Bioavailability of food folates is 80% of that of folic acid[1–3]

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ABSTRACT

Background: The bioavailability of natural food folates is lower than that of synthetic folic acid, but no agreement exists as to the extent of the difference.

Objective: In a 4-wk dietary intervention study, we determined the aggregate bioavailability of food folates from fruit, vegetables, and liver relative to that of folic acid.

Design: Seventy-two healthy adults were randomly divided into 4 treatment groups. Group A ($n = 29$) received a high-folate diet with 369 μg food folate/d and a placebo capsule; groups B, C, and D ($n =$ 14 or 15) received a low-folate diet with 73 μg food folate/d and folic acid capsules. These capsules contained 92 μg folic acid/d for group B, 191 μg for group C, and 289 μg for group D. In addition, all 72 subjects daily ingested a capsule with 58 μg [$^{13}C_{11}$]-labeled folic acid. We measured the percentage of [$^{13}C_{11}$]-labeled folate in plasma folate at the end of the intervention and ascertained the changes in serum folate concentrations over the 4 wk of the intervention.

Results: Bioavailability of food folate relative to that of folic acid was 78% (95% CI: 48%, 108%) according to [$^{13}C_{11}$]-labeled folate and 85% (52%, 118%) according to changes in serum folate concentrations.

Conclusions: The aggregate bioavailability of folates from fruit, vegetables, and liver is ≈80% of that of folic acid. The consumption of a diet rich in food folate can improve the folate status of a population more efficiently than is generally assumed. *Am J Clin Nutr* 2007;85:465–73.

KEY WORDS Food folate, bioavailability, folic acid, stable isotopes

INTRODUCTION

Folate is a generic term for the various biochemical moieties of the B vitamin pteroyl glutamic acid or folic acid. Important sources of folate are fruit, (fortified) breakfast cereals, vegetables, dairy products, and liver products (1–3). Folic acid, the fully oxidized and stable form of this vitamin, is used extensively in dietary supplements and for food fortification, but it does not occur naturally in significant amounts (4).

An inadequate folate intake will lead to a decrease in serum and erythrocyte folate, to increased blood concentrations of homocysteine, and ultimately to macrocytic anemia (5). It may also increase the risk of neurocognitive disease, cancer, and cardiovascular disease (6–9). High intake of folic acid by women of childbearing age reduces the risk of neural tube defects in the infants of these women (10).

Folate bioavailability is defined as the proportion of an ingested amount of folate that is absorbed in the gut and that becomes available for metabolic processes. In human intervention studies, relative bioavailability is usually assessed by comparison with a reference dose of folic acid. The bioavailability of food folate is generally lower than that of folic acid, but the extent of the difference is unclear (11, 12).

Recommended daily allowances for food folate take into account its lower bioavailability. The basis for adapting the US recommendations for folate to the lower bioavailability of food folate (5) was derived from a study by Sauberlich et all (13), whic stated that the bioavailability of food folate was no more than 50% of that of folic acid. Unfortunately, they did not indicate how that finding was obtained.

Other long-term dietary intervention studies found bioavailability estimates between 30% and 98% of those of folic acid (14, 15). Thus, no clear figure exists for the bioavailability of natural folates as a proportion of that of folic acid. We therefore performed a large, long-term dietary intervention study in which we compared the bioavailability of food folate with that of different doses of folic acid; we simultaneously assessed bioavailability from changes in serum folate concentrations and from stable isotope dilution.

SUBJECTS AND METHODS

Subjects

Ninety-three healthy men and women, recruited from among the staff and students of Wageningen University and the population of the city of Wageningen, volunteered to take part in the study. During a screening visit, 4 wk before the start of the trial, they filled out a questionnaire and donated a fasting blood sample from which we assessed the baseline concentrations of serum

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[2] Supported by the Wageningen Centre for Food Sciences [an alliance of major Dutch food industries, the Nederlands Instituut voor Zuivelonderzoek (NIZO), the Nederlandes Organisatie voor toegepast-natuurwetenschappelijk onderzoek (TNO), Maastricht University, Wageningen University, and the Dutch Government].

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Received June 23, 2006.

Accepted for publication October 3, 2006.

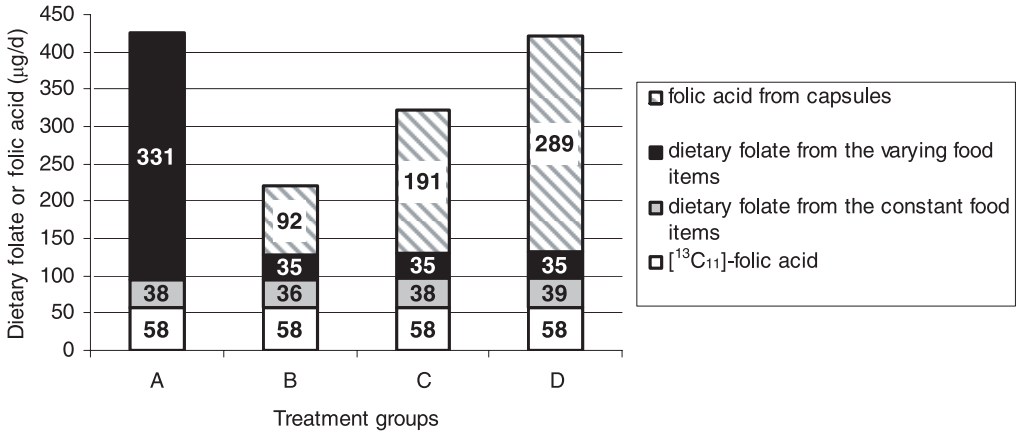

FIGURE 1. Mean intake of food folate and folic acid from food and capsules as analyzed by HPLC. Group A consumed a diet high in food folate and a placebo capsule; groups B, C, and D consumed a diet low in food folate and folic acid capsules. All groups received daily a capsule with [$^{13}C_{11}$]-labeled folic acid. The constant food items were similar for all groups, whereas the varying food items of group A differed from those of groups B through D. For group A, the varying food items were products rich in food folate, and for groups B through D, these products were replaced with similar products that were low in food folate.

vitamin B-12, serum folate (Access Immunoassay; Beckman Coulter, Fullerton, CA), serum creatinine (Synchron LX20; Beckman Coulter), plasma total homocysteine (16), and methylenetetrahydrofolate reductase (MTHFR) 677 C→T genotype (17).

