Is there more to folates than neural-tube defects?

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The purpose of the present paper is to review our current understanding of the chemistry and biochemistry of folic acid and related folates, and to discuss their impact on public health beyond that already established in relation to neural-tube defects. Our understanding of the fascinating world of folates and C_1 metabolism, and their role in health and disease, has come a long way since the discovery of the B-vitamin folic acid by Wills (1931), and its first isolation by Mitchell *et al.* (1941). However, there is still much to do in perfecting methods for the measurement of folate bioavailability, and status, with a high extent of precision and accuracy. Currently, examination of the relationships between common gene polymorphisms involved in C_1 metabolism and folate bioavailability and folate status, morbidity, mortality and longevity is evaluated as a series of individual associations. However, in the future, examination of the concurrent effects of such common gene polymorphisms may be more beneficial.

Folic acid: Bioavailability: C_1 metabolism: Gene polymorphisms: Health and disease

Folate is a generic term for all B-vitamin compounds that exhibit a common vitamin activity based on the parent structure of folic acid (pteroyl-L-monoglutamic acid). Folates are essential for a wide range of biochemical pathways involving C_1 metabolism. In particular, folates play an essential role in cell replication and pregnancy because they are required for the synthesis of purines and pyrimidines, the building blocks of DNA. Marked protection against neural-tube defects has been shown in women supplemented periconceptionally with folic acid (MRC Vitamin Study Research Group, 1991; Czeizel & Dudas, 1992). The remethylation of homocysteine, a S-containing amino acid, intimately involves the metabolism of folate and other B-vitamins, notably vitamin B_{12}. Elevated plasma homocysteine, a consequence of marginal folate deficiency, is an emerging risk factor for vascular disease and certain cancers (Boushey *et al.* 1995; Mason, 1995).

The purpose of the present paper is to review our current understanding of the chemistry and biochemistry of the vitamin, and to discuss its impact on public health. It also identifies research areas where our knowledge is limited or inadequate.

Chemical forms, dietary sources and intake

Folic acid is the most oxidised and stable form of folate and consists of an aromatic pteridine ring linked through a methylene bridge to *p*-aminobenzoic acid, and then to one L-glutamic acid residue. It occurs rarely in nature, but is the form used for vitamin supplementation and food fortification because of its greater stability and lower cost. Folates occurring naturally in body tissues and foods are mainly 5,6,7,8-tetrahydro-pteroylpolyglutamates, which contain a fully-reduced pteridine ring together with additional glutamic acid molecules linked by γ-peptide bonds. The nutritional activity of these reduced polyglutamates is expressed as long as the essential subunit structure of folic acid remains largely intact. Additionally, folates are usually C_1 substituted at the N-5 (e.g. 5-methyl, 5-formyl) or N-10 (e.g. 10-formyl) positions, or have a single C bridge spanning these positions (e.g. 5,10-methylene, 5,10-methenyl). Thus, there are many chemical derivatives of folic acid that exhibit a common vitamin activity (as folates), and these have been well described (Wagner, 1996; Scott, 1999).

The main dietary sources of folate are leafy green vegetables, dairy products and cereal products, especially fortified breads and breakfast cereals. Mainly as a result of the increased consumption of such fortified products, there has been a gradual increase of about 14 % in folate intakes since 1980. The average folate intake from the household food supply in the UK in 1998 was 241 µg/d (Department of Health, 2000). An additional average of 29 µg/d was consumed outside the home, giving a final total average folate intake of 270 µg/d.

Abbreviations: 5-methyl THF, 5-methyltetrahydrofolic acid; MTHFR, methylenetetrahydrofolate reductase.
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In the UK a value for the recommended daily amount (which equates to the current reference nutrient intake) was set for folate in 1979 at 300 μg/d for adults (Department of Health, 1979). This value was later withdrawn because of scientific uncertainties in the food folate data used to calculate intakes. In 1991 the reference nutrient intake, which is sufficient to cover the needs of most population groups, was introduced for folate and set at 200 μg/d for all adults, rising to 300 μg/d during pregnancy and 260 μg/d during lactation (Department of Health, 1991). These reference nutrient intakes were estimated on the basis of the relationship between dietary intakes and markers of folate status such as serum or erythrocyte folate concentrations, and liver folate concentrations at post-mortem examination. However, the current reference nutrient intake does not take into account any differences due to genetic polymorphisms of common folate enzymes.

