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Citation for published version (APA):

Document status and date:
Published: 01/01/2002

DOI:
10.1079/BJN2002613

Document Version:
Publisher's PDF, also known as Version of record

Please check the document version of this publication:

• A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
• The final author version and the galley proof are versions of the publication after peer review.
• The final published version features the final layout of the paper including the volume, issue and page numbers.

Link to publication

Download date: 19 Apr. 2021

Intestinal absorption of different types of folate in healthy subjects with an ileostomy

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Our knowledge on the absorption of folate is incomplete. The deconjugation process as a possible limiting factor in the absorption of folates was investigated. The study also attempted to validate the use of the area under the serum response curve (AUC) from food compared with folic acid as a proxy variable for food folate bioavailability. Folate absorption was determined in healthy ileostomy volunteers (*n* 11) using a single-dose short-term protocol. In a randomised crossover design, volunteers received spinach meals and a supplement. Based on analysis of test meals and ileostomy effluents, there was no difference in folate absorption between spinach with a mono-:polyglutamate ratio 40:60 and the same spinach with a 100:0 ratio. The absolute absorption of spinach folate (79 %) calculated from the difference between folate intake and folate content of ileostomy effluents was approximately equal to the relative absorption (81 %) calculated from the AUC after consumption of spinach meals in relation to the AUC after consumption of the folic acid supplement. We conclude that the deconjugation process is not a limiting factor in the absorption of spinach folates. Comparison of AUC of food folate *v.* folic acid in a short-term protocol may be suitable for assessing food folate bioavailability.

Folates: Bioavailability: Absorption: Area under the curve: Spinach

In recent years, attention has been focused on the role of folic acid–folates in neural tube defects, cardiovascular disease via homocysteine, and colorectal cancer, and has led to fortification of certain food groups, such as cereals with folic acid. Folates are a large family of polyglutamates (usually five to seven glutamyl residues) of pteroic acid and related analogues, which qualitatively exhibit the biological activity of folic acid. Folate polyglutamates need to be cleaved into folate monoglutamates by pteroylpolyglutamate hydrolase (conjugase), present in the intestinal brush border, before they can be absorbed in the intestine. The average bioavailability of folates from foods has been estimated at about 50 % in a long-term, 92 d controlled metabolic study by Sauberlich *et al.* (1987). So far, this is the only study on food folate bioavailability from mixed diets. In spite of many studies, our understanding of possible causes of this poor intestinal absorption of food folate compared with folic acid remains incomplete.

Reisenauer & Halsted (1987) calculated that the human brush border might contain a large amount of conjugase, which would be able to deconjugate polyglutamates even when consumed in quantities far above the recommended daily intake. Certain food components, such as organic acids in orange juice (Wei *et al.* 1996; Wei & Gregory, 1998) or dietary fibre (Bailey *et al.* 1988; Keagy *et al.* 1988) might influence this deconjugation process. Results of *in vitro* experiments have indicated that, for instance, spinach components inhibit porcine conjugase (Bhandari & Gregory, 1990) and human conjugase (Bhandari &

Gregory, 1990; Wei & Hou, 2000). There might be significant differences in the absorption of different folate vitamers (Gregory, 1995). Although many studies have investigated the bioavailability of endogenous folate in selected foods using human subjects or animal bioassay procedures (Tamura & Stokstad, 1973; Babu & Srikantia, 1976; Clifford *et al.* 1990), most methods applied to determine folate bioavailability have procedural or conceptual limitations (Gregory, 1995). Results of *in vitro* or animal studies provide little information on the applicability to the human *in vivo* situation. Labelled folates have often been used to determine folate bioavailability (Gregory *et al.* 1992; Wei *et al.* 1996; Rogers *et al.* 1997). The question remains, however, whether these isolated folates are absorbed in the same way as folates in a food matrix. So far, most methods have yielded information regarding the absorption of total folate relative to folic acid, rather than the absolute absorption of total folate or the various folate vitamers. The bioavailability of folate from dietary sources can be determined with short-term protocols based on measurement of the change in plasma or serum folate as applied in the present study. A limitation of such methods is their relative insensitivity, which makes the protocols suitable only for foods that are relatively high in folate ($>300\,\mu g$ per dose). It is important that a sufficient number of blood samples during an acceptable period of time should be taken to allow reliable estimation of the area under the curve (AUC) (Gregory, 2001). Both of these limitations have to be taken into account. The use of isotopic labelling in studies of folate bioavailability has the main advantages of specificity and clarity of interpretation. However, this approach gives no information about the bioavailability of naturally occurring folates.

Ileostomy subjects with an intact small intestine and without any recent (<1 year) remission of the original disease are pre-eminently suitable for a study of absolute folate absorption, because absorption of folates takes place exclusively in the small intestine (Gregory, 1995). Folate absorption cannot be estimated via normal faecal excretion because the presence of micro-organisms in the colon will influence the amount of folate in faeces.

