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Folate absorption from folate-fortified and processed foods using a human ileostomy model

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Data on folate absorption from food from validated human studies using physiological folate doses are still needed to estimate dietary requirements and to formulate recommendations. The aim of the present work was to study the effects from fortified and processed foods on folate absorption in ileostomy volunteers (*n* 9) using the area under the plasma concentration curve (AUC) and kinetic modelling. Using a standardized single-dose protocol, dairy products fortified with a candidate fortificant (6S)-5-methyltetrahydrofolate ((6S)-5-CH_3-H_4folate), folic acid-fortified bread and a dessert crème containing natural yeast folate polyglutamates were compared with folate supplements. Absorbed folate was estimated by AUC and a kinetic model, and non-absorbed folate by ileostomal folate excretion. Median apparent absorption from test foods ranged from 55 to 86 %. Added folate-binding proteins (FBP) significantly reduced folate absorption from dairy products, as in the absence of FBP, AUC–dose-corrected ratios were increased and ileal folate excretion decreased. After *in vivo* gastrointestinal passage of dairy products containing FBP, up to 43 % of the ingested FBP was found in ileostomal effluent. Folate absorption was similar for (6S)-5-CH_3-H_4folate fortificant from fermented milk and for folic acid from fortified bread. Folic acid, ingested as food fortificant in bread, was significantly less absorbed compared with an isolated supplement. We conclude that all tested foods were suitable matrices for folate fortification. However, dairy products, fortified with the new candidate fortificant (6S)-5-CH_3-H_4folate, are suitable if no active FBP is present.

(6S)-5-Methyltetrahydrofolate/folic acid: Folate-binding proteins: Fortified foods: Human folate absorption

An optimal folate status is linked to several health-protective effects, e.g. diminished risk for neural tube defects (Honein *et al.* 2001; Liu *et al.* 2004) and spontaneous abortions (George *et al.* 2002), decreased risk of occlusive vascular diseases (Wald *et al.* 2002) and improved cognitive or mental functions (Seshadri *et al.* 2002). These reported health benefits have led to increased folate intake recommendations in the USA and some European and Nordic countries (Yates *et al.* 1998; Becker *et al.* 2004).

Information about the extent to which certain foods could contribute to increased folate intake is still incomplete. However, some *in vitro* and *in vivo* trials aimed to determine the effects of food matrix on folate absorption, e.g. folic acid-fortified cereal-based foods (Pfeiffer *et al.* 1997; Malinow *et al.* 1998; Johansson *et al.* 2002) or dairy products which contain folate-binding proteins (FBP; Arkbåge *et al.* 2003; Verwei *et al.* 2003). Different human models have been used to determine long-term (Malinow *et al.* 1998; Johansson *et al.* 2002; Vahteristo *et al.* 2002) or short-term (Pfeiffer *et al.* 1997; Prinz-Langenohl *et al.* 1999; Finglas *et al.* 2002; Konings *et al.* 2002; Witthöft *et al.* 2003) folate bioavailability or absorption. Long-term protocols are tedious, and also most short-term protocols have certain requirements and limitations as reviewed elsewhere (Witthöft *et al.* 1999; Gregory, 2001), e.g. lack of sensitivity demanding high test doses or presaturation of volunteers' body stores as recommended for dual-label stable-isotope protocols (Pfeiffer *et al.* 1997; Rogers *et al.* 1997). The area under the plasma concentration curve (AUC) technique (Prinz-Langenohl *et al.* 1999; Konings *et al.* 2002) is commonly used to estimate folate absorption by comparing a single oral dose of test food with a known dose of a pharmaceutical folate preparation. This concept was questioned as it was hypothesized that oxidized folic acid and reduced folates have different sites of initial metabolism resulting in a greater liver sequestering of folic acid (Wright *et al.* 2003).

Abbreviations: AUC, $AUC^{0\rightarrow600}$, area under the (plasma concentration) curve, superscript time range (in min); C_{20}, plasma folate concentration, subscript defines time (in min); FBP, folate-binding proteins; (6S)-5-CH_3-H_4folate, (6S)-5-methyltetrahydrofolate; t, t_{20}, time (point), subscript defines time (in min) of folate concentration in plasma.

