Is folic acid good for everyone?[1,2]

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ABSTRACT

Fortification of food with folic acid to reduce the number of neural tube defects was introduced 10 y ago in North America. Many countries are considering whether to adopt this policy. When fortification is introduced, several hundred thousand people are exposed to an increased intake of folic acid for each neural tube defect pregnancy that is prevented. Are the benefits to the few outweighed by possible harm to some of the many exposed? In animals, a folic acid–rich diet can influence DNA and histone methylation, which leads to phenotypic changes in subsequent generations. In humans, increased folic acid intake leads to elevated blood concentrations of naturally occurring folates and of unmetabolized folic acid. High blood concentrations of folic acid may be related to decreased natural killer cell cytotoxicity, and high folate status may reduce the response to antifolate drugs used against malaria, rheumatoid arthritis, psoriasis, and cancer. In the elderly, a combination of high folate levels and low vitamin B-12 status may be associated with an increased risk of cognitive impairment and anemia and, in pregnant women, with an increased risk of insulin resistance and obesity in their children. Folate has a dual effect on cancer, protecting against cancer initiation but facilitating progression and growth of preneoplastic cells and subclinical cancers, which are common in the population. Thus, a high folic acid intake may be harmful for some people. Nations considering fortification should be cautious and stimulate further research to identify the effects, good and bad, caused by a high intake of folic acid from fortified food or dietary supplements. Only then can authorities develop the right strategies for the population as a whole. *Am J Clin Nutr* 2008;87:517–33.

KEY WORDS Folate, folic acid, vitamin B-12, fortification, supplements, cancer, antifolates, cognition, epigenetics, public health

INTRODUCTION

In its recent report, "Folate and Disease Prevention," the UK Standing Advisory Committee on Nutrition (SACN) has recommended (1) that mandatory fortification of flour with folic acid should be introduced, with certain conditions, in the United Kingdom to reduce the number of children born with neural tube defects (NTDs). The report of the SACN provides an expert assessment of available evidence, starting from the premise that folic acid can play a role in disease prevention. We do not challenge the established benefits of fortification with folic acid in the prevention of neural tube defects (2, 3), but we would like to ask a different question: Is the benefit to the relatively few mothers and children sufficient justification for exposing the entire population to an increased intake of folic acid? It has been estimated by the SACN that ≈77–162 NTD pregnancies would be prevented each year by fortification in the United Kingdom at a level of 300 μg folic acid/100 g flour. Thus, between 370 000 and 780 000 people in the United Kingdom will be exposed to extra folic acid for each infant saved. Can we be sure that, out of three-quarters of a million people, less than one person will not suffer serious harm, that $<$100 people will not suffer intermediate adverse effects, and that $<$1000 people will not suffer mild adverse effects?

It is now almost 10 y since mandatory folic acid fortification was introduced in the United States and Canada. Is there any evidence since then that an increased intake of folic acid might have caused harm in some people? The purpose of this article is to consider the theoretical basis for potential harm and to review selected observational studies that are consistent with possible harm; it is not meant to be a systematic review of the evidence, but rather to highlight issues for further discussion. We wish to stimulate an open debate on these issues, with a view to better informing policy makers in countries that are considering fortification. We will argue that what is urgently needed now is targeted research.

The plan of the article is as follows: after a brief review of the biochemistry of folates and a comparison of folic acid with natural folates, we will look at the evidence that elevated concentrations of folates might cause harm in relation to anemia, cognition, the balance between folate and vitamin B-12, natural killer (NK) cell activity, and cardiovascular disease. We will then review the likely dual role of folate in cancer, followed by a discussion of the interaction between folates and antifolate drugs. The field of epigenetics, in which the methylation of DNA and of histones plays a key role, will be surveyed, because we believe that this has the potential importance for our understanding of the role of folates in health and disease. We conclude with a brief discussion of what blood concentrations of folate might cause harm.

