

The rate of intestinal absorption of natural food folates is not related to the extent of folate conjugation¹⁻³

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ABSTRACT

Background: Evidence is conflicting as to whether the bioavailability of food folates is influenced by the extent of their conjugation.

Objective: The objective was to compare the bioavailability of 3 representative food folate sources with various degrees of glutamylation—ie, egg yolk, spinach, and yeast, whose polyglutamyl folate content measured 0%, 50%, and 100%, respectively.

Design: In a randomized crossover trial, 13 male subjects, after a prestudy folate saturation procedure, received in random order either placebo or 500 μg total folate, which was provided as concentrated freeze-dried extract removed from the normal food matrix of egg yolk, spinach, or yeast. Blood samples ($n = 10$) were collected before and up to 10 h after treatments, which were administered at weekly intervals.

Results: A significant increase from baseline plasma folate concentrations was observed by 0.5 h after treatment with egg yolk folate or spinach folate and by 1 h after treatment with yeast folate, and the concentrations remained significantly elevated for 3–5 h; no plasma folate response was observed after placebo treatment. The overall responses, calculated as plasma folate area under the curve (AUC) for egg yolk, spinach, and yeast folate, were 122.6 ± 23.6 , 136.2 ± 21.4 , and 102.5 ± 21.1 $\text{nmol} \cdot \text{h/L}$, respectively. No significant differences in AUC were seen between monoglutamyl (egg yolk) folate and either of the polyglutamate-containing folates examined.

Conclusion: These results suggest that the ratio of monoglutamate to polyglutamate in natural folates is not a factor that limits the extent of intestinal absorption of food folate. *Am J Clin Nutr* 2006;84:167–73.

KEY WORDS Food folate, polyglutamylation, plasma folate, folate bioavailability

INTRODUCTION

Folate is required for one-carbon transfer reactions involving purine and pyrimidine biosynthesis and for amino acid interconversions. Optimal folate status is considered to be protective against certain cancers (1, 2) and to help maintain normal plasma homocysteine, elevated concentrations of which are considered to be an independent risk factor for cardiovascular disease (3). However, the strongest evidence to date of the health benefits of optimal folate status is provided by clinical trials that showed conclusively that folic acid (FA) supplementation during the periconceptional period prevents both the recurrence (4) and first occurrence (5) of neural tube defects. To prevent the first occurrence of neural tube defects, public health authorities worldwide

recommend the consumption of 400 μg folate or FA/d above current intakes (6–8). However, as a means of enhancing folate status (increasing blood folate concentrations), the consumption of additional folate-rich foods has been shown to be relatively ineffective compared with either taking supplements or consuming foods fortified with FA, the synthetic form of the vitamin (9). This finding is generally assumed to be the result of the poor bioavailability of the natural folates found in food as compared with the high degree of bioavailability of FA (10). The bioavailability of dietary folates from a mixed diet is considered to be $\leq 50\%$ of that of FA (11), but published estimates of folate bioavailability vary greatly, ranging anywhere from 10% to 98% of that of FA (11–17). Thus, a great deal of uncertainty remains with respect to the bioavailability of natural food folates and the factors influencing that bioavailability (18).

In broad terms, folate bioavailability is considered to be measured by intestinal absorption, tissue uptake, enterohepatic circulation, and the rate of urinary excretion (19). It is, however, widely accepted that intestinal absorption plays the largest role in influencing the bioavailability of food folates (18). The efficiency of intestinal absorption of food folates is, in turn, reported to depend on a number of factors, including entrapment of folates in the food matrix (20, 21), the presence of inhibitors to the intestinal folylpoly- γ -glutamate carboxypeptidase (FGCP) that is required for the deconjugation of polyglutamate to monoglutamate (22, 23), the instability of reduced folates in the digestive system before absorption (24), and the presence of certain dietary constituents that may enhance folate stability (19). However, the relative influence of these factors on overall bioavailability is not known and cannot easily be ascertained. In particular, evidence is conflicting as to whether the extent of conjugation of natural

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TABLE 1Relative polyglutamate content and distribution of the main folate derivatives in foods and the corresponding food folate extracts¹

	Egg yolk		Spinach		Yeast	
	Food ²	Extract ³	Food ²	Extract ³	Food ²	Extract ³
Polyglutamate (%) ⁴	0	0	60 ± 6.7 ⁵	50 ± 3.0	98 ± 0.3	99.6 ± 0.8
5-CH ₃ H ₄ folate (%) ⁶	84.5 ± 5.7	80.6 ± 5.5	43.2 ± 2.1	46.5 ± 8.5	46.2 ± 3.9	47.7 ± 3.8
5-CHO H ₄ folate (%) ⁶	15.5 ± 5.7	14.1 ± 3.4	21.2 ± 2.0	43.0 ± 9.5	23.0 ± 3.2	35.1 ± 5.6
10-CHO H ₄ folate (%) ⁶	ND	ND	3.0 ± 1.2	ND	11.1 ± 2.3	5.4 ± 2.5
DHF (%) ⁶	ND	5.3 ± 2.0	20.0 ± 4.5	4.6 ± 0.1	11.6 ± 1.4	9.2 ± 1.6
THF (%) ⁶	ND	ND	9.2 ± 4.4	4.2 ± 1.2	5.5 ± 0.6	1.0 ± 0.1
5,10-CH H ₄ folate (%) ⁶	ND	ND	3.3 ± 0.6	1.7 ± 0.5	2.6 ± 0.5	1.6 ± 0.8

¹ 5-CH₃ H₄ folate, 5-methyltetrahydrofolate; 5-CHO H₄ folate, 5-formyltetrahydrofolate; 10-CHO H₄ folate, 10-formyltetrahydrofolate; DHF, dihydrofolate; THF, tetrahydrofolate; 5,10-CH H₄ folate, 5,10-methenyltetrahydrofolate; ND, not detected.

