Plasma Homocysteine and Glycine Are Sensitive Indices of Folate Status in a Rodent Model of Folate Depletion and Repletion

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The objectives of the current studies included the characterization of the temporal changes in indices of folate status and amino acid concentrations during both folate depletion and repletion phases. In trial 1, a 6 week folate depletion protocol was employed, using 60 weanling rats assigned to receive an amino acid-defined diet with or without 1 mg/kg folic acid. A 4 week folate depletion period was judged to be optimal on the basis of the development of nadirs in both plasma and hepatic folate stores and elevated (>6-fold relative to folate-adequate controls) concentrations of plasma homocysteine and glycine. In trial 2, 54 weanling rats, previously maintained on a folate-devoid diet for 4 weeks, were assigned to receive 0.25 mg/kg folate as either crystalline folic acid or folate from a folate-enriched egg yolk powder. Both forms of folate supported similar rates of gain, increases in plasma and hepatic folate stores, and reductions in plasma glycine concentrations, whereas the folate in egg yolk powder lowered plasma homocysteine concentrations further than the crystalline folic acid ($P < 0.05$). These data support the use of both plasma glycine and homocysteine as sensitive response criteria for folate status in a rat bioassay of folate depletion and repletion and establish appropriate temporal end-points for such studies.

KEYWORDS: Folate; depletion/repletion protocol; homocysteine; glycine

INTRODUCTION

The introduction of the Dietary Folate Equivalent (DFE), on publication of the Dietary Reference Intake (DRI) estimates for the water soluble vitamins (*1*), confirmed the importance, in human nutrition, of considering the bioavailability of naturally occurring food folates. As established, the DFEs ascribe a constant bioavailability percentage, for all naturally occurring food folates, equivalent to 50% of the bioavailability of crystalline folic acid taken on an empty stomach (*1*). Although the DFEs represent a major advance for ensuring adequate folate nutrition, the use of a single adjustment factor for all foods is not without its drawbacks. The USDA Nutrient Database (*2*) has converted all food folate values in the database to DFEs, using the 50% bioavailability factor. For dietitians and nutritionists to effectively counsel their patients, the database should present a more accurate reflection of the true folate value of individual foods, by using food-specific estimates of folate bioavailability. However, before this can occur, the methodology for determining folate bioavailability in food needs to be reexamined.

A variety of approaches have been used for the estimation of the bioavailability of food folates, including the use of in vitro methods (*3*), studies with human subjects (*4–7*), and rat bioassays (*8–12*). With respect to the rat model, a typical approach to the determination of folate bioavailability would include, first, the development of a folate-depleted state, followed by a repletion phase in which animals received a diet containing either crystalline folic acid or a molar equivalent of food folate. By comparison of the changes, using slope ratio analysis, in the chosen response criteria, a measure of the relative bioavailability (crystalline folic acid set at 100%) of the food folate can be made. Traditional response criteria have included folate concentrations in plasma/serum, red blood cells and liver, and growth of the animal. As indicated by Clifford et al. (*10*), the choice of response criterion can significantly influence the derived estimate of folate bioavailability. These researchers, while observing a high correlation between bioavailability estimates derived from serum folate and growth, also observed consistently lower estimates using serum folate (~20%). This discrepancy likely reflects differences in either sensitivity or temporal responses of the chosen criteria to folate repletion. For the current approach to calculating DFEs to be improved, an understanding of factors contributing to the observed differences is critical before approaches to determine the folate bioavailability of individual foods can be adopted. This may necessitate the use of more sensitive response indices than those traditionally used.

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Plasma homocysteine is well documented in its sensitivity to folate status (*13*−*16*), due to the role that this vitamin plays in homocysteine remethylation to methionine. Whereas other dietary factors, including cobalamin (*17*, *18*) and pyridoxine (*19*) status, are known to influence plasma homocysteine levels, Miller et al. (*14*) showed that, if these factors are controlled, homocysteine concentrations respond sensitively to the level of dietary folic acid addition in a folate-depleted rat model. In addition to homocysteine, folate is required for the metabolism of other amino acids, and the potential exists to use changes in amino acid concentrations as new response criteria for the determination of the relative bioavailability of food folate, using a folate depletion−repletion protocol in a rat model. However, before a depletion/repletion protocol can be systematically implemented for the determination of folate bioavailability, appropriate temporal end-points must be established. Therefore, two studies were conducted with the following objectives: (1) the characterization of the temporal changes in measures of folate status and plasma amino acid concentrations in a rat model of folate deficiency and (2) the comparison of the temporal changes in the aforementioned parameters in response to folate repletion, using either crystalline folic acid or a folate-enriched egg yolk powder (*20*).