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BIOCHEMICAL ROLES OF FOLATES

Folates are cofactors and cosubstrates for biological methylation and nucleic acid synthesis and also function as regulatory molecules (**Figure 1**.) They act as mobile cofactors in several key enzymatic reactions, not being tightly bound to the apoenzyme, and carry one-carbon residues. In effect, folates are cosubstrates for the reactions they are involved in. The intracellular concentrations of the different folates are in general much lower than their Michaelis constant values for the enzymes, and so the rate or steady state of the reaction can change over quite a large range of cellular folate concentrations (4). Measurements of the concentration of plasma total homocysteine, which reflects the intracellular concentration of homocysteine, can be used as a surrogate marker of the possible range. Homocysteine can be converted to methionine by methionine synthase, and 5-methyltetrahydrofolate (5-methyl-THF) is the cosubstrate that donates the methyl group. The concentration of plasma total homocysteine falls as plasma folate concentrations increase from <2 nmol/L to >15 nmol/L (5, 6). Thus, over at least this range, changes in the blood concentration of folates could influence the methylation potential of tissues of the body. There is no convenient marker of the status of nucleic acid synthesis, but it is likely that this can also vary over a similar range of plasma folate concentrations (4). For example, the misincorporation of uracil into DNA, because of the inadequate biosynthesis of thymidine, a folate-requiring step, is inversely related to the blood concentration of folate (7).

An additional function of cellular folates is as regulatory molecules that exert allosteric effects on several enzymes in the folate and methionine cycles, such as methylenetetrahydrofolate reductase (MTHFR), glycine-*N*-methyltransferase, and serine hydroxymethyltransferase (8–10). This regulatory role of folates is less well understood, but it will be influenced not only by the affinity of the enzymes for folate but also by the steady state concentration of folate in the cell (11).

Although the concentration of folates within cells is subject to many regulatory processes (9, 10), plasma concentrations of folates clearly influence the cellular concentrations of folates. Because many enzymes using folates have Michaelis constant values higher than the usual cellular concentration (4, 12), changing the dietary intake of folate, which will be reflected in plasma concentrations, will influence the functioning of processes in the cell that use folates.

Folates enter mammalian cells as monoglutamates, but are rapidly modified by the addition of 4–8 glutamate residues to form long side chains. Polyglutamation greatly increases the affinity of folates as both substrates of their own enzyme and inhibitors of other enzymes in the folate pathway (8, 13). Polyglutamation also constitutes a mechanism to trap folates within mammalian cells because the long-chain folylpolyglutamates are poorly accepted by the membrane carriers responsible for efflux across the cell membrane (9). The consequences of very high intracellular concentrations of folates are not known, but it may be significant that many folate-requiring enzymes are inhibited by excess substrate (4). Thus, we have a situation whereby modest increases in cellular concentrations of folates will activate several folate-dependent enzymes, whereas large increases in concentrations might actually inhibit these and related enzymes.

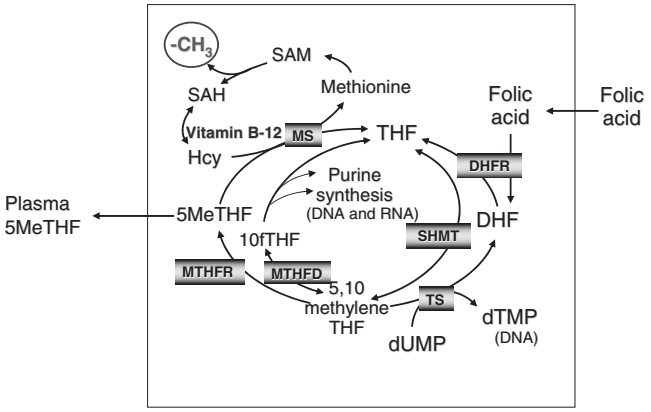

FIGURE 1. The methylation and folate cycles within the cytoplasm (simplified). The methionine cycle is shown at the top left: its function is to regenerate methionine from homocysteine (Hcy) so that the methyl group ($-CH_3$) can be donated, via *S*-adenosylmethionine (SAM), to the many acceptors in the cell (DNA, proteins, lipids, and metabolites). The interconnected folate cycles interconvert the different forms of reduced folates, and their function is to transfer one-carbon units to form methionine, purines, and thymidine. Enzymes: DHFR, dihydrofolate reductase; MS, methionine synthase; MTHFD, 5,10-methylenetetrahydrofolate dehydrogenase; MTHFR, 5,10-methylenetetrahydrofolate reductase; TS, thymidylate synthase. Intermediates: DHF, dihydrofolate; THF, tetrahydrofolate; 5Me, 5-methyl-; 10f, 10formyl-; SAH, *S*-adenosylhomocysteine; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate.