Fig. 1. C_1 metabolism, encompassing both homocysteine remethylation and thymidylate synthesis. SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; MS, methionine synthase; B-12, vitamin B_{12}; THF, tetrahydrofolic acid; MTHFR, methylenetetrahydrofolic acid; dUMP, deoxyuridine monophosphate; TS, thymidylate synthase.

Absorption and metabolism

Before absorption across the intestinal mucosa can take place, dietary folates (which exist predominantly as polyglutamates) need to be hydrolysed to the folate monoglutamate form in the gut lumen by a brush-border γ-glutamyl hydrolase or conjugase (folylpolyglutamate carboxypeptidase; Halsted, 1980). Human brush-border folate conjugase is a Zn-dependent exopeptidase that catalyses the stepwise hydrolysis of folate polyglutamates (Reisenauer *et al.* 1977). The mucosal cells of the proximal small intestine (jejunum) then take up these monoglutamates by a saturable active energy-dependent carrier-mediated process at physiological concentrations, and by passive diffusion at higher concentrations (Selhub *et al.* 1984). Many foods contain inhibitors of the intestinal brush-border folate conjugase enzyme and/or folate transport system, which can reduce the efficiency of absorption (Tamura & Stokstad, 1973; Butterworth *et al.* 1974; Babu & Srikantia, 1976).

The efficiency of deconjugation and absorption in the gut lumen can vary considerably with the folate form, the presence of other dietary constituents and various physiological factors. Absorption is not affected by the aging process (Bailey *et al.* 1984), but it is markedly influenced by pH, with a maximum at pH 6·3 and a sharp decline between 6·3 and 7·6 (Russell *et al.* 1979). Some foods, such as milk and other dairy foods, contain folate-binding proteins (Ghitis, 1967; Wagner, 1985). Small amounts of folate may also be synthesised by intestinal flora in the body, but in man (unlike the rat) this source of absorbable folate is only a very minor one.

Absorbed folate monoglutamates are converted to the 5-methyltetrahydrofolic acid (5-methyl THF) form during transit through the intestinal mucosa, before onward transport into the hepatic portal vein. From here 5-methyl THF is conveyed to the systemic plasma circulation via the liver, which is thought to initially remove a sizeable proportion of the absorbed dose ('first pass effect'; Steinberg *et al.* 1979). The only folate form usually entering the human circulation from intestinal cells is 5-methyl THF. A review by Selhub *et al.* (1983) concluded that the intestine is capable of both reduction and C_1 substitution (methylation) of physiological doses of
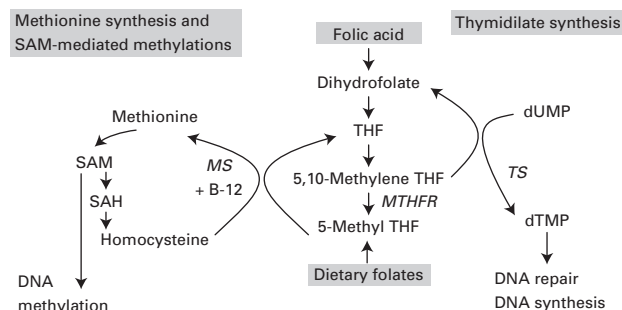
folic acid (e.g. from supplements and fortified foods) before it is transported from the mucosal side to the serosal side. However, this process is limited in capacity, and folic acid uptake is itself saturable, with an additional capacity for passive diffusion. As a result, if enough folic acid is ingested (>280 μg in one dose), unaltered folic acid may appear in the circulation (Kelly *et al.* 1997). With this exception, the folate form generally present in the systemic circulation is 5-methyl THF, which is then taken up by cells via folate transport systems. This form cannot be retained intracellularly, or used as a coenzyme, unless it is first metabolised by the vitamin B_{12}-dependent enzyme methionine synthase to the tetrahydrofolate form (see Fig. 1) and then converted to a polyglutamate. Circulating folic acid, from excess intake, can also be taken up by cells and subsequently utilised after it is initially reduced, via dihydrofolate, to the tetrahydrofolate form. In man folate is mainly stored in the liver (Whitehead, 1973; Hoppner & Lampi, 1980), which is assumed to contain 50 % of the normal total body folate content of 5–20 mg. Folate undergoes substantial entero-hepatic recirculation, with as much as 100 μg folate undergoing biliary re-excretion each day (Herbert & Das, 1993). Although much of this folate is reabsorbed by the small intestine (Weir *et al.* 1985), the efficiency of this re-absorption may be influenced by diet composition if digestion of foods happens to be concurrently in progress. Deficiencies of vitamin B_{12}, vitamin C, Fe and Zn can reduce the efficiency of folate utilisation. Folate absorption is also affected adversely by some drugs (anticonvulsants, anti-inflammatory and anti-cancer drugs, and some oral contraceptives; Institute of Medicine, 2000).