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The present study was carried out to determine effects from differently fortified and processed foods on folate absorption using the AUC technique and a new kinetic modelling method (Kok *et al.* 2004; Wright *et al.* 2005) in human ileostomy volunteers and forgoing body store presaturation (Witthöft *et al.* 2003). Test foods were differently processed dairy and cereal products, which were fortified with folic acid or a new candidate fortificant (6S)-5-methyltetrahydrofolate ((6S)-5-CH_3-H_4folate) or natural yeast folate polyglutamates. Furthermore, effects from dairy FBP on folate absorption and the fate of FBP during *in vivo* gastro-intestinal passage were studied.

Material and methods

Subjects

Nine subjects were recruited (eight males, one female), apparently healthy based on routine haematological and biochemical measurements and a physical examination. They had a mean age of 62 (SD 9·3, range 51–79) years, a mean BMI of 28·9 (SD 4·3, range 22·6–38·4) kg/m^2, were non-smokers, and did not use any medication or vitamin supplements affecting folate metabolism. They underwent proctocolectomy 12–37 years earlier as a result of ulcerative colitis with a maximal resection of 5–10 cm (except one volunteer: 25 cm) of the distal ileum and possessed a conventional well-established ileostomy with no signs of inflammation. Volunteers were screened for fasting serum folate, serum cobalamin and erythrocyte folate concentrations to ensure normal folate and vitamin B_{12} status. The protocol was approved by the Ethical Committee of Umeå University Hospital.

Study design

All volunteers underwent nine independent study days each 2–4 weeks apart in random order. They received, after overnight fast, either a single dose of test food or a pharmaceutical preparation of the naturally occurring diastereoisomer (6S)-5-methyltetrahydrofolate ((6S)-5-CH_3-H_4folate) or folic acid (Table 1). On one day they received no folate to allow for estimation of baseline folate excretion into stomal effluent. During the several months' long trial, volunteers' folate status was standardized by presaturation of body stores with a daily dose of 0·96 mg folic acid from day 9 to day 2 prior to each study day (Witthöft *et al.* 2003). A standardized low-folate and low-fat lunch (Witthöft *et al.* 2003) was consumed at 4 h 5 min post-dose, providing 2556 kJ, 13·6 g fat and 18·1 μg folate. A snack of 8 g unsalted rice-cake and 15 g pasteurized apple crème, providing 163 kJ, 0·2 g fat and 3·4 μg folate was consumed at 7 h 5 min post-dose.

Table 1. Pharmaceutical preparations and test foods

Treatment		Folate dose/portion*	Further details
I	Intramuscular injection	194–266 μg (422–579 nmol)† 5-CH_3-H_4folate	Injection solution (1 ml) prepared from (6S)-Ca-5-CH_3-H_4 folate (Merck Eprova AG, Schaffhausen, Switzerland) according to Witthöft *et al.* (2003).
O	Pharmaceutical preparation	192 μg (418 nmol) (6S)-5-CH_3-H_4folate	Gelatine capsule by Merck Eprova AG.
C	Pharmaceutical preparation	199 μg (451 nmol) folic acid	Gelatine capsule by Merck Eprova AG.
U	Fermented milk	187–234 μg (406–509 nmol) (6S)-5-CH_3-H_4folate	Commercial fermented milk product (Filmjölk®; Arla Foods, Stockholm, Sweden; 0·5 % fat), addition of (6S)-Ca-5-CH_3-H_4 folate injection solution 30 min prior to consumption; 400 g/portion.
F	Fermented milk with FBP	180–205 μg (392–445 nmol)‡ (6S)-5-CH_3-H_4folate	Commercial fermented milk product (Filmjölk®; Arla Foods; 0·5 % fat), addition of (6S)-Ca-5-CH_3-H_4 folate injection solution and whey protein concentrate WPC 65 (Arla Foods) 30 min prior to consumption; providing 156–442 nmol FBP/400 g portion.
P	Pasteurized milk with FBP	249 μg (542 nmol) (6S)-5-CH_3-H_4folate	Pasteurized skimmed milk (0·5 % fat) with strawberry taste, fortified with 262 nmol FBP/portion by addition of whey protein concentrate, WPC 65 (Arla Foods). Prior to consumption, the milk was defrosted in a refrigerator overnight and mixed (400 g/portion).
B	Bread	217 μg (491 nmol) folic acid	Wheat bread, fortified with folic acid (Merck Eprova), baked by Cerealia (Järna, Sweden), stored at −20°C. The bread was thawed in a refrigerator overnight, the crust removed and portions of 50 g weighed out 20 min prior to consumption, providing 12 μg (26 nmol) endogenous 5-CH_3-H_4folic acid/portion.
Y	Yeast crème	68–75 μg (147–162 nmol) yeast 5-CH_3-H_4folate polyglutamates	Lemon mousse 'fresta' (citronfromage; Ekströms, Procordia Food, Eslöv, Sweden) with yeast flakes (Edelhefe-Flocken auf Melasse Basis, Tartex + Dr Ritter GmbH, Freiburg, Germany); portions (about 170 g) were prepared 30–45 min prior to consumption.

