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# FOLATES AND ONE-CARBON METABOLISM IN PLANTS AND FUNGI

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**Key Word Index**—Plants; fungi; microorganisms; folate derivatives; folate biosynthesis; folate-dependent enzymes; one-carbon metabolism.

**Abstract**—Folate-dependent pathways of one-carbon metabolism are essential for the synthesis of purines, formylmethionyl-*t*RNA, thymidylate, serine and methionine. These syntheses use a cellular source of one-carbon substituted, tetrahydrofolate polyglutamate derivatives which are the preferred substrates of most folate-dependent enzymes. In the last decade, there have been major advances in the folate biochemistry of animal, bacterial, fungal and plant systems. These have included the refinement of methods for folate isolation and characterization, basic work on key enzymes of folate biosynthesis and the detailed characterization of proteins that catalyze the generation and utilization of one-carbon substituted folates. 

## INTRODUCTION†

One-carbon metabolism has importance in the formation of purines, thymidylate, serine, methionine and formylmethionyl-*t*RNA [2, 3]. In these syntheses, single carbon groups, ranging in oxidation state from formyl to methyl, are donated by tetrahydrofolate polyglutamates ($H_4PteGlu_n$), which contain pteridine, *p*-aminobenzoate and glutamate entities (Fig. 1). The pathway for $H_4PteGlu_n$ synthesis (Fig. 2) involves the generation of pteridine precursors from GTP, the formation and reduction of $H_2PteGlu$, and finally the glutamyl conjugation of tetrahydrofolate [2, 3].

Living cells contain a number of one-carbon substituted $H_4PteGlu_n$ derivatives [2, 4], which actively participate in folate-dependent pathways (Fig. 3). Enzymes catalyzing these reactions have received detailed study [5–9]. Recently, work in this field has intensified with the development of new and improved methods for folate isolation and characterization. Important advances have also been made by the cloning, sequencing and site-directed mutagenesis of genes that encode the major enzymes of folate metabolism.

The nutritional and clinical importance of folates has provided special impetus for work on mammalian and bacterial cells. Several topics related to these fields have been reviewed. These include human folate deficiencies [10], folate-binding proteins [11, 12], folate and antifolate transport [13], the interaction of enzymes with folylpolyglutamates [14], the development of new antifolates [15–17], folate status and carcinogenesis [18], the metabolic role of leucovorin (5-HCO-$H_4PteGlu$) [19], the biosynthesis and regulatory roles of folylpolyglutamates [20], and the compartmentation of one-carbon metabolism [21].

The present review focuses on investigations of folate biochemistry in fungi and higher plants, with

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†Abbreviations for folate derivatives are those suggested by the IUPAC-IUB Commission as summarized by Blakley and Benkovic [1]; e.g. $H_4PteGlu_n$ = 5,6,7,8-tetrahydropteroylpoly-γ-glutamate, where $n$ = the number of L-glutamate moieties; 10-HCO-$H_4PteGlu_n$ = 10-formyltetrahydropteroylpolyglutamate; 5,10-$CH_2$-$H_4PteGlu_n$ = 5,10-methylenetetrahydropteroylpolyglutamate; 5,10-$CH^+$ - $H_4PteGlu_n$ = 5,10 - methenyltetrahydropteroylpolyglutamate. other abbveviations: DHFS = dihydrofolate synthetase; FPGS = folylpolyglutamate synthetase; DHFR = dihydrofolate reductase; GDC = glycine decarboxylase; GGH = γ-glutamyl hydrolase; $MTXGlu_n$ = methotrexate polyglutamate; PMSF = phenylmethylsulphonylfluoride; SHMT = serine hydroxymethyl transferase; TS = thymidylate synthase.

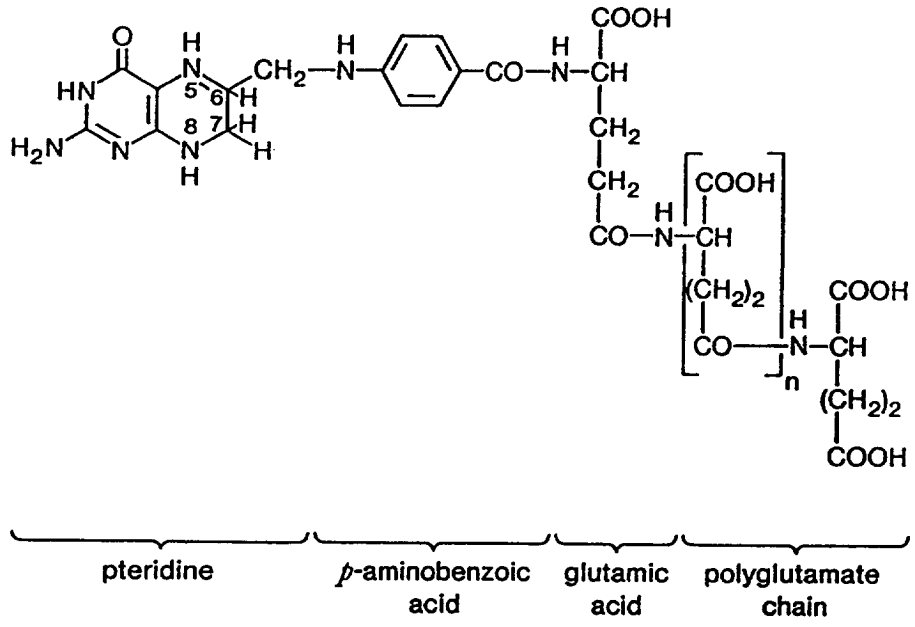


Fig. 1. Structure of the $H_4PteGlu_n$ molecule. Cellular folates are C-1 substituted at the N-5 and N-10 positions to give 5-formyl-, 5-methyl-, 10-formyl-, 5,10-methenyl- and 5,10-methylene derivatives. The polyglutamate chain usually contains 5-8 γ-glutamyl residues.

emphasis on a selection of research that has been published since 1987. Besides identifying aspects of this subject that require further study, the authors have cited basic work on animal and bacterial cells

**Guanosine 5′-triphosphate**

↓ [1]

**L-*threo*-neopterin 2′,3′-cyclic phosphate**

↓

↓

**6-hydroxymethyl dihydroneopterin**

↓ [2]

**2-amino-4-hydroxy-6-hydroxymethyl dihydropteridine**

ATP ↓ [3]

**2-amino-4-hydroxy-6-pyrophosphoryl-methyl dihydropteridine**

p-ABA ↓ [4]

**Dihydropteroate ($H_2Pte$)**

ATP, glutamate ↓ [5]

**Dihydrofolate ($H_2PteGlu$)**

NADPH ↓ [6]

**Tetrahydrofolate ($H_4PteGlu$)**

ATP, glutamate ↓ [7]

**Tetrahydrofolate polyglutamate ($H_4PteGlu_n$)**

Fig. 2. Principal steps in the pathway for $H_4PteGlu_n$ biosynthesis. The dihydropteroate precursor, 2-amino-4-hydroxy-6-pyrophosphoryl-methyl dihydropteridine, condenses with *p*-aminobenzoate in a reaction catalyzed by dihydropteroate synthase [4]. Other key enzymes are dihydrofolate synthetase [5], dihydrofolate reductase [6], and folylpolyglutamate synthetase [7].

