Synthesis and turnover of folates in plants

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Abbreviations: ADC aminodeoxychorismate
C1 one-carbon
DHF dihydrofolate
EST expressed sequence tag
DHN dihydroneopterin
GTPCHI GTP cyclohydrolase I
HMDHP hydroxymethyldihydropteroate
PABA p-aminobenzoate
THF tetrahydrofolate

Key words: Compartmentation, folate biosynthesis, folate metabolism, genomics
Summary of recent advances

Folates are essential cofactors for one-carbon transfer reactions, which are central to plant metabolism. Plants synthesize folates de novo, and are key sources of dietary folate for humans. Plant folate research therefore impacts human nutrition. Biochemical progress, the sequencing of the Arabidopsis genome, and EST databases are now painting a clear picture of the plant folate synthesis pathway and its surprising compartmentation. Moreover, new analytical advances will help elucidate plant folate turnover and transport, which are practically unexplored.

Introduction

It is hard to overstate the biological importance of tetrahydrofolate (THF) and its derivatives, collectively termed folates. The metabolism of plants and all other organisms involves generation, interconversion, and donation of one-carbon (C1) units, and folates are essential cofactors for these reactions. THF is a tripartite molecule, comprising pteridine, p-aminobenzoate (PABA) and glutamate moieties, to which C1 units at various oxidation levels are attached at N5 of the pteridine ring, N10 of the PABA unit, or bridged between the two (Figure 1). Plant folates, like those of other organisms, have a short, γ-linked chain of glutamyl residues attached to the first glutamate, and most folate-requiring enzymes prefer such polyglutamylated forms [1*,2**]. Folates are present at trace levels in plants (typically ≤5 nmol g−1 fresh weight) and are all to varying degrees unstable, particularly to oxidative cleavage into pteridine and PABA-glutamyl fragments [2**]. This oxidative degradation is promoted by light. Folates are, however, stabilized in vivo when they are bound to proteins [2**].

Despite their low abundance and lability, plant folate pools support huge metabolic fluxes. In a normally photorespiring C3 leaf [3,4], if 30% of total leaf folate participates in the THF-mediated
glycine → serine reaction in mitochondria [5•], then a folate pool of ~1 nmol g⁻¹ must carry a one-carbon flux of 1-2 µmol g⁻¹ min⁻¹, i.e., the C₁ unit must turn over ~20-30 times per second. This is several times faster than the rate of ATP turnover in leaves, which is itself very rapid [6].

Besides being vital for plants, folates are required in human diets because – unlike plants – humans lack a complete folate synthesis pathway. As plant foods are the single most important folate source for humans, plant folate metabolism is of major nutritional significance. This is driving interest in engineering plants to raise dietary folate intake in rich and poor countries alike [2••,7]. Accordingly, this review covers recent progress in elucidating the plant folate synthesis pathway. We also highlight incipient developments in folate compartmentation, transport, and catabolism, and point to analytical advances that will help investigations of these crucial topics in plants.

**Enzymes and genes of folate synthesis**

The steps in the plant folate synthesis pathway are not all known, but are probably the same as in bacteria (Figure 2). The pteridine moiety – hydroxymethylglycerapterin – is formed from GTP, and PABA is formed from chorismate. The pteridine and PABA units are condensed, glutamylated, and reduced to give tetrahydrofolate, and the polyglutamyl tail is then added [8]. Of the eleven steps in Figure 2, nine are mediated by specific enzymes [8,9]. Plant genes and enzymes for the final five steps have been characterized and the enzymes have been shown to be mitochondrial, which contrasts with their primarily cytosolic location in other eukaryotes [4,10••,11,12].

