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## **<sup>13</sup>C Enrichment of Carbons 2 and 8 of Purine by Folate-Dependent Reactions After [<sup>13</sup>C]Formate and [2-<sup>13</sup>C]Glycine Dosing in Adult Humans**

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### **Abstract**

The 10-formyl moiety of 10-formyltetrahydrofolate is the source of carbons at the positions 8 (C<sub>8</sub>) and 2 (C<sub>2</sub>) of the purine ring, originating from formate and a few amino acids. Uric acid is the final catabolic product of purines. In adult humans, we independently measured the <sup>13</sup>C enrichment of the C<sub>2</sub> and C<sub>8</sub> positions of urinary uric acid after an oral dose of [<sup>13</sup>C]sodium formate and that of the C<sub>2</sub> and C<sub>8</sub> plus C<sub>5</sub> positions after [2-<sup>13</sup>C]glycine. A liquid-chromatography mass-spectrometric method was used to measure the <sup>13</sup>C enrichment of uric acid in urine which was collected for 3 - 4 days. Purine catabolism to uric acid does not alter the positions of carbons in the ring. After the formate dose, the <sup>13</sup>C-enrichment at C<sub>2</sub> was greater than that at C<sub>8</sub>, and a circadian rhythm was observed in the enrichment at C<sub>2</sub>. After the glycine dose, the C<sub>8</sub> plus C<sub>5</sub> positions were enriched, whereas no significant enrichment at C<sub>2</sub> was found. These <sup>13</sup>C enrichment patterns are not consistent with previous accepted metabolism. To our knowledge, this is the first study to investigate <sup>13</sup>C enrichment from formate and glycine independently into the C<sub>2</sub> and C<sub>8</sub> positions of purine in the same subjects. Possible mechanisms explaining our findings are discussed. Oral [<sup>13</sup>C]formate or [2-<sup>13</sup>C]glycine dosing and urine collection can be used to study purine biosynthesis in humans.

### **Keywords**

<sup>13</sup>C isotope; formate; glycine; purine nucleotide biosynthesis; humans

## **1. Introduction**

Purine *de novo* nucleotide biosynthesis is a fundamental process producing building blocks of DNA and RNA. Glycinamide ribotide (GAR) and aminoimidazolecarboxamide ribotide (AICAR) transformylases utilize folate coenzymes to introduce carbons 8 (C<sub>8</sub> in red, Fig. 1) and 2 (C<sub>2</sub> in blue, Fig. 1) into the purine ring, respectively [1].

Formate is one source of the formyl C (green, Fig. 1) of 10-formyltetrahydrofolate (10-HCO-H<sub>4</sub>folate), which is a substrate for these two enzymes. 10-HCO-H<sub>4</sub>folate synthetase forms this substrate from tetrahydrofolate (H<sub>4</sub>folate), formate and ATP [2]. Under normal conditions, human plasma formate concentrations range 20 - 250 μM, which is about 50% of those in

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erythrocytes, and formate is derived from many sources [3,4]. In animals,  $^{14}\text{C}$ -labeled formate given *in vivo* is predominantly found at  $\text{C}_2$  and  $\text{C}_8$  of purines, whereas only a small amount appears in the other positions [5-10], and the  $^{14}\text{C}_2/^{14}\text{C}_8$  ratio was reported to be about 1.0 [6-8]. However, this ratio in humans has never been documented.

The second C of glycine (green, Fig. 1) is also potentially incorporated into  $\text{C}_2$  and  $\text{C}_8$  of purines by folate-dependent reactions and into  $\text{C}_5$  by folate-independent metabolism. Glycine in the presence of  $\text{H}_4\text{folate}$  is metabolized to 5,10-methylenetetrahydrofolate (5,10- $\text{CH}_2\text{-H}_4\text{folate}$ ),  $\text{CO}_2$  and  $\text{NH}_3$  by the glycine-cleavage system (GCS) [11]. The second C of glycine (green, Fig. 1), now the methylene C of 5,10- $\text{CH}_2\text{-H}_4\text{folate}$ , can be converted to 10- $\text{HCO-H}_4\text{folate}$  by 5,10- $\text{CH}_2\text{-H}_4\text{folate}$  dehydrogenase and 5,10-methenyltetrahydrofolate (5,10- $\text{CH=H}_4\text{folate}$ ) cyclohydrolase [2].

Uric acid is the final catabolite of purines in humans, and the catabolic process does not alter the C positions of purines [1]. In mammals,  $\text{C}_2$  and  $\text{C}_8$  of purines are not more labile to simple isotope exchange *in vivo* than the other Cs of purines [12,13]. The peak in labeled urinary uric acid occurs 1 - 3 days after a dose of labeled formate or glycine in humans [14-19], representing catabolism of newly synthesized purines [14]. We measured the  $^{13}\text{C}$  enrichment independently at  $\text{C}_2$  and  $\text{C}_8$  of uric acid by expanding the *in vivo* formate pool with an oral [ $^{13}\text{C}$ ]formate dose using a liquid-chromatography mass spectrometry (LC/MS/MS) method [20]. We also measured the  $^{13}\text{C}$  enrichment at  $\text{C}_2$  and  $\text{C}_8$  plus  $\text{C}_5$  after expanding the glycine pool with [ $2\text{-}^{13}\text{C}$ ]glycine.

