British Journal of Nutrition (2007), **98**, 667–675
doi: 10.1017/S0007114507777140

Review Article

Folic acid metabolism in human subjects revisited: potential implications for proposed mandatory folic acid fortification in the UK

Anthony J. A. Wright*, Jack R. Dainty and Paul M. Finglas

Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, UK

(Received 6 December 2006 – Revised 21 May 2007 – Accepted 24 May 2007)

Following an introduction of the importance of folates and the rationale for seeking to estimate fractional folate absorption from foods (especially for countries not having a mandatory folic acid fortification policy), scientific papers covering the mechanisms of folate absorption and initial biotransformation are discussed. There appears (post-1983) to be a consensus that physiological doses of folic acid undergo biotransformation in the absorptive cells of the upper small intestine to 5-methyltetrahydrofolic acid (as happens for all naturally-occurring reduced 1-carbon-substituted folates). This 'validates' short-term experimental protocols assessing 'relative' folate absorption in human subjects that use folic acid as the 'reference' dose. The underlying scientific premise on which this consensus is based is challenged on three grounds: (i) the apparent absence of a 5-methyltetrahydrofolic acid response in the human hepatic portal vein following absorption of folic acid, (ii) the low dihydrofolate reductase activity peculiar to man and (iii) the implications derived from recent stable-isotope studies of folate absorption. It is concluded that the historically accepted case for folic acid being a suitable 'reference folate' for studies of the 'relative absorption' of reduced folates in human subjects is invalid. It is hypothesised that the liver, and not the absorptive cells of the upper small intestine, is the initial site of folic acid metabolism in man and that this may have important implications for its use as a supplement or fortificant since human liver's low capacity for reduction may eventually give rise to saturation, resulting in significant (and potentially deleterious) unmetabolised folic acid entering the systemic circulation.

Pteroylmonoglutamic acid: Folic acid: Folate: Absorption: Metabolism: Human subjects: Supplementation: Fortification

Introduction

Folic acid (pteroylmonoglutamic acid), though occurring rarely in nature, is the most oxidised and stable form of a vitamin used extensively for supplements and food fortification purposes. Folate is a generic term for the related family of water-soluble B-group vitamins found widely in foodstuffs, mainly reduced methyl and formyl folylpolyglutamates[1], that have similar nutritional properties and chemical structures to those of folic acid[2,3]. Reduced tetrahydrofolates, carrying 1-carbon substitutions at positions 5 and/or 10, are crucial for methionine and nucleotide biosynthesis[4,5]. A significant reduction in the incidence and recurrence of neural tube defects, such as spina bifida, has been shown when women undertake periconceptual supplementation with folic acid[6,7]. Low folate status is associated with elevated plasma homocysteine, a risk factor for CVD and stroke[8,9], and has been linked to dementia and Alzheimer's disease[10]. Low folate status is additionally associated with altered methylation of DNA that may affect gene expression and uracil induced genomic instability, both of which may increase cancer risk[11,12].

Fortification of food with physiological levels of folic acid may be expected to reduce the prevalence of folate-related diseases. Some countries have mandatory folic acid fortification of flour programmes (USA 1·4 mg/kg from 1998; Canada 1·5 mg/kg from 1998; Chile 2·2 mg/kg from 2000), but many European countries do not permit the fortification of foodstuffs with folic acid at all. Only a thorough knowledge of the fractional absorption of folate from a variety of folate supplements, fortified foods and natural food folates would allow us to answer the question of whether optimal folate status is easily achievable in countries that do not have a mandatory folic acid fortification policy. An international workshop concluded that the absorption of different folate vitamers from foods and isolates is not well understood[13].

Mechanism of folate absorption

In human subjects, dietary folate polyglutamates are deconjugated at the mucosal epithelial cell brush border by folyl-poly-glutamate carboxypeptidase (EC 3.4.17.21) to the corresponding monoglutamate forms[14]. Folic acid and reduced monoglutamyl folates are absorbed mainly in the proximal

Abbreviations: AUC, area under the curve; DHF, dihydrofolic acid; DHFR, dihydrofolic acid reductase.

* **Corresponding author:** Mr A. J. A. Wright, fax +44 1603 507723, email tony.wright@bbsrc.ac.uk

small intestine (jejunum) by a saturable, carrier-mediated, pH and energy-dependent transport mechanism which, unlike other epithelial tissues, appears to be unique in its lack of hierarchy of transport, having a similar affinity for both oxidised (e.g. folic acid) and reduced folate forms[15,16]. Oral doses of folic acid in excess of about 260–280 μg (589–634 nmol) have been reported to lead to the direct appearance of untransformed folic acid in the systemic circulation of man[17]. This indicates a saturation point, is evidence that intestinal conversion is not a prerequisite for transport, and is arguably indicative of the dividing line between physiological and non-physiological oral doses of folate. Absorbed folate, which may undergo biotransformation in the absorptive mucosa (see next section), is subsequently transferred *via* the mesenteric veins to the hepatic portal vein and carried to the liver where an extensive amount (liver 'first-pass') is removed[18,19]. The liver has a high affinity for the removal of folic acid[20], but a lower one for the removal of 5-methyltetrahydrofolic acid which allows a reasonable fraction of this folate form to proceed uninterrupted into the systemic circulation where it usually appears, under fasting conditions, as the predominant plasma folate form for passage to tissues. Folates removed by the liver, some of which may have undergone further biotransformation, are partially released into the bile allowing significant re-absorption of folate from the small intestine and subsequent delivery of 5-methylterahydrofolic acid, *via* systemic circulating plasma, to tissues[21]. This process of 'enterohepatic recirculation' is responsible for maintaining baseline plasma folate levels[20]. Physiological doses of pure folate compounds seem to be well absorbed ($\geq$90 %) in both man[22,23] and rats[24].

Site of biotransformation of newly absorbed folates, and folate transport into the hepatic portal vein

