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Hyperglycinemia due to folate deficiency in rats: evidence for the lack of involvement of the hepatic glycine cleavage system☆

Tyra M. Dickson[a], Erica Edison[c], John T. Brosnan[c], James D. House[a,b,*]

[a]Department of Animal Science, University of Manitoba, Winnipeg, MB, Canada R3T 2N2
[b]Department of Human Nutritional Sciences, University of Manitoba, Winnipeg, MB, Canada R3T 2N2
[c]The Department of Biochemistry, Memorial University of Newfoundland, St. John's, NL, Canada A1B 3X9

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Abstract

In addition to a well-recognized hyperhomocysteinemic state, folate deficiency also leads to profound hyperglycinemia. To further characterize the latter observation, two trials were conducted using a folate-deficient rat model to (1) determine the sensitivity of plasma glycine to folate repletion and (2) test the hypothesis that hyperglycinemia results from a reduced flux through the folate-dependent glycine cleavage system (GCS). Weanling male Sprague–Dawley rats were used, and they consumed an amino acid-defined diet with either 0 (FD) or 1 (FA) mg/kg of crystalline folic acid. In Trial 1, 30 rats consumed the FD diet for 28 days. Rats then consumed diets containing 0.1, 0.2, 0.3 or 0.4 mg/kg of folic acid for 14 days before termination. In Trial 2, 16 rats were allocated to receive either the FA ($n=8$) or FD ($n=8$) diet for 30 days before termination. Liver mitochondria were isolated and flux through the GCS (measured as $^{14}CO_2$ production from 1-^{14}C-glycine) was determined. Plasma from blood collected at termination was analyzed for folate, homocysteine and glycine. In Trial 1, both homocysteine and glycine responded linearly to increased dietary folic acid (milligrams per kilogram) levels ($P<.05$). In Trial 2, plasma folate (FA=25.85 vs. FD=0.66; S.E.M.=1.4 μM), homocysteine (FA=11.1 vs. FD=55.3; S.E.M.=1.7 μM) and glycine (FA=564 vs. FD=1983; S.E.M.=114 μM) were significantly affected by folate deficiency ($P<.0001$). However, glycine flux through hepatic GCS was not affected by folate deficiency ($P>.05$). These results provide evidence that in a folate-deficient rat model, both homocysteine and glycine are sensitive to dietary folic acid levels; however, the observed hyperglycinemia does not appear to be related to a reduced flux through the hepatic GCS.

Keywords: Folate deficiency; Homocysteine; Glycine; Glycine cleavage system

1. Introduction

The depletion/repletion protocol has been used extensively for the measurement of folate bioavailability in animal models, most notably rats [1,2]. With the rodent model, rats are typically made folate deficient through consumption of crystalline amino acid diets devoid of folate and containing 1% succinylsulfathiazole for a period of approximately 28 days [3–5]. After attainment of a folate-depleted state, the ingredient containing folate is included back into the diet at a given level of folate and rats are allowed to consume the diets for a period in excess of 7 days. By comparison of the changes in either plasma or liver folate concentrations relative to changes observed in rats consuming an equal amount of crystalline folic acid, a measure of the bioavailability (crystalline folic acid set at 100%) of the food folate can be made through the use of slope ratio analysis. In addition to folate concentrations, plasma homocysteine has been shown to be sensitive to folate repletion [6]. Previously, we characterized the temporal changes in folate and plasma homocysteine concentrations in response to a single level of folate repletion (0.25 mg/kg diet) and used these response criteria to determine that the relative bioavailability of folate in egg yolk was ≥100% [7]. In addition to using homocysteine as a sensitive marker of folate status, we showed that plasma glycine concentrations were acutely sensitive to folate status

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* Corresponding author. Department of Animal Science, University of Manitoba, Winnipeg, MB, Canada R3T 2N2. Tel.: +1 204 474 9523; fax: +1 204 474 7628.