The present study was designed to determine folate absorption in healthy ileostomy volunteers, using spinach as a food matrix in a short-term protocol. In a pilot study to design the present protocol, we were able to show that folates in cooked spinach were mainly present as polyglutamates (approximately 85%), and were mainly equally distributed between two folate vitamers: 5-methyltetrahydrofolate ($5\text{-}CH_3\text{-}H_4$folate) and 10-formyldihydrofolate ($10\text{-}HCO\text{-}H_2$folate). Based on a previous study on the folate concentration of different food products it was decided to select spinach since it has a high total folate concentration (approximately $1000\,\mu g/kg$), it is an excellent source of the various folate vitamers and a suitable vehicle to study the absorption of the various folate vitamers from food compared with folic acid (Konings *et al.* 2001). Thus, the purpose of the present study was to address the issue of deconjugation of folate vitamers and to examine the absorption of the folate vitamers. Furthermore, it provided an opportunity to validate the use of the serum folate response AUC from food in a single-dose short-term protocol as a suitable proxy variable for food folate bioavailability compared with supplemental folic acid.

Subjects and methods

Subjects

Twelve ileostomy volunteers were recruited (six men and six women). The mean age of the men was 56 (range 36–69) years and that of the women 48 (range: 37–58) years. The mean BMI of the men was 26·1 (range 23·0–27·8) kg/m^2 and that of the women 26·9 (range 20·9–34·7) kg/m^2. Reasons for performing an ileostomy in subjects varied from colitis ulcerosa (n 7) to polyposis (n 1), Crohn's disease (n 3) or a neurological defect of the pelvic floor (n 1). In all subjects, no more than 20 cm of terminal ileum had been removed and led out onto the anterior abdominal wall as a fistula. All subjects had had their well-functioning ileostomies for 5 years or longer and were not hospitalised at the time of the study. Subjects with Crohn's disease had been in remission for at least 1 year. No medication that could affect the folate absorption process, such as anti-inflammatory drugs, was used. There were no indications of malabsorption. Plasma folate levels and erythrocyte folate concentrations among ileostomists have been found to be normal (Nilsson *et al.* 1979; Kennedy *et al.* 1982). Mean serum folate concentration was 18·3 (SE 5·5) nmol/l. None of the subjects in the present study had serum folate concentrations $<6·8$ nmol/l. Individuals with serum concentrations below this level are considered to be folate deficient (Sauberlich, 1995), which might affect the amount of folate absorbed (Pietrzik *et al.* 1990). One man and one woman smoked. The suitability of each of the volunteers was tested by having them complete a medical questionnaire and through assessment by a gastroenterologist. The protocol was approved by the Maastricht University Hospital Ethical Committee and was fully explained to the participants, who all gave their written informed consent.

Study design

In a randomised crossover design with a wash-out period of 2 weeks, volunteers received a standard meal followed 1 h later by a test meal. The standard meal was given in portions every 2 h during the test days to prevent increasing blood folate levels due to disturbed enterohepatic circulation as described by Pietrzik *et al.* (1990) and Cahill *et al.* (1998). Fasting seems to interrupt the enterohepatic circulation by blocking a potential elimination route for plasma folate. Administering an energetically adequate but folate-free meal during the trial kept this and other potential systems for folate excretion intact. Volunteers came to the laboratory early in the morning after an overnight fast. The standard meal, administered 1 h before the test meal and every 2 h up to 10 h thereafter, consisted of a mixture of formula diet, soft curd cheese and water, containing 648 kJ (155 kcal; 15% energy as protein, 58% as carbohydrate, 27% as fat), and 35 g milk chocolate as a

source of energy (5 % energy as protein, 37 % as carbohydrate, 58 % as fat), resulting in a total intake of 8·63 MJ (2064 kcal) during the experimental day. The standard meals provided 31 μg folates. Subjects were instructed not to consume folate-rich foods, such as liver products, marmite, spinach or supplements, according to a food list, during the 8 d preceding each test day. One hour after the consumption of the first standard meal, one of the three test meals was given. In order to keep folate amounts equal, slightly different quantities of spinach were given (test meal A 308 g spinach, test meal B 367 g spinach), to account for folate losses during preparation. Subjects were allowed to drink unlimited amounts of water, tea and coffee (without sugar or milk).

On each test day, blood samples were drawn before the first standard meal (fasting blood sample), before the test meal (pre-dose blood sample) and during 9 h after the consumption of the test meal.

Immediately after the consumption of the test meal, volunteers were asked to empty their ileostomy bags. From that moment on, these bags were emptied every 2 h into a container stored in dry ice (-79°C), during the 9 h after the consumption of the test meal. After these 9 h, the volunteers went home, having been instructed not to eat until the next morning and to collect their ileostomy effluents until that time in a container stored in dry ice. All frozen ileostomy effluents of each subject were combined and homogenised in liquid N_2, yielding the final sample over a 24 h period after the test meal, which was then used for analysis. The samples were stored at -20°C.

To confirm that there was no serum folate enhancement by the standard meal, another three ileostomy volunteers completed a test day and consumed all standard meals, but not the test meal.

Test meals

Fresh spinach was washed and subsequently dried in a kitchen centrifuge, after which it was chopped in a food processor. All spinach came from one batch. For test meal A, chopped spinach was immediately heated in a microwave for 5 min at 900 W and stored at -20°C. The chopping process took 15–20 min. For test meal B, chopped spinach was stored for 24 h in a refrigerator at 4°C. After this storage period the spinach was given the same treatment as test meal A: heating in a microwave for 5 min at 900 W and storage at -20°C. For test meal C, a folic acid supplement was given as a tablet containing 500 μg folic acid. The volunteers were instructed to swallow this tablet with some water.