**Formate**

ATP, $H_4PteGlu_n$ ↓↑ [1]

**Purines ← 10-HCO-$H_4PteGlu_n$ → Formylmet-tRNA**

$-H_2O$ ↓↑ [2]

**5,10-$CH^+$-$H_4PteGlu_n$**

NADPH ↓↑ [3]

**dTMP [6] ← 5,10-$CH_2$-$H_4PteGlu_n$ ↔ Gly [7] & Ser [8]**

NADH ↓ [4]

**5-$CH_3$-$H_4PteGlu_n$**

Homocysteine ↓ [5]

**Methionine**

Fig. 3. Major reactions for the generation and utilization of one-carbon units. The major enzymes are: [1], 10-formyltetrahydrofolate synthetase; [2], 5,10-methenyltetrahydrofolate cyclohydrolase; [3], 5,10-methylenetetrahydrofolate dehydrogenase; [4], 5,10-methylenetetrahydrofolate reductase; [5], methionine synthase; [6], thymidylate synthase; [7], glycine decarboxylase; and [8], serine hydroxymethyltransferase.

where new information may provide avenues for future research on plants.

## IMPROVEMENTS TO METHODS FOR FOLATE ASSAY

### *The microbiological assay of folates*

A sensitive, microbiological assay of naturally occurring folates has been widely used for over 40 years. The procedure, modified for use in clinical and nutritional studies [4, 22, 23], remains the most specific and reliable assay of folates in tissue extracts [24]. This method has also been used in assays of plant extracts

[2, 4] where the major folates are glutamyl conjugates of $H_4$PteGlu [4]. Three assay bacteria, *Lactobacillus casei* ATCC 7469, *Enterococcus hirae* ATCC 8043, and *Pediococcus acidilactici* ATCC 8081 are generally employed. Growth of these strains requires small quantities of folate and modern assays can detect as little as 10 femtamoles of PteGlu. These bacteria generally show a variable, non-quantitative response to folylpolyglutamates [4]. Consequently, these folates are usually hydrolyzed to monoglutamates prior to assay [24]. Recent modifications to this assay include the use of 96-well microplates, plate readers and computerized processing of data to facilitate measurement of bacterial growth [24–26]. Cryo-protected *L. casei* cells have also been used to provide rapid and reproducible responses to added folate [25, 26]. Modified assays of this type have been used in studies of mammalian folate deficiencies [27]. Other recent work has used the standard microbiological assay to examine dietary folate requirements [28–30], the bioavailability of folates in man [31] and the synthesis of folates by *Euglena gracilis* [32, 33]. The microbiological assay has also been used to quantify mammalian folates [34, 35], following their separation by HPLC.

Crosti *et al.* [36] employed the microbiological assay to measure folates in cultured carrot cells. Cells were incubated with [$^3$H]-PteGlu and polyglutamyl folates were examined during the cell cycle. Although the major folates were highly conjugated, the polyglutamate/monoglutamate ratio decreased as the stationary phase was approached. Inhibitors of folate biosynthesis such as methotrexate and sulphanilamide also affected polyglutamate chain lengths. These folate changes during the cell cycle prompted the authors to suggest that derepression or repression of enzymes in the folate biosynthetic pathway must precede cell replication.

Imeson *et al.* [37] used a differential microbiological assay to determine the nature of C-1 substituted folates in whole-leaf and bundle sheath cell extracts of corn and sorghum. Bundle sheath cells of both species contained mainly (70–83%) formyl- and unsubstituted derivatives of $H_4PteGlu_n$. In whole-leaf extracts, these derivatives accounted for 35–40% of the total folate pool and 5-$CH_3$-$H_4PteGlu_n$ was the principal folate. This latter derivative was a minor component in the bundle sheath cells which had little ability to synthesize methionine from formate, glycine or serine [37].

### *HPLC analyses of folate derivatives*

In the last decade, HPLC methods have been used for the isolation and assay of folate derivatives. Initially, these methods were of limited value as the folate pools of most species were below the detection limits of standard HPLC systems [3, 4]. However, recent modifications allow the concentration of endogenous folates prior to HPLC. For example, folates of cultured mammalian cells were concentrated on a $C_{18}$ Sep-Pak cartridge prior to HPLC on a $C_8$ μBondapak column [38]. Selhub and coworkers [39–41] used affinity chromatography to concentrate and purify biological folates prior to HPLC analyses. In this work, a purified milk folate-binding protein, covalently linked to Sepharose, was used to selectively retain folylmono- and folylpolyglutamates [39]. Quantitative recovery from the column was achieved with 0.02 M trifluoroacetic acid and HPLC analysis used reverse-phase ion pair chromatography with diode array peak detection [40]. Absorption signals at 350 nm identified PteGlu and $H_2$PteGlu derivatives whereas those at 258 nm were used for 10-HCO-$H_4$PteGlu derivatives. This method has been used to examine the distribution of folylpolyglutamates in rat tissues [41]. A comprehensive review of this combined affinity, ion pair chromatographic procedure [42] suggests that it could be effective in the analysis of plant folates.

Analysis of folate polyglutamates has also been facilitated by the development of methods for folate cleavage to the corresponding *p*-aminobenzoylpolyglutamate (*p*-ABAGlu$_n$) derivatives [3, 4]. The method of Shane [43] employs cleavage conditions that have been optimized for each type of one-carbon derivative found in biological samples. The resulting *p*-ABAGlu$_n$ derivatives are separated by HPLC after being purified as azo dyes of naphthylethylene diamine. The folate pools of plants [44–46] and fungi [47] have been examined using this method. For example, Imeson *et al.* [44] reported that the major polyglutamates of pea cotyledons, leaves and chloroplasts were tetra- and pentaglutamates. Similar data were obtained for pea leaf mitochondria [45]. A survey of different plant tissues [46] showed that polyglutamate chain lengths are variable. Thus extracts of cauliflower and broccoli florets contained folates ranging from di- to octaglutamates. In carrot root, about 70% of the recovered folates were diglutamates. Carboxypeptidase treatments, prior to cleavage and HPLC analysis, showed that the folates, like those of other species [7], contained γ-glutamyl linkages. A differential cleavage procedure [48] showed that the formyl- and methyl-folates of broccoli were mainly (80%) diglutamates, but the methylene and unsubstituted folates were principally (78%) hexaglutamates [46]. This suggests that plant folates of differing degrees of glutamyl conjugation may participate in different pathways of one-carbon metabolism. This possibility warrants further study as it could be an important basis for regulation of one-carbon flux through the folate-dependent pathways of the plant cell.

HPLC analyses have also aided studies of folate biosynthesis in plants. In peas seeds, [$^{14}$C]*p*-ABA is readily incorporated into pentaglutamyl folates [44], but in broccoli seedlings this folate precursor does not fully attain equilibrium with the polyglutamate pools after a 48-hr pulse feeding [46]. In cultured *Datura* cells, [$^{14}$C]*p*-ABA is mainly incorporated into hexa-

glutamyl folates and this is not affected when the growth media is supplemented with products of one-carbon metabolism [49].

*Folate assays based on enzyme-catalyzed reactions*

Folates have recently been examined by highly specific and sensitive enzyme-based assays. A method developed by Priest and coworkers [50, 51] depends on the separation of ternary complexes, formed by reaction of 5,10-$CH_2$-$H_4$PteGlu$_n$ with thymidylate synthase and fluorodeoxyuridylate (FdUMP). Other reduced folates are estimated after enzymic conversion to 5,10-$CH_2$-$H_4$PteGlu$_n$. The use of [$^3$H]-FdUMP provided a sensitive quantitative assay, that for cultured hepatoma cells, was in close agreement with data obtained by HPLC [52]. Schirch's laboratory [53] has developed an enzyme assay for measuring the concentration of various $H_4$PteGlu$_n$ derivatives. Initially, folates in biological extracts were converted enzymically into derivatives that are substrates of mammalian $C_1$-THF synthase and serine hydroxymethyltransferase. The latter proteins, added in excess, catalyze a cycle of reactions that generates substrate quantities of NADPH. The rate of NADP reduction was shown to be a linear function of $H_4$PteGlu$_n$ concentration between 10 and 200 nM. This assay showed that 5-HCO-$H_4$PteGlu$_n$, the principal folate of *Neurospora* conidiospores, was rapidly converted into other folates during spore germination [53].