We excluded 18 subjects on the basis of the following criteria: use of B vitamins within the period 3 mo before the study; body mass index (BMI; in kg/m^2) <18 or >30; use of drugs that interfere with folate metabolism; serum vitamin B-12 concentrations <119 pmol/L; serum creatinine concentrations >125 μmol/L; plasma total homocysteine concentrations >26 μmol/L; and the presence of cardiovascular disease, cancer, rheumatoid arthritis, epilepsy, or gastrointestinal disorders.

The remaining 75 subjects were stratified by serum folate, *MTHFR 677C→T* genotype, and sex. Within each stratum, randomized blocks assigned the subjects to 1 of the 4 intervention groups.

All subjects gave written informed consent. The Medical Ethical Committee of Wageningen University approved the study.

Design

The trial included 4 parallel intervention groups, as shown in **Figure 1**. Group A (the food folate group) consumed a diet high in food folate and a placebo capsule. Groups B, C, and D (the folic acid groups) all received a diet low in food folate plus a daily capsule, which contained 92, 191, and 289 μg folic acid, respectively. In addition, all 75 subjects received a capsule with 58 μg [$^{13}C_{11}$]-labeled folic acid/d. The folic acid groups (B through D) served to calibrate our outcome measurements—namely. the percentage of [$^{13}C_{11}$]-labeled folate in the total pool of plasma folate and the change in serum folate in terms of folic acid intake. We constructed calibration lines of the percentage of [$^{13}C_{11}$]-labeled folate in the total pool of plasma folate and of the change in serum folate as a function of folic acid intake in groups B through D. We then projected the mean percentage of labeled folate in plasma folate in group A and the mean change in serum folate in group A on the corresponding calibration line to assess the dose of folic acid to which the food folate in group A was equivalent. The study lasted 31 d; the folate intervention started on day 3 and ended on day 31. Two fasting blood samples were drawn at baseline (days 1 and 3) and after the 4-wk intervention (on days 29 and 31).

Diet

Dietitians from the Division of Human Nutrition of Wageningen University estimated the habitual energy intake of the subjects with a validated food-frequency questionnaire before the start of the intervention (18). We provided the subjects with ≈90% of their total daily energy requirement during the intervention. Subjects daily had a limited free choice from a list of products that provided the remaining 10% of energy. These free-choice items were mainly nonalcoholic drinks, alcoholic drinks (≤1 beer/d), candies, and sweet sandwich fillings. The items contained a low amount of folate and fat. Subjects kept a diary in which they recorded which free-choice items they consumed, whether they took their capsules, illnesses, use of medication, and any deviations from the diet.

The diets we supplied consisted of constant food items and varying food items. The constant part of the diet consisted of whole-wheat bread; margarine; sweet sandwich fillings; cheese, ham, or both; cookies; milk; boiled potatoes, rice, or pasta; and meat or a meat replacement. These constant food items provided ≈9 MJ/d (≈2100 kcal/d) for a typical participant and were similar for all groups. The varying food items provided ≈2 MJ/d (≈480 kcal/d) and differed between group A and groups B through D (**Table 1**).

Although the constant food items were similar for all groups, the amounts of food we supplied varied dependent on the energy requirement of the subjects. The food folate in the constant part of the diet was as low as possible—≈100 μg folate/9 MJ constant foods—as calculated from food tables (19).

The varying food items for group A were products rich in food folate—≈350 μg per 2 MJ of varying foods—as calculated from food tables; for groups B through D these products were replaced by similar products low in food folate—≈30 μg per 2 MJ—as calculated from food tables (Table 1). Vegetables, fruit juices, liver paste, and fruit contributed most to the folate intake in the high-folate group (**Figure 2**). The varying part of the diet was nearly the same for all 29 subjects in group A, independent of the subject's energy requirement; only the amounts of dressing,

TABLE 1
Varying food items of the diets in a study of the bioavailability of food folate versus folic acid[1]