Before the consumption of test meals A and B, portions were defrosted for 20 min in a microwave and subsequently heated for 5 min at 900 W.

Analytical methods

An HPLC method (Konings, 1999) was used to analyse the standard meal, test meals (spinach), and ileostomy effluent samples. Briefly, folates were extracted by homogenising in a 2-(cyclohexylamino)ethanesulfonic acid (Ches)–Hepes buffer (pH 7·8) containing ascorbic acid and 2-mer-captoethanol as antioxidants. This homogenate was subjected to heat treatment (10 min in a boiling water-bath). A first aliquot was analysed without addition of any enzymes (treatment 1) to estimate the monoglutamate contents of the samples. In a second aliquot, folate concentrations were quantified after addition of rat plasma conjugase (treatment 2) to establish the sum of mono- and polyglutamates. In a third aliquot, folate concentrations were determined after treatment with rat plasma conjugase as well as protease and amylase (treatment 3). The difference between the folate amounts assayed in treatments 1 and 2 represents the folate polyglutamate content. The difference between the folate amounts determined in treatments 2 and 3 reflects matrix-bound folates. After purification by affinity chromatography, folate monoglutamates were determined using an HPLC method with fluorescence and diode array detection (Konings, 1999). All analyses were performed under subdued light. This procedure was used to quantify the levels of the most abundant folate forms naturally present, including tetrahydrofolate, $5\text{-CH}_3\text{-H}_4$folate, 10-HCO-H_2folate, 5-formyltetrahydrofolate, 10-formyl-folic acid and folic acid. Tetrahydrofolate, $5\text{-CH}_3\text{-H}_4$folate, and 10-formyl-folic acid were detected by fluorescence detection. 10-HCO-H_2folate, 5-formyltetrahydrofolate and folic acid were quantified from u.v. chromatograms. Peak identification was based on matching of retention times of external standards with those of the sample peaks, and to confirm identity, u.v. spectra of all sample peaks were matched with u.v. spectra of the external calibration standards.

The folate content of spinach samples (n 2) was also determined by microbiological assay based on the method of Williams (1984) for comparison with HPLC results. The growth response of *Lactobacillus casei*, subspecies rhamnosus to extracted folates at pH 6·2 was followed turbidimetrically and was compared with that of folic acid calibrant solutions with known concentration. The folate contents of spinach meals given to the volunteers were determined in all portions taken from the defrosted and heated spinach just before consumption. Samples were homogenised in liquid N_2 before analysis.

An automated fluoroimmunoassay (Wallac Inc, Akron, OH, USA), a fast routine method, was used to determine folate concentrations in serum samples. This folate assay is a solid phase fluoroimmunoassay based on the competitive reaction between Eu-labelled folic acid, and sample folates for a limited number of binding sites on folate binding protein (FBP). Anti-FBP antibody is first incubated to the anti-mouse immunoglobulin G-coated microtitration plate. In a second incubation, Eu-labelled folate tracer, folate sample or standard, and FBP are added. The FBP is captured by the anti-FBP antibody, while labelled and unlabelled folate compete for binding sites on the FBP in a competitive manner. Eu ions are dissociated from the labelled folate forming highly fluorescent chelates. The fluorescence is inversely proportional to the quantity of folate in the sample.

Analytical quality control was implemented by use of certified reference materials for folates supplied by the Institute for Reference Materials and Measurement in Geel, Belgium. A vegetable mix (CRM 485) was used as

control in each HPLC series of sample analysis. For the fluoroimmunoassay procedure, internal and external (certified) plasma control samples were used in each series of sample analysis.

Statistical evaluation

To verify any presence of folates in ileostomy effluents not originating from the test meals, folates other than supplemental folic acid were determined in ileostomy fluids after the consumption of test meal C and used as an individual's background level. Absorption of spinach folates was determined on the basis of residual folates in ileostomy fluids, after correcting for the total weight of the ileostomy effluents collected and the folate background levels. In fact, the 'apparent absorption' is calculated because of no corrections for physiological losses in the present study. Absorption (%) = ((intake + individual background level − excretion in ileostomy bags)/ intake) × 100 %. Absorption of folates from test meals A and B was compared by means of a paired Student's *t* test at a significance level of 0·05. Folate concentrations of extracts treated with amylase and protease (treatment 3) in the analysis were compared with folate concentrations of extracts that had not been treated with these enzymes (treatment 2) by means of a paired Student's *t* test at a significance level of 0·05.

The serum response AUC was determined using 'CurveExpert' version 1.3 (D.G. Hyams, Starkville, MS, USA). Curves were integrated from each individual pre-dose serum folate level (at 1 h) to the serum folate level at 10 h. Individual pre-dose plasma folate concentrations of each test day were used as baseline for the calculation of AUC. When plasma folate concentration fell below the individual pre-dose level, the positive AUC was used. This means that when the folate concentration falls below this level, the increment was taken as zero, rather than negative.

AUC of different test meals were compared by means of a Student's *t* test at a significance level of 0·05.

Pre-dose serum folate levels of all subjects on three different test days were compared by means of ANOVA at a significance level of 0·05. Pre-dose serum folate levels and final serum folate levels 9 h after the consumption of the test meals were compared by means of a paired Student's *t* test at a significance level of 0·05. Results are expressed as mean values and standard deviations. For statistical analysis, the software SPSS for Windows (version 7.5; SPSS, Chicago, IL, USA) was used.