E-mail address: j_house@umanitoba.ca (J.D. House).

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in this model, increasing by more than fourfold during folate depletion and decreasing during repletion. The temporal changes in glycine concentrations were determined at a single level of folate repletion; therefore, the sensitivity of the changes in plasma glycine concentrations in response to different levels of folate repletion must be determined.

The factors leading to hyperglycinemia during folate deficiency are poorly characterized. As depicted in Fig. 1, folate serves as a cofactor in several reactions of glycine metabolism [8]. However, the principal route of glycine oxidation is believed to be through the folate-dependent glycine cleavage system (GCS), a mitochondrial multiprotein system consisting of P-protein, T-protein, H-protein and L-protein (dihydrolipoamide dehydrogenase) [9]. These four proteins catalyze the oxidative breakdown of glycine to carbon dioxide and ammonia with the transfer of a one-carbon unit to tetrahydrofolate (THF) to form 5,10-methylenetetrahydrofolate. Elevated levels of glycine in the blood and cerebrospinal fluid are characteristic of an inherited deficiency of the GCS leading to nonketotic hyperglycinemia [10,11]. However, it remains to be determined as to what extent flux through the GCS is impaired during folate deficiency. Therefore, the main objectives of this folate depletion/repletion study are (1) to determine the sensitivity of plasma glycine to varying degrees of folate repletion in the folate-deficient rat and (2) to test the hypothesis that hyperglycinemia due to folate deficiency results from a reduced flux through the folate-dependent hepatic GCS.

2. Materials and methods

2.1. Chemicals

All chemicals, unless otherwise indicated, were obtained from Sigma Chemical (Oakville, ON, Canada). 1-^{14}C-glycine was obtained from American Radiolabelled Chemicals (St. Louis, MO, USA) and NCS-II tissue solubilizer was obtained from Amersham Biosciences (Piscataway, NJ, USA).

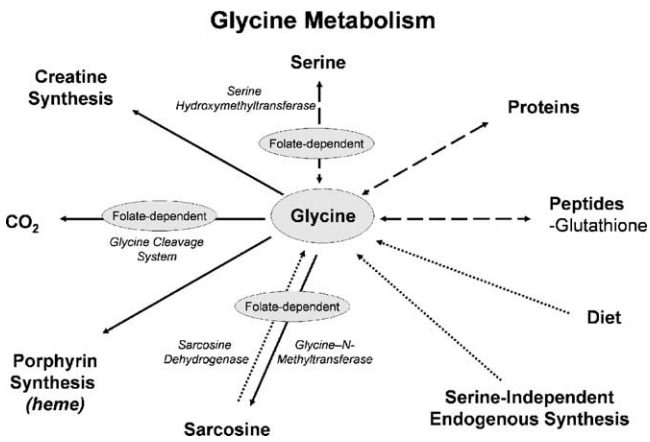

Fig. 1. Pathways of folate-dependent and folate-independent glycine metabolism.

2.2. Animals

Weanling male Sprague–Dawley rats with an initial weight of 70–80 g (Experiment 1: Central Animal Care, University of Manitoba, Winnipeg, Manitoba; Experiment 2: Memorial University's Central Animal Breeding) were used. They were housed in a humidified (45%) temperature-controlled (21°C) environment with a 12-h photoperiod (lights on from 08:00 to 20:00). Rats were individually housed in stainless steel wire-bottomed cages. Each cage was equipped with a glass feeder, a glass water bottle and a stainless steel drinking nipple. All rats had ad libitum access to feed and water. All animals were treated with ethical consideration as directed by the guidelines for experimental animals set by the Canadian Council of Animal Care [12] and as enforced by the respective institutions' animal care committees.

2.3. Basal diet

The basal diet used in both trials was a folic acid-devoid, amino acid-defined diet from a commercial diet formulator (Harlan Teklad, Madison, WI, USA), the composition of which was described previously [7], with cobalamin concentrations increased to meet NRC requirements for rats (50 μg/kg diet) [13]. The diet included 1% succinylsulfathiazole to inhibit intestinal folate synthesis.