Foods and groups	Amount per day	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Boiled vegetables								
Group A	200 g	Spinach	Brussels sprouts and red pepper	Broad beans and corn	Broccoli	Spinach, corn, and red pepper	Sugar peas	Broccoli and cauliflower
Groups B–D	100 g	Green beans	Peas and carrots	Mushrooms, leek, and carrots	Red cabbage	Corn, carrots, green pepper, and mushrooms	Carrots	White cabbage, green pepper, and mushrooms
Salad								
Group A	50 g	Chinese cabbage, green pepper, tangerines, and cashews	Endive, red pepper, tangerine, and hazelnuts	Lettuce, chick peas, pineapple, and walnuts	Pakchoi, red pepper, peach, and cashews	Iceberg lettuce, chick peas, dates, and hazelnut	Broccoli, corn, peach, and walnuts	Kohlrabi, corn, dates, and hazelnuts
Groups B–D	50 g	Blanched celery, tomato, and dates	Cucumber, onion, tomato, and pineapple	Chicory, apple, and raisins	Fennel, cucumber, tomato, and mixed fruit	White cabbage, tomato, pickle, and peach	Radish, cucumber, onion, and pineapple	Carrot, cucumber, and raisins
Fruit								
Group A	2 pieces	1 orange and 1 banana or kiwi	1 orange and 1 banana or kiwi	1 orange and 1 banana or kiwi	1 orange and 1 banana or kiwi	1 orange and 1 banana or kiwi	1 orange and 1 banana or kiwi	1 orange and 1 banana or kiwi
Groups B–D	1 piece	Apple	100 g grapes	Apple	100 g melon	Pear	Apple	Pear
Fruit juice								
Group A	400 mL	Orange juice	Orange juice	Orange juice	Orange juice	Orange juice	Orange juice	Orange juice
Groups B–D	400 mL	Apple or grape juice	Apple or grape juice	Apple or grape juice	Apple or grape juice	Apple or grape juice	Apple or grape juice	Apple or grape juice
Liver paste[2]								
Group A	25 g	Liver paste	Liver paste	Liver paste	Liver paste	Liver paste	Liver paste	Liver paste
Groups B–D	—	—	—	—	—	—	—	—
Salad dressing[3]								
Group A	15 g (10–18 g)[4,5]	Dressing	Dressing	Dressing	Dressing	Dressing	Dressing	Dressing
Groups B–D	17 g (14–20 g)[5]	Dressing	Dressing	Dressing	Dressing	Dressing	Dressing	Dressing
Sauce or gravy[2]								
Group A	53 g (41–68 g)[5]	Sauce or gravy	Sauce or gravy	Sauce or gravy	Sauce or gravy	Sauce or gravy	Sauce or gravy	Sauce or gravy
Groups B–D	69 g (60–75 g)[5]	Sauce or gravy	Sauce or gravy	Sauce or gravy	Sauce or gravy	Sauce or gravy	Sauce or gravy	Sauce or gravy
Dessert								
Group A	140 g (115–155)[5]	Flavored yogurt	Flavored yogurt	Flavored yogurt	Flavored yogurt	Flavored yogurt	Flavored yogurt	Flavored yogurt
Groups B–D	110 g (83–135 g)[5]	Flavored custard	Flavored custard	Flavored custard	Flavored custard	Flavored custard	Flavored custard	Flavored custard

[1] Group A, the food folate group (fed foods and drinks high in folate); Groups B–D, the folic acid groups (fed equivalent foods and drinks low in food folate plus various doses of folic acid).
[2] Vegetarians received 12.5 g/d of a yeast-based vegetarian spread (Tartex; Tartex, Freiburg, Germany).
[3] Salad dressing and sauce or gravy were prepared with egg yolk for group A and with egg white for groups B–D.
[4] Median; 25th and 75th percentiles in parentheses (all such values).
[5] The amount of dressing, sauce, and dessert differed by subject, according to the energy requirement of that subject.

sauce, and dessert were adjusted to individual energy requirements (Table 1). Likewise, the varying part of the diet for groups B through D was nearly the same for all 43 subjects in those groups.

During weekdays, we served hot lunches to all subjects; subjects consumed these meals under our supervision at the Division of Human Nutrition of Wageningen University. We weighed out all foods and beverages for each subject. After lunch, subjects received their package with foods and beverages for their evening meal and for breakfast. On Fridays, subjects received a package with food and beverages for the weekend breakfasts, lunches, and hot dinners, plus instructions for the preparation of these foods.

Meals were prepared from conventional foods and drinks. Folic acid fortification is not mandatory in the Netherlands, and the foods and beverages that we used did not contain added folic acid.

Measurement of nutrients in food samples

We collected a total of 4 duplicate diets on each day of the trial: 2 duplicate diets representative of group A and 2 representative of groups B through D; the energy content of these duplicates was 11 MJ (≈2600 kcal) according to food tables. On weekdays, we collected the duplicates every day at lunchtime. We also collected a duplicate of a package with foods and beverages for the weekend. This was prepared for consumption during the actual weekend and then worked up as described below. We collected, pooled, and analyzed the items of the constant part of the diet separately from the varying food items. For the food folate analyses, hot items were cooled to ≈10 °C immediately after collection in a blast

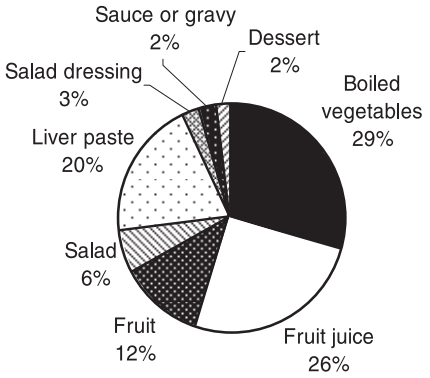

FIGURE 2. Comparison of the contributions of the various food items to the additional food folate in the diet of group A with those in the diets of groups B through D.

chiller before they were added to the cold items. We added 250 mL of chilled CHES/HEPES buffer (pH 7.85) with 2% ascorbic acid and 0.2 mol 2-mercaptoethanol/L per kg of food, homogenized it in a blender under a flow of nitrogen, and stored the samples at −80 °C until they were analyzed.

Food folate was analyzed in a selection of the duplicate diets: we selected one random Monday duplicate out of the 4 Mondays of the study of group A and analyzed food folate in both the constant and the varying food items. The same step was taken for all other weekdays. We repeated this selection for diets from groups B through D. In this way, we avoided the thawing and homogenization that would have been necessary if we had pooled the diets of all 4 Mondays of the study. We decided not to analyze folate in all samples, because food folate analysis is time- and labor-intensive. Food folate was analyzed with an HPLC method (20). In short, duplicate diets were thawed, extracted with CHES/HEPES buffer in a boiling water bath for 10 min, and cooled in a water bath at 0 °C. Samples were subjected to trienzyme treatment, purified on an affinity column (Folate Binding Protein; Scripps, San Diego, CA), and analyzed by HPLC with fluorescence and ultraviolet detection. All analyses were performed in duplicate by splitting the sample after it had been thawed.

Duplicate diets for the analyses of energy in the diets were stored at −20 °C. After the study, we thawed these duplicates, pooled the constant parts of each diet per week, pooled the varying food items of each diet per week, and homogenized the pooled items. Samples were stored at −20 °C until they were analyzed. Total fat, protein, dry matter, ash, and fiber were analyzed in these duplicates. From these analyses, we calculated the amount of fat, protein, carbohydrates, fiber, and energy in the diets actually eaten by the subjects.