² Dried yeast, fresh spinach, and raw egg yolk were purchased from a local retail outlet (Tesco, Coleraine, United Kingdom).

³ Food folate extracts were produced on an industrial scale as described in detail elsewhere (26).

⁴ A percentage of total folate content (based on 3 independent analyses).

⁵ $\bar{x} \pm SD$ (all such values).

⁶ A percentage of the sum of all folate derivatives detected (based on 3 independent analyses).

folates is a limiting factor influencing folate bioavailability (25). The aim of the current study was to focus on the effect of folate conjugation on folate absorption after control, as far as possible, for the other factors likely to affect folate absorption. We compared the intestinal absorption of 3 representative food folate sources of equivalent total folate content but with various degrees of glutamylation—egg yolk, spinach, and yeast, whose measured polyglutamyl folate content is 0%, 50%, and 100%, respectively. This comparison was achieved by providing each of the 3 food folates in the form of a concentrated freeze-dried extract removed from the normal food matrix. In addition, ascorbate was added to the extract to protect the reduced folates during transit through the digestive tract.

SUBJECTS AND METHODS

Subjects

Male volunteers were recruited from the staff and the student population of the University of Ulster. Subjects were asked to provide information on their general health and use of supplements or medicines and to provide a blood sample for screening purposes. Subjects were invited to participate in the study if they had no history of hepatic, gastrointestinal, renal, vascular, hematologic, or psychiatric disease; were not taking FA-containing supplements or medicines known to interfere with folate metabolism; were without B vitamin deficiency or an elevated plasma homocysteine concentration; and were nonhomozygous for the C677T (so-called thermolabile) variant of the methylenetetrahydrofolate reductase (*MTHFR*) gene. These exclusion criteria were implemented to avoid the inclusion of participants who could potentially give an abnormal response to ingested dietary folates.

Subjects gave written informed consent. The Research Ethics Committee of the University of Ulster granted ethical approval for the study.

Study design and treatments

In a crossover design, subjects received each of the following 4 treatments: placebo and 500 μ g total folate provided as egg yolk folate, spinach folate, and yeast folate. The treatments were

administered in random order, with an interval of 1 wk between each test day, so that, at the end of the 4-wk study period, all participants had received all 4 treatments. The food folates were provided in the form of concentrated extracts removed from their normal food matrix (Loughry College—The Food Centre, Agri-food Development Service, Cookstown, United Kingdom). The protocol for the production of the extracts was designed to preserve both the monoglutamate-to-polyglutamate ratio and the distribution of the main folate derivatives of the original foods and to use only reagents that were safe for human consumption. Briefly, this process involved freeze-drying the original food, performing thermal extraction of the food folates, and then freeze-drying the extract. The folate extracts were kept frozen at -20°C for the duration of the study. Full details of the production protocol and information on total folate content, folate derivative distribution, and the monoglutamate:polyglutamate of each food folate extract have been reported in detail elsewhere (26) and are summarized here (Table 1).

The treatments were provided as a reconstituted drink in 200 mL sugar-free lemonade (in a disposable plastic cup) that the subjects consumed under supervision midway through their breakfast, which was eaten early in the morning after an overnight fast. The drinks were freshly prepared before each administration as follows: 50 g egg folate extract, 21.7 g spinach folate extract, or 13.5 g yeast folate extract (each providing 500 μ g total folate) was added to 100 mL sugar-free lemonade; the drink was mixed vigorously; another 100 mL sugar-free lemonade was added, and the drink was mixed again. The placebo drink consisted of 200 mL sugar-free lemonade with no other ingredient. Any residue remaining in the cup was rinsed with a small amount of lemonade, which the subject was required to drink to ensure the ingestion of the entire treatment dose. Duplicate test drinks (each containing one of the food folate extracts or placebo) were analyzed for total folate content.

During the period of measurement on each of the test days, subjects consumed an identical, specially prepared low-folate diet (breakfast, lunch, and 2 snacks), which provided two-thirds of the energy requirements for male subjects with a sedentary lifestyle (27). The lunch meal was prepared in advance in one batch and kept frozen at -20°C for the duration of the study. To

decrease the folate content of the test day menu, all foods included in the lunch meal were boiled 3 times (the water was discarded after each boiling) and then seasoned and stir-fried before serving to increase palatability as detailed elsewhere (28). Duplicate portions of the low-folate diet were analyzed for total folate content.

Subjects were placed on an FA saturation regimen before commencement of the study (5 mg FA/d for 1 wk followed by 2 FA supplement-free days) and during the intervals between treatments (5 mg FA/d for 5 d followed by 2 FA supplement-free d). The subjects were advised to take the supplement each morning during breakfast. FA supplements were provided in a 7-d tablet-organizer box (Shantys Ltd, Dagenham, United Kingdom/Carepac), and compliance was monitored by counting the tablets in the returned boxes. The rationale for placing the subjects on high-dose FA before and between treatments was to saturate the tissues to provoke a measurable plasma folate response to the administered treatments. This approach not only avoided the problems experienced with earlier acute folate bioavailability protocols, in which, without the use of a prestudy saturation protocol, a very high variability in folate measurements was observed, even when stable isotopes were used (29), but also helped to minimize intersubject differences in baseline plasma concentrations.