## 2. Methods

### 2.1. Human study

The study was approved by the Institutional Review Board for Human Use at the University of Alabama at Birmingham. Three healthy adult males collected urine at each void for 72 - 96 hours after an oral dose of 14.5 mmol [ $^{13}\text{C}$ ]sodium formate (1.0 g,  $^{13}\text{C}$  99%, Cambridge Isotope Laboratories, Andover, MA) with 100 mL of water at about noon. The volume of each void was measured and urine samples were stored at  $-80^\circ\text{C}$  until analysis. In addition, as baseline experiments, similar urine collections for 72 hour were performed without a [ $^{13}\text{C}$ ]sodium formate dose (once in subject B and twice in subjects A and C). With a minimum of a 12-month interval, the identical procedure was repeated in two subjects using 41.7 mmol of [ $2\text{-}^{13}\text{C}$ ]glycine (2.5 g,  $^{13}\text{C}$  99%, Cambridge Isotope Lab.). Subjects were asked to maintain regular life style including their diet and physical activity during the study.

### 2.2. Measurement of $^{13}\text{C}$ enrichment

Independent  $^{13}\text{C}$  enrichment from [ $^{13}\text{C}$ ]formate into the  $\text{C}_2$  and  $\text{C}_8$  positions of uric acid was measured by the LC/MS/MS method [20] followed by the calculation as described below. The area-under-the-curve (AUC) of the extracted ion chromatograms (XIC) for  $m/z$  168  $\rightarrow$  124 and 168  $\rightarrow$  125 and 169  $\rightarrow$  125 represents the amount of  $^{13}\text{C}$  at  $\text{C}_2$ ,  $\text{C}_8$  and both  $\text{C}_2$  and  $\text{C}_8$ , respectively. The AUC of XIC for  $m/z$  167  $\rightarrow$  124 represents uric acid containing only  $^{12}\text{C}$ ,  $^1\text{H}$ ,  $^{14}\text{N}$  and  $^{16}\text{O}$ . The percentage of  $^{13}\text{C}$  at  $\text{C}_2$  (%  $^{13}\text{C}_2$ ) and  $\text{C}_8$  (%  $^{13}\text{C}_8$ ) in total uric acid was estimated using the following formulae: %  $^{13}\text{C}_2 = (\text{AUC for XIC } m/z \text{ 168 } \rightarrow \text{124}) \div (\text{AUC for XIC } m/z \text{ 167 } \rightarrow \text{124} + \text{AUC for XIC } m/z \text{ 169 } \rightarrow \text{125}) \times 100$ ; and %  $^{13}\text{C}_8 = (\text{AUC for XIC } m/z \text{ 168 } \rightarrow \text{125}) \div (\text{AUC for XIC } m/z \text{ 167 } \rightarrow \text{124} + \text{AUC for XIC } m/z \text{ 169 } \rightarrow \text{125}) \times 100$ .

Similar calculations were made to estimate the %  $^{13}\text{C}$  after a [ $2\text{-}^{13}\text{C}$ ]glycine dose. Although the LS/MS/MS method allows to measure %  $^{13}\text{C}$  at  $\text{C}_2$  cleanly, %  $^{13}\text{C}$  at  $\text{C}_8$  also includes that at  $\text{C}_5$ [20]. The amount of  $^{13}\text{C}$  at both  $\text{C}_2$  and  $\text{C}_8$  is included in the denominator because it is

unlikely that both positions would be simultaneously enriched by a [ $^{13}\text{C}$ ]formate or [2- $^{13}\text{C}$ ] glycine dose. The  $^{13}\text{C}$  enrichment at  $\text{C}_2$  and  $\text{C}_8$ (plus  $\text{C}_5$ ) following [ $^{13}\text{C}$ ]formate or [2- $^{13}\text{C}$ ] glycine dosing was calculated for each void by subtracting baseline %  $^{13}\text{C}_2$  and %  $^{13}\text{C}_8$  values that were paired for the subject and time of d when void was collected from the values obtained after the dose. The values of %  $^{13}\text{C}_2$  and %  $^{13}\text{C}_8$  from baseline experiments were subtracted from each other in two subjects (A and C) to measure the variability of baseline values. It is important to note that the measurements of baseline values are essential for such a study, since life style or dietary habit can affect the  $^{13}\text{C}$  enrichment of human samples [21,22].