MATERIALS AND METHODS

Materials. All chemicals, unless otherwise indicated, were obtained from Sigma Chemical Co. (Oakville, ON, Canada).

Animals. Weanling, male Sprague−Dawley rats (Central Animal Care, University of Manitoba, Winnipeg, MB) were used. They were housed in a humidified (45%), temperature-controlled (21 °C) environment with a 12 h photoperiod (lights on 08:00 a.m.−8:00 p.m.), at the University of Manitoba's Animal Care facility. Rats were individually housed in stainless steel, wire-bottomed cages. Each cage was equipped with a glass feeder, a plastic water bottle, and a stainless steel drinking nipple. All rats had ad libitum access to deionized water. All animals were treated with ethical consideration as directed by the guidelines for experimental animals set by the Canadian Council of Animal Care (*21*), as enforced by our institution's Animal Care Committee.

Basal Diet. The basal diet used in both trials was a folic acid-devoid, amino acid-defined diet, in powder form, from a commercial diet formulator (Harlan Teklad Co., Madison, WI). The composition of the diet was as follows: crystalline amino acids, consisting of 0.35% L-alanine, 1.21% L-arginine, 0.6% L-asparagine, 0.35% L-aspartic acid, 0.35% L-cystine, 4.0% L-glutamic acid, 2.33% glycine, 0.45% L-histidine HCl·H$_2$O, 0.82% L-isoleucine, 1.11% L-leucine, 1.80% l-lysine·HCl, 0.82% L-methionine, 0.75% L-phenylalanine, 0.35% L-proline, 0.35% L-serine, 0.82% l-threonine, 0.18% L-tryptophan, 0.5% L-tyrosine, 0.82% L-valine, 35.4% sucrose, 15% cornstarch, 15% maltodextrin, 8% soybean oil, 3% cellulose, 3.5% AIN-76A mineral mix, 1% succinylsulfathiazole (to inhibit endogenous folate synthesis), 0.82% calcium phosphate, and 0.002% *tert*-butylhydroquinone (THBQ; antioxidant); vitamins (in mg/kg), choline bitartrate, 250; nicotinic acid, 33.9; calcium panthothenate, 18.1; pyridoxine hydrochloride, 7.9; thiamin hydrochloride, 7; ribloflavin, 6.8; D-biotin, 0.3; vitamin B12 (0.1% in mannitol), 28.3; dl-α-tocopherol acetate (500 IU/g), 169.5; vitamin A palmitate (500000 IU/g), 9; vitamin D3 palmitate, 2.3; phylloquinone, 1. When present, crystalline folic acid was provided at either the established requirement level for rats of 1 mg/kg diet (*22*) in trial 1 or at 0.25 mg/kg (trial 2). Upon procurement, weanling rats (∼75 g) were adapted to the basal, folate-supplemented diet (1 mg/kg) prior to their introduction into the trials.

Trial 1−Folate Depletion Protocol. Sixty rats were randomly assigned to receive one of two diets: (1) folate-devoid diet−the basal diet containing 0 mg/kg crystalline folic acid (*n* = 30)−or (2) folate-adequate diet−the basal diet containing 1 mg/kg crystalline folic acid (*n* = 30). Rats receiving the folate-adequate diet were matched (by body weight) to corresponding folate-devoid rats and received a diet allotment equivalent to the intake of the corresponding match from

the previous day, using a pair-feeding protocol. Feed intake was measured daily, and body weights were measured on a weekly basis. At weekly intervals for 6 weeks, 10 rats (5 folate-adequate; 5 folate-devoid) were euthanized as indicated below (Sampling Procedure).

Trial 2−Folate Repletion Protocol. On the basis of the results from trial 1, 54 rats were maintained on a folate-devoid diet for 4 weeks. Following the 4 week depletion period, 6 rats were killed for the establishment of baseline biochemical values. The remaining 48 rats were randomly assigned to receive the amino acid-defined diet containing 0.25 mg/kg of either crystalline folic acid or folate (expressed as folic acid equivalents) from folate-enriched egg yolk powder. At weekly intervals, 12 rats (6/treatment group) were euthanized as indicated below (Sampling Procedure).

The folate-enriched egg yolk powder was produced as described by House et al. (*20*). Briefly, eggs were collected from laying hens that had been consuming a ration containing 4 mg/kg folic acid−a level previously shown to maximize egg folate content. To be representative of a form that eggs would be consumed, they were placed in boiling water for 10 min and then cooled, and the yolks were collected and lyophilized. The folate content of the pooled dried egg yolk powder was determined, on eight replicate samples, according to the reverse-phase high-pressure liquid chromatography (rp-HPLC) method of Vahteristo et al. (*23*). 5-Methyltetrahydrofolate was used as a standard, as this form of folate is the only form that was found in the enriched egg yolk, a fact substantiated by others (*3*). Samples were analyzed immediately following extraction. The folate content of the egg powder was expressed as milligrams of folic acid per gram, using the molecular weight of folic acid (441.4) as the conversion factor. The level of folate in the enriched, freeze-dried egg yolk powder permitted an inclusion rate of 3.24% in the final diet, at the expense of an equivalent amount of the complete diet.