COMPARISON OF FOLIC ACID WITH NATURAL DIETARY FOLATES

The form of folate that is used in food fortification is synthetic and is called folic acid (pteroylmonoglutamate); it is different from the predominant forms of naturally occurring folates in our diet because it is in the oxidized state and contains only one conjugated glutamate residue (14). The folates that are used as coenzymes and regulatory molecules in the body are all in the reduced form (tetrahydrofolates; THF) and are mainly polyglutamated.

Folic acid has a substantially higher bioavailability than do natural folates, being rapidly absorbed across the intestine (15). Even in countries without mandatory fortification, some members of the population, including infants, have detectable unmetabolized folic acid in their blood, probably because of the voluntary fortification of foods (16) or intake of supplements containing folic acid (17). After mandatory fortification in the United States, unmetabolized folic acid is present in the blood of most individuals. A study conducted in the United States, carried out after fortification, found that 78% of fasting postmenopausal women had unmetabolized folic acid in the blood (18). Another study reported that unmetabolized folic acid accounts for 16% of the folates in the blood of persons whose total folates are >50 nmol/L (19). Both of these studies were conducted in small groups of people and need to be extended to larger populations.

Theoretically, folic acid could interfere with the metabolism, cellular transport, and regulatory functions of the natural folates that occur in the body by competing with the reduced forms for binding with enzymes, carrier proteins, and binding proteins. For example, the folate receptor has a higher affinity for folic acid than for methyl-THF—the main form of folate that occurs in the blood. The transport of folates into the brain is carried out by the folate receptor in the choroid plexus, and so folic acid in the blood

might inhibit the transport of methyl-THF into the brain. Transport of unmetabolized folic acid into cells can also occur via the folate receptor, as well as by several transporters (20), but little is known about the intracellular effects of folic acid itself. A recent report has described the down-regulation of folate transporters in the membranes of human intestinal and renal cells cultured with excess folic acid (21).

Folic acid is not a normal metabolite and must be reduced, first to dihydrofolate and then to tetrahydrofolate, probably in the liver (22), before it can enter the folate cycle (Figure 1). The same enzyme, dihydrofolate reductase (DHFR), catalyzes both these reactions but has been little studied in humans. It appears that the activity of DHFR is much lower in humans than in other animals (22–24) and that the activity varies markedly between individuals (24). Thus, it is possible that the plasma concentration of unmetabolized folic acid (and perhaps of dihydrofolate) will vary between individuals according to their DHFR activity.

Other unresolved issues include whether the increased uptake of folic acid rather than 5-methyl-THF during cell division could reduce the methionine supply at a critical time and whether the long-term intake of relatively high doses of folic acid can change gene expression of the folate-dependent enzymes or influence metabolic flux in the pathways involving one-carbon units.

IS THERE EVIDENCE THAT ELEVATED BLOOD FOLATE CONCENTRATIONS MAY CAUSE HARM?

Fortification will raise the concentration of total folates in the body, not just unmetabolized folic acid, above that occurring with normal diets (25, 26). In a significant proportion of the population, the concentrations are likely to be particularly high because of dietary habits. These groups include children and the elderly, for whom bread and breakfast cereals are a major part of their diet, and the increasing number who take multivitamin supplements (*see* section on folate levels below). The safe upper limit for the intake of folate is not known, but it is usually considered to be 1 mg/d for adults (27), and there is no consensus about a safe upper concentration of blood folate. Serum folate concentrations >45 nmol/L are often considered supraphysiologic. After folic acid fortification, such supraphysiologic concentrations were found in 23% of the US population, including 43% of children aged ≤5y and 38% of the elderly (26). There is little research directed at the question: Do these high concentrations of serum folate have any harmful effects? Some examples of possible harmful effects are given below. For quantitative comparisons, we have chosen one country, the United Kingdom, to illustrate some diseases that might be influenced by folate status in the body (**Table 1**).

Relation between folate and vitamin B-12: anemia and cognition

The concern most commonly raised (28) is that high folate concentrations will mask the hematologic signs of overt vitamin B-12 deficiency and lead to a missed diagnosis and to the subacute degeneration of the spinal cord; but, as discussed in the SACN report (1), this is likely to be very rare. Nevertheless, what perhaps should be considered is whether elevated blood concentrations of folate over a period of time might lead to undesirable consequences in persons with a low vitamin B-12 status (28). Theoretically, excess folic acid in persons with low vitamin B-12 status could bypass the metabolic block in nucleic acid synthesis (Figure 1), allowing cell division in bone marrow to continue and thus mask anemia. The consequence will be an increased demand for methyl groups by the growing cells and further depletion of the methylation potential, particularly in nondividing cells in the nervous system (29).