Capsules

The capsules with folic acid (Merck Eprova, Schaffhausen, Switzerland) were specially produced for this study. We ordered capsules containing 100, 200, and 300 μg. To analyze the actual amount of folic acid, we dissolved capsules in CHES/HEPES buffer pH 7.85 with 2% ascorbic acid and 0.2 mol 2-mercaptoethanol/L in a boiling water bath and measured folic acid by using HPLC with ultraviolet detection (20). The amount of folic acid in a random sample of 20 capsules of each dose was ≈95% of the expected dose.

[$^{13}C_{11}$]-Folic acid was synthesized for us by ARC (Apeldoorn, Netherlands). Folic acid was labeled with 6 ^{13}C atoms in the benzoic acid structure and with 5 ^{13}C atoms in the glutamate part (21). These capsules contained a mean (±SD) of 58 ± 11 μg folic acid (n = 20) according to HPLC analysis (20), which was ≈85% of the expected dose.

An independent research assistant from Wageningen University coded the placebo and folic acid capsules: subjects and investigators were blinded to the folic acid treatment. The assistant did not reveal the code until all data had been gathered and double-checked. Subjects ingested their capsules just before the hot lunch.

Measurements in blood

For the measurement of serum folate, blood samples were drawn into coagulation tubes, stored in the dark for 30–120 min at room temperature, and centrifuged at 2600 × g for 10 min at 4 °C. Serum was pipetted off and stored at −80 °C. We measured the serum folate concentration with a chemiluminescence immunoassay (Access Immunoassay; Beckman Coulter) in the samples for days 1, 3, 29, and 31. Baseline values represent the mean of days 1 and 3, and week 4 values represent the mean of days 29 and 31. Intraassay and interassay CVs of this immunoassay were <15%.

For the measurement of labeled folate, blood samples were drawn into EDTA-containing tubes that were immediately placed on ice and centrifuged within 30 min at 2600 × g for 10 min at 4 °C. Plasma was pipetted off and stored at −80 °C. We measured the percentage of labeled folate in the total pool of folate in plasma with liquid chromatography–tandem mass spectrometry [(LC-MS/MS) 21], as in the following equation:

$$\text{Percentage of labeled folate} = 100 \times [^{13}C_{11}]\text{-5-methyltetrahydrofolate}/([^{13}C_{11}]\text{-5-methyltetrahydrofolate} + [^{13}C_{0}]\text{-5-methyltetrahydrofolate}) \quad (1)$$

where the numerator is the area under the curve of the [$^{13}C_{11}$]-5-methyltetrahydrofolate LC-MS/MS peak and the denominator is the sum of the areas under the curve of the [$^{13}C_{11}$]-5-methyltetrahydrofolate and the [$^{13}C_{O}$]-5-methyltetrahydrofolate LC-MS/MS peaks.

Labeled folate was analyzed only in the samples for days 1 and 29. We chose to analyze labeled folate in only one baseline sample and one follow-up sample because we expected that the estimate for bioavailability derived from labeled folate data would be more precise than the estimate derived from serum folate data, even if analyzed only in one sample. The labeled compound does not occur naturally and was not detected in baseline samples. We restricted our measurements to the 5-methyltetrahydrofolate fraction of the plasma, because this is the most abundant folate vitamer in plasma (22).

Calculation of bioavailability

We plotted the individual percentages of labeled folate in the plasma folate of subjects in groups B, C, and D against the intake of supplemental folic acid. We fitted a linear regression line through these points to construct a calibration line of percentage of labeled folate against the intake of supplemental folic acid. The mean percentage of labeled folate in group A was then projected on this calibration line to assess by interpolation the

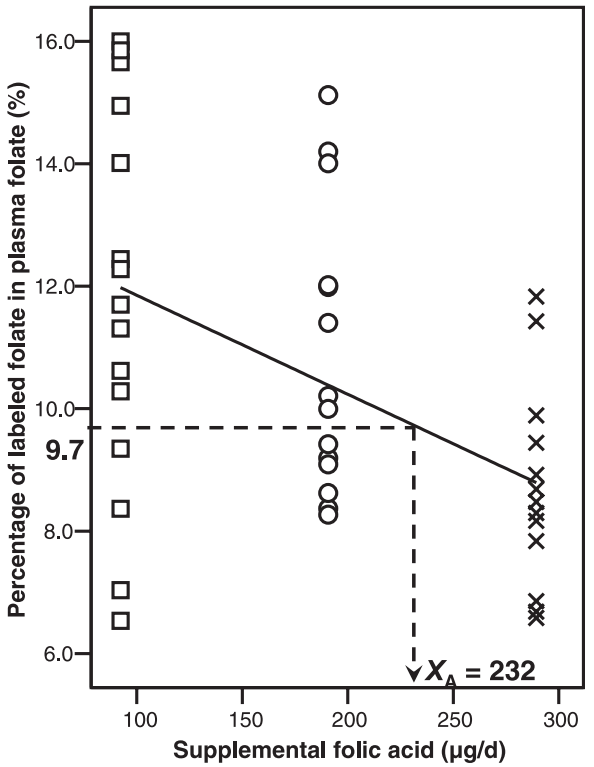

FIGURE 3. Percentage of labeled folate in plasma folate after a 4-wk intervention in folic acid groups (B through D). Each symbol represents 1 subject; □, group B; ○, group C; ×, group D; —, the linear regression line through the individual data points of groups B, C, and D. The mean percentage of labeled folate in group A was 9.7%. This was projected on the regression line and corresponded to an estimated intake of folic acid of 232 μg (broken lines). The R^2 of the regression line was 0.23, and the slope was −0.01616 (95% CI: −0.02550, −0.00683).

