Minireview

Evidence for the hypothesis that 10-formyldihydrofolate is the *in vivo* substrate for aminoimidazolecarboxamide ribotide transformylase

Joseph E Baggott and Tsunenobu Tamura

Department of Nutrition Sciences, University of Alabama at Birmingham, Birmingham, AL 35294, USA
Corresponding author: Joseph E Baggott. Email: tamurat@uab.edu

Abstract

We postulate that 10-formyl-7,8-dihydrofolate (10-HCO-H_2folate), not 10-formyl-5,6,7,8-tetrahydrofolate (10-HCO-H_4folate), is the predominant *in vivo* substrate for mammalian aminoimidazolecarboxamide ribotide (AICAR) transformylase, an enzyme in purine nucleotide biosynthesis *de novo*, which introduces carbon 2 (C_2) into the purine ring. 10-HCO-H_2folate exists *in vivo* as labeled 10-formyl-folic acid (10-HCO-folic acid: an oxidation product of 10-HCO-H_4folate and 10-HCO-H_2folate) and is found after doses of labeled folic acid in humans or laboratory animals. The bioactivity of the unnatural isomer, [6*R*]-5-formyltetrahydrofolate, in humans is explained by its *in vivo* conversion to 10-HCO-H_2folate. The structure and active site of AICAR transformylase are not consistent with other enzymes that utilize 10-HCO-H_4folate. Because 10-HCO-H_4folate is rapidly oxidized *in vitro* to 10-HCO-H_2folate by cytochrome *C* alone and in mitochondria, it is hypothesized that this process takes place *in vivo. In vitro* data indicate that 10-HCO-H_2folate is kinetically preferred over 10-HCO-H_4folate by AICAR transformylase and that this enzyme may not have access to sufficient supplies of 10-HCO-H_4folate. Methotrexate blockage of the AICAR transformylase process in patients with rheumatoid arthritis suggests that dihydrofolate (H_2folate) reductase is involved and is consistent with H_2folate and 10-HCO-H_2folate being the product and substrate for AICAR transformylase. The labeling of purine C_2 by an oral dose of [6*RS*]-5-H[^{13}C]O-H_4folate in a human subject is consistent with 10-H[^{13}C]O-H_2folate formation from unnatural isomer, [6*R*]-5-H[^{13}C]O-H_4folate, and it being a substrate for AICAR transformylase. *In vitro* exchange reactions of purine C_2 using H_4folate coenzymes are not duplicated *in vivo* and is consistent with H_2folate coenzymes being used *in vivo* by AICAR transformylase.

Keywords: AICAR transformylase, 10-formyldihydrofolate, folate, purine nucleotide biosynthesis, *in vivo* coenzymes

Experimental Biology and Medicine 2010; **235**: 271–277. DOI: 10.1258/ebm.2009.009151

Introduction

The vast majority of the purine ring is biosynthesized *de novo* rather than coming from the diet.[1] Two folate-dependent transformylases, glycinamide ribotide (GAR) and aminoimidazolecarboxamide ribotide (AICAR) transformylases, are required for purine nucleotide biosynthesis (PNB) *de novo* in mammals by introducing ring carbons 8 (C_8) and 2 (C_2), respectively.[2] It is assumed that 10-formyl-5,6,7,8-tetrahydrofolate (10-HCO-H_4folate) is the *in vivo* substrate for both enzymes, because it has been shown that only 5,6,7,8-H_4folate is capable of enzymatically acquiring a one-carbon unit.[3,4] Therefore, cellular pools of H_4folate are maintained, because 7,8-dihydrofolate (H_2folate) reductase (DHFR) catalyzes reduction of H_2folate to H_4folate.[5] However, *in vitro* mammalian AICAR transformylase (not GAR transformylase) is able to utilize both 10-HCO-H_4folate and 10-formyl-7,8-dihydrofolate (10-HCO-H_2folate) with H_4folate and H_2folate as products, respectively.[6] In this review, we present a hypothesis that 10-HCO-H_2folate (not 10-HCO-H_4folate) is the actual *in vivo* substrate for mammalian AICAR transformylase. To support this hypothesis, we present *in vivo* and *in vitro* findings, including our previously published data and new analyses of those as well as findings by others.

Presence of 10-formyldihydrofolate *in vivo*

An important prerequisite for 10-HCO-H_2folate being the *in vivo* substrate for AICAR transformylase in PNB is the proof indicating that this compound exists *in vivo*. Researchers have identified isotope-labeled or -unlabeled 10-formyl-folic acid (10-HCO-folic acid) in human bile, portal plasma and

urine within a few hours after a dose of labeled folic acid or 5-formyl-5,6,7,8-tetrahydrofolate (5-HCO-H_4folate).[7–9] These samples were collected in the presence of ascorbate to prevent the oxidation of reduced folates. In order to acquire a one-carbon unit, folic acid must first be reduced to H_4folate. Therefore, in these studies, H_4folate must have acquired a one-carbon unit to form labeled 10-HCO-H_4folate that must have been oxidized to form 10-HCO-folic acid. The obligate intermediate in this two-step-oxidation process is 10-HCO-H_2folate that was in fact tentatively identified in the human bile.[7] The first oxidation of 10-HCO-H_4folate to 10-HCO-H_2folate is more rapid than the second oxidation to 10-HCO-folic acid,[6,10] suggesting that 10-HCO-H_2folate was rapidly formed after the dose of folic acid or 5-HCO-H_4folate. Labeled 10-HCO-folic acid was also identified in the rat liver, bile and urine after the dose of [^{14}C]- and [^{3}H]-labeled folic acid.[11–13] Although labeled 10-HCO-folic acid is detected after a dose of labeled folic acid, it is in relatively small quantities compared with the pool of labeled tetrahydrofolates. Therefore, the *in vivo* concentration of its precursor, 10-HCO-H_2folate, may also be relatively small.[7,11–13]