### 2.3. Statistical analysis

The Wilcoxon paired-sample test was used to detect a significant difference from 0 in mean  $^{13}\text{C}$  enrichment from [ $^{13}\text{C}$ ]formate for each day, where more than eight voids were obtained. For subject C with fewer than eight voids per day, the data from three d were combined [23]. The same test was used to detect the  $^{13}\text{C}$  enrichment from [ $^{13}\text{C}$ ]glycine where data of three days were combined. The runs above-and below-the-median test (“runs test”) was performed to detect rhythmicity in subjects with greater than 25 voids. Significantly fewer than expected runs above- and below-the-median indicate a non-random temporal distribution of the data, suggesting a rhythmic pattern. This runs test avoids having to force fit the data to a cosine function. The details of the principle of runs test is presented by Sokal and Rohlf [23].

## 3. Results

### 3.1. [ $^{13}\text{C}$ ]Formate dose

The  $^{13}\text{C}$  enrichment after a [ $^{13}\text{C}$ ]formate dose did not significantly correlate with the amount of uric acid excreted in each void, uric acid concentration or urine volume. The  $^{13}\text{C}$  enrichment at  $\text{C}_2$  (blue columns) and  $\text{C}_8$  (red columns) from subjects A and B is shown in Fig. 2. The peak %  $^{13}\text{C}$  enrichment from [ $^{13}\text{C}$ ]formate at  $\text{C}_2$  was 0.74 - 5.7% and that at  $\text{C}_8$  was 0.08 - 0.24%, and mean %  $^{13}\text{C}$  enrichment at  $\text{C}_2$  was significantly greater than 0 in all three subjects (Table 1, Fig. 2). Mean %  $^{13}\text{C}$  enrichment at  $\text{C}_8$  was significant greater than 0 in subjects B and C. Lower mean %  $^{13}\text{C}$  enrichments at  $\text{C}_8$  were generally found compared to  $\text{C}_2$ . Thus, contrary to our expectation, the  $^{13}\text{C}$  enrichment ratio of  $\text{C}_2/\text{C}_8$  was far from 1.0, and median  $\text{C}_2/\text{C}_8$  ratios were 6.6, 6.5 and 3.0 for subjects A and B (obtained from the data presented in Fig. 2) and C (data not presented), respectively. Only positive ratios were used to determine the median. We observed that only 11 of 66 voids, where the  $\text{C}_2/\text{C}_8$  ratios were positive, fell in the range 0.5 - 2.0, which could be generously considered close to 1.0.

To verify that our methods did not yield spurious positive enrichments, two paired-baseline values were subtracted from each other in subjects A and C (Table 1). In theory, this subtraction should have yielded 0% enrichment for all voids; however, this subtraction yielded non-significant mean % enrichments or mean % enrichments below 0 at  $\text{C}_2$  due to unavoidable errors in the LC/MS/MS method and calculations [20]. The %  $^{13}\text{C}$  enrichments at  $\text{C}_8$  in subject A was similar to the values that were found when two paired-baseline values were subtracted from each other, indicating no enrichment at  $\text{C}_8$ .

To test rhythmicity (circadian rhythm), the “run test” was applied to the data of subjects A and B [23]. As shown in Fig. 2, %  $^{13}\text{C}$  enrichment at  $\text{C}_2$  had much fewer runs (i.e., 8) than the predicted number of 25 and 23 for these subjects, indicating rhythmicity in the data ( $P < 0.01$ ). In contrast, %  $^{13}\text{C}$  enrichment at  $\text{C}_8$  had 19 and 17 runs for these two subjects, which is consistent with a random pattern, indicating no rhythmicity ( $P > 0.05$ ) [23]. Subject C did not have a sufficient number of voids to perform the test for rhythmicity.

### 3.2. [2-<sup>13</sup>C]glycine dose

There was low <sup>13</sup>C enrichment at C<sub>2</sub> after the [2-<sup>13</sup>C]glycine dose (Table 1, Fig. 2). Of 28 voids collected for each subject, over 80% was 0 or negative <sup>13</sup>C enrichment at C<sub>2</sub>, indicating that the ratio of C<sub>2</sub>/C<sub>8</sub> (plus C<sub>5</sub>) after a [2-<sup>13</sup>C]glycine dose was low. In both subjects, significant <sup>13</sup>C enrichments at C<sub>2</sub> after [2-<sup>13</sup>C]glycine were detected in only several voids that correspond to the timing of high C<sub>2</sub> enrichments from [<sup>13</sup>C]formate (Fig. 2). [2-<sup>13</sup>C]glycine enriched the C<sub>8</sub> plus C<sub>5</sub> (red column) positions, and peak % <sup>13</sup>C enrichments at C<sub>8</sub> plus C<sub>5</sub> were 0.62 and 0.42% in subjects A and B, respectively (Fig. 2, Table 1). Unlike the formate dose, no rhythmicity was found in the <sup>13</sup>C enrichment at C<sub>8</sub> plus C<sub>5</sub>.

## 4. Discussion

We found that [<sup>13</sup>C]formate predominantly enriched the C<sub>2</sub> position of purines, whereas significantly greater than zero <sup>13</sup>C enrichment was only found at the combination of the C<sub>8</sub> and C<sub>5</sub> positions after a [2-<sup>13</sup>C]glycine dose (Table 1, Fig. 2). We shall describe our hypotheses to explain these findings below; however, we realize that these hypotheses (see Fig. 3) may require reevaluation or modification in the future, or they may be incorrect.