(6S)-5-CH_3-H_4folate, (6S)-5-methyltetrahydrofolate; FBP, folate-binding proteins.
* Concentrations in μg as free 5-CH_3-H_4folic acid.
† Range for *n* 8, another = 433 μg/921 nmol.
‡ Range for *n* 8, another = 111 μg/241 nmol.

Folate absorption was estimated by plasma AUC and a kinetic model from venous blood samples collected 10 min predose and at 20, 40, 60, 90, 120 min and 3, 4, 6, 8 and 10 h post-dose. Non-absorbed folate was estimated from folate excretion into stomal effluent samples, collected every second hour over 10 h post-dose. Urine samples from spontaneous bladder emptying during 10 h post-dose were used to ensure that ingested doses did not exceed the kidney threshold. Detailed information of sample collection and storage is given elsewhere (Witthöft *et al.* 2003).

Sample pretreatment for folate analysis

Procedures for extraction and purification of plasma and ileostomy samples by strong anion exchange solid-phase extraction and urine samples by affinity chromatography using bovine FBP (Scripps Laboratories, Cincinnati, OH, USA) are described elsewhere (Witthöft *et al.* 2003). Individual urine samples derived from each subject were pooled beforehand for each test day. Procedures for extraction, deconjugation using hog kidney acetone powder (Sigma Chemical Co., St Louis, MO, USA) and strong anion exchange solid-phase extraction purification of aliquots from food samples (dairy products 5 g, dessert crème with yeast 3·5 g, freeze-dried homogenized lunch and snack samples 2 g) have been described earlier (Witthöft *et al.* 2003). To ensure complete deconjugation of folate polyglutamates in the yeast dessert crème, rat serum (Scanbur, Sollentuna, Sweden) was used according to Patring *et al.* (2005). Freeze-dried bread samples (2 g) were extracted by a tri-enzyme method using thermostable α-amylase (Megazyme International, Cork, Ireland) and protease (Sigma Chemical Co.) according to Johansson *et al.* (2002).

Folate quantification by HPLC

5-CH_3-H_4folate content in test foods, pharmaceutical folate preparations and human samples was quantified by reverse-phase HPLC according to Jastrebova *et al.* (2003) using a HP 1100 series system equipped with a multi-wavelength detector and a fluorescence detector (Agilent Technologies, Waldbronn, Germany) and a Zorbax SB C8, 150 × 4·6 mm, 5 μm (Agilent Technologies, Palo Alto, CA, USA) column. External calibration (n 8) was carried out using the standards (Eprova AG, Schaffhausen, Switzerland) (6S)-H_4folate, (6S)-5-CH_3-H_4folate, (6S)-5-HCO-H_4folate at 290/356 nm (fluorescence detector) and folic acid at 290 and 300 nm (multi-wavelength detector). The limits of quantification were 0·5 ng/ml for H_4folate, 0·3 ng/ml for 5-CH_3-H_4folate, 4 ng/ml for 5-HCO-H_4folate and 4 ng/ml for folic acid. Calibration was linear over a range of 0·5–100 ng/ml for H_4folate, 0·3–100 ng/ml for 5-CH_3-H_4folate, 4–200 ng/ml for 5-HCO-H_4folate and 4–200 ng/ml for folic acid. Intra-assay CV and relative recoveries for 5-CH_3-H_4folate were: CV of 11·0 % (n 4), 84–105 % recovery in stomal effluent samples and CV of 6·9 % (n 4), 86–94 % recovery in plasma samples, including sample preparation and all analytical steps. An in-house plasma control sample and a milk sample as control for stomal effluent samples were carried through all procedures of sample extraction and purification, resulting in CV of 6·2 % (n 41, plasma) and 5·5 % (n 35, milk) of 5-CH_3-H_4folate concentrations. For folic acid in bread a CV of 0·9 % (n 3) and for 5-CH_3-H_4folate in yeast crème a CV of 7·5 % (n 3) were obtained. Spiking of urine samples with 5-CH_3-H_4folate prior to affinity purification resulted in recoveries of 92–111 % (Witthöft *et al.* 2003). The day-to-day repeatability for affinity procedures and subsequent 5-CH_3-H_4folate quantification resulted in CV of 6·8 % (n 3, urine) and 4·5 % (n 18, standard solution).