Potentially harmful effects of the high concentrations of blood folates that occur in a population exposed to mandatory folic acid fortification have been reported from the United States (51). Whereas elderly people with low vitamin B-12 status and normal folate status had a 70% increased risk of cognitive impairment, those with high folate (>59 nmol/L) and low vitamin B-12 status had an even higher risk of cognitive impairment (OR: 5.1; 95% CI: 2.7, 9.5) and anemia (OR: 5.2; 95% CI: 2.5, 11.0) than did the elderly who had normal vitamin B-12 and normal folate concentrations (52). These findings from a cross-sectional study are consistent with earlier reports on subjects with vitamin B-12 deficiency that even low doses of folic acid may aggravate the neurologic symptoms (53, 54) and that the severity of neurologic impairment increases with rising serum folate concentrations (55). However, in contrast with "masking" of anemia, the new findings (52) suggest that the high folate concentrations could also advance hematologic symptoms. Thus, one has to consider whether such high folate concentrations could impair normal folate function, not only in nerve cells but in proliferating cells as well.

One possible mechanism is that high concentrations of folic acid might act as a folate antagonist after the first step in its metabolism: conversion to dihydrofolate (Figure 1). Accumulation of this folate derivative in its polyglutamated form inhibits thymidylate synthase (56) and hence the formation of dTMP required for DNA synthesis. Dihydrofolate also inhibits the folate-requiring enzymes of purine synthesis (57). In rats, administration of folic acid after partial hepatectomy temporarily slowed DNA synthesis, a finding that was explained by a delay in the normal elevation of thymidylate synthase and thymidine kinase (58). Thus, folic acid may have a dual effect and either inhibit or facilitate normal DNA synthesis by entering the folate cycle outside the normal pathways.

Dihydrofolate is also a potent inhibitor of MTHFR (59); therefore, high concentrations of folic acid could also inhibit the formation of 5-methyl-THF and lead to a decrease in methionine synthesis. In those with poor vitamin B-12 status, methionine synthesis is already compromised, so this mechanism would make it worse, possibly explaining the effect on cognition found by Morris et al (52).

A prospective study in the United States, conducted after fortification, has reported that a high intake of folate, estimated from questionnaires completed by >2000 persons in Chicago, is associated with a risk of cognitive decline in the elderly, especially in those who took vitamin supplements containing >400 μg folic acid/d (60). The rate of cognitive decline in the high-folate-intake group was slower in those who also took supplements containing extra vitamin B-12 than in those consuming the Recommended Dietary Allowance (RDA) for vitamin B-12. This is consistent with the cross-sectional findings of Morris et al (52) that high folate is a risk in persons with a low vitamin B-12 status. On the other hand, other prospective studies on folate intake in the United States (61) and on serum folate concentrations in Italy (62) in the elderly found that low folate intake or status is a risk factor for dementia. In the latter 2 studies, it would be valuable to know the vitamin B-12 status of the populations. In addition, it is

TABLE 1
Some diseases that may be influenced by dietary folate status[1]

Disease	Incidence or prevalence in UK (population: 60 million)	Effect of increasing folate status	Nature of evidence	Key recent references on folate
Neural tube defects	800 pregnancies/y (1)[2]	Protective; may reduce NTDs by up to 162/y (1)	Randomized trials of folic acid; population studies	(1, 3)
Neoplastic disease				
Colorectal polyps	22% prevalence in 55–64-y-olds (30), ≈1.4 million	Dual effects: protective and facilitatory	Prospective cohorts and trial of folic acid	(31, 32)
Colorectal cancer	34 400/y	Dual effects	Prospective cohorts; population study	(31) (33, 34)
Breast cancer	43 000/y	Dual effects	Prospective cohorts	(35, 36)
Prostate cancer	32 800/y	Dual effects	Prospective cohorts and trial of folic acid	(32, 37, 38)
Antifolate drugs				
Antifolate drug use	>10 million prescriptions/y for methotrexate; >6 million prescriptions/y for trimethoprim (antibacterial)	May reduce efficacy of, or increase resistance to, methotrexate and theoretically all other antifolates (anticancer, antimalarial, and antibacterial)	In vitro studies of cancer; clinical trials (*see* below)	(39, 40)
Rheumatoid arthritis	0.8% prevalence, 500 000 (41)	Folic acid may modify response to antifolates	Randomized trials	(42)
Psoriasis	1.5% prevalence, 900 000 (43)	Folic acid may modify response to antifolates	Randomized trial	(44)
Cardiovascular disease				
Stroke	97 000/y (45)	Protective	Meta-analysis of trials; population study	(46, 47)

