the influence of SAM which allosterically inhibits 5,10MTHFR while activating CβS. Therefore, when SAM levels are low, 5CH₃-H⁴PteGlu formation proceeds unabated while cystathionine formation is reduced. Under these conditions, Hcy is conserved for methionine production. In contrast, elevated SAM leads to trans-sulphuration of Hcy due to enhanced CβS activity. Thus, the SAM/SAH ratio is an important determinant of one-carbon metabolism and has other critical regulatory sites.

A metabolic balance between re methylation and trans-sulphuration is, therefore, dependent on SAM levels (SAM/SAH ratio), the concentration of the *de novo* methyl group acceptor Hcy, and specific dietary factors particularly folate and methionine but also vitamins B₁₂ and B₆.
It is known that elevated dietary methionine and consequently SAM lead to inhibition of the synthesis of 5,10-MTHFR and 5CH$_3$-H$_4$PteGlu and, concomitantly, diversion of Hcy into the trans-sulphuration pathway due to SAM stimulated CβS. Thus, valuable folates such as 5CH$_3$-H$_4$PteGlu are conserved. It has been estimated that humans use more methyl groups than they consume from dietary methionine with the shortfall being made up from 5CH$_3$-H$_4$PteGlu and betaine. This demand for active methyl groups as SAM is largely due to creatine formation which consumes more SAM than all other transmethylations combined. In addition to these regulatory mechanisms, methylfolate also acts as a regulatory molecule controlling methionine metabolism. Under conditions where a greater proportion of methyl groups for production of the active methyl compound SAM are derived de novo from 5CH$_3$-H$_4$PteGlu (or preformed from betaine) than from methionine, excess 5CH$_3$-H$_4$PteGlu inhibits glycine-N-methyltransferase (GNMT) and thus utilization of SAM. This leads to conservation of limited active methionine for essential methylation reactions. In this way, the inhibition of 5CH$_3$-H$_4$PteGlu production by SAM, and inhibition by 5CH$_3$-H$_4$PteGlu of SAM utilization via GNMT, links de novo methyl group synthesis with control of the SAM/SAH regulatory ‘switch’ and availability of dietary methionine. Furthermore, since 5CH$_3$-H$_4$PteGlu is a potent inhibitor of porcine SHMT, it may in humans act as another feedback mechanism to further reduce methylfolate production for de novo methionine biosynthesis. Such a mechanism might help partition folates between the re methylation of Hcy and other essential one-carbon transfer reactions.

**COMPARTMENTALIZATION OF FOLATE METABOLISM**

One-carbon metabolic transfers are compartmentalized between the cytosol and mitochondria. It has been shown in liver that folate in these two pools is not in equilibrium. Reduced 5CH$_3$-H$_4$PteGlu and 5CHOH$_3$-H$_4$PteGlu can enter intact mitochondria by a non energy-dependent carrier-mediated mechanism but oxidized folates cannot. This is consistent with the absence of DHFR in the mitochondria.

Table 1.2 lists the most likely site for each folate-dependent enzyme which may be found either exclusively in, or shared between, mitochondria and cytosol. SHMT purified from both compartments have similar activities but are proteins with differing primary structures. Although folate co-enzymes move slowly between compartments, serine, glycine and formate rapidly equilibrate and it is recognized that there is an interdependence in one-carbon metabolism between compartments.

It is thought that serine (the major source of one-carbon units) or dimethylglycine and sarcosine (which are products of choline metabolism) enter mitochondria and
produce $5\text{CH}_2\text{H}_4\text{PteGlu}$ which can generate $10\text{CHOH}_4\text{PteGlu}$ for mitochondrial protein synthesis. Unwanted formate can efflux back to the cytosol via the 10-formyltetrahydrofolate synthase reaction.