Blood sampling and analysis

A cannula was placed and blood taken from each subject before consumption of the test treatments and then at varied intervals over a period of 10 h: 0.5, 1, 1.5, 2, and 2.5 h (before the midmorning snack); 3 and 5 h (before lunch); 7 h (before the afternoon snack); and 10 h. Blood samples were collected into foil-wrapped Monoject EDTA-coated tubes (Sherwood, Ballymoney, United Kingdom). The samples were immediately placed on ice, and plasma was separated by centrifugation ($2000 \times g$ for 10 min at 4°C) within 30 min and stored at -70°C until analysis. All samples from each subject (10×4 d) were analyzed in one assay to minimize measurement variability.

The folate concentrations of plasma and red blood cells (whole blood folate standardized by packed cell volume) were measured by using the *Lactobacillus casei* microbiological assay (30) adapted to a microtiter plate procedure and carried out by a robotic system (Hamilton, Bonaduz, Switzerland). The calibration of the assay was performed by using FA (Sigma Chemical Co, Poole, United Kingdom) as a standard. Under the conditions of the assay in our laboratory (pH 6.7 of the assay medium), *L. casei* has equivalent responses to FA and the major food folate constituents (5-methyltetrahydrofolate and 5-formyltetrahydrofolate). The interassay CV in the folate concentration of quality-control samples ($n = 48$) was 5.5%. Plasma vitamin B-12 concentrations were analyzed by using the method of Kelleher and O'Broin (31). Plasma total homocysteine was measured by Abbott Imx Fluorescence Polarization Immunoassay (Abbott, Ludwigshafen, Germany; 32). Quality control was provided by pooled plasma and erythrocyte samples stored at -70°C in small aliquots. The *MTHFR* 677C $\rightarrow$ T genotype was determined by using polymerase chain reaction amplification followed by *HinFI* restriction digestion (33). The total folate content of the test drinks and the low-folate diet was measured in duplicate portions by using a microbiologic assay (30) after thermal extraction and tri-enzyme treatment according to the procedure of Tamura et al (34).

Statistical analysis

Power calculations were based on the expected difference between responses to polyglutamyl and monoglutamyl folate in the range of $\approx 20\%$ to 50% , which was found in earlier reports (35–38) and on the overall response to $500\text{ }\mu\text{g}$ 5-methyltetrahydrofolate observed in our previous study by using a similar acute bioavailability protocol (39). We estimated that 10–20 subjects per treatment group were required to show a significant difference in the plasma folate responses between the 2 folate forms with 80% power and $\alpha = 0.05$.

The differences in plasma folate concentrations within each treatment (baseline compared with each time point over 10 h) were analyzed by repeated-measures analysis of variance (ANOVA) followed by Dunnett's post hoc test. Responses to treatments were calculated on an individual basis by subtracting the baseline plasma folate value from the value observed at each time point. Responses to different treatments were compared by full repeated-measures ANOVA including time and treatment and the time $\times$ treatment interaction as factors. To account for the fact that, in the case of egg yolk folate, only 9 of the 13 subjects successfully completed the treatment, one observation per subject was created, and random subject effect was included in the model. The Tukey-Kramer test was used for post hoc comparisons within each time point. Plasma folate response and time were plotted and the area under the curve (AUC) was measured by using the trapezoidal method (40). This calculation involved the sum of the trapezoidal area of each time frame. The differences in AUC and maximum response (R_{max}) among treatments were analyzed by 2-factor univariate ANOVA with subject and treatment as factors and Tukey-Kramer post hoc test. All statistical analyses were performed by using SAS software (version 9.1.3; SAS Institute Inc, Cary, NC) and Microsoft EXCEL (version 97 SR-1; Microsoft Corp, Redmond, WA). P values < 0.05 were considered significant.

RESULTS

Folate analysis of drinks and meals

Analysis of the total folate content of the duplicate test drinks showed the following mean ($\pm$ SD) results: $519 \pm 9.3\text{ }\mu\text{g}/200\text{ mL}$ ($n = 3$) for the reconstituted egg yolk folate drink, $503 \pm 20.5\text{ }\mu\text{g}/200\text{ mL}$ ($n = 4$) for the reconstituted spinach folate drink, $509 \pm 18.7\text{ }\mu\text{g}/200\text{ mL}$ ($n = 4$) for the reconstituted yeast folate drink, and undetectable amounts of folates ($n = 4$) for the placebo drink. The diet consumed by the subjects during the 10-h treatment on each test day was found (on the basis of duplicate meal analysis) to have a total folate content of $87\text{ }\mu\text{g}$. The breakfast (during which treatments were administered) contained only $15\text{ }\mu\text{g}$ folate, each of the snacks (midmorning and afternoon) provided $6\text{ }\mu\text{g}$ folate, and the lunch provided the remaining $60\text{ }\mu\text{g}$ folate.

Plasma folate response to treatments

Of 21 recruited and screened subjects, 13 were eligible to take part in the study. The baseline concentrations of plasma folate, vitamin B-12, total homocysteine, and red blood cell folate in these participants were within the normal reference ranges (Table 2). All 13 subjects successfully completed the study protocol except the egg yolk folate treatment, which was found to be unacceptable by 4 subjects (none of whom had reported an



TABLE 2

Subject characteristics and baseline data

	Value
Age (y)	25.6 ± 5.5 ¹
BMI (kg/m ²)	23.1 ± 2.0 (20–25)
<i>MTHFR</i> genotype (<i>CC/CT</i>) ²	9/4
Plasma folate (nmol/L) ³	22.7 ± 8.2 (6.80–45.32)
Red blood cell folate (nmol/L) ³	1612 ± 465 (340–2266)
Plasma total homocysteine (μmol/L) ⁴	8.5 ± 2.0 (≤15.0)
Plasma vitamin B-12 (pmol/L) ³	386.1 ± 150.0 (111–738)

¹ $\bar{x} \pm$ SD; reference range in parentheses (all such values).