[1] Cancer incidence data from Office of National Statistics, 2004 (48). Heart disease was omitted because the evidence is conflicting (49, 50).
[2] This is the middle of an estimate made by Standing Advisory Committee on Nutrition, allowing for underreporting; the actual number of reported NTD pregnancies averaged 472/y from 2000 to 2004 (1).

also possible that the harmful effect of high folate intake in the Chicago study is related to a combination of folic acid fortification and a high intake of folic acid–containing supplements (51). A recent clinical trial showed that those who took folic acid (0.8 mg/d) for 3 y showed improved performance on certain cognitive tests compared with those who took placebo (63). It is notable that subjects were excluded from this trial if they had poor vitamin B-12 status.

After folate fortification in the United States, the proportion of elderly in the cohort studied by Morris et al (52) with a high folate, low vitamin B-12 status is ≈4%. If the same proportion of all elderly in the United States is affected, then ≈1.8 million elderly might be at increased risk of cognitive impairment and anemia because of an imbalance between folate and vitamin B-12. In Canada, the proportion of elderly women that had high serum folate (>45 nmol/L) and vitamin B-12 insufficiency (<165 pmol/L) is 0.61% (64). If similar proportions occur in the United Kingdom after folic acid fortification, then 25 000–170 000 elderly would have this particular combination, and so may be potentially at high risk of anemia and cognitive impairment (52).

Maternal vitamin B-12 and folate

In India, most of the population eats a vegetarian diet. Such a diet lacks not only vitamin B-12 but also often methionine. The Pune Maternal Nutrition Study from India has shown a possible adverse effect of high maternal folate status (65). It was found that the children (aged 6 y) whose mothers, during pregnancy, had a high blood folate concentration had a greater total fat mass than did the children of mothers with lower folate concentrations. Furthermore, children at 6 y of age whose mothers had a combination of high blood folate and low vitamin B-12 concentrations during pregnancy were at greater risk of insulin resistance. This important, though so far isolated, finding raises the possibility that an imbalance between folate and vitamin B-12 during pregnancy could influence imprinting in the embryo, perhaps by an effect on DNA methylation (*see* section on epigenetics below).

Natural killer cell cytotoxicity

A report by Troen et al (18) studied an index of immune function, NK cell cytotoxicity, in postmenopausal women in the United States after folic acid fortification. NK cells are an important part of the nonspecific immune response and can kill tumor cells and virally infected cells. In this study, the authors found an inverse U-shaped relation between total folate intake and NK cytotoxicity. Women in the bottom tertile of dietary intake of folates (<233 μg/d) who took daily supplements containing up to 400 μg folic acid displayed better immune function than did those who took no supplementary folic acid, but women whose dietary folate intake was ≥233 μg/d and who took >400 μg/d in supplements had impaired NK cytotoxicity. Although there was no relation between total plasma folates and NK cytotoxicity, there was a highly significant inverse linear association between the amounts of unmetabolized folic acid in plasma and NK cytotoxicity, particularly in women older than 60 y. The only important sources of folic acid are from fortified foods and dietary supplements. These findings raise the hypothesis that

excess folic acid from supplements or fortification could impair normal immune function. This hypothesis could, and therefore should, be tested.

Cardiovascular disease

Despite evidence from observational epidemiology indicating that low folate concentrations are a risk factor for cardiovascular disease (49), the evidence from clinical trials is not consistent. One trial found that folic acid (together with vitamins B-6 and B-12) reduced the need for revascularization interventions in patients who had had balloon angioplasty (66), whereas another study found that a similar treatment (including 1.2 mg/d folic acid) in patients who had coronary stents increased the degree of restenosis in men, although there was a tendency in the opposite direction for women, diabetics, and those with hyperhomocysteinemia (67). Results from 3 large trials of homocysteine-lowering B vitamins (including folic acid) in cardiovascular disease have now been reported (68–70) with overall negative results, except for a reduction in stroke (46, 69, 71). In 2 of the trials, a trend for an increase in cardiac events was found in patients treated with a combination of folic acid (0.8 mg/d) and vitamins B-12 and B6 (70) or with folic acid (1 mg/d) alone (32). None of these trials has convincingly shown that folic acid and other B vitamins will be beneficial for heart disease. Two of the trials raise a note of caution about the use of folic acid (67, 70). Because the trials to date have been underpowered to show any effect on cardiac outcomes (72), we will have to wait for a meta-analysis of the results from recent and ongoing trials before drawing conclusions about the safety, as well as appropriate doses, of folic acid in the prevention of cardiovascular disease.