*The radioassay of plant folates*

There has been considerable work on the development of reliable folate assays based on the use of binding proteins [4]. When such binding assays include a radiolabelled folate ligand it is possible to detect and quantify the biological levels of folate normally found in blood serum. Several commercial folate radioassay kits are now available and are widely used in clinical laboratories. However, a critical assessment of these methods [54] suggested that they may have limited value in the measurement of polyglutamyl folates. In this regard, the affinity shown by the binding protein for conjugated folates is often distinct from that shown for the radiolabelled, folate ligand. It is therefore important to compare data obtained by radioassay with that obtained by the standard microbiological assay procedure. In recent work, Neuburger *et al.* [55] used a radioassay method to measure the folate content of pea leaves. The average value of 0.5 $\mu$g folate g fresh weight$^{-1}$ was in agreement with earlier data [4] obtained by microbiological assay. These workers also detected significant levels of folate in extracts of pea leaf mitochondria. Although individual folates were not characterized in this study, the size of this folate pool, as measured by radioassay [55], was comparable to that detected in earlier HPLC analyses [45]. Despite the apparent efficacy of this radioassay method, we are of the opinion that a detailed assessment is needed before this procedure can replace standard microbiological assay methods.

*The discovery of novel folates in bacteria*

The majority of species contain folates with the basic structure depicted in Fig. 1. Studies of *Escherichia coli* by Ferone *et al.* [56, 57], however, showed that the polyglutamate chain contains $\alpha$- and $\gamma$-glutamyl linkages. These workers used [$^{14}$C]*p*-ABA feedings, folate cleavage and HPLC to examine the folate pool of this bacterium. Mass spectrometry, chiral amino acid analyses and peptidase digestion experiments were used to examine the structure of the cleavage products. Evidence was obtained for folypolyglutamates with up to eight glutamate residues. The first three of these residues were clearly $\gamma$-glutamyl linked, as in other species; but residues 4–8 were linked at the $\alpha$-carboxyl of the preceding glutamate. A folylpoly-$\alpha$-glutamate synthetase that lacked conventional FPGS activity was subsequently isolated from these *E. coli* cells [57].

The methanogenic archaebacteria contain a modified folate, methanopterin, that acts as a C-1 carrier in the reduction of carbon dioxide to methane [58]. This pterin and other modified folates appear to support the conventional pathways of one-carbon metabolism in these bacteria. In this regard, normal folates are either absent or present at very low levels in these species [59]. On the other hand, White [60] examined several species of thermophilic bacteria and reported elevated levels of normal and modified folates. The chemical structures of the latter were examined in *Thermococcus litoralis* and shown to contain a ribose core whose 5 position was $\beta$-linked to the C-1 of a poly-$\beta$-(1 $\rightarrow$ 4) *N*-acetylglucosamine entity [61]. In *Sulfolobus solfataricans*, a modified folate contains 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane [62].

## STUDIES OF FOLATE BIOSYNTHESIS

*Dihydropteroate synthase (DHPS)*

Knowledge of plant folate biochemistry has been advanced by recent work on the biosynthetic pathway (Fig. 2). The 7,8-dihydropteroate synthase (DHPS) reaction (eqn. (1)) is strongly inhibited by sulphonamides [63]. In some bacteria, resistance to these drugs

2-amino-4-hydroxy-6-pyrophosphoryl-methyldihydropteridine + *p*-ABA = dihydropteroate + $PP_i$ (1)

is associated with R-plasmids encoding sulphonamide-insensitive DHPS proteins [63, 64]. These plasmids have been used to generate transgenic sulphonamide-resistant tobacco plants [65]. In these

studies, the bacterial DHPS protein was fused to the transit peptide of pea Rubisco. When incubated with isolated chloroplasts, the bacterial protein was transported to the stroma and converted to an enzymically active form. Transgenic plants, expressing this gene, grew in the presence of sulphonamide and this ability was transmitted to progeny as a single Mendelian dominant character. It is clear that this chimeric gene has value as a marker of plant transformation. These studies also raise the possibility that a major step of folate biosynthesis in the transgenic plants was mediated by a bacterial form of DHPS. It follows that the DHPS of bacteria and higher plants may have conserved structures that have been little altered by evolution. Base sequencing of these genes would provide information on this possibility.

The DHPS activity of pea leaves and potato tubers is mainly mitochondrial and gel filtration showed that the pea protein coeluted with hydroxmethyldihydropterin pyrophosphokinase (Fig. 2, reaction 3) activity [55]. Further purification is required to determine whether these activities occur as a bifunctional complex as was claimed in earlier work [66].

*Dihydrofolate synthetase (DHFS)*

The formation of dihydrofolate ($H_2$PteGlu) is catalyzed by DHFS (eqn (2)), a reaction that involves an ATP-dependent addition of L-glutamate to dihydropteroate. The bacterial enzyme has been extensively studied [67–70]. In *E. coli* and *Corynebacterium*,

$$H_2\text{Pte} + \text{L-Glutamate} + \text{ATP} = H_2\text{PteGlu} + \text{ADP} + P_i \quad (2)$$

DHFS and folylpolyglutamate synthetase (FPGS) are catalytic entities of a bifunctional protein, encoded by the *folC* gene [68–70]. The enzyme of higher plants has received less study, but is associated with mitochondria [55] and has catalytic properties [71] like those of the bacterial enzyme. However, plant DHFS and FPGS activities are probably not structurally associated as chromatography of pea cotyledon extracts resulted in distinct peaks of DHFS and FPGS activity [72]. In this study, proteolysis was minimized by the inclusion of benzamidine and PMSF in the extraction and isolation buffers.

The DHFS of *Neurospora* was recently purified over 1000-fold [72]. The resulting monomeric protein ($M_r$ = 52 000) lacked ability to glutamyl conjugate $H_4$PteGlu but displayed other properties like those of bacterial and higher plant DHFS. Under the conditions employed, DHFS and FPGS activities did not copurify and a distinct FPGS protein ($M_r$ = 66 000) was isolated which lacked ability to glutamyl conjugate $H_2$Pte. In *Saccharomyces*, separate DHFS and FPGS genes, located on different chromosomes, have been detected [73]. Thus the structural organization of DHFS and FPGS in bacteria and fungi is fundamentally different. The reason for these differences is not clear. Conceivably, the production of separate proteins to mediate these key reactions may facilitate a transcriptional control of folate biosynthesis in fungi.

*Dihydrofolate reductase (DHFR) and its association with thymidylate synthase (TS)*

The formation of $H_4$PteGlu, catalyzed by DHFR (eqn (3)), has received detailed study [74] and represents the target of several drugs used in cancer chemotherapy. In

$$H_2\text{PteGlu} + \text{NADPH} = H_4\text{PteGlu} + \text{NADP} \quad (3)$$

animals, fungi and bacteria, DHFR is of relatively low molecular weight ($M_r$ = *ca* 22 000). The DHFR gene has been cloned and the effects of site-directed mutagenesis have been assessed in a number of laboratories [74–82].