Sampling Procedure. At the designed termination dates and immediately after lights were turned on, rats were weighed and then anaesthetized by an intraperitoneal injection of 60 mg/kg of sodium pentobarbital (65 mg/mL; CDMV, Calgary, AB, Canada). Rats were killed by exsanguination through the collection of blood, via direct cardiac puncture, into evacuated tubes containing sodium heparin. The tubes were placed immediately on ice. Livers were removed, rinsed in ice-cold 0.9% saline solution, and frozen immediately in liquid nitrogen. Plasma was separated from erythrocytes via centrifugation (3000*g* at 4 °C for 20 min). Plasma and liver samples were stored at −80 °C until analyzed.

Biochemical Analyses. Plasma homocysteine concentrations were determined via rp-HPLC, according to the method of Vester and Rasmussen (*24*). Plasma folate concentrations were determined by radioimmunoassay (Quantaphase II; Bio-Rad Laboratories, Mississauga, ON). Liver 5-methyltetrahydrofolate polyglutamates were extracted and the polyglutamates hydrolyzed to monoglutamates, using endogenous hepatic folate conjugase activity, according to the method of Abad and Gregory (*9*). Liver 5-methyltetrahydrofolate monoglutamate was analyzed by rp-HPLC with fluorescence detection, as described by Vahteristo et al. (*23*). 5-Methyltetrahydrofolate alone was measured as a marker of hepatic folate stores, due to the fact that it is has been shown to be acutely sensitive to folate depletion (*25*). Plasma amino acid concentrations were measured, via ion-exchange chromatography (LKB 4151 Alpha-Plus amino acid analyzer), using the method of Blom and Huijmans (*26*).

Statistical Analyses. The studies were designed as completely randomized designs, with the main effects of folate level/source and time. In both trials, data were subjected to ANOVA, using the PROC-GLM feature of SAS (*27*). When evidence of heterogeneity of variance was present, data were log transformed prior to analysis. Data are presented as least-squares means plus standard errors, with differences between treatments assessed using the protected least squares difference procedure. Level of significance was set at an α level of 0.05.

RESULTS

Folate Depletion Study. Values for body weights at study initiation were not different between the two treatment groups

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Table 1. Temporal Changes in Plasma Folate and Liver 5-Methyltetrahydrofolate (5-CH₃-THF) Concentrations in Rats (*n* = 5 per Time Point per Treatment) Consuming Amino Acid-Defined Diets with or without 1 mg/kg Crystalline Folic Acid (Trial 1)[a]

variable	dietary folate (mg/kg)	duration of folate depletion					
		1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks
plasma folate (nmol/L)	0	10.5a	3.7b	2.0c	1.2d	0.9d	0.9d
		(6.8–16.4)	(2.4–5.8)	(1.3–3.2)	(0.8–1.9)	(0.6–1.3)	(0.5–1.6)
	1	50.5*	48.2*	48.0*	48.5*	45.3*	45.4*
		(32.3–78.6)	(30.9–75.1)	(30.8–74.8)	(31.1–75.7)	(29.1–70.6)	(25.6–80.6)
liver 5-CH₃-THF (nmol/g)	0	5.3a	1.9b	1.4b	0.9c	0.7c	0.6c
		(3.5–8.0)	(1.2–2.9)	(0.9–2.2)	(0.6–1.3)	(0.5–1.1)	(0.4–1.1)
	1	9.1*	8.0*	7.3*	7.8*	6.1*	8.9*
		(5.9–13.8)	(5.2–12.1)	(4.8–11.2)	(5.1–11.9)	(4.0–9.2)	(5.2–15.3)

[a] Data are presented as geometric means, with 95% confidence intervals presented in parentheses. Values within a row with different letters are significantly different (*P* < 0.05), as assessed by Fisher's protected LSD procedure. An asterisk denotes that the values obtained for rats consuming the 1 mg/kg dietary folate, within a given week, are significantly different (*P* < 0.05) from the corresponding value obtained from rats consuming 0 mg/kg folate, as assessed by Fisher's protected LSD.