It has been reported that an oral dose of the unnatural isomers, [6*R*]-5-HCO-H_4folate and [6*S*]-5,10-methenyltetrahydrofolate (5,10-CH = H_4folate), were 25% and 50% as bioactive compared with [6*S*]-5-HCO-H_4folate, respectively, in humans.[14] As described, both [6*R*]-5-HCO-H_4folate and [6*S*]-5,10-CH = H_4folate are converted chemically in the gastrointestinal tract to [6*S*]-10-HCO-H_4folate.[14] Oxidation of the pteridine ring of [6*S*]-10-HCO-H_4folate (unnatural isomer) to the 7,8-dihydro-oxidation state (therefore, destroying the chiral center) converts this unnatural isomer to 10-HCO-H_2folate. This folate is now bioactive, because it is a substrate for AICAR transformylase producing H_2folate that can be enzymatically reduced to H_4folate by DHFR.[6] The fact that [6*S*]-5,10-CH = H_4folate was more bioactive than [6*R*]-5-HCO-H_4folate is consistent with the fact that the former is an intermediate in the conversion of [6*R*]-5-HCO-H_4folate to 10-HCO-H_2folate.[14] Chemical formation of [6*S*]-10-HCO-H_4folate and its oxidation to 10-HCO-H_2folate must have been rapid because the experiments lasted only 4 h. Baggott and Tamura[14] were not the first to present data suggesting that [6*R*]-5-HCO-H_4folate is bioactive. Although Baker *et al.*[15] did not report this, re-evaluation of their data using an 8-h follow-up after oral doses of [6*RS*]-5-HCO-H_4folate (believed to be only 50% bioactive) and folic acid indicated that the unnatural isomer, [6*R*]-5-HCO-H_4folate, was ~80% as bioactive compared with folic acid in humans.[16,17] Devito *et al.*[18] also reported that an intravenous dose of [6*RS*]-5-HCO-H_4folate produced 19% more bioactive folates than one-half that of the [6*S*]-5-HCO-H_4folate dose in healthy subjects. Therefore, formation of [6*S*]-10-HCO-H_4folate from [6*R*]-5-HCO-H_4folate and its oxidation to 10-HCO-H_2folate may take place in sites other than the gastrointestinal tract. In addition, using high-performance liquid chromatography with a microbiological assay, Baggott and Tamura[14] identified 10-HCO-H_2folate and 10-HCO-folic acid in 2-h urine samples after doses of either [6*R*]- or [6*S*]-5-HCO-H_4folate,[14] indicating that metabolites of these natural and unnatural isomers must undergo *in vivo* oxidation to 10-HCO-H_2folate. Based on the above data, Baggott and Tamura[14] conclude that 10-HCO-H_2folate exists *in vivo*.

In vitro data supporting our hypothesis

Unique properties of AICAR transformylase

Enzymes that utilize H_2folates include thymidylate synthase, DHFR and AICAR transformylase. Thymidylate synthase catalyzes the production of H_2folate from an H_4folate, and DHFR reduces H_2folate to H_4folate. On the other hand, AICAR transformylase is unique because both substrate and product can be H_2folates. Further, it is important to note that AICAR transformylase has neither structural similarity to GAR transformylase[19] nor amino acid sequence for the 10-HCO-H_4folate binding site that is present in GAR transformylase, 10-HCO-H_4folate synthetase and 10-HCO-H_4folate dehydrogenase of a variety of species such as *Escherichia coli* and humans.[20,21] Site-directed mutagenesis of a suspected 10-HCO-H_4folate binding site with a minor homology to the site in GAR transformylase has no effect on AICAR transformylase activity.[21] Another aspect of the uniqueness in AICAR transformylase is that it is the only folate-dependent enzyme with a homodimer quaternary structure and the active site at the dimmer interface; thus, the monomer is inactive.[19] The pterine ring of folate analogs binds differently to AICAR and GAR transformylases. The ring is more exposed to the bulk water (possibly more exposed to oxidation) in AICAR transformylase.[19] Considering the above, it would be reasonable that folate coenzyme preference by AICAR transformylase evolved with a preference for 10-HCO-H_2folate in a way not similar to other 10-HCO-H_4folate-utilizing enzymes.

In vitro lability of 10-HCO-H_4folate

10-HCO-H_4folate is extremely labile to oxidation *in vitro*. It has been reported that a trace amount of iron in high-grade reagents catalyzes the oxidation of 10-HCO-H_4folate to 10-HCO-H_2folate.[22] Therefore, 10-HCO-H_2folate cannot be prepared *in vitro* without being contaminated with oxidation products.[23]

Oxidized cytochrome *C* reacts with 10-HCO-H_4folate with a second-order rate constant of 1.3×10^4/mol/L × s.[10] As cytochrome *C* concentration is 100–200 μmol/L in the intermembrane space, and mitochondrial folate concentration is 1–5 μmol/L, the pseudo-first-order oxidation rate suggests that 10-HCO-H_4folate would have a half-life of 1 s or less in the intermembrane space.[10] As the protein porin forms channels in the outer mitochondrial membrane that allow free diffusion of molecules of 10,000 Da or less,[24] 10-HCO H_4folate and its polyglutamates found in the cytoplasm could come in contact with cytochrome *C* in the intermembrane space and diffuse out to the cytoplasm as 10-HCO-H_2folate to be utilized by AICAR transformylase. The above process would explain how isolated rat liver mitochondria rapidly form 10-HCO-H_2folate from

10-HCO-H_4folate in the solution outside the organelle.[25] Therefore, 10-HCO-H_4folate must be short-lived if it is formed in the intermembrane space. However, it is possible that 10-HCO-H_4folate may still be able to play a role as a one-carbon donor to AICAR transformylase before it is oxidized. In conclusion, the oxidation of 10-HCO-H_4folate to 10-HCO-H_2folate occurs so readily suggesting the *in vivo* existence of the latter.