The study of plant DHFR has been actively pursued [83] and there is good evidence that many species express DHFR proteins that are monofunctional. These proteins have different properties [83] that may reflect the species and tissue examined or the method of extraction employed. In addition, some higher plants, protozoans and the green alga *Scenedesmus* [55, 83–90], express a bifunctional DHFR/TS protein (eqn. (4)).

$$5,10\text{-}CH_2\text{-}H_4\text{PteGlu}_n + \text{dUMP} = H_2\text{PteGlu}_n + \text{dTMP} \quad (4)$$

As DHFR has a major role in the reduction of $H_2$PteGlu$_n$, that arises during thymidylate biosynthesis [74], it follows that DHFR/TS complexing probably facilitates channeling of folate needed for the generation of 5,10-$CH_2$-$H_4$PteGlu$_n$. For example, a homodimeric DHFR/TS complex (subunit $M_r$ = 62 000) occurs in pea leaf mitochondria [55]. The distribution of these activities suggested that mitochondria may be a major site for folate and thymidylate biosynthesis. This mitochondrial TS activity, like that of other species [3], displays a preference for polyglutamate substrates and is inhibited by $H_2$PteGlu$_5$ when this product accumulates in the absence of NADPH. The *dhfr-ts* genes that encode DHFR/TS complexes in *Arabidopsis thaliana* [84], *Daucus carota* [85] and *Glycine max* [86] have been cloned and sequenced. Coding regions for DHFR and TS are located at the N- and carboxy termini, respectively. The deduced amino acid sequences of these domains show striking similarities to those reported for the monofunctional enzymes of other eukaryotic species. The studies of *A. thaliana* [84] and later work on carrot [89] also provide evidence for two distinct genes each capable of encoding a DHFR/TS complex. As plant cells require DNA precursors in nuclei, mitochondria and chloroplasts it is conceivable that different DHFR/TS complexes may occur in these compartments. Some support for this possibility has been provided by work on carrot where

immunogold was used to demonstrate a plastidial localization of a DHFR/TS complex [89]. In this study, Cella's group also analyzed the 5′ end of carrot *dhfr-ts* gene transcripts and detected a start site of a transit peptide. The use of polyclonal antibodies raised against these interesting proteins [88] and the feasibility of conducting site-directed mutagensis of the *dhfr-ts* genes should stimulate further work in this area.

With the exception of some bacteria [15, 74], DHFR proteins are strongly inhibited by antifolates such as methotrexate (MTX). These antifolates, by blocking DHFR and other folate-dependent enzyme activities, typically result in $H_4PteGlu_n$ deficiencies. In mammalian cells, MTX cytotoxicity is enhanced by its glutamyl conjugation, a reaction catalyzed by FPGS [15]. In contrast, there have been few studies of $MTXGlu_n$ formation by plant cells [49] or of the folate deficiences [36, 37] caused when such cells are exposed to MTX or other antifolates. Despite this, several laboratories have described the isolation and characterization of MTX-resistant plant cell lines [49, 83, 91–95]. As in cultured mammalian cells, MTX resistance in plants may be due to increased DHFR expression [83], decreased transport of the antifolate [92, 93], or the production of MTX-binding proteins [83, 92, 94]. In a cell line of *Datura*, MTX resistance may be associated with increased levels of γ-glutamyl hydrolase [49, 95], an enzyme that may indirectly affect the retention of MTX polyglutamates.

Further studies of MTX resistance in plant cells could provide important information on the possible physiological roles of folate/antifolate binding proteins. These proteins have importance in the assimilation, distribution and retention of folate in a variety of mammalian tissues [11]. There is, however, little information on this topic for plants.

### *Folylpolyglutamate synthetase (FPGS)*

Most folate-dependent enzymes exhibit a preference for γ-glutamyl conjugated folate substrates [7, 14] and these polyglutamates are the principal forms of folate in living cells [4, 20]. The importance of these derivatives in one-carbon metabolism is also supported by studies of mutant cell lines. Thus lesions which affect the expression of FPGS (eqn (5)), result in auxotrophies for products of one-carbon metabolism [96–100].

$$H_4PteGlu + n\text{ATP} + n\text{Glu} = H_4PteGlu_{n+1} + n\text{ADP} + nP_i \quad (5)$$

As noted earlier, in some bacteria, the DHFS and FPGS reactions are catalyzed by a bifunctional protein encoded by the *folC* gene [68–70], but in mammals [101–109], fungi [110] and plants [111–113] polyglutamate biosynthesis is catalyzed by monofunctional, monomeric proteins ($M_r$ = approx. 60 000) that have several properties in common. These include alkaline pH optima, fairly broad specificity for folate substrates, and dependency on Mg-ATP. The extent of glutamyl conjugation is usually affected by incubation time and the concentration of folate substrate [7, 106]. Thus, non-saturating amounts of $H_4PteGlu$ give polyglutamate chain lengths that approach those of the endogenous folate pool. In studies of pea cotyledon FPGS [44], reaction systems containing 100 μM $H_4PteGlu$ were incubated for 2 h at 37° and produced di- (93%), tri- (5%) and tetraglutamates (2%). Longer incubations and folate at 10 μM resulted in a shift in this distribution to favour the longer chain lengths.

The majority of FPGS assays are based on measurement of radiolabelled glutamate incorporated into $H_4PteGlu$, $H_4PteGlu_2$, or $5,10\text{-}CH_2\text{-}H_4PteGlu$ [68, 106, 114] with the labelled polyglutamates being isolated on DEAE-cellulose. Modification of this assay involves conversion of the major product, $H_4PteGlu_2$, to $5,10\text{-}CH_2\text{-}H_4PteGlu_2$, which is incubated with excess TS and FdUMP [115]. The resulting polyglutamate complex is recovered by centrifugation through small columns of Sephadex G-50. A novel *in situ* FPGS assay [100] uses autoradiography and [6-$^3$H]deoxyuridine to indirectly monitor the formation of $5,10\text{-}CH_2\text{-}H_4PteGlu_n$ for the TS reaction.

A number of workers have now cloned and sequenced the FPGS gene. For example, the *E. coli folC* gene that encodes DHFS/FPGS was cloned [68] and used to obtain the highly purified protein. Work on the base sequence of *folC* identified an upstream gene that may regulate FPGS expression [116] and site-directed mutagenesis affected the affinities shown for FPGS substrates [117]. On the other hand, progressive deletions from the 5′ and 3′ ends of *folC* suggested that bacterial DHFS/FPGS has a single catalytic site for both activities [118]. It is clear that mutated forms of *folC* resulted in low levels of FPGS expression and growth requirements for methionine, glycine, thymidine, adenosine and pantothenate [119]. Cloning of the *L. casei* FPGS gene has also allowed extensive work on the purification, crystallization and characterization of FPGS protein [120, 121].

There have been detailed studies of the human FPGS gene [100, 122–126]. In this regard, an FPGS-deficient cell line (AUX B1) was transfected with human genomic DNA [122] and the resulting transformants expressed FPGS activity, forming long-chained polyglutamates *in vivo*. However, these cells and others expressing the *E. coli* FPGS gene had varying abilities to retain folates [100]. Cells expressing the bacterial, $Glu_3$-forming enzyme retained less folate than cells expressing comparable levels of the human enzyme. These AUX B1-*E. coli* transformants were unable to synthesize glycine *de novo* [123] due to a lack of mitochondrial FPGS activity [124]. In contrast, AUX B1-human transformant cells expressed both mitochondrial and cytosolic FPGS activities and synthesized glycine for growth. This suggests that in eukaryotes, both intracellular forms of FPGS are products

of the same nuclear gene. These studies also showed that the mitochondrial and cytosolic folate pools were not in equilibrium. Consequently, the AUX B1-*E. coli* transformants contained mitochondria that were seriously deficient in polyglutamates. Plant cells appear to express cytosolic [127] and mitochondrial [55] FPGS activities, but their relative contributions to folate biosynthesis and one-carbon metabolism have not been elucidated.