² Heterozygous (*CT*) and wild-type (*CC*) genotypes for the 677C → T (thermolabile) variant of methylenetetrahydrofolate reductase (*MTHFR*).

³ Laboratory reference range was developed at the Vitamin Research Laboratory, Trinity College Dublin, Ireland.

⁴ Cutoff for hyperhomocysteinemia defined by Kang et al (41).

aversion to egg at recruitment). Therefore, the egg yolk folate response is based on the results of 9 of the 13 subjects who participated in the study.

Mean plasma folate concentrations at baseline and at each time point during the 10-h test period after treatments are shown in **Table 3**. The plasma folate response (calculated as the difference between the individual plasma folate values at each time point and the baseline value) to each treatment over time is shown in **Figure 1**. Full repeated-measures ANOVA of the plasma folate response showed significant ($P < 0.0001$) effects of time and treatment and a time × treatment interaction. Post hoc comparisons between folate treatments and placebo at each time point showed that plasma folate responses from 0.5 to 3 h after consumption of egg yolk folate, from 0.5 to 5 h after spinach folate treatment, and from 1 to 5 h after yeast folate treatment were significantly higher than those after placebo. At 0.5 h only (ie, no other time point), the plasma folate response to yeast folate was found to be significantly lower than the response to egg yolk folate or spinach folate. $R_{\max}$ was observed between 0.5 and 2.5 h after ingestion of either egg yolk folate or spinach folate and between 1 and 3 h after ingestion of yeast folate. No significant differences were observed in the $R_{\max}$ values between the food folate treatments. Significantly lower plasma folate values (compared with baseline) were observed for all folate treatments and placebo at 10 h.

The overall absorption calculated as plasma folate AUC for egg yolk, spinach, and yeast folate was 122.6 ± 23.6 ; 136.2 ± 21.4 , and 102.5 ± 21.1 nmol · h/L, respectively (**Figure 2**).

Two-factor ANOVA showed a significant treatment effect. Post hoc analysis showed that the AUC for placebo was significantly ($P < 0.001$) lower than that for each of the folate treatments; however, AUC did not differ significantly between the 3 folate treatments. The AUC for spinach and yeast folate was calculated to be 111% and 84%, respectively, of that for egg yolk folate.

DISCUSSION

Several factors are recognized as potentially contributing to the incomplete bioavailability of various food folates, but their relevance in influencing folate bioavailability in a typical mixed diet is poorly understood. To address whether incomplete intestinal deconjugation of polyglutamyl folates could contribute to the incomplete bioavailability of dietary folates, we examined 3 food folate sources whose polyglutamate content ranged from 0% (egg yolk folate) to 50% (spinach folate) to 100% (yeast folate). The results showed no significant difference in short-term bioavailability between these food folates, which indicated that the extent of glutamylation is not a limiting factor in measuring food folate bioavailability.

Dietary folate recommendations that are based on an adjustment for the differences in bioavailability between natural food folates and FA added to food are now emerging, with the introduction in the United States of dietary folate equivalents (42). Dietary polyglutamates, which account for an estimated two-thirds of total folate intake from a mixed unfortified diet (43), have to be hydrolyzed to the monoglutamate form for normal absorption in the proximal small intestine, a process controlled by the intestinal brush-border enzyme FGCP. The question of whether polyglutamyl folates are inherently less bioavailable than are monoglutamyl folates (ie, in the absence of specific inhibitors of deconjugation such as organic acids; 23) is a controversial aspect of folate bioavailability (25). This specific issue has been explored in studies using various approaches, and the results have been inconsistent. Earlier acute studies (35–38) and a chronic study (44), all using synthetic folate forms, indicated that the bioavailability of polyglutamyl folate was 50% to 80% less than that of monoglutamyl folate. In contrast, Wei et al (22) used exogenous deuterium-labeled monoglutamyl and polyglutamyl folates added to various foods and showed equivalent bioavailability for the 2 folate forms. Similar bioavailabilities for the 2 folate forms were also reported in a study using stable isotopes in women (45) and in an acute study in ileostomy subjects that compared folate absorption from spinach containing

TABLE 3

Plasma folate concentrations at baseline and during a 10-h test period in men after egg yolk folate, spinach folate, yeast folate, and placebo treatments administered in random order at weekly intervals¹