A high-quality study, with catheterisation of the hepatic portal vein of three human adult volunteer patients, indicated that the absorptive mucosa simply rearranges the 1-carbon substitution of physiological doses of 5-formyltetrahydrofolic acid to 5-methyltetrahydrofolic acid before transport to the serosal side[25], and transports 5-methyltetrahydrofolic acid unchanged. Using everted sacs of rat small intestine, one group reported no reduction (potentially *via* dihydrofolate reductase to H_2-folic acid and then to H_4-folic acid) and subsequent methylation of physiological doses of folic acid to 5-methyltetrahydrofolic acid[26]. In contrast, others not only reported significant degrees of mucosal bioconversion but also reported concurrently that folic acid above the physiological range was passively transported, appearing in circulating plasma in an untransformed state (i.e. that the mechanism of absorption was 'saturable')[15,27,28]. An explanation for the inability of some investigators to demonstrate the intestinal conversion of folic acid to 5-methyltetrahydrofolic in an alkaline medium was given by Strum[15]. He suggested that this may reflect that the enzyme dihydrofolic acid reductase (DHFR) has an acidic pH optimum for reducing folic acid to H_2-folic acid (in contrast to the much wider acid-to-slightly-alkaline pH range for the reduction of H_2-folic acid to H_4-folic acid) and that whilst the intestinal conversion of folic acid to 5-methyltetrahydrofolic acid is extensive at pH 6·0 it is negligible at pH 7·5. Any previous argument and doubts over the degree to which physiological doses of folic acid could be reduced and methylated in mucosal epithelial cells appeared to be mitigated following publication of the high quality study of Tani & Iwai[29]. These authors reported on their *in situ* investigation of absorption and conversion of folic acid in the small intestine of the rat *in vivo*, with mesenteric vein sampling after jejunal loops were injected directly with a solution of [^{3}H]folic acid, and HPLC investigation of [^{3}H]compounds being transferred into the portal blood. They argued compellingly that their *in vivo* technique was much more useful for studying intestinal functions because more physiological conditions were maintained compared to other techniques *in vitro*. They also commented that the correct ratio of conversion of folic acid was not reported in previous papers since, for low concentrations of [^{3}H]folic acid injected into jejunal loops, as high as about 90 % of [^{3}H]compounds that they identified unequivocally by HPLC in portal blood was [^{3}H]5-methyltetrahydrofolic acid.

The paper of Tani & Iwai[29] appeared to be a watershed. Whilst pre-1983 there was already a reasonable degree of agreement that a significant portion of physiological doses of folic acid would undergo conversion to reduced forms in the intestine[30], it was being accepted post-1983 as the norm, with any lingering doubts muted. In particular, specifically those doubts generated some years earlier by the reported appearance of high concentrations of untransformed folic acid in the hepatic portal vein of human subjects following oral administration of doses of folic acid that were, arguably, non-physiological[31,32]. This watershed was reflected in the consensus of subsequent scientific 'Review Articles' which invariably either overtly stated or implied that when the mucosal extracellular folic acid concentration is low (physiological) the small intestine efficiently reduces and methylates folic acid and, as with absorbed naturally-occurring reduced and 1-carbon-substituted folates, subsequently transfers essentially only 5-methyltetrahydrofolic acid to the hepatic portal vein[16,21,33,34]. Thus, from 1983, short-term experimental protocols assessing 'relative' folate absorption in human subjects, which centred on methods comparing the serum/plasma response to a single oral test-dose relative to that of an equal 'reference' dose of folic acid, appeared to have been 'validated' since all consumed folates would broadly enter the hepatic portal vein as 5-methyltetrahydrofolic acid.

Short-term protocols for the estimation of 'relative' folate absorption

Short-term experimental protocols, which do not allow assessment of slow-turnover folate pools in the body, are specifically designed to elicit an understanding of the kinetics of initial absorption, metabolism and transport of absorbed folates. They cannot predict whole-body folate turnover[33]. A short-term protocol involves monitoring the appearance and subsequent clearance of the 5-methyltetrahydrofolic acid response in the fast-turnover plasma pool arising from absorbed and biotransformed oral test folates. However, since there is extensive but unquantified removal of newly absorbed folate from the hepatic portal vein (liver 'first-pass'), 'absolute absorption' cannot be estimated from plasma response[18,19]. Instead, in an attempt to circumvent this problem, these protocols estimate

'relative absorption' comparative to a similar 'reference' dose of folic acid.

Work with radiolabelled folates has been confined mainly to animal models (usually the rat) that may not be comparable to human subjects. Application of pioneering work with stable-isotope folates in human subjects has generally lacked analytical sensitivity when used in single-dose protocols, especially when administered in the physiological range[33]. To date, the vast majority of short-term protocols attempting to assess 'relative' folate absorption in human subjects have centred on methods comparing the serum/plasma response to a single unlabelled oral test-dose relative to that of an equal unlabelled 'reference' dose of folic acid[19,35]. This may entail either measurement of the rate of increase, or the maximum increase, in plasma folate concentration over 2–3 h[17,36–40], or measurement of the dose-normalised rise in plasma folate concentration AUC (the area under the curve of the increase in plasma 5-methyltetrahydrofolic acid concentration above fasting baseline level) over 6 h or more [41–47]. Short-term protocols using unlabelled test folates have not just been confined to the field of nutrition and have also been used in pharmacokinetic studies[48].

Comparison of the dose-normalised AUC between test (food folate or isolate) and 'reference' folic acid has been accepted as a valuable indicator of absorption, provided the post-dosing plasma measurement test period is long enough to capture $\geq 80\,\%$ of the whole AUC[49]. However, it is dependent on the premise that the initial absorption and metabolism of folic acid satisfies the following four conditions: Condition-1, that physiological doses of folic acid are absorbed by the same mechanism as reduced folates, and with a similar affinity;

Condition-2, that physiological doses of folic acid are initially reduced and then methylated in the epithelial cells of the small intestine and that essentially only 5-methyltetrahydrofolic acid is exported from the mucosa to the hepatic portal vein, as is the case for absorbed physiological doses of all naturally-occurring reduced folates; Condition-3, that the kinetics of plasma 5-methyltetrahydrofolic acid response to folic acid is similar, if not equal, to that elicited for reduced 1-carbon-substituted naturally-occurring folates; Condition-4, that plasma 5-methyltetrahydrofolic acid response derives entirely from (biotransformed) newly absorbed folate.

Until recently, no systematic difference had been reported in the kinetics of plasma 5-methyltetrahydrofolic acid response to physiological concentrations of test folates and folic acid. However, new findings have recently shown that much of the plasma AUC response to oral folate doses is induced by, but is not actually derived from, the dose itself[50]. This phenomenon was reported later to also affect plasma vitamin C response to test doses of vitamin C[51,52]. This raises the interesting question of whether this phenomenon will also be found to affect the plasma response to oral test doses of many other water-soluble vitamins. We interpreted our reported large, variable and unpredictable displacement of tissue 5-methyltetrahydrofolic acid into the plasma pool as contrary to Condition-4 above, thus making comparison of the relative absorption of folate from an unlabelled test dose (food or supplement) to an unlabelled folic acid 'reference' dose unusable[50]. Concurrent examination of the plasma ^{13}C-labelled 5-methyltetrahydrofolic acid response to ^{13}C-labelled (6*S*)-5-formyltetrahydrofolic acid and ^{13}C-labelled folic acid also unmasked an underlying serious discrepancy in plasma responses to these two folates [50]. This was possibly due to a limitation in the rate of initial mucosal reduction of folic acid to H_2-folic acid, prior to further reduction to H_4-folic acid and subsequent methylation, which may result in a slower transport of absorbed folate to the serosal side[53]. This is in complete contrast to Condition-3 above and thus renders direct estimates of 'relative absorption' using even labelled-AUC, which are derived definitively from the oral test-doses, invalid.