2.4. Trial 1: Sensitivity of plasma homocysteine and glycine to folate repletion

Twenty-four rats were made folate deficient through consumption, for 4 weeks, of the basal folate-devoid diet. This period was chosen on the basis of results from previous work [7]. After the folate depletion period, rats ($n=6$ per treatment) were randomly assigned to receive the basal diet, supplemented with 0.1, 0.2, 0.3 or 0.4 mg crystalline folic acid/kg diet for 2 weeks. The repletion doses were chosen to provide folic acid at levels below and above the previously studied level of 0.25 mg/kg diet [7], a dose shown to lead to significant changes in both homocysteine and glycine concentrations. Feed intake was measured daily and body weights were measured on a weekly basis. At the end of the 2-week repletion period, rats were anesthetized and samples were procured, as described below.

2.5. Trial 2: Effect of folate deficiency on flux through the hepatic GCS

Twelve rats ($n=6$ per treatment) were provided the basal diet containing either 0 or 1 mg/kg crystalline folic acid for ad libitum consumption for 30 days under the housing conditions described above. After the 30-day feeding period, rats were anesthetized and samples, including isolated liver mitochondria, were procured as described below.

2.6. Blood sampling and analyses

At the designed termination dates and immediately after lights were turned on, rats were weighed, anaesthetized by an intraperitoneal injection of 60 mg/kg of sodium pentobarbital (65 mg/ml; CDMV, Calgary, AB, Canada) and killed by exsanguination. Blood was collected via direct cardiac puncture into evacuated tubes containing sodium heparin, and the tubes were then placed immediately on ice. Plasma was separated from erythrocytes via centrifugation ($3000\times g$ at 4°C for 20 min) and stored at −80°C until analyzed.

Plasma homocysteine concentrations were determined via reversed-phase HPLC, according to the method of Araki and Sako [14], with modifications as suggested by Gilfix et al [15]. Plasma folate concentrations were determined by radioimmunoassay (Quantaphase II, Bio-Rad Laboratories, Mississauga, ON, Canada). Plasma glycine concentrations were measured, via ion-exchange chromatography (LKB 4151 Alpha-Plus Amino Acid Analyzer), using the method of Blom and Huijmans [16].

2.7. Measurement of flux through GCS in isolated hepatic mitochondria

Mitochondria were isolated from the livers of rats from Trial 2 according to procedures outlined previously [17]. The resultant mitochondrial pellet, obtained after homogenization and centrifugation, was suspended in a medium containing 0.225 M mannitol, 0.075 M sucrose, 5 mM HEPES and 1 mM EGTA (pH 7.2) to give a final protein concentration of 30–40 mg protein/ml, as determined by the biuret method [18]. Flux through the GCS pathway was determined according to published methods [19] by measuring the production of $^{14}CO_2$ trapped in NCS-II in center wells from 1-^{14}C-glycine [20]. The mitochondrial incubation medium (100 mM potassium chloride, 50 mM mannitol, 25 mM HEPES, 20 mM sucrose, 10 mM potassium phosphate, 1 mM ADP, 1 mM magnesium chloride, 0.175 mM pyridoxal phosphate, 0.1 mM EGTA, pH 7.4) contained either 1 or 5 mM glycine and either 0 or 1 mM THF (Sigma Chemical) that had been stabilized with an equimolar concentration of 2-mercaptoethanol. Control mitochondrial incubations showed no effect of 2-mercaptoethanol alone on flux through the GCS at either glycine concentration.