Folate-binding protein quantification

FBP concentrations in dairy products and stomal effluent samples were determined by a two-site ELISA developed for milk according to Højer-Madsen *et al.* (1986) with minor modifications as published by Wigertz *et al.* (1997) using rabbit anti-bovine FBP 24 739 (State Serum Institute, Copenhagen, Denmark), FBP calibrant (Central Hospital Hillerød, Hillerød, Denmark) and the software KinetiCalc 4, version 2.5 for Windows (Bio-Tek Instruments, Winooski, VT, USA). A whey protein concentrate, containing 65 % protein (WPC 65; Arla Foods, Götene, Sweden), was included as in-house reference material into every analysis. The CV between runs did not exceed 15 %.

Kinetic and statistical calculations

Non-absorbed folate from oral doses was estimated by 10 h post-dose stomal effluent. Absorbed folate was estimated using plasma folate net increase above baseline concentrations (pre-dose). When plasma concentration fell below the pre-dose level, the increment was taken as zero. The (positive) $AUC^{0\rightarrow\infty}$ from t_0 to infinity was calculated for each subject using linear and logarithmic trapezoidal rules for ascending and descending plasma concentrations up to the last time-point. If folate concentrations at the last blood sampling point (C_{600}) were still above baseline concentrations (C_0), the AUC beyond t_{600} to infinity ($AUC^{600\rightarrow\infty}$) was extrapolated by log-linear regression analysis using the last three to five plasma concentration data points (choosing the best fit by correlation coefficients).

Relative folate absorption from test foods was compared using AUC–dose-corrected ratios ($AUC_{Testfood}/Dose_{Testfood}$ ((h ng/ml) per mol)) to normalize for differences in individual test portions.

Apparent folate absorption was estimated by assuming a zero-order absorption process in a single compartment model as described by Kok *et al.* (2004) using the following equations for all test foods and doses except bread:

$$C = \frac{M}{VTk}\left(1 - e^{-k(t-t_{lag})}\right) \quad (0 < t < t_{max}) \qquad (1)$$

$$C = \frac{M}{VTk}\left(1 - e^{-kT}\right)e^{-k((t-t_{lag})-T)} \quad (t > t_{max}) \qquad (2)$$

where M is the mass of dose absorbed, t_{lag} is the time during which the plasma enrichment remains at baseline, t_{max} is the time at which the 5-CH_3-H_4folate concentration is a maximum in the plasma, T is the time period for absorption ($t_{max} - t_{min}$), C is the 5-CH_3-H_4folate concentration in the sampled (plasma) compartment, V is the distribution volume of 389 ml/kg body weight as estimated by Loew *et al.* (1987) and k is the elimination rate constant. By fitting the above equations to the

5-CH_3-H_4folate curve (above C_0) over time, M can be calculated. For bread, folate absorption was estimated using the first-order absorption process using the Bateman function:

$$C = \frac{MK_a}{V(K_a - K_e)}\left(e^{-K_e t} - e^{-K_a t}\right) \quad (3)$$

where C is the concentration in the sampled compartment, M is the quantity of the dose that is absorbed, V is the distribution volume (389 ml/kg body weight) and K_a and K_e are rate constants of absorption and elimination, respectively. The apparent folate absorption was calculated according to: apparent absorption (%) $= 100 \times M/\text{Dose}_{\text{oral}}$.