It is thought that the bioavailability of food folates averages about 50 % of that for folic acid (Gregory, 1997; Bailey, 1998). Although the bioavailability of folic acid taken in the form of supplements is high, when it is consumed in the form of fortified foods the bioavailability is thought to be lower and depends on the food vehicle used for fortification. An estimate of 85 % bioavailability from fortified foods was used to calculate the dietary folate equivalents in the USA (Bailey, 1998; Lewis *et al.* 1999). It was further calculated that folic acid taken in the form of

fortified foods, or taken with food, is 1·7 times more bioavailable than food folates.

However, recent studies using a dual-label stable-isotope protocol have suggested that some cereal-based vehicles (especially bran) may inhibit folate absorption, i.e. below the 85 % bioavailability used previously for vehicles (Finglas *et al.* 2002*b*). Another recent study has also found much higher absorption of spinach folate (79 %) relative to a folic acid supplement, using an ilesotomy model in which the difference between folate intake and the folate content of ileostomy effluents was used to calculate absolute absorption (Konings *et al.* 2002).

Methods for assessing folate bioavailability

Amongst short-term protocols, comparison of the serum or plasma response to a single oral folate test dose relative to that of a folic acid reference dose has often been used for the assessment of folate bioavailability in human volunteers (Gregory, 1997, 2001). Such protocols have varied, in that they have entailed either measurement of the rate of increase, or the maximum increase, in plasma folate concentration over a period of 2–3 h (Perry & Chanarin, 1972; Lucock *et al.* 1989; Bower *et al.* 1993; Kelly *et al.* 1997), or measurement of the rise in plasma folate concentration (the area under the curve) over a period of ≥ 7 h (Pietrzik *et al.* 1990; Prinz-Langenohl *et al.* 1999). These protocols have been criticised for their relative insensitivity, and because those protocols based on the rate of increase, or the maximum increase, may be flawed (Gregory, 2001). Additionally, it has not yet been confirmed whether the plasma response to a test dose derives, in its entirety, from the test dose.

The use of stable-isotope-labelling studies of folate bioavailability was developed on the premise that labelled folate molecules appearing in plasma or urine could only be derived from any labelled dose administered (Gregory & Toth, 1988). However, further work showed that the appearance of labelled folate in plasma was greater for intravenous folates than for oral folates. Hence, it was suggested that absolute bioavailability could not be determined readily because of a presumed extensive hepatic uptake of absorbed folates from the oral dose (Rogers *et al.* 1997). Methods were then developed to avoid this limitation, based on a single-dose dual-label approach in which two isotopically-labelled forms of folic acid (^{13}C and ^2H) are administered; one as an oral (^{13}C) dose and one as an intravenous (^2H) dose.

The percentage of the oral and intravenous doses excreted as intact folate in urine over a 24–48 h period was measured and then expressed as the urinary excretion ratio to estimate the fractional absorption of the oral dose (Rogers *et al.* 1997). However, the 'phenomenon' that some oral doses of folic acid exhibit a urinary excretion ratio higher than the theoretical maximum of 1·0 was also reported. Although it was confirmed later that the oral folic acid dose (absorbed and then transferred into the plasma as 5-methyl THF) and the intravenous folic acid dose (introduced into the plasma directly as folic acid) were handled differently, it was thought that this dual oral–intravenous approach would still have much merit once the circulating form of plasma folate,
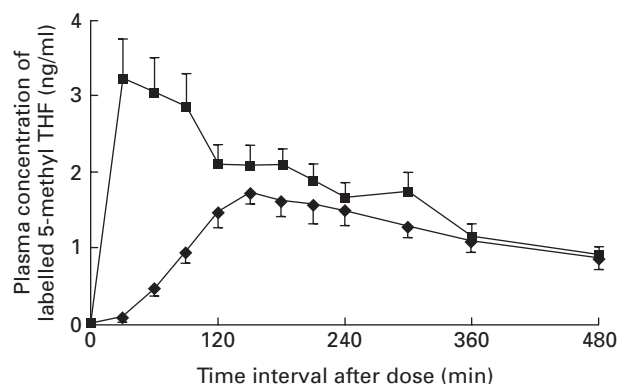