2.8. Statistical analyses

The studies were designed as completely randomized designs with the main effects of folate level. In both trials, data were subjected to ANOVA using the PROC-GLM feature of SAS [21]. When evidence of heterogeneity of variance was present, data were log transformed before analysis. Data are presented as least-square means plus S.E.s, with differences between treatments assessed using Fisher's protected least-squares difference procedure. The level of significance was set at an α level of $P<.05$. In Trial 1, simple linear regression analysis was performed using the PROC-REG feature of SAS [21], with plasma biochemical values serving as the dependent variable and dietary folate level (mg/kg) or plasma folate serving as the independent variable.

3. Results

Feed intake and weight gains were not significantly affected either by the level of folate repletion during Trial 1 or by the level of folate in the diet in Trial 2, consistent with previous findings ([6,7]; data not shown). In Trial 1, both plasma homocysteine and glycine concentrations decreased significantly when dietary folic acid levels increased from 0.1 to 0.3 mg/kg diet (Table 1), with no significant differences observed between 0.3 and 0.4 mg folic acid/kg diet. Plasma folate concentrations increased significantly over the range of folate repletion levels tested (Table 1). As a measure of the sensitivity of the response variables to folate repletion, 74% of the variability in the measures of plasma homocysteine and glycine can be attributed to the level of folate in the diet, whereas the latter accounts for 86% of the variability in plasma folate measurements. Plasma folate accounted for 81% of the variability in plasma

Table 1
Changes in concentrations of plasma folate, homocysteine and glycine in folate-depleted rats in response to graded dietary levels of folate provided as folic acid (Trial 1)

Plasma variable	Dietary folate (mg/kg)*				S.E.M.**	P***	Regression analysis****		
	0.1	0.2	0.3	0.4			Intercept±S.E. (P)*****	Slope±S.E.(P)*****	r^2
Folate (nmol/ml)	1.14[a]	2.13[b]	3.75[c]	4.96[d]	0.26	<.0001	−0.27±0.31 (.39)	13.1±1.1 (<.0001)	.86
Homocysteine (μmol/L)	125.7[a]	58.0[b]	34.1[c]	23.9[c]	7.0	<.0001	142.7±11.3 (<.0001)	−329±41 (<.0001)	.74
Glycine (μmol/L)	3576[a]	2191[b]	1406[c]	894[c]	246	<.0001	4224±309 (<.0001)	−8832±1129 (<.0001)	.74

* Data are presented as least-square means.

** Standard error of the least-square mean.

*** Overall P value due to dietary folate level. Data within rows with different superscripts are significantly different ($P<.05$) as assessed by Fisher's protected least-squares difference procedure.

**** Simple linear regression analyses ($y=a+bx$) of dependent variables plasma folate, homocysteine and glycine, with dietary folate level serving as the independent variable.