All calculations were made using Office Excel 97.SR or 2003 SP1 (Microsoft, Redmond, WA, USA).

All statistical analyses were made using Minitab release 13.32 (Minitab Ltd, Coventry, UK). Continuous variables are presented as median and range.

Normal plots of the residuals after fitting linear models showed that log-transformed response variables: AUC–dose-corrected ratios, apparent folate absorption and relative folate excretion with stomal effluent, were approximately normally distributed. Tukey's method was used to control the simultaneous experimental error when performing pair-wise comparison among the treatments. When comparing the intramuscular injection (day I) with the oral treatments Dunnett's method was used to control the simultaneous experimental error. Wilcoxon signed rank test was used to compare effects of treatments P and F (see Table 1 for treatments) on relative FBP excretion in ileostomal effluent. A two-sided P value less than 0·05 was considered significant in all analyses.

Nutrient content in standardized low-folate and low-fat lunch and snack was calculated using the software MATs 4.05 (2001) (MATs den flexible, version 2.2; Rudans lättdata, Västerås, Sweden).

Results

Effects of ingested doses on folate content in plasma, urine and ileostomal effluent

After ingestion of test foods and pharmaceutical preparations containing 5-CH_3-H_4folate and folic acid, post-dose plasma 5-CH_3-H_4folate concentrations increased above fasted baseline levels, but no folic acid was detected. AUC–dose-corrected ratios after intramuscular injection of pharmaceutical (6S)-5-CH_3-H_4folate (day I) were greater than AUC on days B, P, F ($P<0·0001$) and U ($P=0·0074$), borderline greater than on day C ($P=0·0581$) and similar to days O ($P=0·2898$) and Y ($P=0·2360$). When no folate dose was given to volunteers (day N), no clear increase and subsequent decrease of plasma 5-CH_3-H_4folate concentrations over time was observed. Resulting AUC from t_0 to t_{600} had for all volunteers a mean size of below 10 % of the AUC on day I (data not shown), and were not taken into account for further calculations. AUC–dose-corrected ratios after ingestion of fermented milk without FBP (U) and yeast dessert crème (Y) were higher compared to the other foods (Table 2). This is similar when estimating apparent folate absorption (Table 3). Apparent absorption from fermented milk without FBP (U) is similar to yeast crème (Y) ($P=0·9891$), and both are significantly larger than from pasteurized milk with FBP (P) ($P=0·0137$ and $P=0·0056$, respectively). Apparent folate absorption from bread (B) tends to be larger than from pasteurized milk (P) ($P=0·067$).

Table 2. Area under the plasma concentration curve (AUC)–dose-corrected ratios of plasma 5-methyltetrahydrofolate (5-CH_3-H_4folate) after absorption of 5-CH_3-H_4folate and folic acid from test foods*

(Median values and range for nine subjects)

	AUC–dose-corrected ratio ((h ng/ml) per mol)	
Treatment	Median	Range
I	0·099[bd]	0·034–0·154
O	0·065[cd]	0·033–0·121
C	0·075[cd]	0·023–0·094
U	0·055[c]	0·027–0·087
F	0·030[a]	0·015–0·078
P	0·020[a]	0·014–0·035
B	0·039[a]	0·016–0·052
Y	0·143[b]	0·056–0·177

* For details of treatments and procedures, see Table 1 and p. 182.
[a,b,c,d] Median values with unlike superscript letters were significantly different ($P<0·05$) (Tukey pair-wise comparison among treatments on log-transformed AUC–dose-corrected ratios).

Only small quantities of intact 5-CH_3-H_4folate from below 1 to 20 μg were excreted into urine during 10 h post-dose (data not shown). Highest amounts of intact 5-CH_3-H_4folate excreted into urine corresponded on three occasions to a maximum of 8 %, and on all other occasions to below 5 % of the given dose.