One of the twelve volunteers was unable to consume the complete portion of test meals A and B and was excluded from the statistical analysis.

Results

Spinach

Results of the spinach folate analysis as well as the folate amounts given to the subjects are shown in Table 1. Spinach prepared according to normal household practice (whole-leaf spinach) contained mainly polyglutamates.

When spinach was chopped (test meal A), polyglutamates were partly converted into monoglutamates by endogenous conjugase within the 20 min before the critical denaturation temperature was reached. When chopped spinach was stored for 24 h (test meal B), all polyglutamates were converted to monoglutamates. The folate vitamers in spinach were mainly $5\text{-CH}_3\text{-H}_4\text{folate}$ and $10\text{-HCO-H}_2\text{folate}$, in a proportion of approximately 1:1. After 24 h storage, a large amount of $10\text{-HCO-H}_2\text{folate}$ was oxidised to 10-formyl-folic acid.

Analyses by HPLC and microbiology yielded comparable total folate results for spinach. Treatment of spinach extracts with and without amylase–protease yielded the same results for total folate amounts.

Absorption of folates from small intestine content

Based on analysis of folate concentrations in the ileostomy effluents and spinach consumed, folate amounts absorbed from test meals A, B and C were 316, 309 and 454 μg respectively. These amounts correspond to 73 (SD 21) %, 85 (SD 18) % and 91 (SD 5) % of folate intake respectively. The higher absorption of folates from test meal B compared with that from test meal A was not statistically significant. The mean folate absorption from test meals A and B (79 %) was significantly lower ($P=0\cdot02$) than the absorption from test meal C (91 %).

The absorption of the two most commonly occurring monoglutamates from test meal A ($5\text{-CH}_3\text{-H}_4\text{folate}$ and $10\text{-HCO-H}_2\text{folate}$) was 85 (SD 28) %. The absorption of the two most commonly occurring polyglutamates from test meal A ($5\text{-CH}_3\text{-H}_4\text{folate}$ and $10\text{-HCO-H}_2\text{folate}$) was 72 (SD 46) %. There was no significant difference in the absorption of these mono- and polyglutamates from test meal A. The mean absorptions of all mono- and polyglutamates from test meal A were 81 and 69 % respectively (NS). The mean absorptions of $5\text{-CH}_3\text{-H}_4\text{folate}$ monoglutamate and $10\text{-HCO-H}_2\text{folate}$ monoglutamate from test meal B were 89 (SD 25) % and 88 (SD 17) % respectively (NS). The absorption of 10-formyl-folic acid from test meal B was 74 (SD 23) %, which was not significantly different from the mean absorption of $5\text{-CH}_3\text{-H}_4\text{folate}$ and $10\text{-HCO-H}_2\text{folate}$ monoglutamate from this spinach meal.

Fig. 1 compares total folate amounts in ileostomy fluids collected after consumption of test meals A and B with folate amounts in ileostomy fluid after the consumption of test meal C, excluding non-absorbed folic acid from the supplement. The contribution of ingested folates from the standard meal during the entire day was relatively small (31 μg) compared with ingested spinach folates (362–436 μg). A relatively high amount of folates other than folic acid was found as a background level in ileostomy fluids from test meal C: 197 (SD 175) μg/24 h. The ileal output of the subjects who consumed only the standard meal was 52 (SD 32) μg/24 h (*n* 3). The background level of these subjects after consumption of test meal C was 51 (SD 38) μg/24 h. Treatment of sample extracts with amylase and protease in the analysis of ileostomy effluents yielded 10 % higher folate concentrations ($P<0\cdot0001$) than those found in extracts not treated with

Table 1. Test meals characteristics*

(Mean values and standard deviations)

	Spinach cooked according to normal household practice	Spinach of test meal A		Spinach of test meal B		Test meal C
		Mean	SD	Mean	SD	
Total folates according to HPLC analysis (μg/kg)†	1190	1480	10	1290	60	
Total folates according to microbiological analysis (μg/kg)†	1460	1480	30	1410	40	
Mono-:polyglutamate ratio in spinach	10:90	40:60		100:0		
(Spinach) folate amounts given to subjects (μg)						
Total		436‡	42	362§	23	500‖
Monoglutamates						
5-CH$_3$-H$_4$folate		99	2	111	12	
10-HCO-H$_2$folate		58	14	105	30	
10-HCO-folic acid		12	2	106	11	
5-CHO-H$_4$folate		5	8	40	4	
Polyglutamates						
5-CH$_3$-H$_4$folate		72	8	0		
10-HCO-H$_2$folate		150	39	0		
10-HCO-folic acid		13	4	0		
5-CHO-H$_4$folate		27	15	0		

5-CH$_3$-H$_4$folate, 5-methyltetrahydrofolate; 10-HCO-H$_2$folate, 10-formyldihydrofolate; 10-HCO-folic acid, 10-formyl-folic acid; 5-CHO-H$_4$folate, 5-formyltetrahydro-folate.

* All folate amounts in the table are expressed as folic acid. For details of test meals and analytical procedures, see p. 237.

† This spinach was not given to volunteers, but used for comparison between HPLC and microbiological analysis.