To overcome this, the application of suitable mathematical modelling[54,55], which makes complete allowance for any differences in the kinetics of plasma labelled 5-methyltetrahydrofolic acid response, can be used to estimate 'apparent absorption'. Apart from unlabelled 5-methyltetrahydrofolic acid (the lone folate form we detected in the baseline fasted plasma samples of our unfortified and unsupplemented volunteers), it is important to note that the only enriched folates appearing in plasma after ingestion of physiological doses of labelled $^{13}C_6$ or $^{15}N_{(1-7)}$ folates were $^{13}C_6$ or $^{15}N_{(1-7)}$ 5-methyltetrahydrofolic acid. This was confirmed by liquid chromatography–MS selective ion monitoring for folate monoglutamate forms on the negative $[M\text{-}H]^-$ ion folate masses: from m/z 440 (folic acid, M + 0; having the smallest folate monoglutamate mass) to m/z 479 (5-formyltetrahydrofolic acid, M + 7; having the largest folate monoglutamate mass)[50,55].

Absorption and site of initial metabolism of folic acid in human subjects

The underlying scientific basis on which the post-1983 consensus is based (that physiological doses of folic acid undergo biotransformation to 5-methyltetrahydrofolic acid in the absorptive mucosal cells of the small intestine) is now challenged on three grounds: the apparent absence of a plasma 5-methyltetrahydrofolic acid response in blood sampled directly from the human hepatic portal vein following the mucosal absorption of folic acid; the evidence of an extremely low dihydrofolate reductase activity that seems peculiar to man; the implications derived from mathematical modelling of plasma labelled 5-methyltetrahydrofolic acid responses in human subjects to oral physiological doses of isotopically-labelled folates.

Absence of a 5-methyltetrahydrofolic acid response in the hepatic portal veins of human subjects following an oral dose of folic acid

Whitehead & Cooper[31], who gave an oral dose of 1000 μg (2266 nmol) folic acid, and Meilikian *et al.*[32], who gave a lower oral dose of 500 μg (1132 nmol) folic acid, both reported that human subjects transport folic acid unaltered into the hepatic portal vein. Both groups took blood samples from the hepatic portal vein, the hepatic vein and the systemic blood system for at least 2 h post-dosing. They concluded that folic acid was absorbed unaltered and removed by the liver which they proposed as the initial site of subsequent reduction and methylation. Critics would point to a number of perceived problems with this work: (i) the dose

of folic acid used was non-physiological; (ii) the studies were carried out in patients with either liver disease (cirrhosis) or cancer; (iii) analysis of folate concentrations were undertaken by differential microbiological assay (*L. casei* and *S. faecalis*) in order to distinguish non-methyl folate from total folate. Nevertheless, it cannot be overemphasised that it is not the presence of copious amounts of untransformed folic acid in the hepatic portal vein that is noteworthy, since that would be an inevitable consequence of using a non-physiological dose. On the contrary, it is the almost complete absence of an assayable 5-methyltetrahydrofolic acid response in the hepatic portal vein that is remarkable, particularly when a dose of 500 μg (1132 nmol) was used[32]. This is only twice the dose of 260–280 μg (589–634 nmol) reported much later to be the threshold at which folic acid may even start to appear in the plasma[17]. Hence, at the very least, one may have expected at least half of the dose used by Melikian *et al.*[32] to have appeared as 5-methyltetrahydrofolic acid.

Heterogeneity in the dihydrofolate reductase activity between animals and human subjects

Though initial work with crude enzyme preparations of DHFR had taken place earlier, it was perhaps the work of Zakrzewski & Nichol with highly purified chicken liver DHFR that first provided evidence for a single enzyme reducing folic acid and dihydrofolic acid (DHF; H_2-folic acid)[56]. These authors reported that this enzyme reduced folic acid significantly slower than DHF with a more acidic optimum pH (4·4–4·8 *v.* 5·2–5·6, respectively), and that there was unmeasurable activity above pH 7·0 for folic acid but still reasonable activity for DHF. These conclusions, which indicate the initial reduction of folic acid to DHF to be the rate limiting step in the biotranformation of folic acid, were confirmed by Mathews & Huennekens[53]. Human placental DHFR also showed two pH optima (with unmeasurable activity for folic acid above pH 7·0) with the activity for reducing folic acid seventy-five times lower than DHF at pH 6·2[57]. Nylen *et al.*[58] reported a distinct lack of dihydrofolate reductase activity in human biopsy and autopsy samples compared to cultured cells. Kamen *et al.*[59] noted dihydrofolate reductase activity in normal human tissue and human tumour and leukaemia cells *in vivo* that was several-hundred times lower than present in human cell lines grown *in vitro*, or normal animal liver tissue. One hypothesis put forward for the discrepancy was that the higher level of DHFR activity with *in vitro* cell lines could be due to the high levels of folic acid used historically in culture medium. The apparent heterogeneity in the dihydrofolate reductase activity between animals and human subjects was confirmed in a later paper which concluded that low levels of DHFR activity are a feature peculiar to man, shared in part only by closely related primates (the great apes), which distinguishes him from most animals and birds[60]. The implications of these differences, particularly between rat (the historical experimental animal model used in formulating most of the current understanding of folate-monoglutamate absorption and initial metabolism) and man, have perhaps not been fully appreciated. In comparison to 5-formyltetrahydrofolic acid, the lower effectiveness of folic acid to correct deoxyuridine suppression in megaloblastic marrows (a consequence of folate deficiency) was partly attributed to a delay in reduction of folic acid to tetrahydrofolic acid inside cells[61]. Modern HPLC analytical techniques, capable of accurately measuring DHFR in crude extracts, have indicated that DHFR activity in human liver is about seventy times or more lower than in rat liver[62,63]. Of course a simple comparison of enzyme activity *ex vivo* does not necessarily reflect quantitative differences in the ability to metabolise folic acid, particularly since enzyme activity is pH-dependent and may be compartmentalised within the cell. However, the fact that the clearance of unmetabolised folic acid from the human systemic circulatory system was noted by these authors to be quite slow, and that 78 % of 105 American postmenopausal women have been observed to have measurable concentrations of unmetabolised folic acid in samples of their fasting plasma[64], strongly suggests that DHFR activity in the human mucosa, as well as the liver, must also be quite low. Our reasoning is as follows. A portion of the human systemic blood flow is continuously diverted *via* the splenic and mesenteric arteries where it eventually arrives at the hepatic portal vein (formed from the superior mesenteric and splenic veins) thus allowing the potential for clearance (and subsequent reduction and then methylation) of any systemically circulating folic acid by the human liver, which we now know to have a limited DHFR capacity. When systemically circulating folic acid is presented to the human liver some of it can reappear in bile as unchanged folic acid[65]. The process of enterohepatic re-circulation will allow for the significant re-absorption of biliary folic acid from the small intestine[20,21]; a recent multi-compartment kinetic model indicating a 24-fold higher flux of bile folate than that previously thought[66]. Though human liver is known to have a limited DHFR capacity in comparison to the rat, it cannot be argued that it is still theoretically possible for human subjects to have a good mucosal DHFR activity because enterohepatic re-circulation works in tandem with continuous partial systemic blood flow *via* the hepatic portal vein through the liver. If mucosal DHFR activity in human subjects was much better than that seen in human liver, then the clearance of excess folic acid from the systemic system would not be observed to be quite slow, as it could then be processed alternatively by the mucosal cells of the upper small intestine. The overall implication is that, in comparison to the rat, man is not likely to have the capacity to significantly reduce folic acid in the absorptive mucosa. Thus, most of a physiological dose of folic acid may inevitably be transported by the absorptive mucosa into the hepatic portal vein in an untransformed state to be sequentially removed by the liver for processing (with a much higher affinity than the 5-methyltetrahydrofolic acid usually presented[20]. This would result in a delayed release (*via* entero-hepatic re-circulation) into the systemic blood system of 5-methyltetrahydrofolic acid, its biotransformed metabolite[31]. A delayed plasma response to folic acid compared to a reduced folate was confirmed by the use of isotopically labelled folates[50], though whether this was due to a delay in mucosal or liver biotransformation could not be concluded.