(*P* > 0.05; data not shown). With respect to feed intake, as designed, there was no significant effect due to treatment or time on study, and there was no significant treatment × time interaction (*P* > 0.05). Feed intake was 15.4 vs 15.6, 16.2 vs 16.6, 18.0 vs 19.1, 18.3 vs 18.5, 16.7 vs 16.4, and 13.2 vs 13.0 g/day (pooled SEM = 0.9 g/day) for rats consuming the diets containing 1 vs 0 mg of folic acid/kg, in weeks 1−6, respectively. There was no significant effect (*P* > 0.05) due to treatment on growth of the rats. Body weight increased with time (*P* < 0.05), with weights increasing up to 4 weeks on trial, then reaching a plateau. Body weights were 146 vs 164, 188 vs 195, 226 vs 238, 279 vs 281, 285 vs 287, and 298 vs 285 g (pooled SEM = 12 g) for rats consuming the diets containing 1 vs 0 mg of folic acid/kg, in weeks 1−6, respectively.

The data for plasma folate, liver 5-methyltetrahydrofolate, plasma homocysteine, and plasma glycine were log transformed prior to statistical analyses, due to evidence of heterogeneity of variance. As such, the data are presented as geometric means with the corresponding 95% confidence intervals. In both trials 1 and 2, the incidence of hemolysis in the plasma samples was low (<5% of blood samples obtained). Plasma folate concentrations (**Table 1**) in the folate-adequate animals did not change over time (*P* > 0.05), and the values were significantly higher than the values in the folate-depleted animals at all of the corresponding time points (*P* < 0.05). In the folate-depleted animals, plasma folate dropped significantly (*P* < 0.05) after 1 week on the folate-devoid diet and continued to drop until week 4 of the trial, after which a nadir in plasma folate was reached. Liver 5-methyltetrahydrofolate concentrations (**Table 1**) followed a pattern similar to plasma folate, again reaching a nadir, in the folate-depleted rats, by 4 weeks. Plasma homocysteine concentrations did not change over time (*P* > 0.05) in the folate-adequate animals and remained below 10 *μ*mol/L (**Figure 1A**). Following 1 week of consumption of a folate-devoid diet, plasma homocysteine concentrations were significantly elevated (*P* < 0.05) and increased significantly over 5 weeks on the diet (*P* < 0.05), before reaching a plateau. Plasma glycine concentrations were also increased as a result of rats consuming a folate-devoid diet (**Figure 1B**). Glycine concentrations, at every time point, were higher in the folate-depleted rats relative to the folate-adequate control animals (*P* < 0.05). In the folate-depleted rats, plasma glycine concentrations increased significantly (*P* > 0.05) between weeks 3 and 4 and remained at elevated plateau levels for the remainder of the trial. In the folate-adequate animals, plasma glycine did show marginal changes over time, with the concentrations at week 5 being significantly lower (*P* < 0.05) than the corresponding values at weeks 1 and 3. Rats consuming the folate-devoid diet had
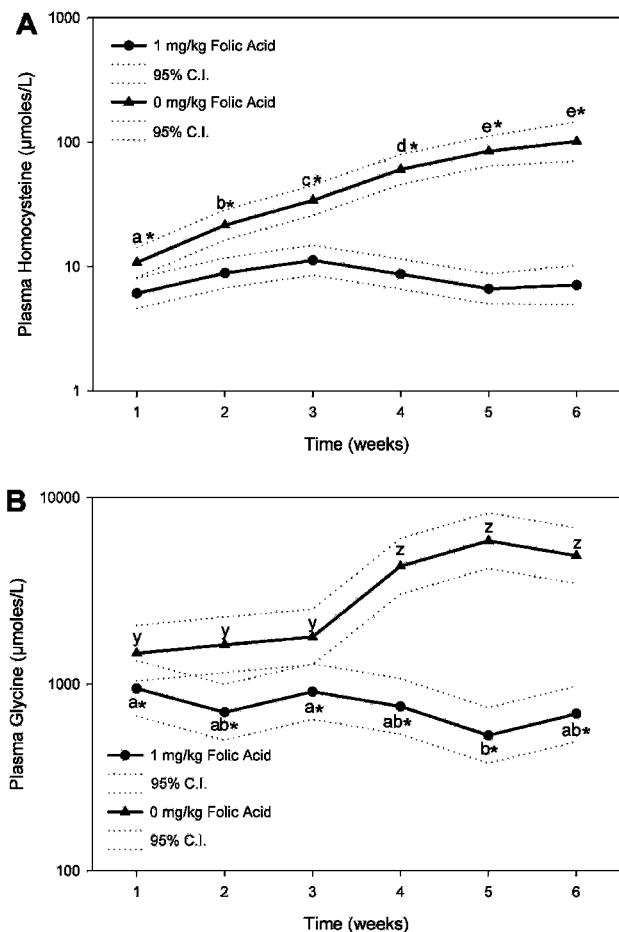
Figure 1. Temporal changes in (A) plasma homocysteine or (B) plasma glycine concentrations of rats consuming amino acid-defined diets with or without 1 mg/kg crystalline folic acid. Data are presented as geometric means with the 95% confidence interval on a linear-log scale. Values within a line with different letters are significantly different (*P* < 0.05) by protected LSD. An asterisk denotes a significant difference (*P* < 0.05), by protected LSD, from the corresponding time point in the rats consuming the diet containing 1 mg/kg folic acid. Main effects of ANOVA: folate level, *P* < 0.05; duration, *P* < 0.05; folate level × duration, *P* < 0.05.