In vitro enzyme kinetics of AICAR transformylase

There is *in vitro* evidence indicating that 10-HCO-H_2folate is the preferred substrate for AICAR transformylase. 10-HCO-H_2folate has a ~5-fold kinetic advantage (V_m/K_m) over 10-HCO-H_4folate for AICAR transformylases in human leukemia cell and rat bone marrow,[6] and in human recombinant AICAR transformylase.[26] This kinetic advantage is largely due to a lower K_m (tighter binding) for 10-HCO-H_2folate, and this is consistent with 10-HCO-H_2folate, rather than 10-HCO-H_4folate being the preferred *in vivo* substrate. However, it is not known whether polyglutamates of 10-HCO-H_2folate would have the same kinetic advantage over polyglutamates of 10-HCO-H_4folate.

It is likely that 10-HCO-H_4folate, which is utilized by GAR transformylase, is supplied *in vivo* by an enzyme complex, originally described *in vitro* by Benkovic and colleagues.[27,28] This complex contains the trifunctional-folate-metabolizing protein (TFM), serine hydroxymethyltransferase (SHMT) and GAR transformylase. The biological purpose of the complex would be to furnish or channel the labile 10-HCO-H_4folate to GAR transformylase immediately after 10-HCO-H_4folate is produced.

Consistent with this channeling hypothesis, Benkovic and colleagues[29] recently reported in cultured human cancer cells that the protein with GAR transformylase activity forms clusters in the cytoplasm. In contrast, AICAR transformylase does not form such clusters, and TFM is evenly distributed in the cytoplasm. Therefore, it is likely that any 10-HCO-H_4folate produced by TFM that is in close proximity to a GAR transformylase cluster must be preferentially utilized by this enzyme and stands little chance of 'finding' AICAR transformylase. This suggests that 10-HCO-H_4folate produced by TFM must diffuse a greater distance with an increased risk of oxidation before its utilization by AICAR transformylase. In addition, Baggott *et al.*[6] found in human leukemia cells that 10-HCO-H_4folate K_m's were 4.9 and 420 μmol/L for GAR and AICAR transformylases, respectively. Thus, GAR transformylase has a higher affinity for 10-HCO-H_4folate than AICAR transformylase.

Taken together, the above data suggest that 10-HCO-H_4folate is channeled to GAR transformylase by forming clusters with a binding affinity advantage over AICAR transformylase; therefore, less 10-HCO-H_4folate is available to AICAR transformylase. It is likely that folate coenzymes are effectively cycled and channeled to each enzyme in the complex (TFM, SHMT and GAR transformylase) at the H_4folate-oxidation state. This series of reactions are shown in Reaction Sequence 1 (Table 1).

No enzyme complex that generates 10-HCO-H_4folate and immediately channels or furnishes it to AICAR transformylase is needed because the first oxidation product of 10-HCO-H_4folate is 10-HCO-H_2folate that is utilized by this transformylase. The oxidation of 10-HCO-H_4folate could occur in a reaction with oxidized cytochrome *C* and is probably not simply an *in vitro* phenomenon, because the oxidation is fast enough to support respiration in isolated rat liver mitochondria.[25] The second-order rate constant for the oxidation of 10-HCO-H_2folate to 10-HCO-folic acid by oxidized cytochrome *C* is slow.[10] Therefore, the relatively stable 10-HCO-H_2folate requires less protection from oxidation than 10-HCO-H_4folate and can be utilized by AICAR transformylase as shown in Reaction Sequence 2 (Table 2).

In conclusion, the data in this section suggest that 10-HCO-H_2folate, an oxidation product of 10-HCO-H_4folate, is the preferred substrate for AICAR transformylase and 10-HCO-H_4folate is the preferred substrate for GAR transformylase. This conclusion appears to be reasonable because it would be biologically wasteful for 10-HCO-H_2folate, an intact folate molecule, to be a metabolically inert dead-end catabolite.

Table 1 Reaction Sequence 1. Utilization of the carbon 3 of serine by GAR transformylase

Enzyme	Reaction
SHMT	Serine + H_4folate ⇄ glycine + 5,10-CH_2-H_4folate
TFM	5,10-CH_2-H_4folate + NADP ⇄ 5,10-CH = H_4folate + NADPH
TFM	5,10-CH = H_4folate + H_2O ⇄ 10-CHO-H_4folate
GAR transformylase	10-CHO-H_4folate + GAR→ formyl-GAR + H_4folate
Net	Serine + NADP + H_2O + GAR → glycine + NADPH + formyl-GAR

GAR, glycinamide ribotide; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; 5,10-CH = H_4folate, 5,10-methenyltetrahydrofolate; TFM, trifunctional-folate-metabolizing protein; SHMT, serine hydroxymethyltransferase

Table 2 Reaction Sequence 2. Formate and 10-HCO-H_2folate utilized by AICAR transformylase

Enzyme	Reaction
TFM	H_4folate + ATP + formate ⇄ 10-HCO-H_4folate + ADP + PO_4
Oxidation*	$Substrate_{OX}$ + 10-HCO-H_4folate → 10-HCO-H_2folate + $substrate_{RED}$
AICAR transformylase	10-HCO-H_2folate + AICAR⇄ formyl-AICAR + H_2folate
AICAR transformylase†	Formyl-AICAR ⇄ IMP + H_2O
DHFR	H_2folate + NADPH → H_4folate + NADP
Net	$Substrate_{OX}$ + ATP + formate + AICAR + NADPH → $Substrate_{RED}$ + ADP + PO_4 + IMP + H_2O + NADP

10-HCO-H_2folate, 10-formyl-7,8-dihydrofolate; AICAR, aminoimidazolecarboxamide ribotide; TFM, trifunctional-folate-metabolizing protein; IMP, inosine 5′-monophosphate; DHFR, 7,8-dihydrofolate (H_2folate) reductase; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; OX, oxidized; RED, reduced

*This could be a reaction with cytochrome *C* or other compounds

†It is assumed that AICAR transformylase also possesses the IMP cyclohydrolase activity

In vivo data in humans and animals supporting our hypothesis

Methotrexate therapy of patients with rheumatoid arthritis

Smoleńska *et al.*[30] measured the change in whole-blood hypoxanthine concentrations in adult patients with rheumatoid arthritis (RA) shortly before (baseline) and 2 h after an oral dose of methotrexate (MTX) (7.5 mg; patient's first MTX dose). There was a significant 36% decrease in mean hypoxanthine from baseline. The mean blood uric acid concentration was also decreased to a significant 32% from baseline, suggesting an overall decrease in PNB.