Treatment	Test period (h)									
	0	0.5	1	1.5	2	2.5	3	5	7	10
	nmol/L									
Egg yolk folate, 500 μg	71.6 ± 6.6	92.6 ± 9.0 ²	98.1 ± 8.8 ²	92.1 ± 7.7 ²	88.9 ± 7.1 ²	97.8 ± 8.5 ²	93.1 ± 6.4 ²	79.4 ± 7.5	71.7 ± 8.0	50.5 ± 5.3 ²
Spinach folate, 500 μg	62.6 ± 5.6	86.0 ± 4.9 ²	93.2 ± 6.6 ²	84.9 ± 4.2 ²	88.1 ± 4.3 ²	83.7 ± 6.0 ²	79.1 ± 4.2 ²	79.1 ± 5.9 ²	63.9 ± 4.5	46.9 ± 3.1 ²
Yeast folate, 500 μg	69.5 ± 5.2	72.6 ± 5.6	87.7 ± 6.0 ²	86.0 ± 4.9 ²	85.1 ± 5.2 ²	87.2 ± 6.3 ²	84.2 ± 7.3 ²	84.5 ± 4.9 ²	68.3 ± 4.6	47.8 ± 3.7 ²
Placebo	67.1 ± 6.7	63.8 ± 4.9	65.6 ± 5.2	61.5 ± 5.7	63.9 ± 5.5	65.0 ± 6.3	62.0 ± 5.3	66.0 ± 4.6	57.8 ± 3.8	44.8 ± 3.9 ²

¹ All values are $\bar{x} \pm$ SEM. All subjects received all 4 treatments (crossover design) except egg yolk folate, in which case only 9 of the 13 subjects successfully completed the treatment; therefore, $n = 9$ for egg yolk folate treatment; $n = 13$ for placebo and spinach and yeast folate treatments.

² Significantly different from time 0 h, $P < 0.05$ (repeated-measures ANOVA followed by Dunnett's post hoc test).

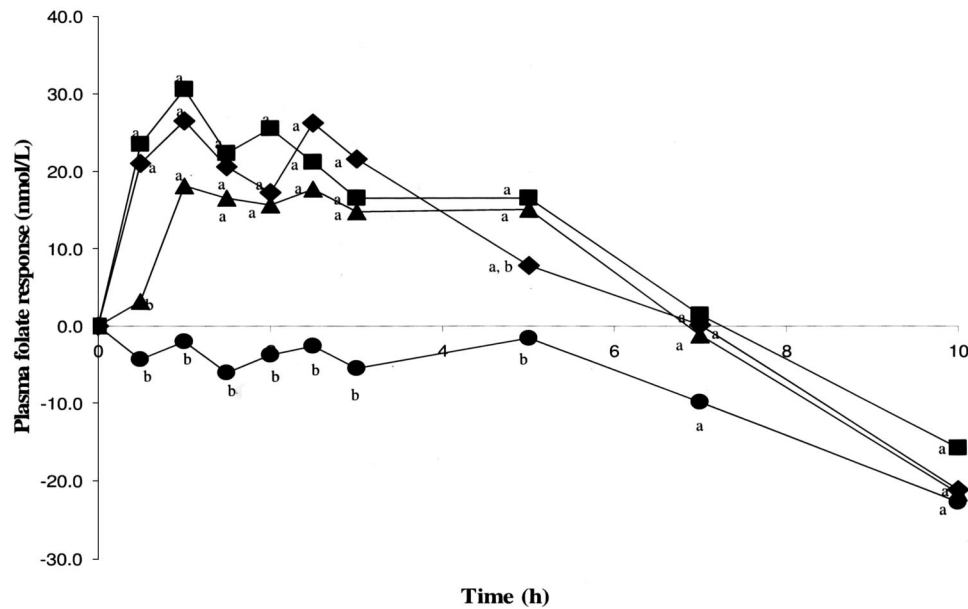


FIGURE 1. Mean plasma folate response (corrected for baseline values) in men after treatment with 500 µg spinach folate (■), 500 µg yeast folate (▲), 500 µg egg yolk folate (◆), or placebo (●) administered in random order at weekly intervals. All subjects received all 4 treatments (crossover design) except egg yolk folate, in which case only 9 of the 13 subjects successfully completed the treatment; therefore, $n = 9$ for egg yolk folate treatment; $n = 13$ for placebo and spinach and yeast folate treatments. Means were compared by full repeated-measures ANOVA with time, treatment, random subject effect, and time $\times$ treatment interaction as factors. Time, treatment, and time $\times$ treatment interaction were significant ($P < 0.0001$). Means with different letters at a given time point are significantly different, $P < 0.05$ (Tukey-Kramer post hoc test).

60% polyglutamate and from spinach treated before ingestion to convert the folates to the monoglutamate form (46). Such findings, showing similar bioavailabilities for polyglutamyl and monoglutamyl folate forms, are consistent with the results from the current study that show no significant difference in the

plasma folate response between natural food folates containing 0%, 50%, or 100% polyglutamyl folate. The current results are in agreement also with earlier observations (47) that indicated that the activity of human jejunal brush-border FGCP exceeded the demands for hydrolysis of polyglutamyl folates within the range of dietary intake and, therefore, was not rate limiting in the absorption process. The reason for the inconsistency of results between the aforementioned studies examining the bioavailability of polyglutamyl folates probably is the differences in the methods and protocols used. The pertinent question in relation to the mixed (unfortified) diet is whether naturally occurring monoglutamyl folates are inherently more bioavailable than are naturally occurring polyglutamyl folates.