### 4.1. Mechanistic explanation of the enrichment at the C position by [<sup>13</sup>C]formate

Our finding differs from the previously reported C<sub>2</sub>/C<sub>8</sub> ratio of about 1.0 in animals [6-8]. We postulate that the liver stops purine biosynthesis *de novo* at AICAR, because isolated mammalian hepatocytes do not metabolize AICAR to IMP or to any other purines [24-28]. This is consistent with the low capacity of the mammalian hepatocytes or liver slices to synthesize purines *in vitro* from radioactive formate or serine [29-31]. The liver cannot enrich C<sub>2</sub> from [<sup>13</sup>C]formate, because AICAR cannot be metabolized to IMP (Fig. 3). Therefore, to explain our data, we tried to identify cells that: a) predominantly utilize formate as a source of one carbons; b) have AICAR transformylase and 10-HCO-H<sub>4</sub>folate synthetase; and c) have an external supply of AICAR. We identified erythrocytes as being a prime candidate that fulfills all these requirements because erythrocytes: a) contain double the amount of formate compared to plasma [3]; b) possess the above two key enzymes but neither GAR transformylase, GCS, nor serine hydroxymethyltransferase (SHMT) [11,32-34]; and c) are exposed to AICAR-riboside, or its base in the circulation, since AICA is a normal constituent of human urine [35]. In fact, AICAR accumulates in erythrocytes in Lesch-Nyhan syndrome, some forms of gout, and a genetic defect of AICAR transformylase [36,37]. Therefore, we hypothesized that the formation of IMP from AICAR with formate and H<sub>4</sub>folate occurs in erythrocytes. The idea of human erythrocytes participating in purine biosynthesis in such a way is not novel. Bertino et al [32] suggested this over 40 years ago based on the findings of abundant activities of 10-HCO-H<sub>4</sub>folate synthetase and AICAR transformylase. In fact, Lowy et al [33] and Wagner and Levitch [34] reported that the *in vitro* incubation of intact erythrocytes with [<sup>14</sup>C]formate and AICA-riboside lead to the formation of IMP, and that this metabolism required H<sub>4</sub>folate, ATP and the formation of AICAR from AICA-riboside. The mass of human erythrocytes, almost equal to that of the liver, should not be underestimated in its ability to metabolize purines [38]. In human erythrocytes, however, the lack of GAR transformylase and most of the other enzymes participating in purine biosynthesis precludes the <sup>13</sup>C enrichment at C<sub>8</sub> [33].

Let us explain our thoughts on how erythrocytes and the liver metabolize and shuttle purines using Fig. 3. Firstly, where does AICAR in erythrocytes come from? We propose that the liver synthesizes AICAR *de novo* and exports into the circulation as AICA-riboside, which is then phosphorylated to AICAR by erythrocyte adenosine kinase. It has been shown that isolated rat hepatocytes cycle AICAR to AICA-riboside and the latter can be released from these cells to the medium [28]. Secondly, what is the fate of erythrocyte IMP, a minor component of its purine pool? Other researchers have established that IMP cannot be converted to AMP in

erythrocytes [33,39]; therefore, it must be exported to the circulation as inosine or hypoxanthine [40]. It has been shown that human erythrocytes loaded with [8-<sup>14</sup>C]IMP *ex vivo* rapidly release radioactivity *in vivo* ( $t_{1/2} = 1$  hour) when re-injected back to their donor [40]. Presumably IMP is released as inosine or hypoxanthine, that are present in blood at 0.1 - 0.5  $\mu\text{M}$  [41]. It is known that AMP is again exported from the liver to the circulation as adenosine that is incorporated back to erythrocytes and metabolized back to AMP by adenosine kinase to maintain adequate erythrocyte ATP concentration [38,39] (Fig. 3). Plasma adenosine concentrations range from 0.1 to 0.5  $\mu\text{M}$  [41].

We now present possible reasons why [<sup>13</sup>C]formate failed to enrich C<sub>8</sub>. This may be due to the dilution of 10-H[<sup>13</sup>C]O-H<sub>4</sub>folate by channeling of 10-H[<sup>12</sup>C]O-H<sub>4</sub>folate to hepatic GAR transformylase (Fig. 3). Avian hepatic GAR transformylase forms a complex with the trifunctional enzyme and SHMT [42,43]. Thus, 5,10-CH<sub>2</sub>-H<sub>4</sub>folate or 5,10-CH=H<sub>4</sub>folate, formed from glycine, serine and histidine, could be channeled to GAR transformylase as 10-H[<sup>12</sup>C]O-H<sub>4</sub>folate resulting in the dilution of the <sup>13</sup>C enrichment at C<sub>8</sub>. Although this is a possible explanation, the existence of such an enzyme complex, however, has not been proven in human liver. In addition, formate is readily oxidized such that about 25% of a tracer dose of [<sup>14</sup>C]formate is lost as <sup>14</sup>CO<sub>2</sub> in 2 hour, whereas only 1% is incorporated into uric acid in 11 days in humans [19]. Some of this oxidation likely takes place in hepatic peroxisomes [44]. Thus, formate is potentially metabolized to CO<sub>2</sub> rather than participating in purine biosynthesis in human liver.