Copies of the *Neurospora* FPGS gene (*met*-6), have been introduced into polyglutamate-deficient mutants of this fungus [47, 128]. The resulting transformants [47] expressed FPGS activity and synthesized long-chained polyglutamates *in vivo*. Sequencing of this gene [129] provided evidence for a product with homology to four other FPGS proteins. DNA sequence analysis of the *met*-6 mutant allele provided evidence for a thymine to cytosine transition in a highly conserved region of the gene [129]. This change would result in replacement of wild type serine for proline in the polyglutamate-deficient *met*-6 mutant. The FPGS gene of higher plants has not been cloned or sequenced.

## THE GENERATION OF ONE-CARBON UNITS

### *The trifunctional* $C_1$*-tetrahydrofolate synthase of yeast*

The generation and interconversion of $C_1$-substituted folates (Fig. 3) involves a number of individual enzyme-catalyzed reactions [3]. In most species, 10-HCO-$H_4$PteGlu$_n$ can be generated by the activation of formate (Fig. 3, reaction 1) or from serine via 5,10-$CH_2$-$H_4$PteGlu$_n$. Besides use in purine and formylmethionyl-*t*RNA biosynthesis, 10-HCO-$H_4$PteGlu$_n$ can be reduced to the corresponding methylene and methyl derivatives (Fig. 3, reactions 2–4). In yeast and vertebrate species, the formation of 10-HCO-$H_4$PteGlu$_n$ and its conversion to 5,10-$CH_2$-$H_4$PteGlu$_n$ are catalyzed by a trifunctional peptide called $C_1$-tetrahydrofolate synthase ($C_1$-THF synthase) [5]. This protein has distinct domains for 10-HCO-$H_4$PteGlu synthetase (eqn. (6)), 5,10-$CH^+$-$H_4$PteGlu cyclohydrolase (eqn. (7)) and 5,10-$CH_2$-$H_4$PteGlu dehydrogenase (eqn (8)) activities [3, 5]. Chemical treatments and immuno-titration studies of the yeast enzyme provided evidence for an overlapping cyclohydrolase:dehydrogenase site that was independent of the synthetase active site [130]. The affinity of $H_4$PteGlu$_n$ for the synthetase site increases with polyglutamate chain length [131]. Thus the monoglutamate ($K_m$ = 265 $\mu$M) is a poorer substrate than the pentaglutamate ($K_m$ = 0.24 $\mu$M). In contrast, the cyclohydrolase and dehydrogenase reactions do not show a preference for polyglutamate substrates [131]. MacKenzie [5] has reviewed the catalytic and physical properties of this trifunctional protein including the channeling of folates between the cyclohydrolase and dehydrogenase reactions.

$$\text{Formate} + H_4\text{PteGlu}_n + \text{ATP} = \text{10-HCO-}H_4\text{PteGlu}_n + \text{ADP} + P_i \quad (6)$$

$$\text{10-HCO-}H_4\text{PteGlu}_n + H^+ = \text{5,10-CH}^+\text{-}H_4\text{PteGlu}_n + H_2O \quad (7)$$

$$\text{5,10-CH}^+\text{-}H_4\text{PteGlu}_n + \text{NADPH} = \text{5,10-CH}_2\text{-}H_4\text{PteGlu}_n + \text{NADP} \quad (8)$$

Yeast $C_1$-THF synthase occurs as a cytosolic protein encoded by the *ade*-3 gene [132–135] and as a mitochondrial protein encoded by the *MIS* 1 gene [21, 136,137]. Both of these genes have been cloned and sequenced [133, 137]. The region of the *ade*-3 gene that encodes the synthetase active site has homology with a bacterial gene that encodes a monofunctional 10-HCO-$H_4$PteGlu synthetase protein [138]. The yeast *MIS* 1 gene [137] has an open reading frame for a protein of $M_r$ = 106 235 that includes a nucleotide sequence (residues 1–34) for a putative mitochondrial signal peptide. Without this leader sequence, the 'mature' protein had a mass in agreement with that determined by electrophoresis [136].

The roles of yeast $C_1$-THF synthase isoenzymes in one-carbon metabolism have also been examined [21, 134–137, 139]. Theoretically, 10-HCO-$H_4$PteGlu$_n$ needed for cytoplasmic purine biosynthesis might arise from 5,10-$CH_2$-$H_4$PteGlu$_n$ or from formate. The metabolic origins of this formate might include the mitochondrial degradation of sarcosine or serine to 10-HCO-$H_4$PteGlu via the intermediary formation of 5,10-$CH_2$-$H_4$PteGlu$_n$ [21]. Conceivably, the mitochondrial isoenzyme, by reversal of its 10-HCO-$H_4$PteGlu synthetase activity, might produce formate for export to the cytoplasm. To assess the importance of this mitochondrial pathway, Shannon and Rabinowitz [137] disrupted the *MSI* 1 gene *in vitro* and introduced copies of the non-functional gene into the yeast chromosome. The resulting mutants grew on a simple medium containing a non-fermentable carbon source suggesting that the mitochondrial form of $C_1$-THF synthase was not essential for high rates of purine synthesis or for the initiation of mitochondrial protein synthesis. On the other hand, deletion of the *ade*-3 gene resulted in an absolute requirement for adenine. However, point mutations that inactivated all three activities of $C_1$-THF synthase did not result in adenine auxotrophy. This raises the possibility that the synthase protein may be a structural component of a metabolon for purine biosynthesis in *Saccharomyces* [139]. These data also imply that 10-HCO-$H_4$PteGlu$_n$, needed for purine synthesis may be derived by a cytosolic pathway involving a monofunctional NAD-dependent 5,10-$CH_2$-$H_4$PteGlu$_n$ dehydrogenase [139–141]. Support for this contention and for catalytic and non-catalytic roles for the soluble $C_1$-THF synthase have recently been reported by Appling's group [139].

The role of $C_1$-THF synthase has also been assessed using $^{13}$C-labelled formate. Yeast cells carrying genetic deletions for either the cytoplasmic or the mito-

chondrial isoenzyme used formate for serine synthesis which occurred primarily in the cytosol [142, 143]. Strains expressing only the mitochondrial isoenzyme produced [2-$^{13}$C]glycine and [3-$^{13}$C]serine from [$^{13}$C]formate. Thus one-carbon units generated from formate by both forms of the synthase, were reduced to 5,10-$CH_2$-$H_4$PteGlu$_n$ prior to incorporation into glycine and serine. Detailed $^{13}$C NMR analysis of purine synthesis showed that the mitochondrial isoenzyme also had an important role in generating formate [144]. Approximately 25% of this formate was transported to the cytosol where it was incorporated into purines via 10-HCO-$H_4$PteGlu$_n$. These workers have also applied $^{13}$C NMR methods to examine the metabolic fates of several other one-carbon donors in yeast [145].