significantly (*P* < 0.05) elevated overall mean concentrations of alanine, aspartate, isoleucine, lysine, methionine, serine, taurine, tyrosine, and valine, relative to the concentrations measured in the plasma of the rats consuming the folate-adequate diet (**Table 2**). Overall mean treatment plasma arginine

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Table 2. Temporal Changes in Plasma Amino Acid Concentrations (Micromoles per Liter) in Rats (*n* = 5 per Time Point per Treatment) Consuming Amino Acid-Defined Diets with or without 1 mg/kg Crystalline Folic Acid (Trial 1)

| | 0 mg/kg folic acid | | | | | | 1 mg/kg folic acid | | | | | | | P values[a] | | |
| | | | | | | | | | | | | | | | | TRT × |
amino acid	week 1	week 2	week 3	week 4	week 5	week 6	week 1	week 2	week 3	week 4	week 5	week 6	SEM	TRT	WK	WK
alanine	944	761	774	703	820	944	705	577	667	704	464	453	74	**	NS	**
arginine	93	88	80	79	82	98	83	125	108	173	114	133	17	**	NS	NS
asparagine	157	89	151	176	212	144	116	102	126	128	130	146	30	NS	NS	NS
aspartate	33	18	23	26	29	65	24	16	25	18	14	19	7	**	**	**
citrulline	82	56	20	32	35	35	81	59	34	31	25	50	10	NS	**	NS
cysteine	131	181	198	186	174	198	150	160	189	185	187	172	10	NS	**	NS
glutamate	160	105	134	109	114	222	183	155	158	160	141	166	31	NS	NS	NS
glutamine	991	953	1085	1066	875	1893	1123	1204	1281	1621	1411	1488	254	NS	NS	NS
histidine	50	46	32	48	45	63	45	53	53	50	46	47	8	NS	NS	NS
isoleucine	111	94	85	71	95	116	71	70	104	99	60	72	12	**	NS	**
leucine	125	104	121	122	124	185	112	120	120	140	103	123	10	NS	**	**
lysine	784	703	580	669	613	793	734	578	648	536	444	382	53	**	**	**
methionine	68	71	71	38	101	118	41	43	69	58	41	51	11	**	**	**
ornithine	63	53	48	32	50	89	50	53	70	59	55	68	10	NS	**	NS
phenylalanine	64	48	68	71	71	92	53	62	66	71	54	79	6	NS	**	NS
proline	120	119	148	140	136	142	160	104	190	147	119	153	21	NS	NS	NS
serine	753	925	968	901	711	634	630	602	766	645	515	600	66	**	**	NS
taurine	379	322	277	241	216	259	307	283	227	230	173	243	32	**	**	NS
threonine	962	1315	1404	969	842	671	735	858	1335	1005	855	784	138	NS	**	NS
tyrosine	136	101	135	131	105	81	128	103	100	88	69	80	14	**	**	NS
valine	154	166	146	152	150	217	131	152	149	158	109	138	11	**	**	**

[a] Main effects of ANOVA: TRT = level of folic acid; WK = week; TRT × WK = treatment by week interaction. ** indicates *P* < 0.05. NS = not significant.

Table 3. Temporal Changes in Plasma Folate, Liver 5-Methyltetrahydrofolate (5-CH₃-THF), Plasma Homocysteine, and Glycine Concentrations in Rats Consuming Diets Containing 0.25 mg/kg Folate from either Crystalline Folic Acid or a Folate-Enriched Egg Yolk Powder (Trial 2)[a]

| variable | dietary folate source | duration of folate repletion (weeks) | | | | | SEM[d] | P values[b] | | |
		baseline[c]	1	2	3	4		TRT	WK	TRT × WK
plasma folate (nmol/L)	folic acid	2.6	2.8	2.9	3.9	4.1	0.4	NS	NS	0.04
	egg folate		3.4	3.2	3.0	2.8				
liver 5-CH₃-THF (nmol/g)	folic acid	1.2	0.8	0.8	0.9	1.1	0.1	NS	NS	NS
	egg folate		0.7	0.8	0.9	1.1				
plasma homocysteine (μmol/L)	folic acid	53.1	51.6	35.0	29.5	25.0	2.8	0.005	<0.0001	NS
	egg folate		36.1	22.9	22.1	20.0				
plasma glycine (μmol/L)	folic acid	3793	4351	2824	2552	989	460	NS	<0.0001	NS
	egg folate		2908	2770	2079	1237				