We observed the circadian rhythm in the <sup>13</sup>C enrichment at C<sub>2</sub>. Erythrocyte adenosine kinase activity parallels blood inosine and hypoxanthine concentrations with a similar circadian rhythm [41]. Therefore, it is possible that the circadian rhythm in adenosine kinase activity could account for rhythmicity in the <sup>13</sup>C enrichment at C<sub>2</sub> (Fig. 3).

#### 4.2. Mechanistic explanation of the enrichment of the C<sub>2</sub>, C<sub>8</sub> and C<sub>5</sub> positions by [2-<sup>13</sup>C] glycine

We postulate that the formation of GAR and formyl-GAR from [2-<sup>13</sup>C]glycine occurs in the liver [45], and enriches both C<sub>8</sub> and C<sub>5</sub> (Fig. 1 and 3). We are forced to report in this way, because our method does not distinguish independent enrichment at C<sub>8</sub> and C<sub>5</sub> [20]. Based on the findings by Pimstone et al [46], this may not impose problems in interpreting our <sup>13</sup>C enrichment data at C<sub>8</sub>. They found that 20% of [2-<sup>14</sup>C]glycine is incorporated into the C<sub>2</sub> and C<sub>8</sub> positions through folate-dependent reactions and the remaining 80% into the C<sub>4</sub> and C<sub>5</sub> positions through the folate independent pathway [46]. They used a method involving degradation of uric acid that does not distinguish between the <sup>14</sup>C incorporation at C<sub>2</sub> and C<sub>8</sub>. Although their data cannot be directly compared to ours, we hypothesize that the majority of this 20% of the <sup>13</sup>C enrichment found in our study is at C<sub>8</sub>. It is unlikely that our method was not sensitive enough to detect <sup>13</sup>C enrichment at the C<sub>2</sub> positions. Our data further agree with those by Heinrich and Wilson [7] who found that there was no labeling at C<sub>2</sub> of guanine in rat carcass after [1,2-<sup>14</sup>C]glycine administration, whereas C<sub>8</sub> was labeled.

The substantial <sup>13</sup>C enrichments at C<sub>2</sub> after [2-<sup>13</sup>C]glycine in subject B corresponded to the timing of high enrichments at C<sub>2</sub> by [<sup>13</sup>C]formate (Fig. 2). This finding suggests that some [<sup>13</sup>C]formate was produced from [2-<sup>13</sup>C]glycine or 10-H[<sup>13</sup>C]O-H<sub>4</sub>folate and enriched C<sub>2</sub>. The [2-<sup>13</sup>C]glycine dose used by us might have made a small but detectable contribution to the [<sup>13</sup>C]formate pool. However, considering many metabolic pathways involving glycine and our larger dose of glycine than formate on molar basis, its pathway to formate may be minor. Various substrates contribute to the formate pool without involving folate coenzymes, including methylthioadenosine, tryptophan, choline, acetate, and others [47-49].

Relatively low  $^{13}\text{C}$  enrichment at  $\text{C}_2$  with  $[2-^{13}\text{C}]$ glycine supports our interpretation of the  $[^{13}\text{C}]$ formate data because erythrocytes with the absence of mitochondria have no GCS activity, whereas the liver has high GCS activity [11]. A possible metabolic pathway for  $[2-^{13}\text{C}]$ glycine includes the following. In hepatic mitochondria, GCS with  $\text{H}_4$ folate cleaves  $[2-^{13}\text{C}]$ glycine to  $5,10-[^{13}\text{C}]\text{H}_2\text{-H}_4$ folate,  $\text{CO}_2$  and  $\text{NH}_3$ . Mitochondrial SHMT in the presence of glycine converts  $5,10-[^{13}\text{C}]\text{H}_2\text{-H}_4$ folate to  $[3-^{13}\text{C}]$ serine, which is then transported to the cytoplasm [2,11] as shown in Fig. 3. Cytoplasmic  $5,10-[^{13}\text{C}]\text{H}_2\text{-H}_4$ folate is formed from  $\text{H}_4$ folate and  $[3-^{13}\text{C}]$ serine catalyzed by cytoplasmic SHMT and further metabolized to  $10\text{-H } [^{13}\text{C}]\text{O-H}_4$ folate by trifunctional enzyme (Fig. 3).

The %  $\text{C}_{13}$  enrichment at  $\text{C}_8$  of 0.19 - 0.39 from  $[2-^{12}\text{C}]$ glycine is less than that at  $\text{C}_2$  of 0.40 - 0.84 from  $[^{13}\text{C}]$ formate in subjects A and B, even though the glycine dose was greater (Table 1). This may be due to channeling of  $^{12}\text{C}$  and dilution of  $^{13}\text{C}$  by  $^{12}\text{C}$  in the GAR transformylase, trifunctional enzyme and SHMT complex as discussed above [42,43].