Mathematical modelling of plasma labelled 5-methyltetrahydrofolic acid responses to oral physiological doses of isotopically-labelled folates

The advent of stable-isotope-labelled folates allows folate absorption to be tracked not only with sensitivity but, more importantly, with specificity. This way, folate can be followed and differentiated from the natural store of folate already present in the body. The recent use of mathematical modelling[55], which bypasses the need for adherence to Condition-3 because it completely takes into account differences in the kinetics of plasma response, infers that sequestration of physiological doses of folic acid to the liver is the main cause of a delayed plasma response. This conclusion is incompatible with current theory that only 5-methyltetrahydrofolic acid enters the hepatic portal vein after oral ingestion of physiological doses of any folate form. Mathematical modelling of stable-isotope-labelled plasma 5-methyltetrahydrofolic acid response was used to estimate the 'apparent absorption' of single oral physiological doses of [$^{13}C_6$]folic acid, (6*S*)-[$^{13}C_6$]5-formyltetrahydrofolic acid and [$^{15}N_{1-7}$]intrinsically-labelled spinach folates in fasting human adults[55]. The 'apparent absorption' of reduced folates was significantly higher than for the 'reference' dose of folic acid; generating 'relative absorptions' significantly in excess of 100 % for both 5-formyltetrahydrofolic acid (158 %) and spinach-folate (183 %). This was unexpected, and biologically impossible, since the 'true absorption' of physiological doses of [^{14}C]folic acid in human subjects has been reported to approximate 90 % or more[22,23]. It was concluded, contrary to current theory, that a significant fraction of absorbed [$^{13}C_6$]folic acid may be entering the hepatic portal vein unchanged in contrast to Condition-2, to be more effectively removed by the liver than reduced 5-methyltetrahydrofolic acid, prior to subsequent biotransformation and (limited) enterohepatic recirculation.

Implications for the use of folic acid as a 'reference folate'

The essential absence of a plasma 5-methyltetrahydrofolic acid response in human blood sampled directly from the hepatic portal vein following a dose of folic acid[31,32], the extremely low dihydrofolate reductase activity that now seems peculiar to man[60,62,63], the recent implications derived from mathematically-modelled 'apparent absorption' of isotopically-labelled folates[55], and the observation of unmetabolised folic acid in plasma of fasted American females[64] are key observations. Collectively, they arguably justify the proposition that, unlike the rat (the chief historical experimental animal model), absorbed physiological doses of folic acid are essentially transferred to the hepatic portal of man in an unmetabolised state to be subsequently removed and metabolised by the liver. The main implication of this is that the currently accepted case for folic acid being a suitable 'reference folate' for studies of the 'relative absorption' of reduced folates in man, particularly in short-term experimental protocols, is invalid.

If subsequent experimentation, using sensitive liquid chromatography–tandem MS techniques[67,68], confirms that physiological doses of stable-isotope-labelled folic acid mainly enter the hepatic portal vein of man in an unmetabolised state, then folic acid should be avoided as the 'reference' folate in 'short-term' studies of the absorption of reduced folates. We suggest that it could be replaced by (6*S*)-5-methyltetrahydrofolic acid (the natural folate form found in circulating blood plasma). Furthermore, it is questionable whether folic acid should be used as the 'reference' folate in longer-term dietary intervention studies where surrogate biological markers of nutritional status or metabolic wellbeing are used as arguments to the relative absorption of folate from basal diets supplemented with natural 'high-folate' foods *versus* those supplemented with an equal amount of folic acid (supplied in either supplemental form or as folic acid-fortified foods). This is because changes in surrogate markers may be influenced by differential tissue distribution of supplemented naturally-occurring reduced folates and folic acid between the liver and other body tissues. This may explain the recent observation that erythrocyte folate concentrations increase more after supplementation with (6*S*)-5-methyltetrahydrofolic acid than with folic acid[69].

Implications for the use of folic acid as a fortificant or supplement

If the initial primary site of folic acid metabolism in human subjects is actually the liver then, because of liver's apparent poor dihydrofolate reductase activity, it would seem entirely logical to hypothesise that regular daily intake of physiological doses of folic acid may eventually result in its chronic appearance in plasma of the systemic circulatory blood system. This may even happen at quite modest physiological doses since poor liver dihydrofolate reductase activity could give rise to eventual saturation of the liver folate-monoglutamate pool with regular intake of doses well below that of the acute threshold dose (260–280 μg; 589–634 nmol) that has been noted to result in its subsequent appearance in plasma[17]. Such an hypothesis could go a long way to explaining observations of the systemic appearance of unmetabolised folic acid observed in both fasting and non-fasting American subjects exposed to what is debatably quite a modest policy of mandatory folic acid fortification[64,70].

Though there have been a variety of concerns expressed regarding the potential negatives of mandatory fortification policies, some of these may arguably result from the generality of an inappropriate exposure to high concentrations of folate *per se*, rather than specifically to folic acid (pteroylmonoglutamic acid); the current exclusive fortificant folate form. The following discussions are solely restricted to potential concerns in human subjects for which there is either metabolic or direct observational arguments that derive uniquely from a systemic exposure to unmetabolised folic acid.

Potential masking of the anaemia of B_{12} deficiency

Effects on cognitive function. Concern that the US policy of mandatory fortification may to a significant degree may 'mask' the anaemia of vitamin B_{12} deficiency, primarily in the elderly population, appears to have been unwarranted as there is no evidence of an increase in the proportion of subjects with low vitamin B_{12} concentration but without anaemia[71]. Whether fortification at 280 μg/100 g flour (double that of the US), as proposed for the UK, would have an impact is unknown. A review on folic acid and cognition in

older persons, which recognised that there may be positive benefits derived from folic acid fortification, concluded that the potential harm is the greater concern[72].