[a] Data are presented as least-squares means (LSMEAN). [b] *P* values of the main effects (TRT = source of folate; WK = time effect) and interaction term (TRT × WK = source of folate × time); NS = not significant (*P* > 0.05). [c] Baseline group following 4 weeks of folate depletion (*n* = 6). [d] SEM = standard error of the LSMEAN (*n* = 6/time point per dietary folate source, weeks 1–4).

concentrations, however, were significantly (*P* < 0.05) depressed by folate deficiency.

Folate Repletion Study. Values for body weights at study initiation were not different between the two treatment groups (*P* > 0.05; data not shown). There was no significant effect (*P* > 0.05), due to source of dietary folate, on the measures of average daily feed intake or body weight. Feed intake was 20.1 vs 19.8, 22.1 vs 22.0, 21.3 vs 22.0, and 22.6 vs 21.6 g (pooled SEM = 1.0 g), for rats consuming the diets containing 0.25 mg of folic acid/kg, from crystalline folic acid versus egg yolk folate, in weeks 1−4, respectively. The level of folic acid used (0.25 mg/kg of diet) allowed for weight gain in the rats, as evident by the significant effect of duration of folate repletion (*P* < 0.05) observed. Body weights were 327 vs 304, 351 vs 337, 370 vs 371, and 417 vs 402 g (pooled SEM = 16 g), for rats consuming the diets containing 0.25 mg of folic acid/kg, from crystalline folic acid versus egg yolk folate, in weeks 1−4, respectively. With respect to the biochemical measurements, the source of dietary folate or duration of folate repletion did not significantly affect the concentration of folate in plasma (**Table 3**); however, a significant (*P* < 0.05) folate source × duration of folate repletion interaction was observed. Compari-

son of the differences in the least-squares mean values revealed that plasma folate concentrations in the rats consuming diets containing crystalline folic acid had slightly, but significantly (*P* < 0.05), higher plasma concentrations at week 4 than those observed in the rats consuming the diets containing folate from egg yolk. Additionally, in the rats consuming the crystalline folic acid diets only, plasma folate concentrations were significantly elevated (*P* < 0.05) after 3 weeks of folate repletion. There were no significant effects (*P* > 0.05) of either folate source, duration of folate repletion, or folate source × duration of folate repletion interaction for liver 5-methyltetrahydrofolate concentrations (**Table 3**).

With respect to plasma amino acid concentrations, there was a significant effect (*P* < 0.05), due to both folate source and duration of folate repletion, with no significant interaction, for plasma homocysteine concentrations (**Table 3**). Rats consuming the diets containing egg yolk folate as the source of dietary folate had significantly lower plasma homocysteine concentrations, overall, than rats consuming diets in which crystalline folic acid was the source of folate. Both groups of rats displayed a significant reduction in plasma homocysteine with increasing duration of folate repletion. There was no significant effect of

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source of dietary folate on mean plasma glycine concentrations ($P > 0.05$; **Table 3**) between the two treatment groups; however, plasma glycine did show a significant decline ($P < 0.05$) as the duration of folate repletion increased. There was no significant interaction, for plasma glycine, of folate source and duration of folate repletion ($P > 0.05$). With respect to other amino acids, there was no significant difference, due to source of dietary folate, on the plasma concentration of any of the other measured amino acids ($P > 0.05$; data not shown).

DISCUSSION

The present studies were conducted to characterize the temporal changes in measures of folate status during both the depletion and repletion phases, with the primary goal of determining the potential for plasma amino acid concentrations to serve as sensitive indices of folate status. Our data support the use by others (9−11) of a 4 week period of folate depletion to produce a marked folate deficiency state, characterized by nadirs in both plasma and hepatic folate levels. Depressions in feed intake and weight gains observed beyond 4 weeks provide further justification for the choice of this depletion period.