The total absorption of oral MTX is ~80%, of which ~70% would be absorbed in 2 h.[31] Thus, the above patients would have absorbed 4.2 mg of the 7.5-mg dose ($7.5\ \text{mg} \times 0.8 \times 0.7$). Excluding urinary MTX excretion, *in vivo* retention would be $\sim 9 \times 10^{-6}$ mol (4.2 mg) or in a 70-kg patient $\sim 1.3 \times 10^{-7}$ mol/kg in 2 h. Morgan *et al.*[32] showed that low-dose MTX therapy significantly increases urinary aminoimidazolecarboxamide (AICA) excretion in 24 h, indicating an interference with AICAR transformylase by the drug. Therefore, it is likely that this interference is the target resulting in a decreased inosine 5′-monophosphate (IMP) biosynthesis, thus decreasing blood hypoxanthine. The question remains as to whether the interference with AICAR transformylase is directly caused by MTX. The competitive inhibition constant (K_i) for MTX of AICAR transformylase is 4.0×10^{-5} mol/L,[33] and it is ~300 times higher than the estimated *in vivo* MTX retention of $\sim 1.3 \times 10^{-7}$ mol/kg, which is negligible compared with this K_i. It is concluded that MTX cannot directly inhibit AICAR transformylase.

Inhibition of an enzyme other than AICAR transformylase is likely to be responsible for decreased blood hypoxanthine[30] and increased urinary AICA excreted in these patients.[32] One logical choice of an enzyme affected by a low MTX concentration is DHFR. The K_i for MTX for human DHFR is 6.1×10^{-12} mol/L,[34] and $\sim 1.3 \times 10^{-7}$ mol/kg is ~20,000 times higher than the K_i. Even such a low MTX concentration would produce potent DHFR inhibition, which could simply reduce the H_4folate-pool leading to an indirect interference with AICAR transformylase. However, this reduction in the H_4folate-pool may not be the actual mechanism, because it would have to occur within 2 h. In fact, Priest and coworkers[35] found that low MTX concentrations do not reduce the H_4folate-pool but increase 10-HCO-H_4folate in cultured cells. Pools of other H_4folate compounds were basically unchanged; therefore, there is no evidence that the intracellular H_4folate-pool was depleted by MTX even though cell growth was reduced. Dervieux *et al.*[36] reported that erythrocyte H_4folate-pools in patients with RA were decreased only 13% from baseline after ~18 weekly doses of MTX (7.5–15 mg/week). Therefore, it is likely that the first MTX dose in patients in the investigation by Smoleńska *et al.*[30] resulted in a trivial reduction in the H_4folate-pool in erythrocytes.

Therefore, an alternative mechanism of MTX interference with AICAR transformylase should be investigated. This may involve the utilization of 10-HCO-H_2folate by the enzyme as shown in Reaction Sequence 2. This sequence is driven towards the biosynthetic direction by the DHFR-catalyzed reduction of H_2folate to H_4folate. The MTX inhibition of DHFR would interfere with AICAR transformylase, as the equilibrium of this step actually lies in the direction of AICAR formation.[26] Reaction Sequence 2 suggests how DHFR, 10-HCO-H_2folate and H_2folate are involved in the overall net reaction. The production of H_2folate by AICAR transformylase would explain how low MTX concentrations interfere with the net reaction. In conclusion, 10-HCO-H_2folate as the *in vivo* substrate for AICAR transformylase explains how low-dose MTX therapy in patients with RA affects this enzyme.

^{13}C enrichment at C_2 and C_8 of purine after a [6*RS*]-5-H[^{13}C]O-H_4folate dose

In this section, we attempt to explain how 10-HCO-H_2folate is the *in vivo* substrate for AICAR transformylase by re-analyzing our published data of ^{13}C enrichment at C_2 and C_8 of purine after a [6*RS*]-5-H[^{13}C]O-H_4folate dose. The ^{13}C enrichment at C_2 and C_8 of urinary uric acid after a 25-mg oral dose of [6*RS*]-5-H[^{13}C]O-H_4folate was measured in an adult.[37,38] Figure 1 shows the relationship between the log of the smoothed C_2/C_8 enrichment ratios and the void number. This logarithmic transformation of the data was used to readily identify ratios <1.0 (negative numbers). Although considerable variations in the C_2/C_8 ratios existed, there are basically two phases after the dose. The first phase consisted of a period of enrichment ratios <1, and the second phase was a large increase of the enrichment ratio with a peak value of ~10.