‡ Fresh spinach was washed, dried and chopped, immediately heated in a microwave for 5 min at 900 W and stored at −20°C. Before consumption, portions were defrosted and subsequently heated for 5 min at 900 W.

§ Fresh spinach was washed, dried, chopped and stored for 24 h in a refrigerator at 4°C. After storage the spinach was heated in a microwave for 5 min at 900 W and kept at −20°C. Before consumption, portions were defrosted and subsequently heated for 5 min at 900 W.

‖ Folic acid supplement was given as a tablet.

these enzymes. No deconjugation of folic acid triglutamate was found after incubation with ileostomy fluids.

Serum folate concentrations

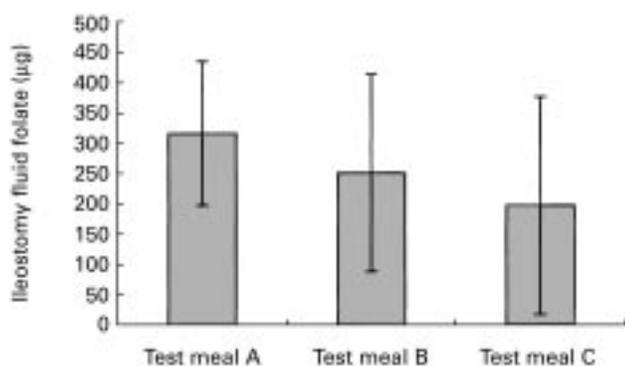Serum folate responses to test meals are shown in Fig. 2.

Fig. 1. Folate in ileostomy fluids collected over a period of 24 h after consumption of various test meals. Test meal A is the standard meal plus 436 μg spinach folate with mono-:polyglutamate ratio 40:60. Test meal B is the standard meal plus 362 μg spinach folate with mono-:polyglutamate ratio 100:0. Test meal C is the standard meal plus a folic acid supplement (500 μg) given as a tablet. The standard meal consisted of a mixture of formula diet, soft curd cheese, water and chocolate. For test meal C, non-absorbed folic acid (46 μg) was not included. Therefore, test meal C represents the mean individual background level. For details of subjects, test meals and procedures, see pp. 236–237. Values are means for eleven subjects with standard deviations shown by vertical bars.

The maximum serum folate concentrations after consumption of test meal A, B and C were significantly higher than pre-dose serum folate concentrations ($P<0.01$). Maximum serum folate concentrations were reached 45 min after the consumption of test meals A and B. Mean maximum folate concentrations were 30·7 (SD 24·3) nmol/l for test meal A and 30·7 (SD 23·1) nmol/l for test meal B. The maximum serum concentration for test meal C (25·7 (SD 22·9) nmol/l) was reached 2 h after consumption. There was no difference in pre-dose serum folate levels between the three test days of each subject: day 1 17·7 (SD 18·9), day 2 18·2 (SD 15·8), day 3 18·5 (SD 21·3) nmol/l. In addition, no significant differences were observed between pre-dose serum folate levels and final serum folate levels 9 h after consumption of test meals A, B or C. The three subjects who consumed only the standard meal had final folate levels (7·5 nmol/l) that were significantly lower than the pre-dose concentration (8·5 nmol/l) ($P<0.001$).

Mean AUC for test meals A, B and C were 31 (SD 11), 26 (SD 28) and 40 (SD 15) nmol/l × h respectively, with no significant differences among them. The relative absorption of spinach folate for test meals A and B, calculated from the AUC after consumption of spinach meals in relation to the AUC after consumption of the folic acid supplement (test meal C), was 80·7 and 81·5 % respectively, with a mean value of 81 %.

Serum samples at maximum concentration levels after the intake of supplemental folic acid (test meal C) were
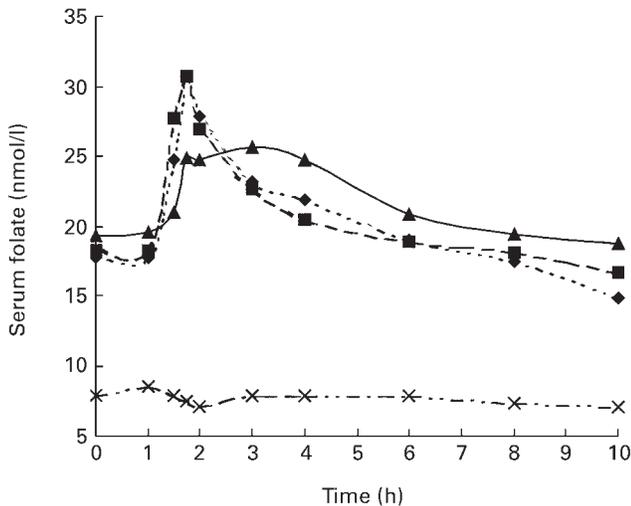
Fig. 2. Serum folate response curves after consumption of different test meals consumed at *t* 1 h. −◆−, Test meal A; −■−, test meal B; −▲−, test meal C, −··×−·, standard meal only. Test meal A is the standard meal plus 436 μg spinach folate with mono-:polygluta-mate ratio 40:60. Test meal B is the standard meal plus 362 μg spinach folate with mono-:polyglutamate ratio 100:0. Test meal C is the standard meal plus a folic acid supplement (500 μg) given as a tablet. The standard meal consisted of a mixture of formula diet, soft curd cheese, water and chocolate. For details of subjects, test meals and procedures, see pp. 236–237. Values are means for eleven subjects.

pooled and analysed by HPLC. The distribution of the vita-mers in serum was as follows: 5-CH$_3$-H$_4$folate 82 %; folic acid 16 %; 10-formyl-folic acid 2 %.