Fig. 2. Appearance of 5-methyltetrahydro[$^{13}C_6$]folic acid in plasma following single oral dosing (600 nmol) of fasted (12 h) human adult volunteers with 5-formyltetrahydro[$^{13}C_6$]folic acid (■); *n* 10) and [$^{13}C_6$]folic acid (◆; *n* 12). Values are means with their standard errors represented by vertical bars. The labelled plasma response to the oral dose of folic acid was slower, and peaked much later (173 (SE 10) min *v.* 41 (SE 9) min), than that to the 5-formyltetrahydrofolic acid test dose.

(6*S*-)5-methyl THF, was used for the intravenous dose (Finglas *et al.* 2002*b*).

The analysis of either isolated plasma or urine folate has been simplified recently. A previous, more cumbersome, GC–MS procedure that involved previous chemical cleavage of folates to their corresponding *p*-aminobenzoylglutamate and subsequent derivatization to a more volatile compound has been replaced by a more direct liquid chromatography–MS technique (Hart *et al.* 2002). Studies using direct measurement of plasma enrichment by the liquid chromatography–MS approach, combined with kinetic modelling of absorption curves, may offer an alternative for the improved quantification of folate bioavailability (Finglas *et al.* 2002*a*). Fig. 2 shows the appearance of 5-methyltetrahydro[$^{13}C_6$]folic acid in plasma following oral dosing of fasted (12 h) adult volunteers with either 5-formyltetrahydro[$^{13}C_6$]folic acid or [$^{13}C_6$]folic acid. The *in vivo* kinetics and absorption profile appears to be different for folic acid compared with 5-formyltetrahydrofolic acid, with the plasma area-under-the-curve response to folic acid being slower, and peaking much later, than the response to the 5-formyltetrahydrofolic acid test dose. This difference would lead to spurious conclusions in bioavailability studies based on comparisons of oral doses of reduced 'labelled' folate (e.g. labelled food folate) *v.* a 'labelled' folic acid reference dose. It is speculated that this phenomenon may be due to a slower rate of mucosal processing of folic acid (involving an initial two-step reduction, first to dihydrofolic acid and then to tetrahydrofolic acid, before methylation) compared with 5-formyltetrahydrofolic acid (methylation only). This difference may result in a slower transfer of 5-methyl THF metabolite to the plasma, which will, when coupled with a similar clearance rate to that of 5-methyl THF metabolite derived from oral 5-formyltetrahydrofolic acid, result in a comparatively smaller labelled plasma area under the curve.

Genetic aspects

Over recent years the enzyme methylenetetrahydrofolate reductase (MTHFR) has received much attention because of the presence of a common gene polymorphism within the population. This polymorphism is a thermo-labile enzyme variant that results in an enzyme activity of about 50 % of the normal value. It is inherited as an autosomal recessive trait and has been identified as a $677C \to T$ mutation resulting in an alanine to valine substitution (Frosst *et al*. 1995). It was found to be associated with increased plasma homocysteine and with possible increased risk of cardio-vascular disease (Kang *et al*. 1991). The MTHFR enzyme, which is dependent on FAD (a riboflavin coenzyme), provides the 5-methyl THF necessary for the vitamin B_{12}-dependent remethylation of homocysteine to methionine (Fig. 1). Thus, it is a crucial enzyme, as it can direct the folate pool towards homocysteine remethylation at the expense of DNA and RNA biosynthesis. The prevalence of the MTHFR $677C \to T$ variant is related to ethnicity; the frequency of homozygosity for the T allele (abnormal TT genotype) is approximately 10–15 % in the UK, 20–30 % in some Italian populations, but only a few percent in Afro-Americans (Schneider *et al*. 1998).

The concentration of erythrocyte folate, used as an index of body stores, varies according to MTHFR $677C \to T$ genotype, and appears to be related to the type of folate assay used. This relationship is explained by the different responses of the intracellular folate species to the various detection systems (Molloy *et al*. 1998), since it has been shown recently that formylated tetrahydrofolate polyglutamates may accumulate in 'TT' (MTHFR $677C \to T$) subjects at the expense of 5-methyl THF, the predominant form in other wild-type (CC) and heterozygous (CT) individuals. These formyl forms may cause re-direction of C_1 metabolism towards the more efficient maintenance of DNA repair under conditions of low folate intake or status (Bagley & Sehub, 1998), since they are more associated with purine and pyrimidine synthesis than with methylation. However, it is clear that there is a lack of heterogeneity within 'TT' subjects, as the percentage of formyl folate may vary from 0 % (i.e. 100 % 'normal' 5-methyl THF) to > 60 %.