dose of folic acid to which the additional amount of food folate in group A ($folate_{additional}$) was equivalent (**Figure 3**); this dose was called X_a. Relative bioavailability of food folate was derived from X_a and $folate_{additional}$ according to the following equation:

$$Bioavailability_{relative} = 100\% \times X_a/folate_{additional} \quad (2)$$

where $folate_{additional}$ was calculated with the use of the following equation:

$$Folate_{additional} = \text{food folate in groupA} - \text{food folate in groups B–D (in } \mu\text{g/d)} \quad (3)$$

The 95% CI associated with this bioavailability estimate was calculated with the use of the following equation:

$$[\text{Lower limit, upper limit}] = [100\% \times (X_a - t_{0.975;n-2} \times S_{X_a})/folate_{additional}, 100\% \times (X_a + t_{0.975;n-2} \times S_{X_a})/folate_{additional}] \quad (4)$$

and the SE of prediction for X_a was calculated with the use of the following equation (23):

$$s_{x_a} = \sqrt{[MSE/b_1^2] \times [1/m + 1/n + (X_a - \bar{X})^2/\sum(X_i - \bar{X})^2]} \quad (5)$$

where MSE = mean square error; b_1 = the slope of the regression line in percentage change in labeled folate per μg folic acid; m = the number of subjects in group A; n = the number of subjects in groups B, C, and D together; $\bar{X}$ = the mean intake of folic acid in groups B, C, and D in μg; and X_i = the individual intake of folic acid of subjects in groups B, C and D in μg.

We also calculated relative bioavailability from changes in serum folate. In an approach similar to that for the labeled folate data, we plotted the individual changes in serum folate of subjects in groups B through D against the intake of supplemental folic acid and fitted a linear regression line through the data points.

RESULTS

One person in group A became ill before the start of the study, and one each in group C and D dropped out for personal reasons within the first 2 wk. Analyses are based on the 72 subjects who completed the study.

The diaries of the subjects did not show any deviations from the provided diets that may have affected the results. Capsule intake was verified by counting the returned capsules and by checking the subjects' diaries: mean capsule intake was 99%, and the lowest intake was 90%. Characteristics measured at screening did not differ significantly between groups (**Table 2**). The mean intakes of energy protein, fat, carbohydrates, and fiber during the trial did not differ significantly between groups (**Table 3**). According to analysis of duplicate diets, the food folate intake in group A was 369 μg/d, and that in groups B through D was 73 μg/d (Figure 1); thus group A had 296 μg/d more food folate than did groups B through D. Homocysteine concentrations decreased slightly with increasing intakes of folic acid (**Table 4**).

As expected, the percentage of labeled folate in the plasma folate decreased with increasing intakes of folic acid in groups B through D (Figure 3). The calibration line was calculated with the use of the following equation:

$$\text{Percentage of labeled folate in plasma folate} = -0.01616 \times (\mu\text{g of supplemental folic acid/day}) + 13.47 \quad (6)$$

The mean percentage of labeled folate in plasma of group A was 9.7% (Table 4). This value was entered into the equation of the calibration line (Figure 3), and it showed that the extra 296 μg food folate ingested by group A subjects was equivalent to 232 μg folic acid. Therefore, the relative bioavailability of food folate calculated from labeled folate data were—ie, (232/296) × 100—was 78% (95% CI: 48%, 108%).

Serum folate concentrations increased linearly with increasing intakes of folic acid in groups B through D (**Figure 4**). The calibration line was calculated with the use of the following equation:

$$\text{Change in serum folate (nmol/L)} = 0.02369 \times (\mu\text{g of supplemental folic acid/day}) - 0.083 \quad (7)$$

The mean increase in serum folate in group A was 5.9 nmol/L (Table 4). This value was entered into the equation of the calibration line (Figure 4), and it showed that the extra 296 μg food folate ingested by group A subjects was equivalent to 252 μg folic acid. Therefore, the relative bioavailability of food folate

TABLE 2
Characteristics of subjects assessed 4 wk before the start of the 4-wk experimental period[1]

	Food folate group	Folic acid groups		
	A (n = 29)	B (n = 15)	C (n = 14)	D (n = 14)
Women (%)	76	73	64	71
Age (y)	22 (19–41)[2]	22 (19–48)	23 (20–49)	24 (19–49)
BMI (kg/m^2)	23 ± 3[3]	22 ± 4	22 ± 2	22 ± 3
Serum folate (nmol/L)	11.3 ± 3.7	11.6 ± 2.9	11.5 ± 4.1	11.9 ± 3.5
Plasma total homocysteine (μmol/L)	10.1 ± 2.6	9.7 ± 2.3	10.3 ± 3.4	10.3 ± 3.2
Serum vitamin B-12 (pmol/L)	219 ± 69	230 ± 68	228 ± 68	191 ± 55
MTHFR genotype (n)				
677CC	15	8	7	8
677CT	13	7	7	6
677TT	1	0	0	0
Vegetarians (%)	17	7	29	14

[1] The group differences were not significant (ANOVA or chi-square test).
[2] Median; range in parentheses (all such values).
[3] $\bar{x}$ ± SD (all such values).

calculated from serum folate data—ie, (252/296) × 100—was 85% (95% CI: 52%, 118%).

DISCUSSION

In a 4-wk dietary controlled study, we found that the bioavailability of food folate was 78% of that of folic acid according to an isotope method and 85% of that of folic acid according to changes in serum folate. The fact that the 2 methods yielded similar estimates strengthens the confidence in our finding. Because we have no reason to believe that one method is superior to the other, we consider their average of 82% to be our best estimate for the bioavailability of natural folates.