Discussion

Conjugase, which is present in vegetables and deactivated by heat treatment, is able to deconjugate folate polygluta-mates and is activated after disruption of the matrix by chopping and homogenisation (Leichter *et al.* 1979). This principle was used in the present study for preparation of the different spinach test meals. Subjects received a portion of spinach containing natural food folate polyglutamates and a portion of the same spinach containing only natural food folate monoglutamates. Absorption differences between mono- and polyglutamates, if any, would thus be revealed without the confounding effects of food matrix, additions or synthetic folates.

As expected, tetrahydrofolate was not present in cooked spinach because of its thermal lability. 10-Formyltetrahy-drofolate cannot be determined by the HPLC method, but, if present in fresh spinach, would completely be oxi-dised to 10-HCO-H$_2$folate and/or 10-formyl-folic acid during preparation. This is supported by the finding that similar amounts of total folate were determined in cooked spinach by microbiological and by HPLC analysis. This strongly suggests that no other folate vitamers were present in the spinach given to the volunteers.

Bailey *et al.* (1988) stated that an intake of 750 μg was necessary to provide consistent response curves. However, other authors showed a distinct response curve at an intake as low as 150 μg folic acid (Pietrzik *et al.* 1990; Prinz-

Langenohl *et al.* 1999). Therefore, the present study was designed with an intake of 362–500 μg folate or folic acid, in order to provide an amount that was a compromise between the disparate amounts used in the previous studies. Although Kelly *et al.* (1997) showed that unaltered folic acid appeared in the blood circulation above a threshold intake of 266 μg folic acid, it was expected that unaltered folic acid would contribute to the total serum folate response in our present study.

The folate absorption from spinach was high. Although the absorption from the folic acid supplement was signifi-cantly higher, the difference was only 10 %. The similarity in the absorption of poly- and monoglutamates from test meals A and B suggests that food components present in spinach do not influence the deconjugation process. *In vitro* experiments have shown inhibition of conjugase activity by spinach components (Bhandari & Gregory, 1990; Wei & Hou, 2000). If these results can be extrap-olated to the *in vivo* situation, this inhibition apparently has no effect on the absorption of folates. Many studies have determined the relative absorption of polyglutamates *v.* corresponding monoglutamates, using isotopically labelled or non-labelled synthetic folates, and found it to range from 50–100 % (Gregory, 1995). The results of the present study showed no differences between the absorp-tion of mono- and polyglutamates from spinach.

The present study indicated a high degree of absorption of 10-formyl-folic acid and 10-HCO-H$_2$folate (>90 %) compared with the absorption of folic acid. Each of these folates contributes to overall folate activity (Spies *et al.* 1948; Gregory *et al.* 1984; Baggott *et al.* 1998; Baggott & Johanning, 1999).

Collection of ileostomy effluents for 24 h after consump-tion of test meals was sufficient because total transit time of stomach and small intestine is approximately 7–9 h. A relatively large amount of folates was determined in ile-ostomy effluents collected on the test days when subjects received the folic acid supplement only as a folate source. This synthetic folic acid is not a common vitamer in man and showed, as well as other vitamers (results not shown), good stability in gastric and duodenal juices. Therefore, the folates present in these ileostomy effluents can be considered as individual background levels. Folates might have ended up in the ileostomy effluents via intest-inal enterocytes. Enterocytes are formed in crypts at the base of the small intestinal villus and migrate along the crypt toward the villus tip, from where they are sloughed into the lumen after a period of 4–7 d. Cell contents might be secreted within the complete enterocyte into the ileostomy effluent (Magee & Dalley, 1986). The standard deviation of the background ileostomy folate was high because of the high inter-individual variation of these amounts. However, the intra-individual variation was small. This justified the determination and correction for this amount individually. The mean folate monoglutamate: folate polyglutamate ratio of the background levels was 11:89. Although no folate polyglutamates were given with test meals C and B, a paired *t* test showed that after collection of ileostomy effluent for 24 h, the excretion of polyglutamates after test meal C was not significantly different from that after test meal B within individuals

($P=0.97$). Folate polyglutamates may have ended up in ileostomy effluents via intestinal enterocytes (Halsted, 1991).

Ileostomy fluids have a unique microbial ecology that is different from the normal microflora of the small and large intestines (Hill, 1976). While aerobes are present in equal numbers as in faeces, anaerobic counts are lower (5 logs) in ileostomy effluent than in faeces (Hill, 1976). Herbert et al. (1984) determined 200 (range 57–577) µg folates (n 6) in 24 h human stools. They hypothesised that it was enteric bacteria that produced these amounts. It is more likely that the folate originated from the enterocytes, as the same mean value and range of folate amounts were determined in our 24 h ileostomy effluents. This is corroborated by the fact that the numbers of anaerobic bacteria, which are major folate producers, are considerably lower in ileostomy effluents than in faeces, especially in view of the frequent exchange of bags and storage on dry ice in our protocol.