After ingestion of test foods containing 5-CH_3-H_4folate, only this folate form was found in stomal effluents and no other folate forms were detected. On day N (baseline), when no folate dose was given, only negligible quantities of 5-CH_3-H_4folate (1·6–6·0 μg/10 h) were excreted, being in the same magnitude as absolute 5-CH_3-H_4folate excretion after intramuscular injection (I) (0·7–11·2 μg/10 h) and after ingestion of folic acid-fortified bread (B) (1·7–15·4 μg/10 h, n 8, for one volunteer peak masked). Relative 5-CH_3-H_4folate excretion increased significantly after ingestion of all test foods containing 5-CH_3-H_4folate (F, P, U, Y and O, all $P<0·0001$) compared with the intramuscular injection (I),

Table 3. Apparent 5-methyltetrahydrofolate (5-CH_3-H_4folate) and folic acid absorption (% of dose) from test foods using kinetic modelling of plasma concentration curves*

(Median values and range for nine subjects)

	Apparent absorption (%)	
Treatment	Median	Range
U	86[a]	29–147
F	62[ab]	37–92
P	55[b]	27–64
B	74[ab]	31–159
Y	80[a]	37–152

* For details of treatments and procedures, see Table 1 and p. 182.
[a,b] Median values with unlike superscript letters were significantly different ($P<0·05$) (Tukey pair-wise comparison among treatments on log-transformed apparent folate absorption).

being significantly higher on days P and Y compared with day U ($P=0{\cdot}0214$ and $P=0{\cdot}0371$, respectively) and day O ($P=0{\cdot}0058$ and $P=0{\cdot}0111$, respectively) (Table 4). After ingestion of folic acid-fortified bread (B), some folic acid traces up to approximately 17 μg/10 h were excreted in stomal effluent, and after pharmaceutical folic acid (C) 1–13 μg/10 h were excreted. This is only a rough estimate due to folate concentrations below the limit of quantification in some of the ileostomal fractions.

Effects from dairy processing and presence of folate-binding proteins on folate absorption

The presence of FBP in dairy products affected folate absorption. AUC–dose-corrected ratios were significantly increased on day U after ingestion of fermented milk without FBP compared to days F ($P=0{\cdot}0243$) and P ($P=0{\cdot}0001$). Median AUC–dose-corrected ratios for both dairy products containing FBP did not differ significantly ($P=0{\cdot}5877$). However, apparent folate absorption on day U was only significantly increased compared to day P ($P=0{\cdot}0137$), but not to day F ($P=0{\cdot}6224$). Plasma results were complemented by data on relative ileostomal folate excretion, which increased significantly on day P ($P=0{\cdot}0214$) compared to day U, but not on day F ($P=0{\cdot}7152$) (Table 4).

After *in vivo* gastrointestinal passage of dairy products fortified with FBP (P and F), FBP was found in ileostomal effluents (Table 5), being significantly higher on day P than day F ($P=0{\cdot}009$). On days without FBP ingestion, e.g. days U and N, no FBP was detected in post-dose effluents, as controlled for four volunteers (data not shown).

Effect of ingested folate form on extent of absorption

Folate absorption by means of AUC–dose-corrected ratios did not differ significantly ($P=0{\cdot}9940$) after oral ingestion of pharmaceutical preparations of (6S)-5-CH_3-H_4folate (O) and folic acid (C). Also total folate excretion after ingestion of both folate forms was similar. After ingestion of 5-CH_3-H_4folate (O), 2–74 μg 5-CH_3-H_4folate were found in stomal effluent during 10 h post-dose, and after ingestion of folic acid (C), 3–41 μg 5-CH_3-H_4folate and an additional 1–13 μg of folic acid were excreted.

Absorption of different folate forms as fortificant, (6S)-5-CH_3-H_4folate monoglutamate in fermented milk (U) compared to folic acid in wheat bread (B) compared to yeast 5-CH_3-H_4folate polyglutamates as 'bio-fortificant' (Y), differed significantly when expressed as AUC–dose-corrected ratios. Y was significantly more absorbed than U ($P=0{\cdot}0001$) and than B ($P<0{\cdot}0001$), and B significantly less than U ($P=0{\cdot}0384$).