Other 'common' inherited enzyme polymorphisms intimately involved in deconjugation, transport and C_1 metabolism are listed in Table 1. These polymorphisms include: a second MTHFR deficiency ($1298A \to C$); methionine synthase deficiency ($2756A \to G$), a vitamin B_{12}-dependent enzyme involved in remethylation of homocysteine to methionine (Silaste *et al*. 2001); methionine synthase reductase deficiency ($66A \to G$), a vitamin B_2-dependent enzyme which regenerates methionine synthase (Wilson *et al*. 1999; Gaughan *et al*. 2001; Rady *et al*. 2002). Additionally, there are 'common' polymorphisms in the

Table 1. Common polymorphisms in folate-related absorption and metabolism (adapted from Molloy, 2002)

Enzyme (gene mutation point)	Mutation (% allele frequency)	Wild-type (%)	Heterozygous defect (%)	Homozygous defect (%)	Known risks or phenotypic differences	Reference
Methylenetetrahydrofolate reductase ($677C \to T$)	T 38	CC 41	CT 41	TT 18	NTD, Down's syndrome, cleft lip or palate, low plasma or erythrocyte folate, high plasma homocysteine, riboflavin-dependent	Molloy *et al*. (1997), Schneider *et al*. (1998), Frosst *et al*. (1995), Kang *et al*. (1991), Chango *et al*. (2000)
Methylenetetrahydrofolate reductase ($1289A \to C$)	C 28	AA 53	AC 37	CC 9	Low plasma or erythrocyte folate, high plasma homocysteine, riboflavin-dependent	Dekou *et al*. (2001), Silaste *et al*. (2001)
Intestinal folate brush-border hydrolase ($1561C \to T$)	T 4	CC 92	CT 8	TT 0 (one in 625)	Low plasma folate, high plasma homocysteine (predict lower absorption)	Devlin *et al*. (2000)
Intestinal reduced folate carrier ($80A \to G$)	G 42	AA 35	AG 47	GG 18	No change in erythrocyte folate or plasma homocysteine (predict lower absorption)	Chango *et al*. (2000), Whetstine *et al*. (2001)
Methionine synthase ($2756A \to G$)	G 24	AA 59	AG 38	GG 3	High plasma folate and erythrocyte folate, reduced plasma homocysteine, vitamin B_{12}-dependent	Silaste *et al*. (2001), Chen *et al*. (2001)
Methionine synthase reductase ($66A \to G$)	G 48	AA 28	AG 49	GG 23	NTD, Down's syndrome, higher plasma homocysteine, riboflavin-dependent	Wilson *et al*. (1999), Gaughan *et al*. (2001), Rady *et al*. (2002)
Cystathionine β-synthase (844ins68)	8	84	16	0 (lethal?)	Higher plasma homocysteine? vitamin B_6-dependent	Tsai *et al*. (2001), Silaste *et al*. (2001)

intestinal folate brush-border hydrolase folylpolyglutamate carboxypeptidase (1561C→T; Devlin *et al*. 2000) and the intestinal reduced folate carrier (80A→G; Whetstine *et al*. 2001).

Although several common genetic polymorphisms in folate-dependent enzymes have been implicated in the development of homocysteinaemia, and increased risk of neural-tube defects, it is not known precisely to what extent these genetic variants contribute to low folate intakes and disease risk in the general population. However, despite the growing body of research on the post-absorptive implications of these polymorphisms, there is very little information available on their influence on folate bioavailability and recommended intakes to maintain an optimal status in a diverse population group.