The significance of conjugase activity in human intestinal fluid is unclear (Gregory, 1995). In our present study, incubation of ileostomy effluents with folic acid triglutamate revealed that there was no conjugase activity in digestive fluids.

The difference between pre-dose serum folate levels of subjects who had only the standard meals and subjects who consumed the test meals was based on coincidence. One person who consumed the test meals used a vitamin B supplement until 8 d preceding each test day and had a serum pre-dose level of 73 nmol folate/l. This influenced the mean serum pre-dose level of all subjects.

The maximum serum folate concentration was reached 45 min after the consumption of test meals A and B. Frequent sampling at about this time is of importance to determine an exact AUC. In a bioavailability study by Prinz-Langenohl et al. (1999) only two blood samples were taken in the first 2 h after spinach consumption. This would definitely have underestimated the AUC. The course of the appearance of folic acid in serum is apparently very different from that of spinach folates, probably because folic acid first has to be reduced to tetrahydrofolate, followed by methylation, before entering the portal blood (Gregory, 1995). This takes some time, resulting in a broader and flatter curve. In addition, the intake as a whole tablet could have led to delayed absorption. This is not likely, however, since Prinz-Langenohl et al. (1999) found a comparable maximum in the serum response curve when folic acid was administered in the form of a solution.

Results from the present study revealed that the absolute absorption of spinach folate (79 %), calculated from the difference between folate intake and folate content of ileostomy effluents, was equal to the relative absorption (81 %) calculated from AUC after consumption of spinach meals in relation to AUC after consumption of the folic acid supplement, provided that the amount of ingested folic acid with the supplement was corrected for non-absorbed folic acid, which was 9 %. This means that comparing AUC of food folate v. supplemental folic acid may be suitable for assessing relative food folate bioavailability. Rogers et al. (1997) described a minimal plasma response of an oral dose of [^{13}C$_5$]folic acid relative to the injected dose of [^{2}H$_2$]folic acid. This could be due to incomplete absorption. However, the apparent bioavailability of folic acid in an oral supplement was estimated to be 76–97 % in human subjects (Schuster et al. 1993). According to Rogers et al. (1997), a more probable explanation of the limited short-term appearance of orally administered [^{13}C$_5$]folate in plasma was the avid uptake of newly absorbed folates by the liver and the active enterohepatic circulation. Steinberg (1984) reported that 10–20 % of reduced folates in portal blood was taken up by the liver during the first pass after absorption in rats, whereas hepatic first-pass uptake of unchanged folic acid is much greater. This does not correspond with our present findings, which show a similar absorption calculated from the difference between folate intake and folate content of ileostomy effluents, or calculated from AUC after consumption of spinach meals in relation to AUC after consumption of the folic acid supplement.

In summary, this is the first study showing absorption of various naturally occurring food folates in absolute amounts. The ileostomy model is a suitable method for studying absorption processes. The study revealed higher than expected absorption of folates from spinach, resulting in a significant enhancement of serum folate concentrations. The deconjugation process is not apparently a limiting factor in the absorption process of spinach folates. The serum folate response AUC from food compared with supplemental folic acid in a single-dose short-term protocol is a suitable proxy variable for food folate bioavailability.

References

Babu S & Srikantia SG (1976) Availability of folates from some foods. American Journal of Clinical Nutrition 29, 376–379.

Baggott JE & Johanning GL (1999) 10-Formyl-dihydrofolic acid is bioactive in human leukemia cells. Journal of Nutrition 129, 1315–1318.

Baggott JE, Johanning GL, Branham KE, Prince CW, Morgan SL, Eto I & Vaughn WH (1998) Cofactor role for 10-formyldihydrofolic acid. Journal of Biological Chemistry 263, 7105–7111.

Bailey LB, Barton LE, Hillier SE & Cerda JJ (1988) Bioavailability of mono and polyglutamyl folate in human subjects. *Nutrition Reports International* **38**, 509–518.

Bhandari SD & Gregory JF III (1990) Inhibition by selected food components of human and porcine intestinal pteroylpolyglutamate hydrolase activity. *American Journal of Clinical Nutrition* **51**, 87–94.

Cahill E, McPartlin J & Gibney MJ (1998) The effects of fasting and refeeding healthy volunteers on serum folate levels. *International Journal for Vitamin Nutrition Research* **68**, 142–145.

Clifford AJ, Jones AD & Bills ND (1990) Bioavailability of folates in selected foods incorporated into amino-acid based-diets fed to rats. *Journal of Nutrition* **120**, 1640–1647.

Gregory JF III (1995) The bioavailability of folate. In *Folate in Health and Disease*, pp. 195–235 [L Bailey, editor]. New York: M. Dekker.

Gregory JF III (2001) Case study: Folate bioavailabilty. *Journal of Nutrition* **131**, 1376S–1382S.

Gregory JF III, Bhandari SD, Bailey LB, Toth JP, Baumgartner TG & Cerda JJ (1992) Relative bioavailability of deuterium-labeled monoglutamyl tetrahydrofolates and folic acid in human subjects. *American Journal of Clinical Nutrition* **55**, 1147–1153.