In the current trial, we carefully controlled the intakes of food folate and folic acid. A problem in studies that aim to compare the bioavailability of food folate and that of folic acid has been the fact that the actual intake of food folate may differ from the targeted dose, because the stability of food folates during cooking is poor and the compliance of subjects with the intervention diet can be low (12). These factors will lead to an underestimation

TABLE 3
Intakes of total energy and natural folate from the diets consumed by all 4 treatment groups and the amount of folic acid measured in the capsules taken by groups B–D[1]

	Food folate group	Folic acid groups		
	A (n = 29)	B (n = 15)	C (n = 14)	D (n = 14)
Energy intake (MJ/d)[2]				
Constant food items	7.6 ± 2.1[3]	8.5 ± 1.8	9.3 ± 2.6	9.2 ± 1.9
Varying food items	2.2 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	1.5 ± 0.1
Total diet	9.8 ± 2.2	9.9 ± 1.9	10.8 ± 2.7	10.7 ± 2.0
Food folate intake (μg/d)[4]				
Constant food items	38 (32–47)[5]	36 (28–49)	38 (30–54)	39 (30–54)
Varying food items	331 (259–426)	35 (14–51)	35 (14–51)	35 (14–51)
Total diet	369 (295–463)	71 (50–101)	73 (52–105)	74 (52–105)
Folic acid (μg/d)[6]				
Capsules	ND	92 ± 6	191 ± 11	289 ± 24

[1] ND, not detectable in the placebo capsules. The constant part of the diet was similar for all groups, but amounts varied with the energy requirement of each subject. The varying food items were high in folate for group A and low in folate for groups B–D; amounts were largely the same for all subjects.
[2] Mean energy intake per group was calculated from food tables (19) and subsequently adjusted for the difference between calculated and analyzed amounts of energy in a diet that provided 11 MJ/d (≈2600 kcal). Values for the constant part of the diet include the amount of energy in the free-choice items, calculated from food tables (19). Protein was 13% of energy, fat was 32–33% of energy, and carbohydrate was 52–53% of energy. Mean fiber content of the diet was 3.7 g/MJ (37 g/d) for group A and 3.4 g/MJ (36 g/d) for groups B–D. No significant differences in energy intake were found between groups by ANOVA.
[3] $\bar{x}$ ± SD (all such values).
[4] The amount of folate was analyzed in both the constant and varying food items 1 time/d from Monday through Sunday in all groups. Thus, each value is the mean of 7 analyses. The amount of food folate varied by day because of the daily variation in menus. All food folate analyses are performed in duplicate diets providing 11 MJ/d (≈2600 kcal/d). The differences between the amounts of folate in the constant part of diets B, C, and D arose because amounts of folate were recalculated to the actual energetic intakes, which differed slightly between the 3 groups.
[5] $\bar{x}$; range in parentheses (all such values).
[6] Folic acid was analyzed in a random sample of 20 capsules/dose.

TABLE 4
Effect of a high-folate diet (group A) and of low-folate diets with increasing amounts of folic acid (groups B–D) on the percentage of labeled folate in plasma folate and on concentrations of serum folate and plasma total homocysteine[1]

	Food folate group	Folic acid groups		
	A (n = 29)	B (n = 15)	C (n = 14)	D (n = 14)
Labeled folate (% of total folate)				
Week 4[2]	9.7 ± 2.0	11.8 ± 3.1	10.9 ± 2.3	8.6 ± 1.7
Serum folate (nmol/L)				
Baseline	12.1 ± 4.0	12.0 ± 3.5	12.8 ± 4.5	12.3 ± 4.9
Week 4	18.0 ± 4.1	15.0 ± 3.4	15.3 ± 3.9	20.0 ± 6.5
Change from baseline to week 4[3]	5.9 ± 3.9	3.0 ± 2.8	2.6 ± 4.1	7.7 ± 3.6
Plasma total homocysteine (μmol/L)				
Change from baseline to week 4[4]	−0.8 ± 1.4	−0.6 ± 1.2	−0.7 ± 1.2	−1.2 ± 1.2

[1] All values are $\bar{x}$ ± SD.
[2] The labeled compound does not occur naturally and was not detected in baseline samples.
[3] Changes in serum folate differed significantly between groups (ANOVA with post hoc Bonferroni tests): group A differed from group C (P = 0.043), group B differed from group D (P = 0.006), and group C differed from group D (P = 0.003).
[4] Changes in homocysteine did not differ significantly between groups (ANOVA).

of the bioavailability of folate from foods. In contrast to food folate, folic acid is very stable, and compliance with taking a pill is likely to be higher than compliance with a prescribed diet. We strictly controlled the intake of both food folate and folic acid in our subjects: the subjects came to our laboratory daily during weekdays to consume a hot lunch plus supplements, and thus a large part of the daily intake of folate and folic acid was supervised. Furthermore, we provided ≈90% of all the foods and beverages for consumption off-site and asked subjects to note in a diary whether they deviated from these supplies. In addition, we based our estimates of bioavailability on the analyzed amounts of folic acid and folate in the capsules and in the duplicates of the diets as actually eaten.

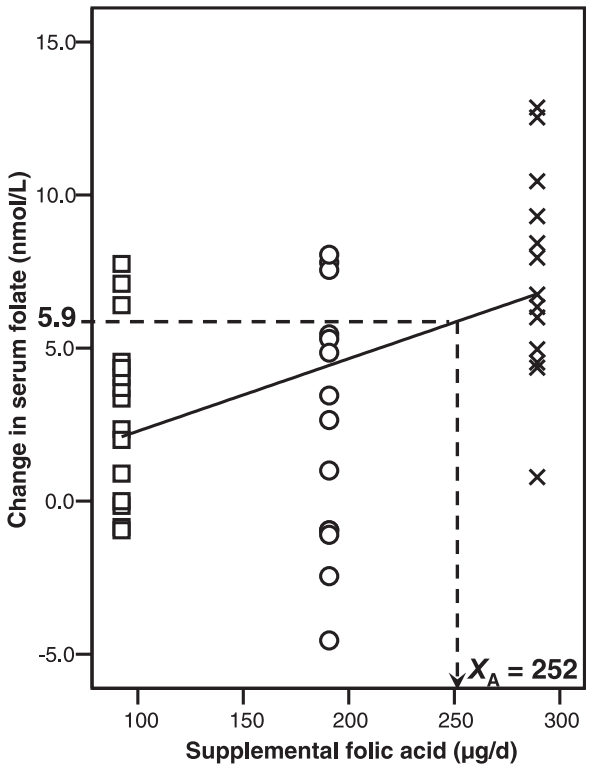

FIGURE 4. Change in serum folate from baseline to week 4 in the folic acid groups (B through D). Each symbol represents 1 subject; □, group B; ○, group C; ×, group D; —, the linear regression line through the individual data points of groups B, C, and D. The mean change in serum folate in group A was 5.9 nmol/L. This was projected on the regression line, and it corresponded to an estimated intake of folic acid of 252 μg (broken lines). The R^2 of the regression line was 0.22, and the slope was 0.02369 (95% CI: 0.00948, 0.03789).