The previously held notion that an equal utilization by both GAR (C_8) and AICAR (C_2) transformylases, of [6*R*]-10-H[^{13}C]O-H_4folate (formed by rapid enzymatic reactions from [6*S*]-5-H[^{13}C]O-H_4folate), is not consistent with the data shown in Figure 1. If an equal utilization were true, the C_2/C_8 ratios should have been 1.0 ($\log = 0$) for all voids. To explain these data, we postulate the following hypotheses. The first phase is the result of the rapid enzymatic formation of [6*R*]-10-H[^{13}C]O-H_4folate that is utilized primarily by GAR transformylase. This folate is channeled to and preferentially utilized by GAR transformylase (see the section '*In vitro* lability of 10-HCO-H_4folate'); hence, C_2/C_8 ratios are <1.0. In the second phase, a slow non-enzymatic formation of 10-H[^{13}C]O-H_2folate from the unnatural isomer [6*R*]-5-H[^{13}C]O-H_4folate predominates,[14] resulting in high C_2/C_8 ratios, since 10-H[^{13}C]O-H_2folate can only be utilized by AICAR transformylase.[6,14] These data resemble what one would predict, if [6*R*]-10-H[^{13}C]O-H_4folate is rapidly formed, channeled to and utilized only by GAR transformylase, and 10-H[^{13}C]O-H_2folate is slowly formed and utilized only by AICAR transformylase. Human data reported by Baggott and Tamura[14] are also consistent with 10-HCO-H_2folate being the *in vivo* substrate for AICAR transformylase.

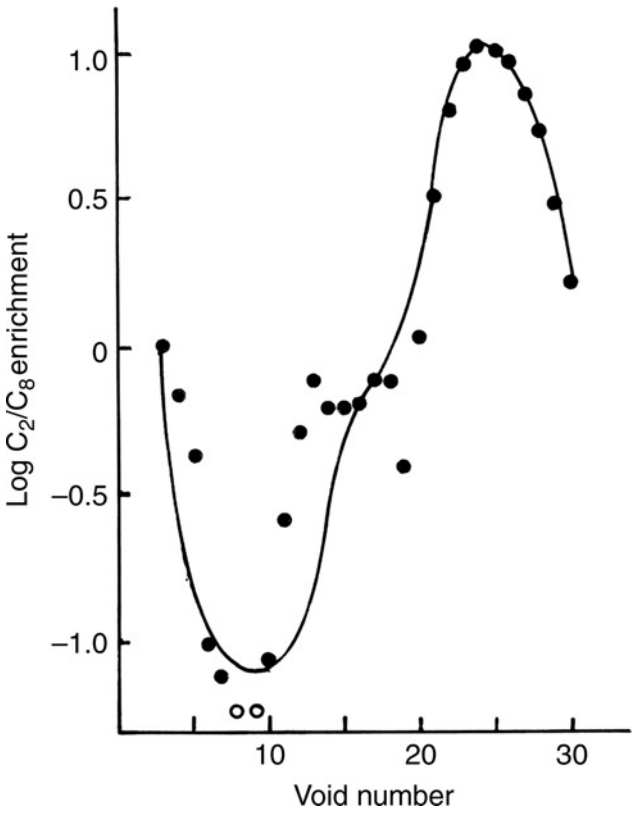

Figure 1 C_2/C_8 ^{13}C enrichment ratios of urinary uric acid after an oral dose of 25 mg [6*RS*]-5-H[^{13}C]O-H_4folate in a human. The smoothed 1:2:3:2:1 weighted running average of the % ^{13}C enrichments of urinary uric acid at the C_2 and C_8 positions were calculated after an oral dose of 25 mg [6*RS*]-5-H[^{13}C]O-H_4folate.[38] Closed circles represent the log of the ^{13}C enrichment ratio (C_2/C_8) that was plotted against the void number. The data from the first two and last two voids were lost in this process. Open circles represent data that were negative numbers, thus presumed to be low ratios (i.e. negative log C_2/C8 enrichment). The line is a best-fit spine curve. C_2, carbon 2; C_8, carbon 8

Carbon exchange at the second position of the purine ring

A C_2 to C_8 exchange in the purine ring was reported by Warren *et al*.[39] When an avian liver preparation containing GAR and AICAR transformylases was incubated with IMP, GAR and H_4folate, both AICAR and formyl-GAR were readily formed (Table 3, Reaction Sequence 3). The C_2 from IMP (in equilibrium with formyl-AICAR) was transferred to H_4folate, and the resulting 10-HCO-H_4folate was utilized to form formyl-GAR. Reaction Sequence 3 shows these reactions for which the apparent equilibrium lies in favor of formyl-GAR and AICAR formation because the GAR transformylase reaction is irreversible. When this formyl-GAR is metabolized to IMP, the former carbon at the C_2 position now appears in the C_8 position. Because GAR transformylase cannot utilize 10-HCO-H_2folate, this exchange requires that H_4folate and 10-HCO-H_4folate are the coenzymes for the AICAR transformylase reaction.[6] In theory, this exchange shown in Reaction Sequence 3 can occur *in vivo*.

Table 3 Reaction Sequence 3. Exchange of carbon 2 of IMP to carbon 8 of IMP

Enzyme	Reaction
AICAR transformylase	IMP + H_2O ⇄ formyl-AICAR
AICAR transformylase	Formyl-AICAR + H_4folate ⇄ 10-HCO-H_4folate + AICAR
GAR transformylase	10-HCO-H_4folate + GAR → formyl-GAR + H_4folate
Net	IMP + H_2O + GAR → AICAR + formyl-GAR

IMP, inosine 5′-monophosphate; AICAR, aminoimidazolecarboxamide ribotide; 10-HCO-H_2folate, 10-formyl-7,8-dihydrofolate; GAR, glycinamide ribotide

Using an avian liver preparation, Flaks *et al*.[40] reported an exchange reaction of C_2 of IMP with the carbon 3 of serine (Reaction Sequence 4, Table 4). This exchange involves TFM, SHMT and H_4folate coenzymes (not H_2folate coenzymes). In theory, this exchange can occur *in vivo*, and the serine formed by this reaction would be readily oxidized to CO_2.

It should be emphasized that exchange reactions (Sequences 3 and 4) require that H_4folate coenzymes function in the AICAR transformylase process. However, it remained to be seen whether these exchange reactions actually occur *in vivo*. Using adenine labeled with ^{13}C at the C_2 position and ^{14}C at the C_8 position, Abrams[41] evaluated the possibility of these exchanges in rats. Adenine is readily metabolized to IMP.[2] The C_2/C_8 labeling ratio was not changed compared with the starting material in liver RNA adenine and guanine days after the dose of the double-labeled adenine. Abrams[41] concluded that C_2 exchanges described above do not occur *in vivo*. However, the fact that no exchange reactions are detected indicates that H_2folate coenzymes are utilized by AICAR transformylase.