Gregory JF III, Ristow KA, Sartain DB & Damron BL (1984) Biological activity of the folacin oxidation products 10-formylfolic acid and 5-methyl-5,6-dihydrofolic acid. *Journal of Agricultural and Food Chemistry* **32**, 1337–1342.

Halsted CH (1991) Jejunal brush-border folate hydrolase, a novel enzyme. *Western Journal of Medicine* **155**, 605–609.

Herbert V, Drivas G, Manusselis C, Mackler B, Eng J & Schwartz E (1984) Are colon bacteria a major source of cobalamin analogues in human tissues? 24-hr stool contains only about 5 μg of cobalamin but about 100 μg of apparent analogue (and 200 μg of folate). *Transactions Association American Physicians* **97**, 161–171.

Hill GL (1976) *Ileostomy: Surgery, Physiology and Management*, pp. 78. New York: Grune & Stratton, Inc.

Keagy PM, Shane B & Oace SM (1988) Folate bioavailability in humans: effects of wheat bran and beans. *American Journal of Clinical Nutrition* **47**, 80–88.

Kelly P, McPartlin J, Goggins M, Weir DG & Scott JM (1997) Unmetabolized folic acid in serum: acute studies in subjects consuming fortified food and supplements. *American Journal of Clinical Nutrition* **65**, 1790–1795.

Kennedy HJ, Callender ST, Truelove SC & Warner GT (1982) Haematological aspects of life with an ileostomy. *British Journal of Haematology* **52**, 445–454.

Konings EJM (1999) A validated LC method for the determination of folates in vegetables, milk powder, liver and flour. *Journal of AOAC INTERNATIONAL* **82**, 119–127.

Konings EJM, Roomans HHS, Dorant E, Goldbohm RA, Saris WHM & van den Brandt PA (2001) Folate intake of the Dutch population according to newly established liquid chromatography data for foods. *American Journal of Clinical Nutrition* **73**, 765–776.

Leichter J, Landymore AF & Krumdieck CL (1979) Folate conjugase activity in fresh vegetables and its effect on the determination of free folate content. *American Journal of Clinical Nutrition* **32**, 92–95.

Magee DF & Dalley AF II (1986) *Digestion and the Structure and Function of the Gut*, pp. 218–219. Basel: Karger.

Nilsson LO, Andersson H, Hultén L, Jagenburg R, Kock NG, Myrfold HE & Philipson B (1979) Absorption studies in patients six to 10 years after construction of ileostomy reservoirs. *Gut* **20**, 499–503.

Pietrzik K, Hages M & Remer T (1990) Methodological aspects in vitamin bioavailability testing. *Journal of Micronutrient Analysis* **7**, 207–222.

Prinz-Langenohl R, Brönstrup A, Thorand B, Hages M & Pietrzik K (1999) Availability of food folate in humans. *Journal of Nutrition* **129**, 913–916.

Reisenauer AM & Halsted CH (1987) Human folate requirements. *Journal of Nutrition* **117**, 600–602.

Rogers LM, Pfeiffer CM, Bailey LB & Gregory JF III (1997) A dual-label stable-isotopic protocol is suitable for determination of folate bioavailability in humans: Evaluation of urinary excretion and plasma folate kinetics of intravenous and oral doses of $[^{13}C_5]$ and $[^2H_2]$ folic acid. *Journal of Nutrition* **127**, 2321–2327.

Sauberlich HE (1995) Folate status of the U.S. population groups. In *Folate in Health and Disease*, pp. 171–194 [L Bailey, editor]. New York: M. Dekker.

Sauberlich HE, Kretsch MJ, Skala JH, Johnson HL & Taylor PC (1987) Folate requirement and metabolism in nonpregnant women. *American Journal of Clinical Nutrition* **46**, 1016–1028.

Schuster O, Weimann HJ, Muller J, Menke A & Menke G (1993) Pharmakokinetik und relative bioverfügbarkeit von eisen und folsäure bei gesunden versuchspersonen (Pharmacokinetics and relative bioavailability of iron and folic acid in healthy volunteers. *Arzneimittelforschung* **43**, 761–766.

Spies TD, Lopez GG, Stone RE, Milanes F, Brandenburg RO & Aramburu T (1948) Further observations on the specificity of the folic acid molecule. *Blood* **3**, 121–126.

Steinberg SE (1984) Mechanisms of folate homeostasis. *American Journal of Physiology* **246**, G319–G324.

Tamura T & Stokstad ELR (1973) The availability of food folate in man. *British Journal of Haematology* **25**, 513–532.

Wei MM, Bailey LB, Toth JP & Gregory JF III (1996) Bioavailability for humans of deuterium-labeled monoglutamyl and polyglutamyl folates is affected by selected foods. *Journal of Nutrition* **126**, 3100–3108.

Wei MM & Gregory JF III (1998) Organic acids in selected foods inhibit intestinal brush border pteroylpolyglutamate hydrolase in vitro: potential mechanism affecting the bioavailability of dietary polyglutamyl folate. *Journal of Agricultural and Food Chemistry* **46**, 211–219.

Wei MM & Hou ML (2000) Effect of spinach components on folate conjugase activity. *FASEB Journal* **14**, Abstr. 166.6.

Williams S (1984) Microbiological methods. In *Official Methods of Analysis of the Association of Analytical Chemists*, 14th ed. pp. 862–868. Arlington: Association of Official Analytical Chemists Inc.