The novelty of the current study relates to the observed temporal changes in plasma amino acid concentrations precipitated by the consumption of a folate-devoid diet. Previous research has documented increases in total homocysteine concentrations in rat plasma from control levels of 6−12 μmol/L (12, 14−16) to levels of ~25 μmol/L, if maintained on a folate-devoid casein-based diet (12, 16), or ~60 μmol/L, if maintained on an amino acid-defined diet (14). However, little work has been done to characterize the temporal changes in plasma homocysteine concentrations in relation to the development of folate deficiency. This information is critical if one is to have confidence in the use of values determined at a single end-point. In the present experiment, plasma homocysteine during folate deficiency was significantly elevated from control levels after 1 week and levels continued to rise after 4 weeks. This elevation should allow for sufficient sensitivity to measure differences in subsequent folate repletion protocols.

As folate is required in the metabolic pathways of several amino acids, including glycine, serine, methione, and histidine, the temporal changes in plasma amino acid concentrations were assessed. In general, the plasma concentrations of most amino acids increased marginally (10−25%) with folate deficiency. This generalized response may reflect either a decreased incorporation of amino acids into protein or an increase in protein degradation due to the impact of reduced folate status on DNA synthesis (28). Of the specific amino acids besides homocysteine known to require folate for their metabolism (**Figure 2**), only glycine exhibited a marked elevation with prolonged deficiency. Previous research has shown that glycine concentrations, following 51 days of folate depletion, were 2.5-fold higher in deficient rats relative to controls (15). These authors also observed increases in plasma serine levels, consistent with the increases observed in the present work, with no impact on the activity of serine hydroxymethyltransferase. The observed increases in plasma glycine are likely related to a reduced glycine flux through the glycine cleavage complex, a critical system for the oxidative catabolism of this amino acid in many tissues, including liver, kidney, and brain. This enzyme complex requires tetrahydrofolate as a substrate (29). In contrast with the other amino acids studied, plasma arginine in the folate deficient rats exhibited a mean reduction of 30%, relative to rats receiving adequate folate. As folate is not generally considered to be required in arginine synthetic pathways, the
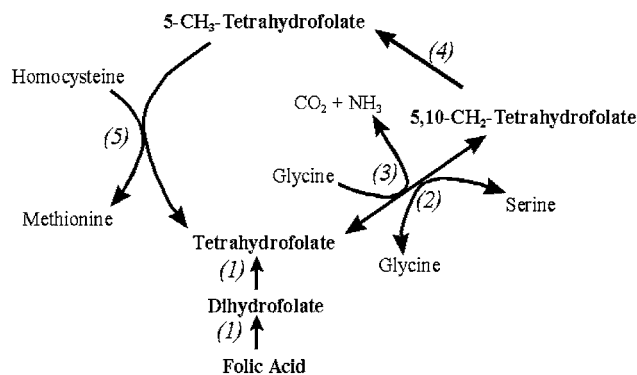
Figure 2. Folate metabolism. Enzymes: *(1)* dihydrofolate reductase (EC 1.5.1.3); *(2)* serine hydroxymethyltransferase (EC 2.1.2.1); *(3)* glycine cleavage system (EC 1.4.4.2; 2.1.2.10); *(4)* methylenetetrahydrofolate reductase (EC 1.7.99.5); *(5)* methionine synthase (EC 2.1.1.13). Adapted from ref *28*.

reduction in plasma arginine concentrations may be an indirect effect of folate, via the presence of a hyperhomocysteinemic state. Therefore, of the amino acids measured, only plasma homocysteine and glycine show promise for use in controlled studies for the assessment of folate bioavailability. This was the objective of the second study conducted.

In the folate repletion study, 0.25 mg/kg folic acid was used, a level equivalent to 25% of the NRC requirement (23) for rats and a midpoint of ranges used previously (10, 14). The use of a lower level of folate repletion (0.025 mg/kg) has been shown to be insufficient to effect measurable biochemical changes in folate status (30). Too high a dose has the potential to return measures of folate status to folate-adequate levels too quickly, thus masking any potential differences in folate bioavailability. Therefore, the target repletion dose should yield sufficient sensitivity to measure marginal changes in folate status. During the folate repletion period, 0.25 mg/kg folic acid proved to be sufficient to prevent the drop in feed intake observed after 4 weeks in trial 1 and to permit weight gains of ~3.5 g/day, irrespective of dietary folate source. The traditional measures of folate status, namely, plasma folate and liver 5-methyltetrahydrofolate concentrations, responded differently to folate repletion. Whereas hepatic 5-methyltetrahydrofolate levels increased with time over the repletion period, plasma folate levels did not, calling into question the usefulness of the latter response criterion.