***** P values testing null hypotheses that estimate 0.

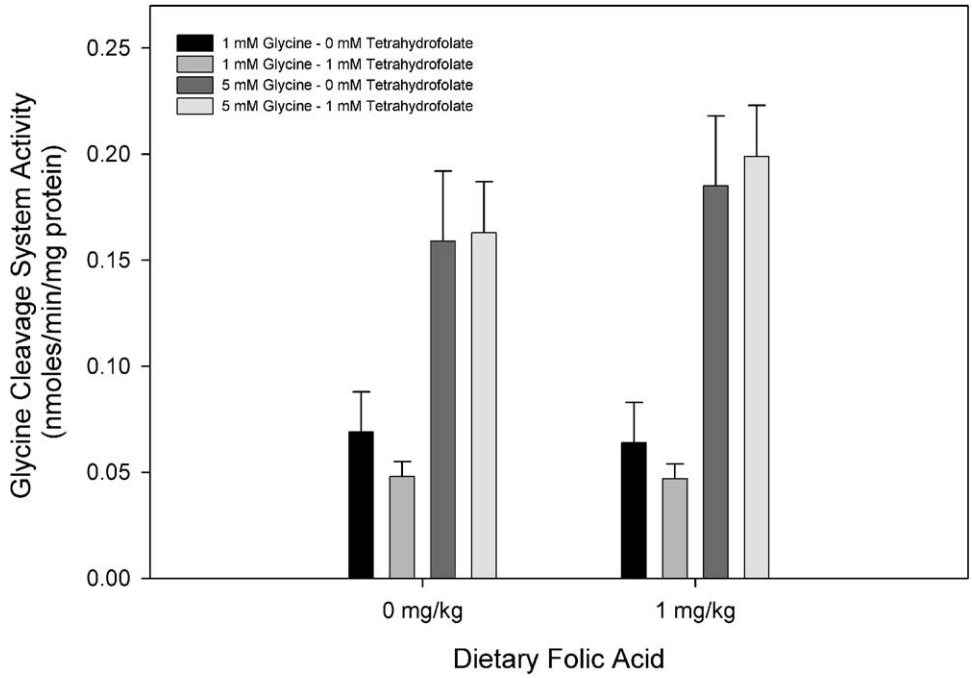

Fig. 2. GCS activity, measured as $^{14}CO_2$ production from 1-^{14}C-glycine, in mitochondria isolated from livers of rats maintained for 30 days on purified diets containing either 0 or 1 mg/kg crystalline folic acid.

glycine concentrations [intercept (S.E.)=3987 (227); slope (S.E.)=−658 (67); $P<.0001$] and 69% of the variability in plasma homocysteine concentrations [intercept (S.E.)=128 (11); slope (S.E.)=−23 (3); $P<.0001$; units=μmol/L]. Plasma homocysteine accounted for 83% of the variability in plasma glycine concentrations [intercept (S.E.)=529 (174); slope (S.E.)=24.6 (2.4); $P<.0001$; units=μmol/L].

In Trial 2, rats consuming the folate-deficient diet had significantly ($P<.05$) lower plasma folate (0.66 vs. 25.85 μM; S.E.M.=1.4 μM) and significantly ($P<.05$) elevated plasma homocysteine (55.3 vs. 11.0 μM; S.E.M.=1.7 μM) and glycine (1983 vs. 564 μM; S.E.M.=114 μM) compared with rats consuming diets containing 1 mg/kg folic acid. Despite the marked increased in plasma glycine, flux through the hepatic GCS, measured as mitochondrial $^{14}CO_2$ production from 1-^{14}C-glycine, was not significantly affected by folate status (Fig. 2) at either of the two glycine concentrations tested (1 and 5 mM). While GCS was sensitive to the level of glycine in the incubation media, the addition of the GCS substrate THF to the incubation media did not significantly affect glycine oxidation.

4. Discussion

The results obtained in the current study provide strong evidence that plasma glycine, in addition to plasma homocysteine and folate, is sensitive to the level of folate repletion in the folate depletion/repletion rat bioassay. In our previous study, rats consumed diets during the repletion phase that contained a single level of folate, set at 0.25 mg/kg as either crystalline folic acid or egg yolk folate. This level of folate repletion was chosen based on previous research [6] that documented significant reductions in plasma homocysteine over a range of 0.1–0.5 mg folic acid/kg diet. The current results support a range of 0.1–0.3 mg folate/kg diet, for a 2-week repletion period, for glycine as well as homocysteine, when either is used as the response criterion for bioavailability studies. Beyond 0.3 mg folic acid/kg diet, changes in the concentrations of either homocysteine or glycine in response to additional folic acid reflect a nonlinear portion of the response curve, as indicated by a lack of significant difference between values at 0.3 and 0.4 mg folic acid/kg diet. Despite the lack of significance between the 0.3 and 0.4 mg/kg repletion levels, the concentrations of plasma homocysteine, glycine and folate did not reach control levels seen with the 1.0 mg/kg level of repletion used in our previous study [7] and in Trial 2. Fitting the data set to a simple linear regression equation, as is needed for slope ratio analyses, with dietary folate level serving as the independent variable, yielded similar r^2 estimates (.74) when either glycine or homocysteine was used as the dependent variable. Therefore, both response criteria were equally sensitive to changes in dietary folate levels. The folate level of the diet explained 86% of the variability associated with plasma folate, providing evidence that, in the current study, this response criterion was a slightly more sensitive measure of folate status over the range of folic acid intakes measured.