*Higher plant* 10-*formyltetrahydrofolate synthetase activity*

Higher plant tissues contain relatively high levels of 10-HCO-$H_4$PteGlu synthetase activity [2, 3], but until recently it was not clear if this activity occurred as part of a trifunctional complex as in yeast and vertebrate species. Nour and Rabinowitz [146, 147] isolated the spinach leaf synthetase in the presence of protease inhibitors and obtained evidence for its monofunctional nature. The homogeneous, dimeric protein (subunit $M_r = 67\,000$) lacked 5,10-$CH_2$-$H_4$PteGlu dehydrogenase and 5,10-$CH^+$-$H_4$PteGlu cyclohydrolase activities. Furthermore, antibodies raised to this synthetase protein caused an immuno-inactivation and immuno-precipitation of synthetase activity in crude leaf extracts without affecting dehydrogenase or cyclohydrolase activities. The subsequent isolation and sequencing of a *c*DNA encoding this protein [147] showed 54–65% homology with the synthetase domains of yeast and mammalian $C_1$-THF synthases. Kirk *et al.* [148] purified a monofunctional synthetase protein from pea cotyledons that was homodimeric (subunit $M_r$ of 56 000) and that strongly cross-reacted with polyclonal antibodies raised to the spinach enzyme. The pea synthetase lacked dehydrogenase and cyclohydrolase activites but its affinity for (6S)-$H_4$PteGlu$_n$ substrates increased with the degree of glutamyl conjugation. The affinities for formate and ATP were also enhanced in the presence of these polyglutamate substrates. In this respect, the plant protein has similar properties to the synthetase domain of mammalian and yeast $C_1$-THF synthases.

The synthetase activity of pea cotyledons is mainly associated with the cytosolic fraction but smaller amounts of activity are also detected in the mitochondrial fraction [148]. The latter activity may have importance in the generation of formylmethionyl-*t*RNA [3] and could conceivably contribute 10-HCO-$H_4$PteGlu$_n$ for use in other folate-dependent pathways within the mitochondria. On the other hand, the major metabolic function of the cytoplasmic synthetase may include serine and purine biosynthesis but information on the flux of one-carbon units from the 10-HCO-$H_4$PteGlu pools of plants is still very limited. Thus [$^{13}$C]-formate supplied to whole *Arabidopsis* plants is readily incorporated into [3-$^{13}$C]-serine [149], but the intracellular location of the folates involved in this biosynthesis has not been elucidated.

*A bifunctional* 5,10-*methylenetetrahydrofolate dehydrogenase*:5,10-*methenyltetrahydrofolate cyclohydrolase*

In *E. coli* [150] and *Clostridium thermoaceticum* [151] the 5,10-$CH_2$-$H_4$PteGlu dehydrogenase and 5,10-$CH^+$-$H_4$PteGlu cyclohydrolase reactions (Fig. 3, reactions 2 and 3) are catalyzed by a bifunctional protein. A similar complex, in which the dehydrogenase is NAD-dependent, occurs in the mitochondria of transformed mammalian cells [152–154]. In plants, NADP-dependent dehydrogenase activity is associated with cyclohydrolase [155]. This bifunctional homogeneous protein ($M_r = 38\,500$) from pea seedlings lacks NAD-dependent dehydrogenase and 10-HCO-$H_4$PteGlu synthetase activities. Tryptic digestion of this protein resulted in coordinated losses of dehydrogenase and cyclohydrolase activity with NADP providing protection during short-term treatments. Both activities are competitively inhibited by dihydrofolates with the $K_i$ values of the dehydrogenase reaction showing that $H_2$PteGlu$_5$ is a more potent inhibitor than $H_2$PteGlu. This inhibition may be the basis for a control of 5,10-$CH_2$-$H_4$PteGlu metabolism particularly when demand for this folate in the TS reaction is high. An immunological survey of wheat, barley, corn and bean leaf extracts [155] suggested that this dehydrogenase–cyclohydrolase complex may be of common occurrence. In each of these species it was also clear that 10-HCO-$H_4$PteGlu synthetase activity was associated with a protein distinct from that containing dehydrogenase–cyclohydrolase. Based on these findings it appears that higher plants, unlike other eukaryotic species, do not express a trifunctional $C_1$-THF synthase protein.

*Glycine decarboxylase* (*GDC*)

The GDC reaction (eqn (9)) represents a major source of 5,10-$CH_2$-$H_4$PteGlu$_n$ in most prokaryotic and eukaryotic species [2, 6]. In animals, fungi and higher plants, the reaction is catalyzed by a complex of mitochondrial matrix proteins [6, 156, 157]. These include a dimeric, pyridoxal phosphate-binding protein (P-protein, $M_r = 94\,000$ subunit), a

$$\text{Glycine} + \text{NAD} + \text{H}_4\text{PteGlu}_n = 5,10\text{-CH}_2\text{-H}_4\text{PteGlu}_n + \text{NADH} + \text{CO}_2 + \text{NH}_3 \quad (9)$$

lipoic acid-containing protein (H-protein, $M_r = 15\,000$), a tetrahydrofolate-binding protein (T-protein, $M_r = 41\,000$) and a lipoamide dehydrogenase protein (L-protein, dimeric, $M_r = 60\,000$ subunit).

The importance of this reaction in photorespiration has stimulated work on the structure, function and biogenesis of GDC in higher plant mitochondria [156–159] where the complex contains one L-protein dimer, two P-protein dimers, 27 H-protein units and nine T-protein units [158].

In yeast the GDC reaction is reversible and cells expressing mitochondrial $C_1$-THF synthase activity incorporate [$^{13}$C]formate into [2-$^{13}$C]-glycine [142, 143]. Furthermore, cells supplied with [3-$^{13}$C]serine produced [2-$^{13}$C]glycine and [2,3-$^{13}$C]serine [142]. These data imply that the mitochondrial pool of 5,10-$CH_2$-$H_4$PteGlu$_n$, arising from formate or serine, is in equilibrium with C-2 of glycine and C-3 of serine.

In contrast, the GDC of pea leaf mitochondria favours glycine oxidation and a relatively large pool of 5,10-$CH_2$-$H_4$PteGlu$_n$ is maintained in the matrix space [160]. This folate, which may account for 65–80% of the folate pool, is thought to drive the serine hydroxymethyltransferase (SHMT) reaction in the direction of serine synthesis [161]. Although both enzymes have more affinity for polyglutamate than monoglutamate substrates [45] there is no direct evidence to suggest that channeling of 5,10-$CH_2$-$H_4$PteGlu$_n$ occurs between these closely related reactions [160]. Earlier studies [2] suggest that 2 mol of glycine are consumed for each mole of serine produced during photorespiration. However, it is not clear if part of the matrix pool of 5,10-$CH_2$-$H_4$PteGlu$_n$, formed during photorespiration, is converted to 10-HCO-, 5-HCO- and 5-$CH_3$-$H_4$PteGlu$_n$.

The mitochondria of light-grown pea leaves have 10-fold greater levels of GDC than mitochondria from etiolated leaves and this difference is reflected in the amounts of the four subunit proteins [159, 162]. This latter finding led to the isolation and characterization of the P-, H- and T-proteins of pea leaf mitochondria [163]. In this regard, monoclonal antibodies were used to isolate the L-protein, which had antigenic and molecular weight similarities to the lipoamide dehydrogenase associated with the pyruvate dehydrogenase complex.

The genes encoding GDC subunit proteins have been cloned in several laboratories. For example, Oliver's group [164] cloned and sequenced the *c*DNA encoding the H-protein in peas. The open reading frame encoded a 165 amino acid precursor protein whose N-terminus contained a putative mitochondrial leader sequence. Light-grown seedlings had significantly more *m*RNA for the H-protein than dark-grown plants. Transfer of the latter to white light enhanced the transcription of this mRNA for up to 24 h. Macherel *et al.* [165] also cloned *c*DNA for the H-protein. They identified a 34 amino acid, mitochondrial targeting peptide, and concluded that a lipoate-binding site occurred at lysine-63. These workers confirmed that light affected transcription of mRNA for the H-protein. The gene (*gdcH*) encoding the H-protein of pea contains three introns in the coding region and has important differences in organization when compared with genes of human and avian origin [166]. These included two transcriptional sites, one of which might be regulated by an 'initiator' element.