In contrast to the results obtained with plasma and liver folate, plasma homocysteine and glycine were markedly decreased with folate repletion at a level of 0.25 mg/kg of diet. With crystalline folic acid as the source of dietary folate, both amino acids appeared to stay elevated after the first week of repletion and then decreased over the remaining 3 weeks. This apparent lag was not observed when folate was provided via the egg yolk powder. The differences in the response profiles observed for homocysteine and glycine may be related to the fact that the egg yolk contains 5-methyltetrahydrofolate monoglutamate as the predominant source of folate (3), the immediate precursor for homocysteine remethylation, in a reaction that forms tetrahydrofolate, the form of folate needed in glycine catabolism (29). Crystalline folic acid needs to be converted via dihydrofolate reductase (EC 1.5.1.3.), in a two-step process, to form tetrahydrofolate. The efficiency of utilization of folic acid to form tetrahydrofolate and 5-methyltetrahydrofolate may be perturbed in folate-deficient animals, but this deserves further attention. On the basis of the findings of the current study, a minimum repletion period of two weeks is required, if plasma

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homocysteine and glycine concentrations are to be used as response criteria, when folate is supplemented at 0.25 mg/kg in the diet. Further research is required to develop optimal temporal end-points if different levels of folate are used in the repletion period.

By comparison of the temporal changes in the biochemical measures of folate status obtained for the rats receiving crystalline folic acid with those receiving folate from egg yolk powder, it is possible to determine the relative bioavailability of the folate in egg yolk. For growth, plasma folate, liver 5-methyltetrahydrofolate, and plasma glycine concentrations, the lack of any significant main effects due to folate source provides strong evidence that the folate in egg yolk is 100% bioavailable, relative to crystalline folic acid. However, the presence of a significant treatment effect, with egg yolk folate producing lower homocysteine concentrations, indicates that egg yolk folate may be more bioavailable than crystalline folic acid. This discrepancy highlights the importance of considering more than one response criterion, but yields a far more interesting question—why do we see this difference? The basal diet, as formulated, provided nutrients, including those known to influence homocysteine concentrations, namely, cobalamin, pyridoxine, riboflavin, and choline, at levels consistent with the AIN-93 formulation (*31*). Additionally, this diet yielded low plasma homocysteine concentrations, when it contained adequate folate (**Figure 1A**). The addition of 3.24% egg yolk diet modified the final diet to a minor extent (i.e., 28.3 vs 29.1 μg of cobalamin/kg in the basal diet and in the basal diet plus egg yolk, respectively). However, there may be yet unappreciated factors in egg yolk which yield a further reduction in homocysteine that are unrelated to the folate content.

With respect to the current study design, considerable debate continues as to the appropriateness of the use of the rat bioassay for drawing inferences related to human folate nutrition (*32*). Differences in folate conjugase activity (*33*) within the small intestine do exist between rats and humans. However, the provision of folate in the monoglutamate form, as was done in the present study, addresses this criticism. Additionally, emerging research has documented a significant up-regulation of rat intestinal brush border conjugase activity, the main form in humans, during folate deficiency (*34*); therefore, it is questionable as to whether folate conjugase activity is rate-limiting for folate uptake in the folate-deficient rat bioassay. Other approaches to the determination of folate bioavailability include direct studies in humans using stable isotopic techniques (*6, 35, 36*), which are currently limited to purified folate sources, and area under the curve studies, which require the consumption of appreciable quantities of food folates to account for endogenous folate levels (*7*). It is clear that the latter studies are not without their own limitations.

Another consideration for future extension of the current protocol relates to the methodology used for the determination of food folate. The use of a trienzyme extraction, including conjugase, amylase, and protease activities, for folate analysis is suggested to yield greater recoveries of folate (*37*) from mixed food composites. As the starch content of egg yolk is negligible, the benefit to using trienzyme extraction for this food source is unclear. A recent Dutch study found no significant differences in the measured values of folate from animal products extracted with traditional techniques versus those extracted using the trienzyme technique; however, higher starch foods showed greater recoveries of folate (*38*). Therefore, for complex food matrices, the use of the trienzyme extraction procedure should be considered. Additional improvements in the measurement

of food folates may arise through the implementation of more sensitive analytical techniques, including stable isotope dilution methods employing liquid chromatography—mass spectrometry (*39*).

Implications. The present data provide a characterization of the temporal changes in biochemical measures of folate status in the folate depletion—repletion rat bioassay. In particular, the data highlight the potential for using plasma homocysteine and glycine as sensitive response measures of folate status. The use of the temporal end-points established in the current studies, coupled with the greater sensitivity observed for homocysteine and glycine relative to traditional measures of folate status, permits the development of a protocol designed for the systematic reevaluation of the bioavailability of folates from natural food sources. Additionally, the current data provide evidence that the folate found in a particular food, namely, enriched egg yolk powder, has a relative bioavailability much greater than 50%, thus highlighting a potential limitation in the current calculations of DFEs. Therefore, continued review of the methodology employed in calculating DFEs is warranted.

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