Less is known for plants about the early steps that produce the pteridine and PABA moieties. However, genomic data show that plants have homologs of the pteridine synthesis enzymes GTP cyclohydrolase I (GTPCHI) and dihydropterin (DHN) aldolase. The deduced proteins apparently lack targeting signals so are presumably cytosolic (Figure 3a). Suprisingly, deduced plant GTPCHI polypeptides are twice the size of those in other organisms and have two diverged
GTPCHI-like domains. The two-domain protein encoded by a tomato cDNA (GenBank AY069920) has been confirmed to have GTPCHI activity by complementation in yeast and by enzyme assays (A Hanson, M Ziemak, E Quinlivan, J Gregory, unpublished data). Genomic data also show that plants have homologs of the first enzyme of PABA synthesis, aminodeoxychorismate (ADC) synthase, and that the plant proteins have domains corresponding to both subunits of the bacterial enzyme. Deduced plant ADC synthases have plastid targeting peptides; this is consistent with chorismate – the substrate for ADC synthase – being made in the chloroplast [13]. There are no obvious plant homologs of the second enzyme of PABA synthesis, ADC lyase, which mediates aromatization (Figure 2), but putative plant ADC synthases have two novel insertions that could contain an aromatization domain.

The enzymes of folate synthesis in *Arabidopsis* are each encoded by one to three genes (see bracketed numbers in Figure 2) and EST data indicate a similar situation in other plants. The abundance of ESTs for folate synthesis enzymes is anomalously high relative to the size of the folate pool. This is illustrated in Figure 3b, which compares folate pathway EST abundances with those for three enzymes each of NAD(P), purine, and pyrimidine synthesis. The folate ESTs are about as abundant as the others even though NAD(P) pools are ~10-fold larger than folate pools [14], and total purine and pyrimidine pools ~10²- to 10³-fold larger [15]. This pattern implies a high capacity for folate synthesis. Consistent with this, the activities of mitochondrial folate synthesizing enzymes are high enough to replace the mitochondrial folate pool around twice per hour [12].

**Folate compartmentation and transport**

Pea leaf folates were recently shown to be distributed in a 30:3:67 ratio between mitochondrial, chloroplast, and (cytosol + nucleus + vacuole) fractions [5*]. This resolves a controversy in which mitochondria were claimed to contain more [12] or much less [16] than 30% of the total. None of
these studies tested whether folates are stored in the vacuole. The folate-dependent mitochondrial enzymes involved in photorespiration (serine hydroxymethyltransferase and glycine decarboxylase) accumulate in green relative to etiolated leaves but the mitochondrial folate content does not rise even though the folate content of the whole leaf increases [5*]. The extra folate was suggested to be cytosolic [5*] but the vacuole is another possibility.

That folates are made solely in mitochondria but occur in other compartments implies there are folate transporters in the mitochondrial envelope. A human mitochondrial folate transporter has been cloned [17] and it is noteworthy that it has an *Arabidopsis* homolog (GenBank NP201439). Similarly, metabolic studies [18,19*] show that plant cells take up folates, implying the existence of plasma membrane transporters, and plants have homologs of mammalian multidrug resistance proteins (MRPs) that mediate folate efflux from cells [20]. An *Arabidopsis* MRP homolog has recently been shown to be a high-capacity folate transporter [21, P Rea, personal communication].

**Folate turnover and homeostasis**

Little is known about the rates and routes of folate breakdown in plants, but there are clues from comparative biochemistry, physiology, and genomics. Folate levels in harvested peas and spinach leaves dropped by 50% in ~5 days at 20°C [2**, which provides a *minimum* estimate of turnover, albeit under conditions highly unlike those in growing plants. It is noteworthy that this rate is more than ten-fold that of total body folates in humans [22*]. Folate catabolites and the reactions producing them have scarcely been studied in plants, but several enzyme-mediated catabolic reactions have been identified in other organisms [23,24] (Figure 1). These include: *(A)* Hydrolysis of the PABA-glutamate linkage, releasing glutamate from folates or PABA-glutamates; *(B)* Enzymatic cleavage between C9 and N10, yielding pterin and PABA-glutamyl moieties (oxidative cleavage also gives these products); and *(C)* Deamination of the pteridine ring.
The enzyme for reaction $A$, folylpoly-$\gamma$-glutamate hydrolase, has been reported from plants [1$^*$] and cloned from soybean [25$^*$]. Unexpectedly, the soybean protein was found to be extracellular, and this may be a general feature of plants as at least two of the three homologs of this protein in the *Arabidopsis* genome (GenBank AAC83041, AAC83042, AAD30570) have predicted secretory pathway signal peptides, and the data on the enzyme [1$^*$] fit with an extracellular location.