Rawsthorne and coworkers [167, 168] used a pea leaf *c*DNA library to isolate, clone and characterize the P-protein. A putative 86 amino acid leader sequence, probably required for mitochondrial import, was detected and the protein showed homology with that of chicken [167]. Strong homology was also apparent when *c*DNA for the P-proteins of *Flaveria pringlei* and pea were compared [168]. Similarly, the L-protein has homology with the lipoamide dehydrogenase that is associated with pyruvate dehydrogenase in yeast, *E. coli* and man [169]. Thus the L-protein may have a common function in the α-ketoglutarate dehydrogenase, pyruvate dehydrogenase and GDC reactions of plant mitochondria. This possibility is supported by Bourguignon *et al.* [170] who isolated and characterized a *c*DNA for the complete L-protein precursor. Immunological criteria as well as extensive Northern and Southern blot analyses showed that the L-protein was a functional component of several mitochondrial enzyme complexes.

Cloning and characterization of the T-protein have been accomplished by screening a λgt11 pea leaf *c*DNA library [171]. The resulting cDNA encoded a polypeptide of 408 amino acids with an N-terminal 30 amino acid presequence. The pure protein did not contain covalently bound folate [171]. However, it was clear that the levels of *m*RNA for this T-protein were 8–10-fold higher in green than etiolated leaves. This *m*RNA species and those for the P- and L-proteins were also detected in root tissues but mRNA for the H-protein was lacking. A cDNA for the T-protein in potato has also been sequenced [172]. In this species, Southern blot analyses of genomic DNA suggest that the protein is encoded by a single gene.

A number of detailed studies have focussed on the structure and reaction mechanism of GDC subunits [173–177]. For example, Neuburger *et al.* [173] isolated two catalytically active forms of the H-protein from pea mitochondria. One of these forms was a stable, methylamine intermediate that was separated, by ion exchange chromatography, from the oxidized form of the H-protein. The primary structure of this protein has been examined by mass spectrometry, tandem mass spectrometry, fast flow atom bombardment and electrospray ionization [174]. These studies identified a lipoic acid cofactor and a modified lysine residue that interacted with different active sites in the enzyme complex. Recently, the H-protein has been successfully crystallized [175] and used in a number of elegant studies to determine its X-ray crystal structure [176, 177].

The organization and expression of the genes encoding pea mitochondrial GDC have been examined by Turner *et al.* [178]. Restriction fragment length polymorphisms were used to determine the chromosomal location of these genes. Genes encoding the H-protein and SHMT showed linkage to the classical

group I marker i while those for the P-protein had linkage to the classical group I marker a. Genes for the L- and T-proteins were linked and probably situated on the satellite of chromosome 7. The mRNA species encoding SHMT and the subunits of GDC were strongly induced when etiolated pea plants were placed in light. In other work, Somerville's laboratory [179] isolated a novel photorespiratory mutant of *Arabidopsis thaliana* that complemented previously described *gld*1 mutants. The latter have lesions affecting the expression of GDC. This new locus, designated *gld*2, mapped to chromosome 5 but it was not ascertained whether it encoded for GDC subunits or for the transport of glycine into the mitochondrion.

*Serine hydroxymethyltransferase (SHMT)*

The SHMT reaction (eqn (10)) results in the interconversion of serine and glycine

$$\text{Serine} + \text{H}_4\text{PteGlu}_n = 5,10\text{-CH}_2\text{-H}_4\text{PteGlu}_n + \text{glycine} \quad (10)$$

and is a major source of 5,10-$CH_2$-$H_4PteGlu_n$ needed in the synthesis of purines, thymidylate, methionine and choline [6]. The enzyme has been purified and characterized from several prokaryotic and eukaryotic sources [3, 6]. Although mono- and polyglutamyl folates are substrates of the enzyme [14], the mammalian [180–182], fungal [53] and higher plant [45] SHMT proteins show greater affinities for conjugated folates.

There have been numerous studies of bacterial and mammalian SHMT proteins. For bacteria, these investigations have included cloning of the SHMT (*glyA*) gene in *Salmonella typhimurum* [183], and characterization of *E. coli* SHMT [184–186]. Mammalian SHMT isoenzymes have also been characterized in terms of catalytic properties, primary structure and sequencing of the encoding nuclear genes [181, 182, 187–195]. The SHMT proteins of *N. crassa* [53, 196, 197] and yeast [198–200] have also been studied extensively. Work on plant SHMT has tended to concentrate on the mitochondrial isoenzyme of photorespiring leaves [45, 160, 200–202]. Some of these major SHMT investigations of fungi and plants are reviewed in the following paragraphs.

In common with other eukaryotes [6], *Neurospora crassa* expresses cytosolic and mitochondrial SHMT proteins [3]. The former activity has been purified to apparent homogeneity, and like the mammalian enzyme, is a tetramer of identical subunits ($M_r$ = 54 000) [196]. The protein contains bound pyridoxal phosphate, catalyzes the cleavage of serine, allothreonine and 3-phenylserine, is stabilized to heat denaturation by 5-formyl-$H_4PteGlu_n$ and forms the latter folate when incubated with glycine and 5,10-$CH^+$-$H_4PteGlu_n$ [196]. This SHMT protein also displays greater affinity for $H_4PteGlu_n$ than the corresponding monoglutamate substrates [53]. Thus while the $K_m$ value for $H_4PteGlu$ is 30$\mu$M, the $K_d$ values for $H_4PteGlu_3$ and $H_4PteGlu_5$ are 0.2 and 0.1 $\mu$M, respectively. It is also clear that 5-formyl-$H_4PteGlu_n$ are slow binding inhibitors of the *Neurospora* SHMT and these folates may therefore have roles in controlling this key reaction of one-carbon metabolism. The *for* locus that encodes cytosolic SHMT in *N. crassa* has been cloned and sequenced [197]. This gene encodes a 479 amino acid peptide that has a primary structure like that of bacterial and mammalian SHMT proteins. There is also evidence for transcription factor binding sites upstream of the *for*$^+$ transcription start site [197].

Bognar and coworkers [198] isolated DNA fragments from *Saccharomyces cervisiae* that encode the mitochondrial (*SHM*1) and cytosolic (*SHM*2) forms of SHMT. A yeast genomic DNA library was screened for the intact genes using primers with sequences corresponding to the SHMT genes of *N. crassa* and mammals. The N-terminus of the *SHM*1 product had a leader sequence indicative of a mitochondrial transit peptide. This mitochondrial isoenzyme has importance in the generation of formate for export to the cytoplasm [199].

A mitochondrial SHMT protein (tetrameric, subunit $M_r$ = 53 000) has been isolated from pea leaves and purified to apparent homogeneity [178]. This matrix protein is associated with GDC so that the production and utilization of 5,10-$CH_2$-$H_4PteGlu$ during glycine oxidation reaches a steady state equilibrium. In leaf mitochondria, glycine cleavage and SHMT activity are probably closely linked via a soluble pool of 5,10-$CH_2$-$H_4PteGlu_n$. Thus both enzymes display a preference for folylpolyglutamates [45].

The SHMT of pea leaf mitochondria occurs as two forms that are not immunologically distinct [200]. This study also revealed a third, non-mitochondrial form of SHMT in leaf extracts. A *c*DNA for mitochondrial SHMT contains a reading frame for a mature protein of 487 amino acids ($M_r$ = 54 000) and for an N-terminal leader sequence of 27–31 amino acids, presumably involved in mitochondrial import. The deduced amino acid sequence of the mature pea SHMT is similar to those of the cytosolic [189] and mitochondrial [193] forms of rabbit SHMT. These homologies are also shared with the mitochondrial SHMT of *Solanum tuberosum* [201].