We measured 2 markers for folate bioavailability: the percentage of labeled folate in the plasma folate and changes in serum folate. We expected that the most precise estimate for bioavailability would result from the labeled folate data. Because the labeled compound does not occur naturally, the percentage of labeled folate in plasma had to be evaluated only after the intervention: this approach eliminated the extra error in subtracting 2 measurements from each other. However, the precision was similar for labeled folate and serum folate: the CIs surrounding both estimates for bioavailability had a similar width. For serum folate, the estimate for bioavailability and the width of the CI did not change when we based our calculations only on the measurements in samples from day 1 and day 29 (data not shown). Therefore, in this experimental set-up, the expensive stable-isotope method yielded no advantage over serum folate measurements. However, the fact that the 2 methods yielded similar numbers emphasizes the internal consistency of the study and strengthens the confidence in our outcome. We used folic acid labeled with 11 ^{13}C atoms in the current intervention, because we had it in stock from earlier experiments. Using cheaper forms of folic acid would not have made the intervention much cheaper 3because the LC-MS/MS analysis is much more expensive than is the use of the labeled compound itself.

The participants ingested the folic acid capsules just before their hot lunch, which may have affected the serum folate responses in groups B through D and, hence, the estimate for relative bioavailability. Pfeiffer et al (24)found that the serum folate response to a folic acid supplement taken together with a light meal was 15% lower than the folate response to a folic acid supplement taken on an empty stomach. The difference was not significant, but if it were valid, then the calibration line as plotted

in Figure 4 would have been steeper and the bioavailability estimate for food folates would have been lower if subjects had ingested the capsules on an empty stomach. We plotted a linear calibration line in Figure 2. However, a lack of fit test showed that the linear model was not the best model with which to describe our data: the addition of a second-order term to the model improved the R^2 and the model's fit to the data (data not shown). Estimation of bioavailability from this second-order regression line produced a slightly higher value for bioavailability of food folate (89%) than did the linear model (85%). This finding reinforces our conclusion that the bioavailability of folate is higher than previously reported. We decided to use a linear regression model to fit our data, because other, larger studies (25, 26) found that the relation between the intake of folic acid and the change in serum folate is linear over the range of intakes that we used.

HPLC analysis of folate in food generally results in lower values than does microbiological analysis (1, 27), and therefore the bioavailability figures for food folate generally will be higher when based on HPLC analysis. Previous bioavailability studies (13–15) analyzed food folate with microbiological assays. Therefore, we also analyzed our food samples microbiologically (28), and that approach yielded a food folate intake in group A of 474 μg/d and 136 μg/d in groups B through D; the food folate in group A was therefore 338 μg/d greater than that in groups B through D. This finding resulted in a bioavailability of (232/338) $\times$ 100 = 68% (95% CI: 42%, 95%) according to labeled folate data or (252/338) $\times$ 100 = 75% (95% CI: 45%, 103%) according to changes in serum folate. Thus, the estimate for bioavailability was $\approx$10% lower with microbiologically derived figures than when based on HPLC analysis.

Our findings disagree with a statement by Sauberlich et al (13), who said that "when compared with synthetic PGA (pteroyl glutamic acid), dietary folates appeared to be no more than 50% available." Unfortunately, those authors did not indicate how this finding was obtained. In our opinion, the less-than-suitable design and small size of this study—with 3–4 subjects per treatment group—and the absence of a folic acid group make it impossible to compare the bioavailability of natural folates with that of folic acid from these data. Our results also differ from those of Hannon-Fletcher et al (15). In that study, subjects consumed a folate-depleted carrier meal to which food folates extracted from spinach or from yeast were added or they consumed the carrier meal together with folic acid in a tablet; meals were provided daily for 30 d. On the basis of changes in serum folate, the bioavailability of spinach folate and yeast folate was 36% and 62%, respectively, of that of folic acid. However, the addition of these 2 sources of folate to meals is not representative for folates from whole diets: in whole diets, folates originate from various sources and matrixes of foods. Furthermore food intake was not controlled. These factors limit the usefulness of the bioavailability findings in that study. The findings in the current study are, however, in excellent agreement with those of Brouwer et al (14), who conducted a 4-wk, highly controlled intervention study and found that bioavailability of food folate from fruit and vegetables was 78% of that of folic acid, according to changes in plasma folate.

We composed a diet high in folate by selecting fruit and vegetables rich in folate, by providing liver paste as sandwich filling, and by adding egg yolk to sauces and salad dressings. Fruit and vegetables were the main source, providing 73% of the additional folate in group A (Figure 2). Besides fruit and vegetables, unfortified bread and cereals are important sources of folate in the general population (1, 2). However, it was not feasible to include these foods in the varying food items of group A, because we could not replace them with similar products low in folate for groups B through D. Bioavailability of folates from food is influenced by a number of food-related factors, eg, the species of folate in the food, the number of glutamate residues attached to the folate molecule, and the food matrix (29). Because cereals and fruit and vegetables are all plant foods, similarities in factors that affect bioavailability for these food groups are likely to exist. Therefore, we speculate that our findings will also be applicable for diets in which cereals are an important folate source, but this possibility requires confirmation.