Bennett and Karlsson[42] studied the exchange of 2-[^{14}C]adenine and 8-[^{14}C]adenine in mice. Measuring hepatic RNA adenine- and guanine-specific activities, they found no difference comparing C_2 to C_8 in experiments that lasted for 2 weeks, and concluded that C_2 was not involved in exchange reactions described above. Of particular interest is the measurement of expired $^{14}CO_2$ from the C_2- and C_8-labeled compounds in mice. After 20 h, $^{14}CO_2$

Table 4 Reaction Sequence 4. Exchange of carbon 2 of IMP to carbon 3 of serine

Enzyme	Reaction
AICAR transformylase	IMP + H_2O ⇄ formyl-AICAR
AICAR transformylase	Formyl-AICAR + H_4folate ⇄ 10-HCO-H_4folate + AICAR
TFM	10-HCO-H_4folate + H_2O ⇄ 5,10-CH = H_4folate
TFM	5,10-CH = H_4folate + NADPH ⇄ 5,10-CH_2-H_4folate + NADP
SHMT	Glycine + 5,10-CH_2-H_4folate ⇄ serine + H_4folate
Net	IMP + H_2O + NADPH + glycine ⇄ AICAR + NADPH + serine

IMP, inosine 5′-monophosphate; AICAR, aminoimidazolecarboxamide ribotide; 10-HCO-H_2folate, 10-formyl-7,8-dihydrofolate; TFM, trifunctional-folate-metabolizing protein; 5,10-CH = H_4folate, 5,10-methenyltetrahydrofolate; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; SHMT, serine hydroxymethyltransferase

production from both compounds was small, and there was no marked difference in $^{14}CO_2$ production from 2-[^{14}C]adenine compared with 8-[^{14}C]adenine. These findings are also evidence indicating that the exchange reactions discussed above do not occur *in vivo* in mice.

In humans, however, data are limited. Baggott *et al.*[43] conducted a human study to measure incorporation of [^{13}C]formate into C_2 and C_8 of urinary uric acid. In three-day experiments, two subjects had little incorporation into C_8, but C_2 incorporation was significant. Therefore, the C_2 to C_8 exchange (Reaction Sequence 3) was small. However, a subject incorporated significant amounts into the C_8 position as well as into C_2. Therefore, the data in this subject could be used to test the possible C_2 to C_8 exchange. If C_8 incorporation (all or part) was the result of the exchange from C_2, more enrichment at C_8 should occur in a time-dependent manner as C_2 becomes more enriched. Thus, both C_8 enrichment and the C_8/C_2 enrichment ratio should have increased with time. Figure 2 shows both of these values plotted over a three-day period after the [^{13}C]formate dose, and linear regression of the data in both plots indicates negative slopes. Thus, there was no evidence of C_2 to C_8 exchange in this subject. The findings in humans by Baggott *et al.*[43] are, therefore, in agreement with the animal studies.[41,42] It is possible that *in vitro* exchange reactions are not operable *in vivo*, because enzymes are not in close proximity and do not exchange folate coenzymes or other factors. However, if H_2folate coenzymes are the *in vivo* substrate for AICAR transformylase, these exchanges should not occur *in vivo*.

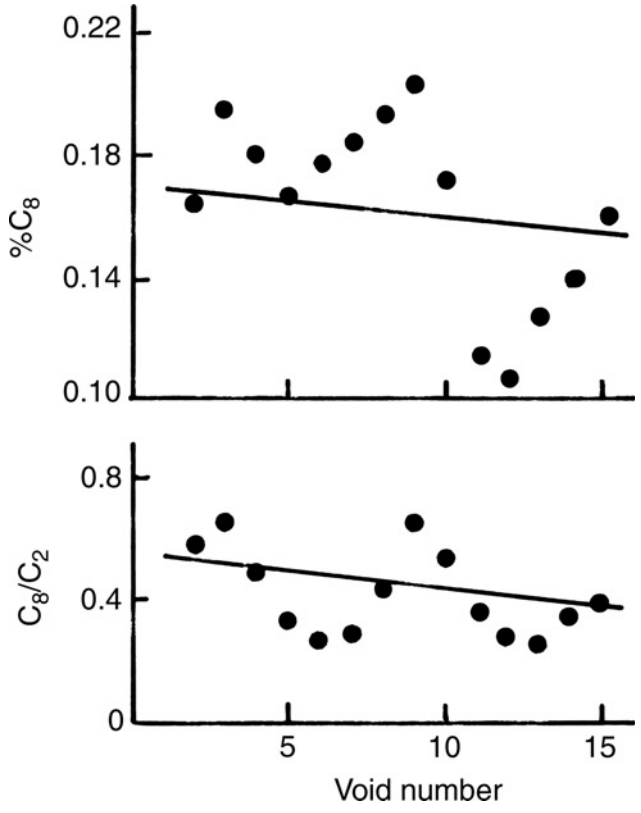

Figure 2 C_8/C_2 ^{13}C enrichment ratios and % ^{13}C enrichment at the C_8 position of urinary uric acid after an oral dose of 1.0 g of sodium [^{13}C]formate in a human. The smoothed 1:2:1 weighted running averages of % enrichment at the C_2 and C_8 positions (%C_8) and the enrichment ratio (C_8/C_2) of urinary uric acid after an oral dose of 1.0 g of sodium [^{13}C]formate were plotted against the void number for subject C.[38] The data from the first and last void were lost in this process. Linear regression lines are negative, but are not significant ($P > 0.05$)