It was recently discovered that rat ferritin promotes reaction $B$ *in vitro* and *in vivo* in animal cells [26], and as plastids contain ferritin [27] this process could occur in plants. More recently still, it has been found that reaction $B$ is a secondary catalytic activity of mammalian 5-formyltetrahydrofolate cycloligase, an enzyme of folate-mediated $C_1$ metabolism [28]. Plants also have this enzyme [29], putatively located in mitochondria [30]. Lastly, it must be stressed that reaction $B$ can readily occur via chemical oxidation, so that reactive oxygen species or other oxidants formed in mitochondria or chloroplasts have the potential to cause significant folate breakdown. Moreover, since light promotes oxidative degradation of folates, leaf folates may be particularly vulnerable.

Folate homeostasis in plants is well documented in the sense that plant organs are known to have characteristic folate contents [1$^*$,2$^{**}$,5$^*$,31]. For instance, leaves typically have 2-5, roots 0.2-2, and fruits 0.05-0.5 nmol folate g$^{-1}$ fresh weight. But nothing is known about the balance between synthesis and breakdown that maintains folate levels or about how plants sense their folate status.

**Analytical advances**

Folate determination is complicated by the various possible $C_1$ substituents, polyglutamyl tail lengths, and states of oxidation of the pteridine ring. Moreover, many folates are susceptible to interconversion and oxidative degradation during extraction and analysis. The validity of analytical results depends heavily on use of appropriate methods of sample extraction, extract process-
ing, and folate quantification. Folates can be measured by ligand binding methods, high performance liquid chromatography (HPLC), and microbiological assays [2**,32,33]. Ligand-binding methods are widely used for clinical determination of plasma folate. However, as illustrated by Gambonnet et al. [5*], these methods are unsuited to determining total folate in plants due to differences among the various folates in affinity for the folate-binding protein used in the assay. Several well-validated HPLC methods allow purified folates to be separated and individually quantified by UV-absorbance [34,35,36,37,38], fluorescence [39,40], or electrochemical response [41**], or by microbiological assay of collected fractions [42,43**]. HPLC gives important quantitative and qualitative information, but suffers the problem of preparing reliable standards for unstable folates. Microbiological assays using *Lactobacillus rhamnosus* (formerly *L. casei*) are perhaps the most generally useful methods for routine assay of total folate because *L. rhamnosus* responds similarly to essentially all folate forms. It is prudent to verify that equivalent response occurs among the folate forms likely to be present. This is crucial in studies of folate partitioning among subcellular compartments whose predominant folates differ [16].

Regardless of the assay method used, effective extraction of folates is essential. Achieving this can be especially difficult for plants because of the potential for entrapment of folates in cell walls and starchy residues. Extraction in a neutral or slightly alkaline buffer containing ascorbate plus a thiol reagent appears to be most efficient for solubilization and stabilization of folates [35,44]. Standard composition tables [e.g., 31] may underestimate folate contents of plant materials because of inefficient extraction procedures [44]. Certain samples show a significant increase in measured folate after treatment with α-amylase and a nonspecific protease [37,38,45,46]. Enzymatic deglutamylation of polyglutamyl folates is another critical step of many folate assays. *L. rhamnosus* responds mainly to folates with ≤3 glutamate residues so that extracts must be
thoroughly deglutamylated to achieve a full assay response. Similarly, many HPLC methods require complete conversion to the monoglutamyl state [37,38,39]. Because plant constituents can inhibit the folylpoly-\(\gamma\)-glutamate hydrolases frequently used in folate assays [2,47] preliminary studies should be conducted to optimize incubation time and enzyme concentration.

A major advance is use of affinity chromatography with milk folate binding protein to purify and extract folates, followed by HPLC to allow their measurement as intact polyglutamates [34]. This basic approach has been extended by Selhub and associates [35,36,41**]. Multiwavelength UV absorbance detection permits reliable analysis of folates in many biological materials [34, 35,36], and multichannel electrochemical detection greatly increases sensitivity [41**]. In applications of these methods to plants, HPLC analysis has shown that banana fruit contains 5-methyl-THF in a range of polyglutamyl forms, while lima beans contain formyl, methyl and unsubstituted THF species almost exclusively in pentaglutamyl form [36]. Little is currently known about the role of polyglutamylation of folates in the overall folate metabolism of plants, and analyses of this type will be important in clarifying this phase of folate biochemistry.