Based on the results from our previous work, coupled with the current data, a repletion period of 2 weeks after a 4-week depletion period [7] and a folate repletion dose range of 0.1–0.3 mg folate/kg diet are recommended for

future studies using the rat bioassay for measuring folate bioavailability when plasma glycine as well as homocysteine and folate are used as response criteria. This repletion dose range is consistent with that used by other groups using liver or serum folate as a response criterion [2,6]. The choice of response criterion can have a significant impact on the estimate of folate bioavailability obtained in depletion/repletion bioassays. Clifford et al. [2] observed a high correlation between bioavailability estimates derived from serum folate and growth; however, they also observed consistently lower estimates of folate bioavailability using serum folate as a response criterion (approximately 20%). Folate-enriched egg yolk powder, when compared with crystalline folic acid, elicited greater reductions in plasma homocysteine (relative bioavailability > 100%) but produced similar reductions in plasma glycine and folate in rats (relative bioavailability = 100%; [7]). The latter observation is likely explained by the fact that plasma folate accounted for 81% of the variability in plasma glycine and 69% in plasma homocysteine, suggesting a tighter relationship between plasma glycine and folate measures than that which exists between plasma homocysteine and folate in the current model system. Consistent with the findings of Stabler et al. [22], a strong relationship between the plasma glycine and homocysteine concentrations was observed.

The use of plasma glycine as a response criterion for folate status necessitates further examination of the factors that control its concentration during a folate deficiency. Plasma glycine levels broadly reflect a balance between glycine production and utilization. As indicated in Fig. 1, folate participates in a number of pathways of glycine metabolism including (1) the serine hydoxymethyltransferase reaction (SHMT; EC. 2.1.2.1.), (2) the sarcosine dehydrogenase reaction, (3) the glycine-*N*-methyltransferase reaction (GNMT; EC 2.1.1.20) and (4) the T-protein (EC 2.1.2.10) component of the GCS reaction. In clinical settings, hyperglycinemia normally presents due to a disruption in one or more of the components of the GCS. In ketotic hyperglycinemia, elevated plasma glycine levels are believed to arise as a result of the inhibition of the H-protein component of the enzyme complex, secondary to organic acidemia (i.e., propionic acidemia and methylmalonic acidemia) [23]. In nonketotic hyperglycinemia, glycine oxidation is inhibited due to a genetic mutation in either the T-protein or the P-protein component of the GCS [11], rendering the enzyme system inactive, with a concomitant reduction in glycine oxidation. THF serves as a cofactor for the T-protein of the mitochondrial GCS [10]. Previous research has shown that mitochondrial THF levels are reduced by 90%, relative to control levels, in livers from rats made folate deficient using a similar model [24]. Therefore, we hypothesized that the folate-induced hyperglycinemia arose due to a reduced flux through the hepatic mitochondrial GCS. However, this was not observed in the current study. GCS flux rates did not differ as a result of folate status, nor did they respond to added THF in the incubation media, which may reflect a limited capacity for the mitochondrial uptake of reduced folates [25]. The fact that glycine oxidation rates increased in response to increasing the glycine concentration in the incubation media from 1 to 5 mM provides evidence that the lack of difference was not a result of exceeding the metabolic capacity for glycine oxidation.