Summary

We conclude that 10-HCO-H_2folate is the *in vivo* predominant substrate for mammalian AICAR transformylase, and summarize as follows. The *in vivo* existence of 10-HCO-H_2folate is likely due to the lability to oxidation of 10-HCO-H_4folate, suggesting that 10-HCO-H_2folate must have a metabolic role rather than being a dead-end catabolite. AICAR transformylase does not have a 10-HCO-H_4folate binding site that is present in other 10-HCO-H_4folate-utilizing enzymes and has a unique active site formed by a homodimer. Enzyme-kinetic data suggest the utilization of 10-HCO-H_2folate by AICAR transformylase and utilization of only 10-HCO-H_4folate by GAR transformylase. The labile 10-HCO-H_4folate is likely channeled to GAR transformylase *in vivo* and the relatively stable 10-HCO-H_2folate is utilized by AICAR transformylase. These findings are consistent with labeling patterns in the purine ring after a dose of [6*RS*]-5-H[^{13}C]O-H_4folate in a human. The unique sensitivity of AICAR transformylase to the low-dose MTX therapy in patients suggests that formation of H_2folate from 10-HCO-H_2folate and inhibition of DHFR are involved in the transformylation process. The facile *in vitro* exchanges of the C_2 position of purine in the presence of H_4folates and enzymes are not duplicated *in vivo*, suggesting that H_2folates (which cannot produce facile *in vitro* exchanges), not H_4folates, are involved in the *in vivo* AICAR transformylase (C_2) process.

Finally, it may be difficult, if not impossible, to prove that 10-HCO-H_2folate is the *in vivo* substrate for mammalian AICAR transformylase; however, we can turn the table on this argument. Simply stated, at present, we believe that it is difficult (if not impossible) to design experiments proving that 10-HCO-H_4folate is the one and only substrate for AICAR transformylase *in vivo* and we are not aware of any experiments that attempted to prove this. It would be biologically wasteful for 10-HCO-H_2folate to be a metabolically inert dead-end catabolite. It is possible that mammalian AICAR transformylase evolved with metabolic flexibility to utilize both 10-HCO-H_2folate and 10-HCO-H_4folate in order to metabolically 'salvage' former utilizing the PNB pathway. Changing the relatively *in vivo* amounts of 10-HCO-H_4folate and 10-HCO-H_2folate could be a regulatory mechanism in the PNB pathway.

REFERENCES

1 Berthold HK, Crain PF, Gouni I, Reeds PJ, Klein PD. Evidence for incorporation of intact dietary pyrimidine (but not purine) nucleotides into hepatic RNA. *Proc Natl Acad Sci USA* 1995;**92**:10123–7

2 Garrett RH, Grisham CH. The synthesis and degradation of nucleotides. In: *Biochemistry*. 3rd edn. Belmont, CA: Thomson Brooks/Cole, 2005:853–78

3 Jaenicke L, Brode E. Untersuchungen über Einkohlenstoffkörper. I. Die Tetrahydrofolatformylase aus Taubenleber Reinigung und Mechanismus. *Biochem Zeitsch* 1961;**334**:108–32

4 Blakley RL. The reaction of tetrahydropteroylglutamic acid and related hydropteridines with formaldehyde. *Biochem J* 1959;**72**:707–15

5 Blakley RL. Dihydrofolate reductase. In: Blakley RL, Benkovic SJ eds. *Folates and Pterins*. Vol. 1. New York: John Wiley, 1984:191–253

6 Baggott JE, Johanning GL, Branham KE, Prince CW, Morgan SL, Eto I, Vaughn WH. Cofactor role for 10-formyldihydrofolic acid. *Biochem J* 1995;**308**:1031–6

7 Pratt RF, Cooper BA. Folates in plasma and bile of man after feeding folic acid-^{3}H and 5-formyltetrahydrofolate (folinic acid). *J Clin Invest* 1971;**50**:455–62

8 Whitehead VM, Pratt R, Viallet A, Cooper BA. Intestinal conversion of folinic acid to 5-methyltetrahydrofolate in man. *Br J Haematol* 1972;**22**:63–72

9 McLean A, Chanarin I. Urinary excretion of 5-methyl-tetrahydrofolate in man. *Blood* 1966;**27**:386–8

10 Baggott JE, Robinson CB, Johnston KE. Bioactivity of [6*R*]-5-formyltetrahydrofolate, an unnatural isomer, in humans and *Enterococcus hirae*, and cytochrome *c* oxidation of 10-formyltetrahydrofolate to 10-formyldihydrofolate. *Biochem J* 2001;**354**:115–22

11 Murphy M, Keating M, Boyle P, Weir DG, Scott JM. The elucidation of the mechanism of folate catabolism in the rat. *Biochem Biophys Res Commun* 1976;**71**:1017–24

12 Barford PA, Blair JA. Effect of an implanted Walker tumour on metabolism of folic acid in the rat. *Br J Cancer* 1978;**38**:122–9

13 Pheasant AE, Connor MJ, Blair JA. The metabolism and physiological disposition of radioactively labelled folate derivatives in the rat. *Biochem Med* 1981;**26**:435–50

14 Baggott JE, Tamura T. Bioactivity of oral doses of unnatural isomers, [6*R*]-5-formyltetrahydrofolate and [6*S*]-5,10-methenyltetrahydrofolate, in humans. *Biochim Biophys Acta* 1999;**1472**:323–32

15 Baker H, Frank O, Feingold S, Ziffer H, Gellene RA, Leevy CM, Sobotka H. The fate of orally and parenterally administered folates. *Am J Clin Nutr* 1965;**17**:88–95

16 Baggott JE, Tamura T, Baker H. Reevaluation of the metabolism of oral doses of racemic carbon-6 isomers of formyltetrahydrofolate in humans. *Br J Nutr* 2001;**85**:653–7