DOES FOLATE PLAY A DUAL ROLE IN CANCER?

Folate is critically required for cell division and growth because it is a cofactor in the de novo synthesis of purines and thymidylate and thus in nucleic acid synthesis (Figure 1). It is also required for DNA repair processes. In cancer cells, where DNA replication and cell division occur at a rapid rate, removal of folate or a blockade of its metabolism causes inhibition of tumor growth. This is the basis of the use of antifolate drugs in cancer chemotherapy (*see* below). It therefore seems paradoxical that epidemiologic evidence suggests that raised intakes or concentrations of folate protect against the development of several different types of cancer (73–75). The epidemiology is supported by evidence from in vitro, animal, and human studies showing that low folate status is associated with DNA strand breaks, impaired DNA repair, increased mutations, and aberrant DNA methylation. Some of these defects can be corrected or prevented by folate supplementation (76, 77). However, animal studies on colorectal cancer have shown that the timing and dose of folate intervention are critical:

1) If folate supplementation is started before the establishment of neoplastic foci, the development and progression of the tumor is suppressed.
2) If folate supplementation is started after the neoplastic foci are established, it enhances their growth and progression (78–80).

Thus, it appears that folate plays a dual role: it may protect against the initiation of cancer, but facilitate the growth of preneoplastic cells (**Figure 2**) (33, 73–75, 81).

Extension of the concept of a dual role for folate in carcinogenesis in humans is subject to all the usual caveats. The

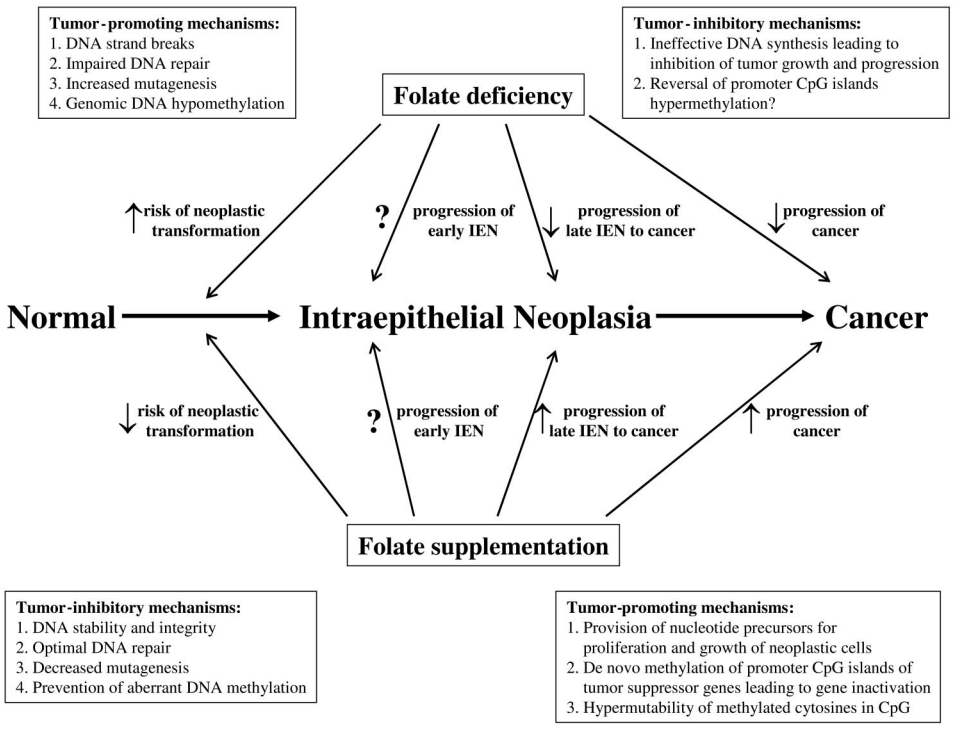

FIGURE 2. Postulated dual modulatory role of folate in carcinogenesis. IEN, intraepithelial neoplasia.

epidemiologic evidence of a protective role of folate intake against the development of cancer (82–85) is largely based on estimates of folate intake from questionnaires and thus is subject to multiple potential confounders (86). There have been fewer prospective studies of the association between the blood concentrations of folate and later development of cancer. Although these studies are also subject to confounding, they are closer to the disease process than food-frequency questionnaires. Accepting the body of evidence of a protective effect of folate under certain conditions, we asked the question: Is there any evidence of a harmful effect of elevated blood concentrations of folate in any of the human studies?