After ingestion of folic acid as fortificant within a bread matrix (B), AUC–dose-corrected ratios were significantly smaller ($P=0{\cdot}0041$) compared with a supplement (C). Ileostomal folic acid excretion was estimated to be 4–24 μg on day B and 1–13 μg on day C. (6S)-5-CH_3-H_4folate given as supplement (O) was similarly absorbed as when given as fortificant in fermented dairy matrix (without FBP, U), based on AUC–dose-corrected ratios ($P=0{\cdot}7822$).

Table 4. Relative excretion of 5-methyltetrahydrofolate (5-CH_3-H_4folate) (% of dose) with stomal effluent over 10 h post-dose*

(Median values and range for nine subjects)

	5-CH_3-H_4folate excretion (%)	
Treatment	Median†	Range
I	2[a]	0–5
O	8[b]	2–39
U	7[b]	4–25
F	16[bc]	5–29
P	20[c]	13–51
Y	23[c]	13–37

* For details of treatments and procedures, see Table 1 and p. 182.
† Median for Y (n 8) as peak for one volunteer masked, median for I (n 8) as amount for one volunteer traces only.
[a,b,c] Median values with unlike superscript letters were significantly different ($P<0{\cdot}05$) (Tukey pair-wise comparison among treatments on log-transformed relative folate excretion in stomal effluent).

Table 5. Relative excretion of folate-binding proteins (% of dose) with stomal effluent over 10 h post-dose*

(Median values and range for nine subjects)

	FBP excretion (%)	
Treatment†	Median	Range
F	4[b]	0–32
P	24[a]	3–43

* For details of treatments and procedures, see Table 1 and p. 182.
† No folate-binding proteins found in ileostomal effluent on other days (N, U).
[a,b] Median values with unlike superscript letters were significantly different ($P=0{\cdot}009$) (Wilcoxon signed rank test).

Discussion

Effects of dairy processing and presence of folate-binding proteins on folate absorption

New information on effects of presence of FBP on folate absorption in human subjects was provided by the present study. Plasma results demonstrated that (6S)-5-CH_3-H_4folate, a candidate compound for food fortification and the dominant native food folate form, is bioavailable from all tested dairy matrices. Median AUC–dose-corrected ratios are significantly reduced in the presence of FBP (Table 2). Also apparent 5-CH_3-H_4folate absorption from pasteurized milk with FBP (P) is smaller than from fermented milk without FBP (U) (Table 3) and conversely relative folate excretion with ileostomal effluent is higher for P than U (Table 4). The present findings suggest that FBP is reducing folate absorption. Folate is better absorbed from fermented milk than from pasteurized milk due to the absence of native FBP.

The present study is the first to prove intestinal 'survival' of FBP in man. Using *in vitro* methods simulating the upper human intestinal tract, Verwei *et al.* (2003) and Arkbåge *et al.* (2003) reported that between 0 and 34 % of FBP from the dose was recovered after 'digestion' of milk and yoghurt fortified with (6S)-5-CH_3-H_4folate or folic acid. Interestingly, this reflects our findings *in vivo* with FBP survival (Table 5).

Arkbåge *et al.* (2003) reported a significantly decreased bioaccessibility, from yoghurt fortified with FBP, of both 5-CH_3-H_4folate and folic acid. In agreement, we conclude that dairy products might be a suitable matrix for folate fortification if no active FBP is present.

Folate fortificants and supplements

Folate absorption from pharmaceutical folic acid and (6S)-5-CH_3-H_4folate supplements did not differ significantly, but more folic acid was absorbed as a supplement than from a bread matrix (Table 2). However, as median apparent absorption from folic acid-fortified bread was high at 74 % (Table 3), bread is a suitable matrix for folic acid fortification. Data from a previous study (Johansson *et al.* 2002) showed that folic acid fortification of bread results in a significant improvement of volunteers' folate status after just 4 weeks of intervention. Of interest is the comparison of the already-established folic acid fortification practice of cereal-based food with alternative fortificants and matrices, e.g. by using as a new candidate fortificant the biologically active form of (6S)-5-CH_3-H_4folate in dairy matrices, or natural yeast folate polyglutamates for 'biofortification'. Folic acid is used as food fortificant because it is inexpensive and relatively stable, but, in contrast to reduced folates, a high intake can delay diagnosis of an underlying vitamin B_{12} deficiency. Around 80 % of all folate 'fortificants', yeast polyglutamates from dessert crème (Y), (6S)-5-CH_3-H_4folate from fermented milk without FBP (U) and folic acid from bread, were absorbed (Table 3). Median apparent absorption of yeast folate from the dessert crème in the present study (86 %) was much higher than the estimate of folate bioavailability of a yeast drink of 59 % in the intervention study of Hannon-Fletcher *et al.* (2004). Our HPLC method, allowing quantification of four different folate forms, might have led to an underestimation of the total folate content in that particular test food when other folate forms were present. In theory, this could result in an overestimation of folate absorption. As high folate-producing yeast strains could be an alternative for folate enrichment, future investigation of this interesting matrix is warranted.