We and others have documented the responsiveness of the mitochondrial GCS to regulation by hormonal, nutritional and metabolic signals. Flux through the mitochondrial GCS is sensitive to the oxidation state of pyridine nucleotides [20], with oxidizing conditions promoting higher rates of glycine decarboxylation. Furthermore, the work of Hampson et al. [20] clearly showed that the GCS was the predominant route whereby the 1-carbon of glycine is released as CO_2. Evidence that plasma glycine concentrations are reduced in rats consuming high-protein diets [26] provided the backdrop for studies in which the flux through the GCS was shown to be higher in mitochondria isolated from rats consuming high (60% casein) versus standard (15% casein) diets [27]. The hormone glucagon, known to be elevated in rats receiving high-protein diets [28], stimulates flux through the mitochondrial GCS via calcium-dependent mechanisms [17,19,29]. The fact that, in Trial 2, mitochondrial GCS was not reduced in folate-deficient rats despite a marked hyperglycinemia (3.5-fold higher than controls) necessitates the examination of other explanations. While clearly sensitive to hormonal and redox changes, the measured flux through the mitochondrial GCS may be overestimated for folate-deficient animals if the cause of the reduced GCS flux is mediated through other cellular metabolites. For example, the isolation and incubation of mitochondria in defined media may remove potential mediators of glycine flux including inhibitors of glycine transport or factors that may influence the distribution of mitochondrial folate pools. The examination of GCS activity in whole cells such as incubated hepatocytes may provide more insight.

With the lack of evidence to support a role for the hepatic GCS in contributing to folate deficiency-induced hyperglycinemia, the consideration of other potential explanations is warranted. As depicted in Fig. 1, the enzyme GNMT is a major folate binding protein that is responsible for the transfer of a methyl group from *S*-adenosylmethionine to glycine, forming sarcosine [30]. The enzyme is thought to play a critical role in conditions of methyl group excess as high levels of 5-methyltetrahydrofolate pentaglutamate inhibit the activity of GNMT [30]. In folate deficiency, the activity of this enzyme is increased [24,31] and therefore unlikely to account for the observed hyperglycinemia.

The enzyme SHMT is folate dependent and exists in both a cytosolic and a mitochondrial isoform. With respect to the mitochondrial isoform, previous research has provided evidence that it proceeds predominantly in the direction of glycine synthesis [32]. Folate deficiency would likely lead to reductions in mitochondrial glycine production and

therefore be unlikely to explain the hyperglycinemia. However, a role for the cytosolic SHMT must be considered. In our previous study, we observed that plasma serine concentrations were moderately increased during folate deficiency [7], but the percentage increase (approximately 20% relative to controls) was similar to that observed for a number of other amino acids, yielding little additional insight into potential mechanisms. Stabler et al. [22] examined the impact of folate, pyridoxine and cobalamin deficiencies, either alone or in combination, on the serum metabolite profiles of rats maintained for 51 days on diets similar to the diet used in the current study. In a folate-deficient state, serum glycine and homocysteine were elevated over controls by 2.5- and 7-fold, respectively, and other metabolites including methylglycine and dimethylglycine were elevated. However, folate deficiency did not impact SHMT activity and led to increases in serum serine values, consistent with our previous findings. In pyridoxine-deficient rats, SHMT activity was reduced to 22% of control values and serum glycine levels were increased by 2.5-fold; however, serine levels were only modestly decreased. Therefore, if reduced flux through the cytosolic SHMT pathway is responsible for the observed hyperglycinemia of folate deficiency, plasma/serum serine levels are unlikely to be a sensitive marker. Additional experiments, including the use of intact hepatocyte preparations, will help clarify the role of the GCS and SHMT in the development of hyperglycinemia due to folate deficiency.

In conclusion, in a rat model of folate depletion and repletion, plasma glycine serves as a sensitive response criterion for the assessment of folate status, and the data presented here establish the folate repletion dose over which sensitive changes in plasma glycine, homocysteine and folate were observed. However, the observed hyperglycinemia due to folate deficiency does not appear to be mediated via a reduced flux through the hepatic mitochondrial GCS; we must therefore accept the null hypothesis. The investigation of other indices of glycine metabolism may shed light on the specific component that is perturbed by folate deficiency. The measurement of changes in plasma glycine concentrations, as well as those for homocysteine and folate, may prove useful as a marker of folate status in controlled studies.

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