**Table 1.2 – Subcellular localization of folate related enzymes.**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cellular localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Dihydrofolate reductase</td>
<td>#</td>
</tr>
<tr>
<td>2. Thymidylate synthase</td>
<td>#</td>
</tr>
<tr>
<td>3. Phosphoribosylgly cinamide transformylase</td>
<td>#</td>
</tr>
<tr>
<td>4. Aminocarboxamide ribotide transformylase</td>
<td>#</td>
</tr>
<tr>
<td>5. 10 Formyltetrahydrofolate dehydrogenase</td>
<td>#</td>
</tr>
<tr>
<td>6. 10 Formyltetrahydrofolate synthase</td>
<td>#</td>
</tr>
<tr>
<td>7. Methionyl t-RNA formyltransferase</td>
<td>#</td>
</tr>
<tr>
<td>8. 5,10 Methylenetetrahydrofolate cyclohydrolase</td>
<td>#</td>
</tr>
<tr>
<td>9. Dimethylglycine dehydrogenase</td>
<td>#</td>
</tr>
<tr>
<td>10. Sarcosine dehydrogenase</td>
<td>#</td>
</tr>
<tr>
<td>11. Glycine cleavage system(forward reaction only)</td>
<td>#</td>
</tr>
<tr>
<td>12. Serine hydroxymethyltransferase</td>
<td>#</td>
</tr>
<tr>
<td>13. Serine hydroxymethyltransferase minor reaction</td>
<td>#</td>
</tr>
<tr>
<td>14. 5,10 Methylenetetrahydrofolate synthetase</td>
<td>#</td>
</tr>
<tr>
<td>15. Formiminotetrahydrofolate cycloaminase</td>
<td>#</td>
</tr>
<tr>
<td>16. Glutamate formiminotransferase</td>
<td>#</td>
</tr>
<tr>
<td>17. Methionine synthase</td>
<td>#</td>
</tr>
<tr>
<td>18. 5,10 Methylene tetrahydrofolic reductase</td>
<td>#</td>
</tr>
<tr>
<td>19. 5,10 Methylene tetrahydrofolic dehydrogenase</td>
<td>#</td>
</tr>
<tr>
<td>24. Glycine $N$-methyltransferase</td>
<td>#</td>
</tr>
</tbody>
</table>

**FOLIC ACID AND HEALTH**

**Pathogenesis of elevated homocysteine**

Plasma Hcy exists in sulphhydril and mixed disulphide form. The plasma Hcy concentration in normal subjects is quoted as 7–24 mmol/l with urinary levels being within the same range. Elevated plasma and urinary Hcy levels can result from several inherited and nutritional diseases that directly or indirectly affect the pathways of Hcy re methylation and trans-sulphuration. Table 3 summarises the causes of elevated Hcy.

There are two particular clinical situations in which folate nutrition and elevated Hcy levels may have profound implications: occlusive vascular disease and neural tube defects.

**Oclusive vascular disease**

Carson and Neill first described homocystinuria as an inborn error of metabolism.
These pioneering findings were later characterized by Mudd et al. who showed a deficiency of C\(\beta\)S in liver biopsy specimens taken from individuals suffering from homocystinuria. Following this discovery, other rare enzyme deficiencies leading to elevated Hcy were reported. Homozygotes for this defect suffer from mental retardation, thromboembolism and premature occlusive vascular disease which may present at any age including infancy. Considerable \textit{in vivo} and \textit{in vitro} experimental data now link Hcy levels with vascular pathology: for example, arteriosclerosis has been produced in rabbits and baboons by parenteral administration of Hcy derivatives. In baboons, sustained treatment resulted in changes resembling those observed in early human arteriosclerosis.

Experimental data are also supported by numerous clinical studies which are remarkably consistent in their findings. They indicate that patients with occlusive vascular disease have higher blood Hcy than individuals with no disease, though most patients have values within what has been considered a normal range. Furthermore, it has been shown that the risk of occlusive vascular disease is independent of serum cholesterol and hypertension. Since a strong reciprocal

\begin{table}
\centering
\caption{Inherited and acquired defects in folate metabolism.}
\begin{tabular}{ll}
\hline
1. & Inherited defects \\
& a) Enzyme deficiencies \\
& \quad i) Cystathionine \(\beta\)-synthase \\
& \quad ii) Methylenetetrahydrofolate reductase (MTHFR) \\
& \quad iii) Thermolabile MTHFR \\
& \quad iv) Methionine synthase (Cbl E, Cbl G) \\
& \quad v) Cobalamin co enzyme synthesis (Cbl C, Cbl D) \\
& b) Transport defects \\
& \quad i) Transcobalamin II deficiency \\
& \quad ii) Cobalamin lysosomal transporter (Cbl F) \\
& 2. & Acquired defects \\
& a) Nutritional \\
& \quad i) Cobalamin deficiency \\
& \quad ii) Folic acid deficiency \\
& \quad iii) Pyridoxine deficiency \\
& b) Metabolic \\
& \quad i) Chronic renal disease \\
& \quad ii) Hypothyroidism \\
& c) Drug-induced \\
& \quad i) Methotrexate and other folate antagonists \\
& \quad ii) Nitrous oxide and other cobalamin antagonists \\
& \quad iii) Azarbine and other pyridoxine antagonists \\
& \quad iv) Oestrogen antagonists \\
& \quad v) Anti convulsants \\
\hline
\end{tabular}
\end{table}

Information adapted from Green and Jacobsen (1996).