17 Baggott JE, Tamura T. Metabolism of 10-formyldihydrofolate in humans. *Biomed Pharmacother* 2001;**55**:454–7

18 DeVito JM, Kozloski GD, Tonelli AP, Johnson JB. Bioequivalence of oral and injectable levoleucovorin and leucovorin. *Clin Pharmacol* 1993;**12**:293–9

19 Wolan DW, Greasley SE, Wall MJ, Benkovic SJ, Wilson IA. Structure of avian AICAR transformylase with a multisubstrate adduct inhibitor β-DADF identifies the folate binding site. *Biochemistry* 2003;**42**:10904–14

20 Cook RJ, Lloyd RS, Wagner C. Isolation and characterization of cDNA clones for rat liver 10-formyltetrahydrofolate dehydrogenase. *J Biol Chem* 1991;**266**:4965–73

21 Rayl EA, Moroson BA, Beadsley GP. The human *pur*H gene product, 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase. Cloning, sequencing, expression, purification, kinetic analysis, and domain mapping. *J Biol Chem* 1996;**271**:2225–33

22 Baggott JE, Robinson CB, Eto I, Johanning GL, Cornwell PE. Iron compounds catalyze the oxidation of 10-HCO-5,6,7,8 tetrahydrofolic acid to 10-HCO-dihydrofolic acid. *J Inorg Biochem* 1998;**71**:181–7

23 Poe M, Benkovic SJ. 5-Formyl- and 10-formyl-5,6,7,8-tetrahydrofolate. Conformation of the tetrahydropyrazine ring and formyl group in solution. *Biochemistry* 1980;**19**:4576–82

24 Garret RH, Grisham CH. Electron transport and oxidative phosphorylation. In: *Biochemistry*. 3rd edn. Belmont, CA: Thomson Brooks/Cole, 2005:640–73

25 Brooks PS, Baggott JE. Oxidation of 10-formyltetrahydrofolate to formyldihydrofolate by complex IV of rat mitochondria. *Biochemistry* 2002;**41**:5633–6

26 Wall M, Shim JH, Benkovic SJ. Human AICAR transformylase: role of the 4-carboxamide of AICAR in binding and catalysis. *Biochemistry* 2000;**39**:11303–11

27 Caperelli CA, Benkovic PA, Chettur G, Benkovic SJ. Purification of a complex catalyzing folate cofactor synthesis and transformylation in *de novo* purine biosynthesis. *J Biol Chem* 1980;**255**:1885–90

28 Smith GK, Mueller WT, Wasserman GF, Taylor WD, Benkovic SJ. Characterization of the enzyme complex involving the folate-requiring enzymes of *de novo* purine biosynthesis. *Biochemistry* 1980;**19**:4313–21

29 An S, Kumar R, Sheets ED, Benkovic SJ. Reversible compartmentalization of *de novo* purine biosynthetic complexes in living cells. *Science* 2008;**320**:103–6

30 Smoleńska Ż, Karnowska Z, Zarówny D, Simmonds HA, Smoleński RT. Effect of methotrexate on blood purine and pyrimidine levels in patients with rheumatoid arthritis. *Rheumatology* 1999;**38**:997–1002

31 Fox RI, Morgan SL, Smith HT, Robbins BA, Choc MG, Baggott JE. Combined oral cyclosporin and methotrexate therapy in patients with rheumatoid arthritis elevates methotrexate levels and reduce 7-hydroxymethotrexate levels when compared with methotrexate alone. *Rheumatology* 2003;**42**:989–94

32 Morgan SL, Oster RA, Lee JY, Alarcón GS, Baggott JE. The effect of folic acid and folinic acid supplements on purine metabolism in methotrexate-treated rheumatoid arthritis. *Arthr Rheum* 2004;**50**:3104–11

33 Allegra CJ, Drake JC, Jolivet J, Chabner BA. Inhibition of phosphribosylaminoimidazolecarboxamide transformylase by methotrexate and dihydrofolic acid polyglutamates. *Proc Natl Acad Sci USA* 1985;**82**:4881–5

34 Jarabak J, Bachur NR. A soluble dihydrofolate reductase from human placenta: purification and properties. *Arch Biochem Biophys* 1971;**142**:417–25

35 Bunni M, Doig MT, Donato H, Kesavan V, Priest DG. Role of methylenetetrahydrofolate depletion in methotrexate-mediated intracellular thymidylate synthesis inhibition cultured L1210 cells. *Cancer Res* 1988;**48**:3398–404

36 Dervieux T, Greenstein N, Kremer J. Pharmacogenomic and metabolic biomarkers in the folate pathway and their association with methotrexate effects during dosage escalation in rheumatoid arthritis. *Arthr Rheum* 2006;**54**:3095–103

37 Gorman GS, Tamura T, Baggott JE. Mass spectrometric method for detecting carbon 13 enrichment introduced by folate coenzymes in uric acid. *Anal Biochem* 2003;**321**:188–91

38 Baggott JE, Gorman GS, Morgan SL, Tamura T. ^{13}C-enrichment at carbons 8 and 2 or uric acid after ^{13}C-labeled folate dose in man. *Biochem Biophys Res Commun* 2007;**361**:307–10

39 Warren L, Flaks JG, Buchanan JM. Integration of enzymatic transformylation reaction. *J Biol Chem* 1957;**229**:627–40

40 Flaks JG, Warren L, Buchanan JM. Further studies of the inosinic acid transformylase system. *J Biol Chem* 1957;**228**:215–29

41 Abrams R. Stability of the adenine ring structure in the rat. *Biochim Biophys Acta* 1956;**21**:439–40

42 Bennett EL, Karlsson H. Metabolism of adenine in mice. *J Biol Chem* 1957;**229**:39–50

43 Baggott JE, Gorman GS, Tamura T. ^{13}C Enrichment of carbons 2 and 8 of purine by folate-dependent reactions after [^{13}C]formate and [2-^{13}C]glycine dosing in adult humans. *Metabolism* 2007;**56**:708–15