Using  $[2-^{13}\text{C}]$ glycine, we unavoidably tested the  $^{13}\text{C}$  enrichment as if we used  $[3-^{13}\text{C}]$ serine. As we discussed previously,  $[2-^{12}\text{C}]$ glycine and  $[2-^{13}\text{C}]$ glycine can form  $[3-^{13}\text{C}]$ serine in the presence of GCS and SHMT in hepatic mitochondria [11], and  $[3-^{13}\text{C}]$ serine can be transported to the cytoplasm (Fig. 3) [2].

### 4.3. Conclusion

The  $^{13}\text{C}$  enrichment at  $\text{C}_2$  of uric acid was greater than at  $\text{C}_8$  after a  $[^{13}\text{C}]$ formate dose, and a circadian rhythm was seen in the  $^{13}\text{C}$  enrichment at  $\text{C}_2$  in humans. After a  $[2-^{13}\text{C}]$ glycine dose, however, no significant  $^{13}\text{C}$  enrichment at  $\text{C}_2$  was found. To our knowledge, this is the first study to measure  $^{13}\text{C}$  enrichment from  $^{13}\text{C}$ -labeled formate and glycine independently into the  $\text{C}_2$  and  $\text{C}_8$  positions of purine in the same subjects. Although the number of subjects was small, the specificity and consistency of our data are compelling. However, it is necessary to stress that this research is in its infancy, and further investigations are required to confirm our findings and to prove our hypotheses.

Contrary to popular belief, our data suggest that  $^{13}\text{C}$  from these sources behave differently, and the incorporation of  $^{13}\text{C}$  of formate and the second  $^{13}\text{C}$  of glycine into purines may require coordination, which could be made not only on the molecular level but also on the organ level (erythrocytes and the liver). We take it for granted that purine metabolism presented in textbooks obtained mostly using uricotelic animals and microorganisms can be extrapolated to humans; however, our data suggest how fragile and precarious such an assumption is. Our non-invasive method of oral  $[^{13}\text{C}]$ formate or  $[2-^{13}\text{C}]$ glycine dosing and urine collection, although expensive, could be used to better understand human purine biosynthesis.