Our findings and those of Brouwer et al (14) indicate that bioavailability of food folates is higher than generally assumed—namely, for folate as measured by HPLC, it is 80% of that of folic acid from capsules taken with a meal. Subjects in group A consumed a total of 369 μg folate/d from foods, which is equivalent to 295 μg folic acid. Their diet did include 25 g liver paste/d and 400 mL orange juice/d, which may not be to everyone's liking. Nevertheless, we think it is quite feasible for a healthy varied diet to provide the equivalent of 300 μg folic acid/d. The value of 50% bioavailability for folates from food, as used in the construction of recommended daily allowances (5), underestimates the bioavailability of food folates. The use of this low number could lead to skepticism about the importance of a healthy diet as a source of folate: our data suggest that such skepticism may be unwarranted. Our data show that consumption of a diet rich in folate can improve the folate status of a population more efficiently than is generally assumed.

The late Clive West was one of the initiators of this study and contributed greatly to its design. We thank the subjects for their enthusiastic participation in the trial; Alida Melse-Boonstra and Jan Burema for their advice on design and statistical analysis; Lena Leder and the dietitians for their assistance during the trial; and the Pharmacy of the Gelderse Vallei Hospital for manufacturing capsules. This study was conducted under ClinicalTrials.gov Identifier no. NCT00130585.

All authors were involved in the design of the study; RMW wrote the manuscript, and IAB, ES, MK, and PV reviewed and edited the manuscript; ES designed the diets; and IAB, ES, and RMW contributed to the performance of the study. None of the authors had a personal or financial conflict of interest.

REFERENCES

1. Konings EJ, Roomans HH, Dorant E, Goldbohm RA, Saris WH, van den Brandt PA. Folate intake of the Dutch population according to newly established liquid chromatography data for foods. Am J Clin Nutr 2001; 73:765–76.
2. National Food Survey Committee. National Food Survey 2000: annual report on food expenditure, consumption and nutrient intakes. London, United Kingdom: Ministry of Agriculture Fisheries and Food, 2000;161.
3. Tucker KL, Selhub J, Rosenberg IH, Wilson PWF. Dietary intake pattern relates to plasma folate and homocysteine concentrations in the Framingham Heart Study. J Nutr 1996;126:3025–31.
4. Brody T, Shane B. Folic acid. In: Rucker R, Suttie J, McCormick D, eds. Handbook of vitamins. New York, NY: Academic Press Marcel Dekker, 2001:427–63.
5. Institute of Medicine. Folate. Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline. Washington, DC: National Academy Press, 2000: 196–305.
6. Clarke R. Homocysteine-lowering trials for prevention of heart disease and stroke. Semin Vasc Med 2005;5:215–22.

7. Lucock M. Is folic acid the ultimate functional food component for disease prevention? BMJ 2004;328:211–4.
8. Selhub J, Bagley LC, Miller J, Rosenberg IH. B vitamins, homocysteine, and neurocognitive function in the elderly. Am J Clin Nutr 2000; 71(suppl):614S–20S.
9. Seshadri S, Beiser A, Selhub J, et al. Plasma homocysteine as a risk factor for dementia and Alzheimer's disease. N Engl J Med 2002;346:476–83.
10. Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. MRC Vitamin Study Research Group. Lancet 1991; 338:131–7.
11. Gregory JF III. The bioavailability of folate. In: Bailey LB, ed. Folate in health and disease. Gainesville, FL: Marcel Dekker, 1995:195–236.
12. McNulty H, Pentieva K. Folate bioavailability. Proc Nutr Soc 2004;63: 529–36.
13. 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.
14. 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.
15. 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.
16. Ubbink JB, Hayward Vermaak WJ, Bissbort S. Rapid high-performance liquid chromatographic assay for total homocysteine levels in human serum. J Chromatogr 1991;565:441–6.
17. Frosst P, Blom HJ, Milos R, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995;10:111–3.
18. Hiemstra G, de Roos N, de Vries J, et al. Anamnesemethode: snel en doelmatig? (Method of anamnesis: quick and efficient?) Ned Ttijdschr Dieti 2005;60:88–96 (in Dutch).
19. Stichting-NEVO. Dutch Food Composition Table 2001.The Hague, Netherlands: Voedingscentrum, 2001.
20. Konings EJ. A validated liquid chromatographic method for determining folates in vegetables, milk powder, liver, and flour. J AOAC Int 1999; 82:119–27.
21. Melse-Boonstra A, Verhoef P, West CE, et al. A dual-isotope-labeling method of studying the bioavailability of hexaglutamyl folic acid relative to monoglutamyl folic acid in humans by using multiple orally administered low doses. Am J Clin Nutr 2006;84:1128–33.
22. Herbert V, Larrabee A, Buchanan J. Studies on the identification of a folate compound of human serum. J Clin Invest 1962;41:1134–8.
23. Neter J, Kutner MH, Nachtsheim CJ, Wasserman W. Applied linear statistical models. 4th ed. New York, NY: McGraw-Hill, 1996:152–75.
24. 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.
25. van Oort FV, Melse-Boonstra A, Brouwer IA, et al. Folic acid and reduction of plasma homocysteine concentrations in older adults: a dose-response study. Am J Clin Nutr 2003;77:1318–23.
26. Wald DS, Bishop L, Wald NJ, et al. Randomized trial of folic acid supplementation and serum homocysteine levels. Arch Intern Med 2001; 161:695–700.
27. Arcot J, Shrestha A. Folate: methods of analysis. Trends Food Sci Technol 2005;16:253–66.
28. DeVries JW, Rader JI, Keagy PM, et al. Microbiological assay-trienzyme procedure for total folates in cereals and cereal foods: collaborative study. J AOAC Int 2005;88:5–15.
29. Brouwer IA, Dusseldorp MV, West CE, Steegers-Theunissen RPM. Bioavailability and bioefficacy of folate and folic acid in man. Nutr Res Rev 2001;14:267–94.