In most plant tissues, 5-methyl-THF is the major folate form; however, the detection of 10-formyl-dihydrofolate and 10-formyl-folic acid in many plant-derived foods suggests that 10-formyl-THF is also prevalent [37,48]. For samples in which 10-formyl-THF is a significant portion of total folate, selection of preparative and HPLC separation steps that minimize acidic conditions becomes essential. Some methods of sample preparation favor extensive interconversion of 10-formyl-, 5,10-methenyl-, and 5-formyl-THF, and this should be examined carefully. Also, HPLC methods of folate analysis under acidic conditions yield excellent separation of most folates, but do not allow individual quantification of 5-formyl-THF and 10-formyl-THF [37,38,39]. HPLC separations conducted near neutral pH allow separation and analysis of both of these formyl fol-
ates [34,35,42]. HPLC of folates in subcellular fractions of pea leaves, coupled with microbiological assay, indicated that 5-methyl-THF was the major cytosolic folate, while 5-formyl-THF and THF were the major mitochondrial folates [16]. Another important analytical advance with major implications is a recent method that allows quantification of 5,10-methylene-THF [43**].

Conclusions and future directions

The enzymes of plant folate synthesis are (or soon will be) known and partially characterized. But we know nothing about how flux in the whole pathway is controlled, and such knowledge is critical for future engineering. It seems likely \textit{a priori} that substantial control resides in the committing enzymes of the pathway’s pteridine and PABA branches (i.e., GTP cyclohydrolase I and ADC synthase) and it is therefore interesting that in plants these proteins have unusual structures.

The transport of folates and their precursors within and between cells is clearly a frontier for investigation. The role of polyglutamylation – which in animals favors folate retention in cells but not mitochondria [17,20] – is especially intriguing given the finding that folate deglutamylation may occur extracellularly in plants [25*]. It is crucial to find whether folates can be transported into plant vacuoles. Such a process might be engineered to permit stockpiling of folates to high levels without metabolic side-effects. Folate turnover is another frontier. Calculations show that the C$_1$ unit of the mitochondrial folate pool turns over extremely fast in a photorespiring leaf. If, as seems likely [2**, folates are not channeled between glycine decarboxylase and serine hydroxymethyltransferase, then they may be significantly exposed to the mitochondrial matrix during photorespiration and hence be prone to chemical oxidation. Moreover, light itself enhances folate degradation. So perhaps folates are rapidly degraded in illuminated leaves, which would imply that they are also rapidly synthesized. The high EST abundance and relatively high activity of folate synthesis enzymes is consistent with this hypothesis.
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References and recommended reading

A historical perspective on plant folate research and an excellent overview of problems that remain to be solved. Valuable compilations of analytical data on plant folate pools are included.

Besides being an authoritative introduction to folate chemistry, analysis, biosynthesis, and metabolism, this article summarizes the nutritional aspects of folates and puts forward various metabolic engineering strategies for enhancing plant folate content.


Microbiological assays yielded data on folate levels in pea plant organs and on folate distribution between mitochondria, chloroplasts, and the rest of the cell. Mitochondria were shown to contain ~30% of the total folate in green leaves. Etiolated leaves and roots contained less total folate, but their mitochondrial folate levels were similar to green leaves.


Complementation of yeast mutants, enzyme assays, subcellular fractionation, and GFP fusions established that *Arabidopsis* has one DHF synthetase gene, whose product is mitochondrial, and three folylpolyglutamate synthetase genes, encoding mitochondrial, cytosolic, and chloroplastic isoforms.


Besides reporting cloning and characterization of novel methylenetetrahydrofolate reductases from *Arabidopsis* and maize, this study demonstrated that 14C-methyl labeled methylenetetrahydrofolate is readily absorbed and metabolized by *Arabidopsis* shoots.

20. Zeng H, Chen ZS, Belinsky MG, Rea PA, Kruh GD: **Transport of methotrexate (MTX) and folates by multidrug resistance protein (MRP) 3 and MRP1: effect of polyglutamylation on MTX transport.** *Cancer Res* 2001, **61**:7225-7232.