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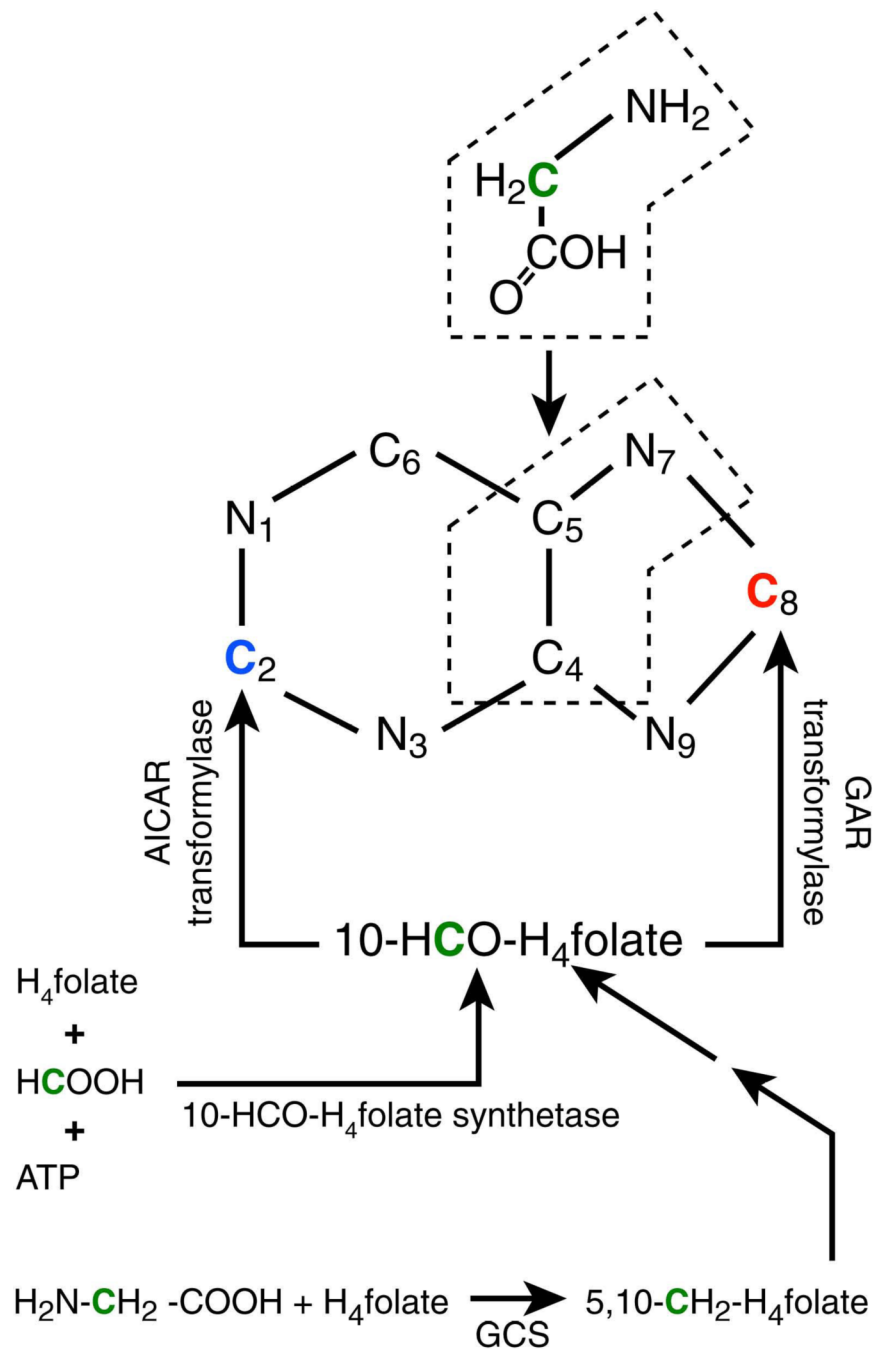
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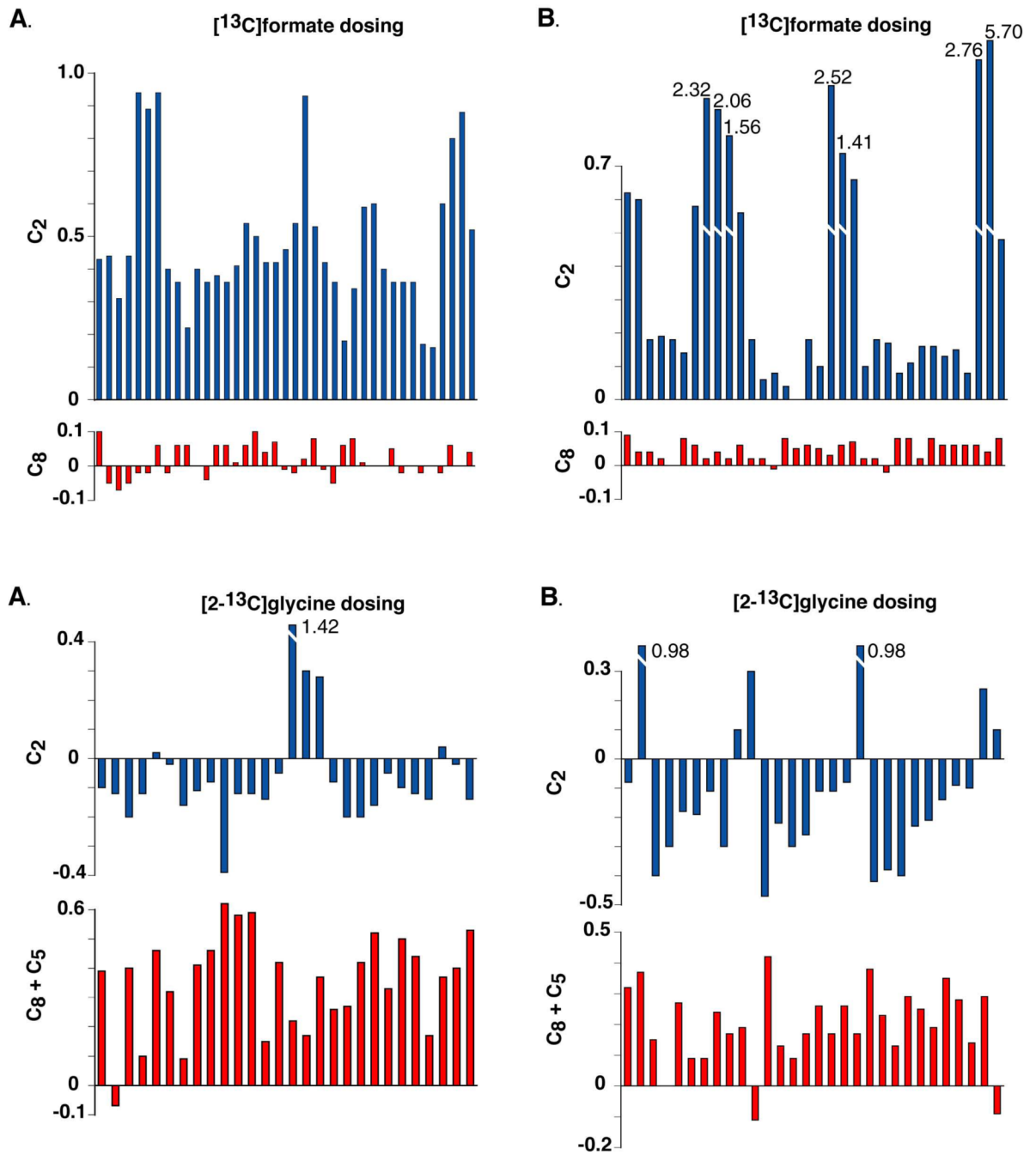
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**Fig1.** The origin of C<sub>2</sub>, C<sub>4</sub>, C<sub>5</sub>, C<sub>8</sub> and N<sub>7</sub> atoms of the purine ring from formate and glycine. The C of formate may be incorporated into C<sub>2</sub> and C<sub>8</sub> via the 10-HCO-H<sub>4</sub>folate synthetase, and GAR and AICAR transformylases. The first and second C and N of glycine are incorporated directly to C<sub>4</sub>, C<sub>5</sub> and N<sub>7</sub>, respectively (in broken lines). The second C of glycine may be incorporated into C<sub>2</sub> and C<sub>8</sub> via GCS and GAR and AICAR transformylases. All carbons potentially incorporated into C<sub>2</sub> and C<sub>8</sub> are in green, and C<sub>2</sub> and C<sub>8</sub> are in blue and red, respectively, which are matched with those of bars in Figure 2.