A high intake of folate (mainly as a result of the intake of folic acid) has been associated with accelerated cognitive decline in older persons, with the hypothesis that this may be related to low vitamin B_{12} status[73]. In contrast, results from the Folic Acid and Carotid Intima-media Thickness (FACIT) Trial on B_{12} replete adults aged 50–70 years suggested that folic acid supplementation may improve domains of cognitive function that tend to decline with age[74]. A contemporary paper[75], examining American seniors exposed to mandatory folic acid fortification, confirmed that when vitamin B_{12} status is normal, high serum folate is associated with protection against cognitive impairment. However, in seniors with low vitamin B_{12} status it also confirmed the hypothesis that high serum folate is associated with cognitive impairment; with the suggestion that this effect may be due to unmetabolised folic acid in the circulation. Thus folic acid supplementation/fortification may be a 'double-edged sword' capable of exhibiting polar 'Jekyll and Hyde' characteristics, depending on vitamin B_{12} status. An accompanying editorial succinctly listed some of the challenging research questions that may need to be addressed if this dichotomous interaction between folic acid and B_{12} is confirmed[76].

Effect on cancer. Arguably, it is important here to distinguish between the relationship between (naturally-occurring) folate and cancer and the potential effects of appreciable concentrations of systemically-circulating unmetabolised folic acid and cancer. Low folate status may be a risk factor for cancer, possibly through uracil induced genomic instability[11] and/or altered methylation of DNA[12], and having an adequate folate status (achievable through sufficient intake of folate, including folic acid) may thus be beneficial.

As compared to the activity in fresh human cells *ex vivo*, similar cells cultured *in vitro* with folic acid as the source of folate can have their DHFR activity increased 100-fold or more[59]. If an up-regulation of DHFR activity can be also induced *in vivo* this may be accompanied by increased thymidylate synthase activity since the transcription of both these genes is co-regulated by the same E2F-1 transcription factor[77,78]. Mathematical modelling indicates that this would increase pyrimidine production (the rate-limiting step for DNA synthesis) without significantly affecting the rest of folate metabolism[79]. It could thus be hypothesised that, in contrast to an increased exposure to the naturally circulating folate (6*S*)-5-methyltetrahydrofolic acid, exposure to unmetabolised folic acid may increase cells' capacity for division, thus predisposing to an 'accelerating' effect which may be detrimental in the context of cancer. Of course if, with prolonged exposure to folic acid, DHFR is inducible in small intestine mucosa and liver then it could potentially somewhat mitigate systemic exposure to unmetabolised folic acid. However, direct evidence of the appearance of folic acid in serum of both fasting [64] and non-fasting[70] American adults many years after the 1998 introduction of mandatory folic acid fortification indicates that any such alleviation must be of limited consequence.

Recent research findings, in human subjects, suggest that folic acid supplements may increase the risk of multiple colorectal adenomas (Asprin–Folate Polyp Prevention Trial)[80] and that plasma folate concentrations are associated with colorectal cancer in a U-shaped manner such that high, as well as low, folate concentrations may increase colorectal cancer risk[81]. Additionally, a recent paper, reporting on a prospective study of US subjects, suggests that a high intake of folate 'generally attributable to supplemental folic acid' may increase the incidence of breast cancer in postmenopausal women[82].

In respect of hyperplasia, supplementation with folic acid at only 1 mg/d adversely increases the risk and rate of in-stent restenosis in men, and the need for target-vessel revascularisation[83]. For the vast majority of patients undergoing coronary intervention, stenting (as opposed to balloon angioplasty) was reported as the current method of choice; for which proliferation of smooth-muscle cells is one of the most important mechanisms leading to restenosis. It was concluded that previously reported positive effects of folic acid on coronary restenosis appeared to be predominantly in patients who were treated with balloon angioplasty alone, where thrombus formation within the intimal cracks and vascular remodelling are of predominant importance to the process of restenosis; these changes being potentially more susceptible to the folate-induced effects of homocysteine lowering.

Effect on anti-folate chemotherapy. A further area of concern is the potential for negative effects on chemotherapy using the anti-folate drug, methotrexate. Its mode of action is to limit folate availability to cells by reducing (by substrate competition) the activity of dihydrofolate reductase, an enzyme whose gene could also conceivably be up-regulated through exposure to high concentrations of folic acid. *Post-hoc* analysis from two randomised, controlled studies has indicated that folic acid reduces the degree of improvement of methotrexate-treated rheumatoid arthritis patients[84]. Additionally, a recent study in a US population has indicated that high serum folate levels above about 50 nmol/litre (approximately 22 ng/ml), a similar concentration above which unmetabolised folic acid makes up about 15 % of total folate[70], significantly increases the failure of ectopic pregnancy treatment with single-dose methotrexate[85].

Effect on multiple births. We accept the conclusions of Li *et al.*, that previous claims, from European studies, that folic acid supplementation may increase the risk of multiple births may have been affected by the use of ovarian stimulation[86]. However, more and more individuals (currently about 14 % in Europe) seek medical advice for infertility, of which about half undergo *in vitro* fertilisation. It has recently been reported that high folate status (attributable to folic acid supplementation) increases the likelihood of multiple births after *in vitro* fertilisation, with its associated increased risks of maternal and infant mortality and morbidity, at a rate similar to that seen in the USA after mandatory folic acid fortification[87].

Effect on immune function. New research has indicated that the concentration of unmetabolised folic acid (but not the concentration of natural circulating folate, (6*S*)-5-methyltetrahydrofolic acid) correlates to a reduction in the cytotoxicity of natural killer cells.[64]. Since experimental and clinical evidence supports a role of natural killer cells in tumour cell destruction[88], and that they may be considered a first line of host defence against carcinogenesis, it was hypothesised that this would suggest another way in which excess folic acid (but not circulating high concentrations of 'natural' 5-methyltetrahydrofolic acid) might promote existing premalignant and malignant lesions.

Conclusions

Our recent results from stable-isotope-folate absorption studies and a re-appraisal of historical literature, strongly suggests that the initial site of folic acid biotransformation in human subjects is the liver, and not the mucosal absorptive cells of the upper small intestine. Such a conclusion invalidates the historical use of folic acid as a 'reference dose' in studies of the 'relative absorption' of reduced folates in man. It can be concluded that previous attempts to gauge the absorption of synthetically-produced 'nature-identical' (6*S*)-5-methyltetrahydrofolic acid (the natural circulating folate form, which arguably should be the form generally used as the 'reference folate' in most future acute and long-term folate-absorption studies), by expressing the plasma response 'relative' to that from an equal dose of folic acid, are untenable. This is not only because unlabelled folate test doses have been used, but because even the use of labelled folates and mathematical modelling cannot overcome the inherent flaw of using folic acid as the 'reference' folate, as we currently argue in this paper. It is suggested that the absolute absorption of oral physiological doses of (6*S*)-5-methyltetrahydrofolic acid in human subjects can only be estimated using ^{14}C-label protocols and accelerator MS, as it has for folic acid[23].