In the current study, we compared polyglutamyl and monoglutamyl folates in their natural forms. The food folates were provided as concentrated extracts of egg yolk, spinach, and yeast folate, which were removed from their normal food matrix and freeze-dried. The protocol for the production of these folate extracts, which has been described in detail elsewhere (26), was developed by us specifically for use in folate bioavailability studies. This approach not only provided natural food folates of predetermined total folate and polyglutamyl folate content in sufficiently concentrated amounts to illicit a measurable plasma folate response but also allowed other factors known to affect food folate bioavailability—such as entrapment of folate in the food matrix and degradation of reduced folates during digestion—to be controlled. Ascorbate, which was used in our folate extract protocol to protect the food folates from degradation, has been reported in some *in vitro* experiments (23) to act as a competitive inhibitor of FGCP. On the basis of stable isotope studies that showed that human FGCP had sufficient activity to tolerate at least moderate amounts of such inhibitors (22), however, we

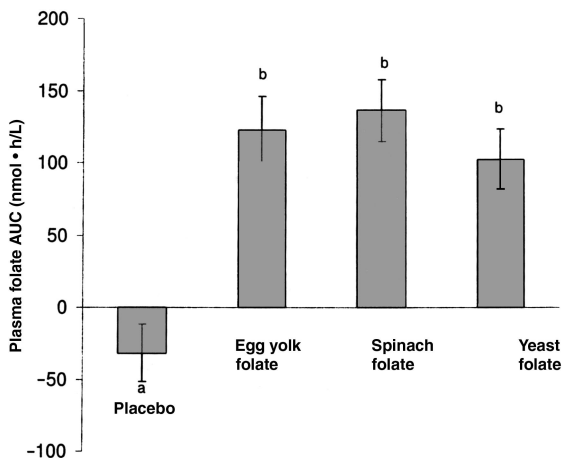


FIGURE 2. Mean ($\pm$ SEM) area under the curve (AUC) for the plasma folate response over a 7-h posttreatment period. All subjects received all 4 treatments (crossover design) except egg yolk folate, in which case only 9 of the 13 subjects successfully completed the treatment; therefore, $n = 9$ for egg yolk folate treatment; $n = 13$ for placebo and spinach and yeast folate treatments. Means were compared by 2-factor univariate ANOVA with subjects and treatments as factors. There was a significant treatment effect ($P < 0.0001$). Means with different letters are significantly different, $P < 0.001$ (Tukey-Kramer post hoc test). The AUC was calculated by using the following equation: $(AUC) = I \times (T_1 + T_2) \times 0.5 + I \times (T_2 + T_3) \times 0.5 + I \times (T_3 + T_4) \times 0.5 + I \times (T_4 + T_5) \times 0.5 \dots I \times (T_8 + T_9) \times 0.5$, where I is the interval (h) between time points, and T is the plasma folate response at each timepoint.

do not anticipate any effect of ascorbate on FGCP activity at the concentration used in the current study.

Another feature of the current protocol is that we used numerous time points (10 in the course of 10 h), which allowed us to monitor as fully as possible the magnitude of the folate peak and the changes in plasma folate concentrations until their return to baseline. In addition, we fed the subjects a standardized, specially prepared low-folate diet on each test day, and treatments were administered midway through breakfast. The low-folate standardized diet not only avoided the need for fasting (reported to provoke an increase in plasma folate concentrations by interrupting the enterohepatic folate circulation; 48) but ensured that the conditions were physiologic and identical for each of the 4 test days. We are confident that the amount of folate in this standard diet did not exert any effect on the folate response, because there was no increase in plasma folate concentrations as a result of the placebo treatment (in fact, values decreased).

Finally, we used a protocol that for experimental reasons required the saturation of tissues with folate before commencement of the study and between treatments to provoke a measurable folate response to treatments (29) and to minimize differences in baseline folate concentrations. It is possible that the supraphysiologic dose of FA (5 mg/d) used may not have been completely cleared during the 2 FA supplement-free days before each treatment, and the resulting continuing clearance of the high-FA dose during the measurement period may have contributed to the drop in folate below baseline observed 10 h after all 4 treatments. However, we do not consider that this could have had any effect on our overall findings in relation to relative folate bioavailability among the 3 folate sources, because the treatments were administered in random order and the conditions between and during the test days, as outlined above, were kept consistent, apart from the administered folate treatments.

Our results show no significant difference between the plasma folate response (AUC) to polyglutamate-containing folate sources and the response to an equivalent intake of natural monoglutamyl (egg yolk) folate: responses for spinach folate and yeast folate were estimated to be 111% and 84%, respectively, of the response for egg yolk folate. The explanation for the slightly (not significantly) lower response of yeast folate may have to do with a specific inhibition of intestinal FGCP by yeast components (49). It is unlikely, however, to be related to the extent of polyglutamyl folate (100%) present, given the lack of a consistent trend in the response of the 2 other folates, despite major differences in their polyglutamyl folate contents, ie, 0% (egg yolk folate) versus 50% (spinach folate). Recent reports have raised questions about the validity of using short-term protocols with AUC calculations to examine folate bioavailability using FA as a reference (50). Those authors examined the rate and extent of appearance of labeled and unlabeled folates after oral doses of FA and natural folates and found differences in the extent of displacement of unlabeled folate between FA and the reduced folate forms, which are indicative of a different hepatic first-pass effect between the different folate forms. However, these reports, which question the interpretation of some previously published bioavailability studies (25), relate specifically to short-term AUC protocols in which FA was used as the reference to estimate relative bioavailability (51). For these reasons, we did not use FA as the reference for comparison with food folates but rather compared responses among the 3 food folates, all of which provided the vitamin in the reduced form and differed only in the

extent of conjugation of the glutamate moiety. Although we did not measure the folate derivative profile of the food folate extracts as part of the current study, we described it in detail elsewhere (26), and the predominant form in all cases is 5-methyltetrahydrofolate. This is known to be one of the most stable of the reduced folate derivatives and therefore is unlikely to have undergone any major interconversion under the experimental conditions of the current protocol.