The mRNA encoding pea leaf mitochondrial SHMT is strongly induced by light and a decline in this mRNA species occurs when mature plants are placed in the dark [178]. Clearly, light controls SHMT expression in leaves but the existence of other regulatory mechanisms is uncertain. Leaves contain 5-HCO-$H_4PteGlu_n$ [2, 4] and this folate, formed from 5,10-$CH^+$-$H_4PteGlu_n$ by SHMT in presence of glycine [190], is a strong inhibitor of bacterial, fungal and mammalian SHMT proteins [53, 191]. Conceivably, a mitochondrial pool of 5-CHO-$H_4PteGlu_n$ in plants might exert some control over the SHMT reaction. There is, however, very little information on the one-

carbon substitution status of mitochondrial folates in plants or of the sensitivity of plant SHMT protein to inhibition by 5-HCO-$H_4$PteGlu$_n$. A report from Rao's laboratory [202] suggests that mung bean seedlings contain a protein-bound SHMT inhibitor. This compound, which contains carbohydrate, an *O*-amino group and vicinal diol groups, inhibited the SHMT proteins of sheep liver and mung bean seedlings. The occurrence of this compound in other plants and its intracellular location should be examined.

*Folate-dependent enzymes of purine synthesis*

The *de novo* synthesis of purines requires 10-HCO-$H_4$PteGlu$_n$ for the formylation of purine ring precursors [3, 203–205]. These folate-dependent reactions are catalyzed by glycinamide ribonucleotide (GAR) transformylase (eqn. (11)) and 5-phosphoribosyl-5-amino-4-imidazole carboxamide (AICAR) transformylase (eqn (12)), respectively.

5-Ribosylglycinamide + 10-HCO-$H_4$PteGlu$_n$
= 5-Phosphoribosyl-N-formylglycinamide
+ $H_4$PteGlu$_n$ (11)

5-phosphoribosyl-5-amino-4-imidazole carboxamide
+ 10-HCO-$H_4$PteGlu$_n$
= 5-Phosphoribosyl-5-formamido-
4-imidazole carboxamide + $H_4$PteGlu$_n$ (12)

In *Escherichia coli* and *Bacillus subtilis* two distinct GAR transformylases are expressed during *de novo* purine biosynthesis. A protein encoded by the *pur*N gene, catalyzes a 10-HCO-$H_4$PteGlu$_n$-dependent transformylation of GAR [206–208]. Both of these bacteria also express a formate-dependent GAR transformylase [207, 209, 210] that is encoded by the *pur*T gene. A 10-formyltetrahydrofolate hydrolase appears to generate formate for this latter reaction [211]. Sequence analyses of *pur*N and *pur*T suggest that the derived proteins are both monofunctional and distinct in primary structure [210].

In eukaryotes, 10-HCO-$H_4$PteGlu$_n$-dependent GAR transformylase activity is commonly associated with other enzymes of *de novo* purine biosynthesis [204, 205]. For example, an avian cDNA encodes GAR synthetase, GAR transformylase and 5′-phosphoribosylaminoimidazole (AIR) synthetase [212]. A similar trifunctional complex occurs in *Drosophila* [213]. In yeast there is genetic evidence that the *ade*-8 locus encodes a monofunctional GAR transformylase with the AIR and GAR synthetase activities being encoded by the *ade* 5,7 locus [204, 214].

Recent work on *Arabidopsis* suggests these three enzymes of purine biosynthesis are encoded by separate genes. Thus a *c*DNA library of *A. thaliana* provided separate clones for GAR transformylase, AIR synthetase and GAR synthetase [215]. Sequence analyses indicated that each gene product was monofunctional. In addition, the deduced amino acid sequence of each enzyme had a high degree of homology with the corresponding proteins of bacteria.

The enzymes of purine biosynthesis have also been recently examined in root nodules of *Vigna unguiculata* [216]. These workers showed that the mitochondria and plastids have a full complement of enzymes, including the two folate-dependent steps, to convert ribose-5-phosphate to inosine monophosphate. A cDNA encoding GAR transformylase has also been successfully isolated, cloned and sequenced from this plant source (Atkins 1996, personal communication).

To date there have been very few reports of AICAR transformylase (eqn. (12)) in higher plants. This enzyme occurs in mitochondrial and plastid extracts of cow pea nodules [216] as well as other legumes [3], but a detailed characterization of this plant protein has not been reported. It would also be of interest to determine whether plant AICAR transformylase activity is associated with inosine monophosphate cyclohydrolase activity [217–219] or with other enzymes of one-carbon metabolism [220] as reported for animal cells.

*Studies of other folate-dependent enzymes*

There have been several recent studies of 5,10-$CH^+$-$H_4$PteGlu$_n$ synthetase (eqn (13)) as it represents a metabolic route by which 5-HCO-$H_4$PteGlu$_n$ can participate in one-carbon metabolism [221]. As noted earlier, there is also evidence that the folate substrate of this irreversible reaction may be a regulator of one-carbon metabolism [222]. The synthetase is a monomeric protein ($M_r$ = 28 000) and has been purified to homogeneity from several eukaryotic and prokaryotic sources [222]. The rabbit liver enzyme has a

5-HCO-$H_4$PteGlu$_n$ + Mg-ATP
= 5,10-$CH^+$-$H_4$PteGlu$_n$ + Mg-ADP + $P_i$ (13)

polyglutamate binding site at lysine-18 [223]. To date, this enzyme has not been reported for plant species.

Living cells contain $\gamma$-glutamyl hydrolases (GGH) that cleave the polyglutamate chain of naturally occurring folylpolyglutamates [7]. In mammals, these enzymes have importance in the dietary uptake of folate by the small intestine. In other cells these enzymes may have roles in folate turnover [224]. A GGH protein has recently been characterized from pea seeds [225]. This cytosolic protein ($M_r$ = 55 000) was purified over 10 000-fold and shown to catalyze an endopeptidase reaction (eqn (14)) in which

$$PteGlu_3 + H_2O = PteGlu + Glu\text{-}Glu \quad (14)$$

$PteGlu_3$ was converted to PteGlu and diglutamyl peptide. Hydrolysis of the triglutamate substrate was strongly inhibited by $PteGlu_5$. The physiological roles of this enzyme in pea seeds have not been fully

assessed. These might include mobilization of folyl-polyglutamates, hydrolysis of regulatory folates such as 5-HCO-$H_4$PteGlu$_n$, and the hydrolysis of non-folate, $\gamma$-glutamyl peptides.

The regulation of several key folate-dependent enzymes has been examined in *Aspergillus nidulans* by Paszewski and coworkers [226–228]. Mutants impaired in the main pathway of cysteine synthesis had low levels of related folate-dependent enzymes such as DHFR, SHMT, methionine synthase and 5,10-$CH_2$-$H_4$PteGlu dehydrogenase [226]. It was suggested that regulatory mechanisms governing the formation of S-amino acids and folate metabolism were interrelated in this fungus. In other work [227], it was clear that the levels of these folate-dependent enzymes were elevated when homocysteine accumulation was favoured. On the other hand, elevated levels of methionine in the culture medium caused repression of these enzymes. Methionine supplements also lowered the levels of FPGS in a wild type strain but homocysteine, at 3 mM, raised FPGS activity by approximately 2.5-fold. Methionine supplements did not appear to affect GGH activity in this fungus. These studies draw attention to the central roles of methionine and homocysteine in regulation of folate-dependent pathways in *Aspergillus*. In contrast, there have been very few studies of methionine or formylmethionyl-tRNA biosynthesis in higher plants. The folate-dependent nature of these important reactions and their physiological control appears to warrant investigation.

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