**Fig 2.** The % <sup>13</sup>C enrichment at C<sub>2</sub> and C<sub>8</sub> or C<sub>8</sub> plus C<sub>5</sub> of uric acid in each void after [<sup>13</sup>C]formate or [2-<sup>13</sup>C]glycine dosing. Bars represent <sup>13</sup>C enrichment at C<sub>2</sub> (blue) and C<sub>8</sub> or C<sub>8</sub> plus C<sub>5</sub> (red) in voids collected for 72 - 96 hours after [<sup>13</sup>C] formate or [2-<sup>13</sup>C]glycine dosing in subjects A and B. The x-axis is time after dosing; however, the intervals between voids were different. Therefore, we did not show specify time of the void. The numbers next to the bars are % enrichments that were out of range on the y-axis.



C<sub>8</sub> plus C<sub>5</sub> are labeled by [2-<sup>13</sup>C]glycine (see Fig. 1). The liver converts inosine that is exported from erythrocytes to AMP. This AMP is then returned to erythrocytes as shown in order to maintain ATP concentrations. The coordinated IMP and AMP metabolism in the liver and erythrocytes has been previously established [38,39].

Mean ( $\pm$  SD)%  $^{13}\text{C}$  enrichment at the  $\text{C}_2$  and  $\text{C}_8$  positions of uric acid after [ $^{13}\text{C}$ ]formate dose and at the  $\text{C}_2$  and  $\text{C}_8$  plus  $\text{C}_5$  positions of uric acid after a [ $^{13}\text{C}$ ]glycine dose

Table 1

Subject [ $^{13}\text{C}$ ]formate	Dose (mmol)	Day 1 % Enrichment at $\text{C}_2$ (number of voids analyzed)	Day 2 % Enrichment at $\text{C}_2$ (number of voids analyzed)	Day 3 % Enrichment at $\text{C}_2$ (number of voids analyzed)	Day 1 % Enrichment at $\text{C}_8$ plus $\text{C}_5$ (number of voids analyzed)	Day 2 % Enrichment at $\text{C}_8$ plus $\text{C}_5$ (number of voids analyzed)	Day 3 % Enrichment at $\text{C}_8$ plus $\text{C}_5$ (number of voids analyzed)
A	14.5	0.58 $\pm$ 0.25 (9) <sup>d</sup>	0.42 $\pm$ 0.09 (12) <sup>d</sup>	0.47 $\pm$ 0.19 (10) <sup>d</sup>	0.00 $\pm$ 0.06 (9)	0.03 $\pm$ 0.04 (12)	0.02 $\pm$ 0.04 (10)
B	14.5	0.84 $\pm$ 0.78 (10) <sup>d</sup>	0.51 $\pm$ 0.78 (10) <sup>d</sup>	0.40 $\pm$ 0.73 (12) <sup>d</sup>	0.04 $\pm$ 0.03 (10) <sup>d</sup>	0.04 $\pm$ 0.03 (10) <sup>d</sup>	0.05 $\pm$ 0.03 (12) <sup>d</sup>
A	0	-0.06 $\pm$ 0.05 (8)	0.02 $\pm$ 0.04 (9)	0.03 $\pm$ 0.06 (9)	-0.01 $\pm$ 0.09 (8)	0.04 $\pm$ 0.08 (9)	0.04 $\pm$ 0.08 (9)
C	14.5	0.43 $\pm$ 0.18 <sup>d</sup> (16)	0.03 $\pm$ 0.16 (16)		0.16 $\pm$ 0.04 <sup>d</sup> (16)		
C	0				0.01 $\pm$ 0.09 (16)		

	% Enrichment at $\text{C}_2$ (number of voids analyzed)	% Enrichment at $\text{C}_8$ plus $\text{C}_5$ (number of voids analyzed)
A	41.7	0.39 $\pm$ 0.17 <sup>d</sup> (28)
B	41.7	0.19 $\pm$ 0.13 <sup>d</sup> (28)

<sup>d</sup>Significantly greater than 0 by the Wilcoxon-paired sample test ( $P < 0.05$ ).