In conclusion, the current study shows equivalent bioavailabilities for 3 natural food folate sources comprising 0%, 50% or 100% polyglutamyl folate. The findings indicate that, in the absence of specific inhibitors of deconjugation in certain foods, the extent of conjugation of natural folates per se is not a limiting factor in ascertaining folate bioavailability in a mixed diet. Our study therefore suggests that other factors, such as the entrapment of folates in the food matrix or the instability of reduced folates in the digestive system before absorption (or even folate losses before ingestion), are more likely to explain the well-established incomplete bioavailability of natural food folates. 

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DJM and JG were responsible for execution of the study. DJM and JG were responsible for data collection; DJM, LH, and MHF were responsible for the laboratory analysis; DJM, LH, and KP were responsible for data analysis; all authors assisted in writing and revising the manuscript; HM was responsible for planning the study; HM, JMS, JJS, and KP were responsible for the study design; HM, JMS, and KP were responsible for data interpretation; IB was responsible for the statistical analysis; JG was responsible for subject recruitment; and RM, JA, and BKP were responsible for the production of food folate extracts (industrial scale). None of the authors had any personal or financial conflict of interest.

REFERENCES

- Choi SW, Mason JB. Folate and carcinogenesis: an integrated scheme. *J Nutr* 2000;130:129–32.
- Kim YI, Pogribny IP, Basnakian AG, et al. Folate deficiency in rats induced DNA strand breaks and hypomethylation within the p53 tumor suppressor gene. *Am J Clin Nutr* 1997;65:46–52.
- Wald DS, Law M, Morris JK. Homocysteine and cardiovascular disease: evidence on causality from a meta-analysis. *BMJ* 2002;325:1202–6.
- Medical Research Council Vitamin Study Research Group. Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. *Lancet* 1991;338:131–7.
- Czeizel AE, Dudas I. Prevention of first occurrence of neural tube defects by periconceptional vitamin supplementation. *N Engl J Med* 1992;327:1832–5.
- Department of Health (United Kingdom). Report from an expert advisory group. Folic acid and the prevention of neural tube defects. London: HM Stationery Office, 1992.
- National Health and Medical Research Council (Australia). Revised statement on the relationship between dietary folic acid and neural tube defects such as spina bifida, 115th session. Canberra, Australia: National Health and Medical Research Council, Australia, 1993.
- Recommendations for the use of folic acid to reduce the number of cases of spina bifida and other neural tube defects. *MMWR Morb Mortal Wkly Rep* 1992;41:1–7.
- Cuskelly GJ, McNulty H, Scott JM. Effect of increasing dietary folate on red-cell folate: implications for preventions of neural tube defects. *Lancet* 1996;347:657–9.
- Gregory JF III. Bioavailability of folate. *Eur J Clin Nutr* 1997;51:S54–9.
- Sauberlich HE, Kretsch MJ, Skala JH, Johnson HL, Taylor PC. Folate requirement and metabolism in nonpregnant women. *Am J Clin Nutr* 1987;46:1016–28.
- Baker H, Jaslow SP, Frank O. Severe impairment of dietary folate utilization in the elderly. *J Am Geriatr Soc* 1978;26:218–21.
- Tamura T, Stokstad ELR. The availability of food folate in man. *Br J Haematol* 1973;25:513–32.