Critical appraisal of the ileostomy–area under the curve model: limitations and advantages

The model enables the direct estimation, by comparison of AUC–dose-corrected ratios and kinetic models, of the extent of folate absorption after ingestion of different test foods.

Some noise in plasma folate concentrations was visible in the form of minor AUC on day N with no test food application, which may reflect effects from fasting, enterohepatic circulation and ingestion of the standardized low-folate, low-fat meals on plasma folate levels. We decided not to correct for them when interpreting plasma results due to strict standardization of the study protocol (regarding sampling, fasting periods and test food ingestion), as we expect possible confounding effects to be similar on all days. Another possible confounder regarding plasma data is the hepatic first pass effect (Pfeiffer *et al.* 1997; Rogers *et al.* 1997). In line with their recommendation, we presaturated, and therefore standardized, volunteers' body stores. Using the plasma AUC approach (Prinz-Langenohl *et al.* 1999; Konings *et al.* 2002), folate absorption from a test food is usually estimated by comparison with an oral reference dose of folic acid; but hereby it is not guaranteed that the oral reference dose is completely absorbed. To overcome this problem, the concept of an intramuscular reference dose was developed (Witthöft *et al.* 2003), where relative absorbed folate from an oral test dose was estimated using a reference dose of (6S)-5-CH_3-H_4folate administered by intramuscular injection (day I). Using labelled folate compounds, Wright *et al.* (2003, 2005) observed concurrent displacement of endogenous (unlabelled) liver folates after an oral folate test dose and hypothesized differences in metabolism of oxidized and reduced folates. It was suggested earlier that different folate forms (oxidized compared to reduced) and administration (oral compared to intravenous injection) could result in different handling in the body (Finglas *et al.* 2002). This may lead to the conclusion that the quantification of absorbed folate from a test food by comparison with any reference dose might be unsuitable when no labelled compounds are used. Therefore, we decided to avoid estimation of relative folate absorption by a reference dose, but rather determine effects of processed and fortified food on folate absorption by direct comparison of AUC–dose-corrected ratios.

Plasma results are complemented by data from ileostomal folate excretion, and estimated absorbed (by AUC) and non-absorbed (by stomal excretion) folate should in theory amount to approximately 100 % (Witthöft *et al.* 2003). Overestimation of total recovery could be caused by overestimating plasma AUC due to a bad curve fit when extrapolating or during kinetic modelling, when estimating the distribution volume V using the factor (of 389 ml/kg body weight) from Loew *et al.* (1987), which was estimated after a single intravenous dose of oxidized folic acid of pharmacological magnitude. Underestimation of the model's overall recovery can mainly be caused by incomplete collection of ileostomal effluent.

The small quantities of 5-CH_3-H_4folate in 10 h post-dose urine are in line with earlier findings (Pfeiffer *et al.* 1997; Witthöft *et al.* 2003). Thus, the given doses can be considered to be of physiological size and that the kidney threshold was not reached.

In conclusion, this new human model was used to compare folate absorption from differently processed and fortified foods. As each volunteer was randomly participating in the nine strictly standardized study days, intra-individual as well as inter-individual comparison of folate absorption was possible. The presented model would be strengthened by combination with stable-isotope techniques, as differentiation of plasma folate deriving from the exogenous dose and from endogenous body stores is of importance when studying folate absorption and elimination kinetics by AUC.

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