Additionally, since human subjects uniquely amongst mammals and birds have a reduced dihydrofolate reductase activity (and a poor ability to reduce folic acid), it is hypothesised that even a modest regular daily intake of physiological doses of folic acid could eventually saturate the preliminary liver folate-monoglutamate pool. This would result in the subsequent chronic appearance of unmetabolised folic acid in the systemic circulatory blood system which arguably, according to circumstance, increasingly looks as though it can induce polar 'Jekyll and Hyde' health effects. Before mandatory folic acid fortification is introduced to the UK, it is suggested that a thorough appraisal of all potential concerns that derive uniquely from the systemic circulation of unmetabolised folic acid should be addressed methodically to ascertain a true picture of risk/benefit of fortification.

Acknowledgements

The authors are supported by a core strategic grant from the UK Biotechnology and Biological Sciences Research Council. A. J. A. W., J. R. D. and P. M. F. contributed collectively to the final manuscript. No financial or personal interests are declared. The Institute of Food Research is a not-for-profit company with charitable status sponsored by the UK Biotechnology & Biological Sciences Research Council.

References

1. Perry J (1971) Folate analogues in normal mixed diets. *Br J Nutr* **21**, 435–441.
2. Scott JM & Weir DG (1976) Folate composition, synthesis and function in natural materials. *Clin Haematol* **5**, 547–568.
3. Suh J-R, Herbig AK & Stover PJ (2001) New perspectives on folate catabolism. *Annu Rev Nutr* **21**, 255–282.
4. Shane B (1995) Folate chemistry and metabolism. In *Folate in Health and Disease*, pp. 1–22 [LB Bailey, editor]. New York: Marcel Dekker.
5. Scott JM (1999) Folate and vitamin B_{12}. *Proc Nutr Soc* **58**, 441–448.
6. Anonymous (1991) Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. *Lancet* **338**, 131–137.
7. Czeizel AE & Dudas I (1992) Prevention of the first occurrence of neural tube defects by periconceptual vitamin supplementation. *N Engl J Med* **327**, 32–35.
8. Wald DS, Law M & Morris JK (2002) Homocysteine and cardiovascular disease: evidence on causality from a meta-analysis. *Br Med J* **325**, 1202–1208.
9. Casas JP, Bautista LE, Smeeth L, Sharma P & Hingorani A (2005) Homocysteine and stroke: evidence on a causal link from mendelian randomisation. *Lancet* **365**, 224–232.
10. Seshadri S, Beiser A, Selhub J, Jacques PF, Rosenberg IH, D'Agostino RB, Wilson PW & Wolf PA (2002) Plasma homocysteine as a risk factor for dementia and Alzheimer's disease. *N Engl J Med* **346**, 476–483.
11. Choi S-W & Mason JB (2002) Folate status: effects on pathways of colorectal carcinogenesis. *J Nutr* **132**, 2413S–2418S.
12. Blount BC, Mack MM, Wehr CM, MacGregor JT, Hiatt RA, Wang G, Wickramasinghe SN, Everson RB & Ames BN (1997) Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: Implications for cancer and neuronal damage. *Proc Natl Acad Sci USA* **94**, 3290–3295.
13. Sanderson P, McNulty H, Mastroiacovo P, McDowell IFW, Melse-Boonstra A, Finglas PM & Gregory JF lll (2003) Folate bioavailability: UK Food Standards Agency workshop report. *Br J Nutr* **90**, 473–479.
14. Chandler CJ, Wang TTY & Halsted CH (1986) Pteroylpolyglutamate hydrolase from human jejunal brush borders. *J Biol Chem* **261**, 928–933.
15. Strum WB (1979) Enzymatic reduction and methylation of folate following pH-dependant, carrier-mediated transport in rat jejunum. *Biochim Biophys Acta* **554**, 249–257.
16. Mason JB (1990) Intestinal transport of monoglutamyl folates in mammalian systems. In *Contemporary issues in clinical nutrition, 13. Folic acid metabolism in health and disease*, pp. 47–63 [MF Picciano, ELR Stokstad and JF Gregory, editors]. New York: Wiley-Liss Inc.
17. 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. *Am J Clin Nutr* **65**, 1790–1795.
18. Rogers LM, Pfeiffer CM, Bailey LB & Gregory JF (1997) A dual-label stable-isotopic protocol is suitable for determination of folate bioavailability: Evaluation of urinary excretion and plasma folate kinetics of intravenous and oral doses of [$^{13}C_5$] and [$^{2}H_2$]folic acid. *J Nutr* **127**, 2321–2327.
19. Gregory JF (2001) Case Study: Folate Bioavailability. *J Nutr* **131**, 1376S–1382S.
20. Steinberg SE, Campbell CL & Hillman RS (1979) Kinetics of the normal folate enterohepatic cycle. *J Clin Invest* **64**, 83–88.
21. Steinberg SE (1984) Mechanisms of folate homeostasis. *Am J Physiol* **246**, G319–G324.
22. Krumdieck CL, Fukushima K, Fukushima T, Shiota T & Butterworth CE (1978) A long-term study of the excretion of folate and pterins in a human subject after ingestion of ^{14}C folic acid, with observations on the effect of diphenylhydantoin administration. *Am J Clin Nutr* **31**, 88–93.
23. Clifford AJ, Arjomand A, Dueker SR, Schneider PD, Buchholz BA & Vogel JS (1998) The dynamics of folic acid metabolism