14. Colman N, Green R, Metz J. Prevention of folate deficiency by food fortification. II. Absorption of folic acid from fortified staple foods. *Am J Clin Nutr* 1975;28:459–64.
15. Prinz-Langenohl R, Bronstrup A, Thorand B, Hages M, Pietrzik K. Availability of food folate in humans. *J Nutr* 1999;129:913–6.
16. Brouwer IA, van Dusseldorp M, West CE, et al. Dietary folate from vegetables and citrus fruit decreases plasma homocysteine concentrations in humans in a dietary controlled trial. *J Nutr* 1999;129:1135–9.
17. Hannon-Fletcher MP, Armstrong NC, Scott JM, et al. Determining bioavailability of food folates in a controlled intervention study. *Am J Clin Nutr* 2004;80:911–8.
18. Gregory JF. Case study: folate bioavailability. *J Nutr* 2001;131:1376S–2S.
19. Ball GFM. Folate. In: Ball GFM, ed. *Bioavailability and analysis of vitamins in foods*. London, United Kingdom: Chapman & Hall, 1998: 439–96.
20. Castenmiller JJ, van De Poll CJ, West CE, Brouwer IA, Thomas CM, van Dusseldorp M. Bioavailability of folate from processed spinach in humans. Effect of food matrix and interaction with carotenoids. *Ann Nutr Metab* 2000;44:163–9.
21. Van het Hof KH, Tijburg LB, Pietrzik K, Weststrate JA. Influence of feeding different vegetables on plasma levels of carotenoids, folate and vitamin C. Effect of disruption of vegetable matrix. *Br J Nutr* 1999;82: 203–12.
22. Wei MM, Bailey LB, Toth JP, Gregory JF. Bioavailability for humans of deuterium labelled monoglutamyl and polyglutamyl folates is affected by selected foods. *J Nutr* 1996;126:3100–8.
23. Wei MM, Gregory JF. Organic acids in selected foods inhibit porcine jejunal brush border pteroylpolyglutamate hydrolase: potential mechanism affecting the bioavailability of dietary polyglutamyl folate. *J Agric Food Chem* 1998;46:211–9.
24. Seyoum E, Selhub J. Properties of food folates determined by stability and susceptibility to intestinal pteroylpolyglutamate hydrolase action. *J Nutr* 1998;128:1956–60.
25. Gregory JF, Quinlivan EP, Davis SR. Integrating the issues of folate bioavailability, intake and metabolism in the era of fortification. *Trends Food Sci Tech* 2005;16:229–40.
26. McKillop DJ, Pentieva K, Strain JJ, et al. The development of food folate extracts for use in human bioavailability studies. *J Agric Food Chem* 2003;51:4382–8.
27. Dietary reference values for food energy and nutrients for the United Kingdom. London, United Kingdom: The Stationery Office, 1991. (Department of Health Report on Health and Social Subjects 41.)
28. Pentieva K, McKillop D, Duffy N, et al. Acute absorption of folic acid from a fortified low fat spread. *Eur J Clin Nutr* 2003;57:1235–41.
29. Pfeiffer CM, Rogers LM, Bailey LB, Gregory JF III. Absorption of folate from fortified cereal-grain products and of supplemental folate consumed with or without food determined by using a dual-label stable-isotope protocol. *Am J Clin Nutr* 1997;66:1388–97.
30. Molloy AM, Scott JM. Microbiological assay for serum, plasma, and red cell folate using cryopreserved, microtiter plate method. *Methods Enzymol* 1997;281:43–53.
31. Kelleher B, O'Broin S. Microbiological assay for vitamin B12 performed in 96-well microtitre plates. *J Clin Pathol* 1991;44:592–95.
32. Leino A. Fully automated measurement of total homocysteine in plasma and serum on the Abbott Imx Analyser. *Clin Chem* 1999;45:569–71.
33. Goyette P, Frosst P, Rosenblatt DS, Rozen R. Seven novel mutations in the MTHFR gene and genotype/phenotype correlations in severe MTHFR deficiency. *Am J Hum Genet* 1995;56:111–3.
34. Tamura T, Mizuno Y, Johnston K, Jacob R. Food folate assay with protease, amylase, and conjugase treatments. *J Agric Food Chem* 1997; 45:135–9.
35. Halsted CH, Reisenauer AM, Shane B, Tamura T. Availability of monoglutamyl and polyglutamyl folates in normal subjects and in patients with celiac sprue. *Gut* 1978;19:886–91.
36. Gregory JF, Bhandari SD, Bailey LB, Baumgartner TG, Cerda JJ. Relative bioavailability of deuterium-labeled monoglutamyl and hexaglutamyl folates in human subjects. *Am J Clin Nutr* 1991;53:736–40.
37. Godwin HA, Rosenberg IH. Comparative studies of the intestinal absorption of [³H]pteroylmonoglutamate and [³H]pteroylheptaglutamate in man. *Gastroenterology* 1975;69:364–73.
38. Keagy PM, Shane B, Oace SM. Folate bioavailability in humans: effect of wheat bran and beans. *Am J Clin Nutr* 1988;47:80–8.
39. Pentieva K, McNulty H, Reichert R, et al. The short-term bioavailabilities of [6S]-5-methyltetrahydrofolate and folic acid are equivalent in men. *J Nutr* 2004;134:580–5.
40. Altman D. Serial measurements. In: Altman D, ed. *Practical statistics for medical research*. London, United Kingdom: Chapman & Hall, 1991: 426–33.
41. Kang SS, Wong PW, Malinow MR. Hyperhomocyst(e) inemia as a risk factor for occlusive vascular disease. *Annual Rev Nutr* 1992;12:279–98.
42. Institute of Medicine. Panel on Folate, Other B Vitamins, and Choline. Dietary reference intake; thiamine, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline. Washington, DC: National Academy Press, 1998.
43. Melse-Boonstra A, de Bree A, Verhoef P, Bjorke-Monsen AL, Verschuren WMM. Dietary monoglutamate and polyglutamate folate are both associated with plasma folate concentrations in Dutch men and women aged 20–65 y. *J Nutr* 2002;132:1307–12.
44. Melse-Boonstra A, West CE, Katan MB, Kok FJ, Verhoef P. Bioavailability of heptaglutamyl relative to monoglutamyl folic acid in healthy adults. *Am J Clin Nutr* 2004;79:424–9.
45. Boddie AM, Dedlow ER, Nackashi JA, et al. Folate absorption in women with a history of neural tube defect-affected pregnancy. *Am J Clin Nutr* 2000;72:154–8.
46. Konings EJM, Troost FJ, Castenmiller JJM, Roomans HHS, van den Brandt PA, Saris WHM. Intestinal absorption of different types of folate in healthy subjects with an ileostomy. *Br J Nutr* 2002;88:235–42.
47. Halsted CH. Intestinal absorption of dietary folates. In: Picciano MF, Stokstad ELR, Gregory JF, eds. *Folic acid metabolism in health and disease*. New York, NY: Wiley-Liss Inc, 1990:23–45.
48. Pietrzik K, Hages M, Remer T. Methodological aspects in vitamin bioavailability testing. *J Micronutrient Anal* 1990;7:207–22.
49. Rosenberg IH, Godwin HA. Inhibition of intestinal glutamate carboxypeptidase by yeast nucleic acid: an explanation of variability in the utilization of dietary polyglutamyl folate. *J Clin Invest* 1971;50:78a (abstr).
50. Wright AJA, Finglas PM, Dainty JR, et al. Single oral doses of 13C forms of pteroylmonoglutamic acid and 5-formyltetrahydrofolic acid elicit differences in short-term kinetics of labelled and unlabelled folates in plasma: potential problems in interpretation of folate bioavailability studies. *Br J Nutr* 2003;90:363–71.
51. McNulty H, Pentieva K. Folate bioavailability. *Proc Nutr Soc* 2004;63: 529–36.