Association of folate status and human health

A low circulating folate concentration gives rise to an elevated plasma homocysteine concentration, which is an independent risk factor for several types of vascular disease and stroke (Kang *et al*. 1992; Boushey *et al*. 1995; Alfthan *et al*. 1997; Vollset *et al*. 2001; Quere *et al*. 2002) and neuro-psychiatric disturbances, including depression and dementia (Botiglieri, 1996). In one of a few 'prospective studies' the risk in Finnish men in the top third of serum folate concentrations was only 30 % of the risk in the lowest third of serum folate concentrations (Voutilainen *et al*. 2000). Whilst elevated homocysteine has been accepted for some time as a 'risk marker', plausible metabolic mechanisms are beginning to emerge that may soon see its acceptance as a 'risk-factor'. It is currently suggested that elevated homocysteine may have direct proatherogenic effects mediated via cholesterol dysregulation (Li *et al*. 2002) and by the enhancement of monocyte and T-cell adhesion to human aortic endothelial cells (Koga *et al*. 2002). Although folic acid supplementation or fortification may be effective in lowering homocysteine concentrations, it is suggested that concurrent ingestion of vitamin B_{12} would be much more effective (Quinlivan *et al*. 2002).

Not only is low maternal folate status associated with increased risk for neural-tube defects (Daly *et al*. 1995) and cancer, e.g. colo-rectal cancer (Ryan & Weir, 2001), but so is the common 'TT' polymorphism in the MTHFR 677C→T gene (Ou *et al*. 1996), particularly when combined with low folate status (Christensen *et al*. 1999). Both MTHFR 677C→T and methionine synthase reductase 66A→G have been implicated in the incidence of Down's Syndrome (James *et al*. 1999; Hobbs *et al*. 2000).

The MTHFR 677C→T mutation may potentially have both positive and negative effects. It might enhance availability of methylenetetrahydrofolate in the DNA synthesis pathway, thus reducing misincorporation of uracil into DNA, which might otherwise result in double-strand breaks during uracil excision repair processes (Blount *et al*. 1997). Also, it might reduce both homocysteine remethylation and DNA methylation, which plays a role in genome stability and gene expression (Choi & Mason, 2000; Friso *et al*. 2002). The ultimate question, of course, is whether such a common mutation reduces overall longevity. As yet, there has been little study of this aspect, and no consensus. There is both evidence of no overall effect (Brattstrom *et al*. 1999), and conflicting evidence that it reduces longevity in men in middle and old age, but not in women (Heijmans *et al*. 1999).

Folic acid fortification

In the UK over half the pregnancies are unplanned, and recommendations for women to increase their folate intakes, together with uncertainties of folate bioavailability, are difficult to implement. In the USA the fortification of all enriched grain products, such as flour, has resulted in a decrease of about 20 % in the prevalence of neural-tube defects (Honein *et al*. 2001). It has also resulted in a population-wide increase in plasma and erythrocyte folate concentrations, together with a fall in plasma homocysteine of 48 % in subjects with an elevated homocysteine concentration of > 13 μmol/l (Lawrence *et al*. 1999; Jacques *et al*. 1999). In 1998, following fortification, there was a 3·4 % reduction in mortality from stroke and heart attack (Martin *et al*. 1999), which could possibly be linked to the fall in plasma homocysteine concentrations.

In the UK the Committee on Medical Aspects of Food and Nutrition Policy (Department of Health, 2000) has recommended a fortification policy of 2400 μg/kg flour, but this recommendation has not yet been implemented. The potential risk–benefits of introducing mandatory fortification of flour were recently reviewed (Wright *et al.* 2001) and it was considered that there are still a number of issues to be resolved; such as claims of an increased risk of dichorionic twin pregnancies and increases in MTHFR 667C→T gene frequency in newborns. Although folic acid is considered to be a very safe compound with no toxicity threshold (Campbell, 1996), it has been estimated that 20 % of young children may exceed the newly-recommended tolerable upper intake level of 1 mg/d for their age-group (Lewis *et al*. 1999).

Conclusions

The discovery that folic acid supplementation before conception markedly reduces the risk of neural-tube defects represents a major achievement since the vitamin was discovered > 70 years ago. There has been considerable progress in understanding the role of folic acid in health and disease, but there is still much to do in perfecting methods for measuring folate bioavailability, and status. Optimal folate status may confer a protective effect against many chronic diseases, and increasing folate intakes may be expected to reduce the prevalence of these folate-related diseases. However, the bioavailability of different folate vitamers is still not well understood. There are still a number of outstanding research issues that need to be resolved before a re-evaluation of optimal folate intakes can be undertaken. These issues include: (1) is folate status increased best by the intake of folate supplements, fortified foods or natural food folates; (2) is optimal folate status easily achievable in countries that do not permit the folic acid fortification of foodstuffs; (3) what are the health consequences of some sections of the population consuming large amounts of folic acid.

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