Stable isotope labeling data were combined with kinetic modeling to show, *inter alia*, that catabolism is a more important route of folate turnover than excretion. This paper illustrates the integrative and predictive value of models in studies of folate metabolism.


A folylpoly-γ-glutamate hydrolase-like protein was found in intercellular wash fluids of young soybean leaves, and shown to be partly bound to cell walls. Enzyme activity was not assayed. The corresponding cDNA encoded a secretory pathway signal peptide.


This is the latest in a series of adaptations of the powerful method in which folates are purified and concentrated by affinity chromatography, then analyzed in intact polyglutamyl form by HPLC. Monitoring with a multichannel electrochemical detector yields the same type of qualitative information for folate peaks as obtained using multiwavelength UV absorbance detection, but with far greater sensitivity.

**43.** Horne DW: **High-performance liquid chromatographic measurement of 5,10-methyl-enetetrahydrofolate in liver.** *Anal Biochem* 2001, **297**:154-159.

The highly labile 5,10-methylene-THF is stabilized by extraction under alkaline conditions, chemically reduced to 5-methyl-THF, and analyzed by HPLC. By comparison of 5-methyl-THF results following this procedure with those obtained without stabilization and reduction of 5,10-methylene-THF, specific quantification of this critically important intermediate of one-carbon metabolism is obtained.

44. Gregory JF, Engelhardt R, Bhandari SD, Sartain DB, Gustafson SK: **Adequacy of extraction techniques for determination of folate in foods and other biological materials.** *J Food Comp Anal* 1990, **3**:134-144.


46. Rader JI, Weaver CM, Angyal G: **Use of a microbiological assay with tri-enzyme extraction for measurement of pre-fortification levels of folates in enriched cereal-grain products.** *Food Chem* 1998, **62**: 451-465.

47. Engelhardt R, Gregory JF: **Adequacy of enzymatic deconjugation in quantification of folate in foods.** *J Agric Food Chem* 1990, **38**:154-158.

Figure legends

**Figure 1.** Chemical structures of tetrahydrofolate and its C1-substituted derivatives. Plant folates and those of other organisms exist mainly as polyglutamylated forms in which a γ-linked chain of up to about eight residues is added to the glutamate moiety. The numbered orange triangles mark the sites of attack of various catabolic enzymes, known mainly from bacteria and animals.

**Figure 2.** The tetrahydrofolate biosynthesis pathway. The enzymes that have not yet been cloned from plants are highlighted in black. The numbers of genes that encode, or putatively encode, each enzyme in *Arabidopsis* are shown in parentheses. Enzyme abbreviations: HPPK, hydroxymethyldehydropterin pyrophosphokinase; DHPS, dihydropteroate synthase; DHFS, dihydrofolate synthetase; DHFR, dihydrofolate reductase; FPGS, folylpolyglutamate synthetase. HPPK and DHPS are asterixed to indicate that in plants they are parts of a bifunctional protein. DHFR is also part of a bifunctional protein in plants, the other part being thymidylate synthase.

**Figure 3.** (a) Probable compartmentation of the folate synthesis pathway in plant cells. Enzyme numbering: 1, GTP cyclohydrolase I; 2, DHN aldolase; 3, ADC synthase; 4/5, HPPK/DHFS; 6, DHF synthetase; 7, DHF reductase/thymidylate synthase; 8, folylpolyglutamate synthetase. The locations of enzymes 1 – 3 are based on targeting predictions for proteins deduced from ESTs and the *Arabidopsis* genome; those of enzymes 4/5 – 8 have been demonstrated by biochemical methods [4,10**,11,12]. (b) Abundance in GenBank (as of December, 2001) of ESTs corresponding to the enzymes of folate synthesis (1 – 8), and to three enzymes each from the biosynthetic pathways of NAD(P) (A-C), purines (D-F), and pyrimidines (G-I). Enzyme letter code: A, aspartate oxidase; B, quinolinate phosphoribosyl transferase; C, quinolinate synthase; D, GAR transformylase; E, GAR synthetase; F, amidophosphoribosyl transferase; G, dihydroorotase; H, aspartate carbamoyl transferase; I, UMP synthase.
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