in an adult given a small tracer dose of ^{14}C-folic acid. *Adv Exp Med Biol* **445**, 239–251.
24. Bhandari SD & Gregory JF (1992) Folic acid, 5-methyl-tetrahydrofolate and 5-formyl-tetrahydrofolate exhibit equivalent intestinal absorption, metabolism and *in vivo* kinetics in rats. *J Nutr* **122**, 1847–1854.
25. Whitehead VM, Pratt R, Viallet A & Cooper BA (1972) Intestinal conversion of folinic acid to 5-methyltetrahydrofolic acid in man. *Br J Haematol* **22**, 63–72.
26. Smith ME, Matty AJ & Blair JA (1970) The transport of pteroylglutamic acid across the small intestine of the rat. *Biochim Biophys Acta* **219**, 37–46.
27. Selhub J, Brin H & Grossowicz N (1973) Uptake and reduction of radioactive folate by everted sacs of rat small intestine. *Eur J Biochem* **33**, 433–438.
28. Olinger EJ, Bertino HJ & Binder HJ (1973) Intestinal folate conversion. II. Conversion and retention of pteroylmonoglutamate by jejunum. *J Clin Invest* **52**, 2138–2145.
29. Tani M & Iwai Z (1983) High-performance liquid chromatographic separation of physiological folate monoglutamate compounds. Investigation of absorption and conversion of pteroylglutamic acid in the small intestine of the rat *in situ*. *J Chromatogr* **267**, 175–181.
30. Rosenberg IH (1976) Absorption and malabsorption of folates. *Clin Haematol* **5**, 589–618.
31. Whitehead VM & Cooper BA (1967) Absorption of unaltered folic acid from the gastro-intestinal tract in man. *Br J Haematol* **13**, 679–686.
32. Melikian V, Paton A, Leeming RJ & Portman-Graham H (1971) Site of reduction and methylation of folic acid in man. *Lancet* **2**, 955–957.
33. Gregory JF & Quinlivan EP (2002) *In vivo* kinetics of folate metabolism. *Annu Rev Nutr* **22**, 199–200.
34. Selhub J, Dhar GJ & Rosenburg IH (1983) Gastrointestinal absorption of folates and antifolates. *Pharmacol Ther* **20**, 397–418.
35. Gregory JF (1997) Bioavailability of folate. *Eur J Clin Nutr* **51**, S54–S59.
36. Perry J & Chanarin I (1970) Intestinal absorption of reduced folate compounds in man. *Br J Haematol* **18**, 329–339.
37. Perry J & Chanarin I (1972) Observations on folate absorption with particular reference to folate polyglutamate and possible inhibitors to its absorption. *Gut* **13**, 544–550.
38. Brown JP, Scott JM, Foster FG & Weir DG (1973) Ingestion and absorption of naturally occurring pteroylmonoglutamates (folates) in man. *Gastroenterology* **64**, 223–232.
39. Lucock MD, Wild J, Smithells RW & Hartley R (1989) *In vivo* characterization of the absorption and biotransformation of pteroylmonoglutamic acid in man: a model for future studies. *Biochem Med Metab Biol* **42**, 30–42.
40. Bower C, Stanley FJ, Croft M, De Clerk NH, Davis RE & Nicol DJ (1993) Absorption of pteroylpolyglutamates in mothers of infants with neural tube defects. *Br J Nutr* **69**, 827–834.
41. Markkanen T (1968) Absorption tests with natural folate material in controls and in gastrectomized patients. *Am J Clin Nutr* **21**, 473–481.
42. Bailey LB, Barton LE, Hillier SE & Cerda JJ (1988) Bioavailability of mono and polyglutamyl folate in human subjects. *Nutr Rep Int* **38**, 509–518.
43. Fenech M, Noakes N, Clifton P & Topping D (1999) Aleurone flour is a rich source of bioavailable folate in humans. *J Nutr* **129**, 1114–1119.
44. Prinz-Langenohl R, Bronstrup A, Thorand B, Hages M & Pietrzik K (1999) Availability of food folate in humans. *J Nutr* **129**, 913–916.
45. Konings EJM, Troost FJ, Castenmiller JJM, Roomans HHS, van den Brandt PA & Saris WHM (2002) Intestinal absorption of different types of folate in healthy subjects with an ileostomy. *Br J Nutr* **88**, 235–242.
46. Rychlik M, Netzel M, Pfannebecker I, Frank T & Bitsch I (2003) Application of stable isotope dilution assays based on liquid chromatography-tandem mass spectrometry for the assessment of folate bioavailability. *J Chromatogr B Analyt Technol Biomed Life Sci* **792**, 167–176.
47. Pentieva K, McNulty H, Reichert R, *et al.* (2004) The short-term bioavailabilities of (6*S*)-5-methyltetrahydrofolate and folic acid are equivalent in men. *J Nutr* **134**, 580–585.
48. Willems FF, Boers GHJ, Blom HJ, Aengevaeren WRM & Verheugt FWA (2004) Pharmacokinetic study on the utilisation of 5-methyltetrahydrofolate and folic acid in patients with coronary artery disease. *Br J Pharmacol* **141**, 825–830.
49. Pietrzik K, Hages M & Remer T (1990) Methodological aspects in vitamin bioavailability testing. *J Micronutr Anal* **7**, 207–222.
50. Wright AJA, Finglas PM, Dainty JR, Hart DJ, Wolfe CA, Southon S & Gregory JF (2003) Single oral doses of ^{13}C forms of pteroylmonoglutamic acid and 5-formyltetrahydrofolic acid elicit differences in short term kinetics of labelled and unlabelled folates in plasma: potential problems in interpretation of folate bioavailability studies. *Br J Nutr* **90**, 363–371.
51. Bates CJ, Kerry SJ & Bluck LJC (2004) Stable isotope-labelled vitamin C as a probe for vitamin C absorption by human subjects. *Br J Nutr* **91**, 699–705.
52. Bluck LJC, Jones KS, Coward WA & Bates CJ (2005) The 'anomolous' absorption of labelled and unlabelled vitamin C in man. *Br J Nutr* **93**, 627–632.
53. Mathews CK & Huennekens FM (1963) Further studies on dihydrofolate reductase. *J Biol Chem* **238**, 3436–3442.
54. Kok RM, Smith DEC, Dainty JR, van den Akker JT, Finglas PM, Smulders YM, Jacobs C & de Meer K (2004) 5-Methyltetrahydrofolic acid and folic acid measured in plasma with liquid chromatography tandem mass spectrometry: applications to folate absorption and metabolism. *Anal Biochem* **326**, 129–138.
55. Wright AJA, Finglas PM, Dainty JR, Wolfe CA, Hart DJ, Wright DM & Gregory JF (2005) Differential kinetic behaviour and distribution of pteroylglutamic acid and reduced folates: a revised hypothesis of the primary site of PteGlu metabolism in humans. *J Nutr* **135**, 619–623.
56. Zakrzewski SF & Nichol CA (1960) Evidence for a single enzyme reducing folate and dihydrofolate. *J Biol Chem* **235**, 2984–2988.
57. Jarabak J & Bachur NR (1971) A soluble dihydrofolate reductase from human placenta: purification and properties. *Arch Biochem Biophys* **142**, 417–425.
58. Nylen PA, Abelson HA, Whitehead VM, Dolnick B, Petersen DW & Kamen BA (1984) Quantitation and lack of dihydrofolate reductase (DHFR) in human tissue in comparison to cultured human and animal cell lines *in vitro* and *in vivo*. *Proc Amer Assoc Cancer Res* **25**, 309.
59. Kamen BA, Nylen PA, Whitehead VM, Abelson HT, Dolnick BJ & Pterson DW (1985) Lack of dihydrofolate reductase in human tumor and leukaemia cells *in vivo*. *Cancer Drug Deliv* **2**, 133–138.
60. Whitehead VM, Kamen BA & Beaulieu D (1987) Levels of dihydrofolate reductase in livers of birds, animals, primates and man. *Cancer Drug Deliv* **4**, 185–189.
61. Ganeshaguru K & Hoffbrand AV (1978) The effect of deoxyuridine, vitamin B_{12}, folate and alcohol on the uptake of thymidine and on the deoxynucleoside triphosphate concentrations in normal and megaloblastic cells. *Br J Haematol* **40**, 29–41.
62. Bailey SW, Syslo MC & Ayling JE (2002) An assay for dihydrofolate reductase in human tissues by HPLC with fluorometric detection. *FASEB J* **16**, A267 (abstr. # 217.4).
63. Bailey SW, Manilow MR, Hess DL, Duell PB, Upson BM, Graf EG, Ivin-Jones A, Syslo MC & Ayling JE (2003) Unreduced

folic acid in plasma of subjects consuming either folic acid or 5-methyltetrahydrofolate. *FASEB J* **17**, A311 (abstr. # 191.16).
64. Troen AM, Mitchell B, Sorensen B, *et al.* (2006) Unmetabolised folic acid in plasma is associated with reduced natural killer cell cytotoxicity among postmenopausal women. *J Nutr* **136**, 189–194.
65. Lavoie A & Cooper BA (1974) Rapid transfer of folic acid from blood to bile in man, and its conversion into folate coenzymes and into a pteroylglutamate with little biological activity. *Clin Sci Mol Med* **46**, 729–741.
66. Lin Y, Dueker SR, Follett JR, *et al.* (2004) Quantitation of *in vivo* human folate metabolism. *Am J Clin Nutr* **80**, 680–691.
67. Garratt LC, Ortori CA, Tucker GA, Sablitzky F, Bennett MJ & Barrett DA (2005) Comprehensive metabolic profiling of mono- and polyglutamated folates and their precursors in plant and animal tissue using liquid chromatography/negative ion electrospray ionisation tandem mass spectrometry. *Rapid Commun Mass Spectrom* **19**, 2390–2398.
68. Smith DEC, Kok RM, Teerlink T, Jacobs C & Smulders YM (2006) Quantitative determination of erythrocyte folate vitamer distribution by liquid chromatography-tandem mass spectrometry. *Clin Chem Lab Med* **44**, 450–459.
69. Lamers Y, Prinz-Langenohl R, Bramswig S & Pietrzik K (2006) Red blood cell folate concentrations increase more after supplementation with (6*S*)-5-methyltetrahydrofolic acid than with folic acid in women of childbearing age. *Am J Clin Nutr* **84**, 156–161.
70. Pfeiffer CM, Fazili Z, McCoy L, Zhang M & Gunter EW (2004) Determination of folate vitamers in human serum by stable-isotope-dilution tandem mass spectrometry and comparison with radioassay and microbiologic assay. *Clin. Chem.* **50**, 423–432.
71. Mills JL, Von Kohorn I, Conley MR, Zeller JA, Cox C, Williamson RE & Dufour DR (2003) Low vitamin B-12 concentrations in patients without anaemia: the effect of folic acid fortification of grain. *Am J Clin Nutr* **77**, 1474–1477.
72. Schneider JA, Tangney CC & Morris MC (2006) Folic acid and cognition in older persons. *Expert Opin Drug Saf* **5**, 511–522.
73. Morris MC, Evans DA, Bienias JL, Tangney CC, Hebert LE, Scherr PA & Schneider JA (2005) Dietary folate and vitamin B_{12} intake and cognitive decline among community-dwelling older persons. *Arch Neurol* **62**, 641–645.
74. Durga J, van Boxtel MPJ, Schouten EG, Kok FJ, Jolles J, Katan MB & Verhoef P (2007) Effect of 3-year folic acid supplementation on cognitive function in older adults in the FACIT trial: a randomised, double-blind, controlled trial. *Lancet* **369**, 208–216.
75. Morris MS, Jacques PF, Rosenberg IH & Selhub J (2007) Folate and vitamin B-12 status in relation to anemia, macrocytosis, and cognitive impairment in older Americans in the age of folic acid fortification. *Am J Clin Nutr* **85**, 193–200.
76. Smith AD (2007) Folic acid fortification: the good, the bad, and the puzzle of vitamin B-12. *Am J Clin Nutr* **85**, 3–5.
77. Slansky JE, Li Y, Kaelin WG & Farnham PJ (1993) A protein synthesis-dependent increase in E2F1 mRNA correlates with growth regulation of the dihydrofolate reductase promoter. *Molec Cell Biol* **13**, 1610–1618.
78. Obama K, Kanai M, Kawai Y, Fukushima M & Takabayashi A (2002) Role of retinoblastoma protein and E2F-1 transcription factor in the acquisition of 5-fluorouracil reistance by colon cancer cells. *Int J Oncol* **21**, 309–314.
79. Nijhout HF, Reed MC, Budu P & Ulrich CM (2004) A mathematical model of the folate cycle. *J Biol Chem* **279**, 55008–55016.
80. Cole BF, Baron JA, Sandler RS, *et al.* (2007) A randomised trial of folic acid for the prevention of colorectal adenomas. *JAMA* (In the Press).
81. Van Guelpen B, Hultdin J, Johansson I, Hallmans G, Stenling R, Riboli E, Winkvist A & Palmqvist R (2005) Low folate levels may protect against colorectal cancer. *Gut* **55**, 1461–1466.
82. Stolzenberg-Solomon RZ, Chang SC, Leitzmann MF, Johnson KA, Johnson C, Buys SS, Hoover RN & Zeigler RG (2006) Folate intake, alcohol use, and postmenopausal breast cancer risk in the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial. *Am J Clin Nutr* **83**, 895–904.
83. Lange H, Suryapranata H, de Luca G, Borner C, Dille J, Kallmeyer K, Pasalary MN, Scherer E & Dambrink J-HE (2004) Folate theraphy and in-stent restenosis after coronary stenting. *N Engl J Med* **350**, 2673–2681.
84. Khanna D, Park GS, Paulus HE, Simpson KM, Elashoff D, Cohen SB, Emery P, Dorrier C & Furst DE (2005) Reduction of the efficacy of methotrexate by the use of folic acid: post hoc analysis from two randomised controlled studies. *Arthritis Rheum* **52**, 3030–3038.
85. Takacs P & Rodriguez L (2005) High folic acid levels and failure of single-dose methotrexate treatment in ectopic pregnancy. *Int J Gynecol Obstet* **89**, 301–302.
86. Li Z, Gindler J, Wang H, Berry RJ, Li S, Correa A, Zheng J-C, Erickson JD & Wang Y (2003) Folic acid supplements during early pregnancy and likelihood of multiple births: a population-based cohort study. *Lancet* **361**, 380–384.
87. Haggarty P, McCallum H, McBain H, Andrews K, Duthie S, McNeill G, Templton A, Haites N, Campbell D & Bhattacharya S (2006) Effect of B vitamins and genetics on success of *in-vitro* fertilisation: prospective cohort study. *Lancet* **367**, 1513–1519.
88. Janeway CA, Travers P, Walport MJ & Capra JD (1999) *Immunobiology: the immune system in health and disease*, 4th ed. New York: Elsevier Science Ltd/Garland Publishing.

British Journal of Nutrition