It should be recalled that the introduction of antifolate drugs to cancer therapy by Farber followed the observation that administration of folic acid in the form of pteroyltriglutamate to children with acute leukemia led to an acceleration of the disease process (87). Some modern studies on folate concentrations and cancer illustrate the complexity of the association. Whereas the New York Women's Health Study, carried out before fortification, found that concentrations of serum folate >31 nmol/L were associated with a reduced risk of colorectal cancer (88), a study of Finnish male smokers found no significant association with serum folate, although there was a tendency for an increased risk of rectal cancer in those with folate concentrations in the second to fourth quartiles (89). A striking result has been reported from a large population-based, nested, case-control study in Sweden: a significant association was found between plasma folate concentrations and colorectal cancer, with the lowest concentrations being protective and higher concentrations being related in a bell-shaped manner to increased risk (90). The novel finding in this study was that low folate status may inhibit colorectal carcinogenesis in humans, whereas high folate status might promote carcinogenesis (74).

Some other recent studies also report associations between elevated folate status and cancer. Another Swedish study found that folate concentrations in the top quartile were associated with an increased risk of prostate cancer in men older than 59 y who had been followed up for >4.9 y (OR: 2.0; 95% CI: 1.1, 3.7) (38). Results from a large prospective study of 24 500 postmenopausal women show a 19% higher risk of breast cancer in women who reported taking supplementary folic acid (>400 μg/d) and a 32% increased risk in women in the highest quintile of total folate intake (>853 μg/d) (35). It is noteworthy that no association was found in the latter study between breast cancer and folate derived solely from food. Furthermore, the women in this study had a higher total folate intake (≈660 μg/d), particularly from folic acid–containing supplements, than did other cohorts (≈300–350 μg/d), in whom a protective association with folate intake was found (36, 75, 91). In the Nurses' Health Study, there was a trend for an association between ovarian cancer and increased intake of total folate (but not dietary folate) in 80 254 women followed-up for up to 22 y: the multivariate RR of cancer for women in the top quintile versus those in the bottom quintile was 1.21 (95% CI: 0.94, 1.63; P for trend 0.05), but this risk was attenuated when further adjusted for intake of other nutrients (92).

More definitive evidence of beneficial or harmful effects of folic acid on carcinogenesis will come from large-scale and long-term randomized trials, but most of these have not yet been completed. Smaller trials for shorter periods have produced inconclusive results (82). A follow-up of a trial of folic acid (5 mg/d) in pregnancy, although not originally designed to study cancer, showed a higher rate of deaths from cancer (OR: 1.7; 95% CI: 1.06, 2.72) and a trend for higher breast cancer deaths (OR: 2.02; 95% CI: 0.88, 4.72) (93). Two large trials of B vitamins in relation to cardiovascular disease have also reported outcomes for cancer. The NORVIT trial originally reported (94) a marginally significant increase in incident cancer (RR: 1.4; 95% CI: 1.0, 2.0; $P = 0.08$) in those treated with folic acid (0.8 mg) and vitamin B-12 (0.4 mg) for 40 mo, but in the final article, the risk ratio was reported to be 1.22 (95% CI: 0.88, 1.70) (70), probably because the national cancer registry was used for case ascertainment in the latter report. A similar nonsignificant trend was reported for colon cancer in the Heart Outcomes Protection Evaluation-2 trial (69), in which the RR was 1.36 (95% CI: 0.89, 2.8) in those treated with a combination of 2.5 mg folic acid, 1 mg vitamin B-12 and 50 mg vitamin B-6 for 5 y. It will be important to monitor the future incidence of cancer in these trial subjects.

The results from the first randomized trial of folic acid for the prevention of colorectal cancer in genetically predisposed patients (32) showed that treatment with folic acid (1 mg/d) for up to 6 y did not prevent the recurrence of colorectal adenomas. On the contrary, at the second follow-up, there was a 67% increased risk of advanced lesions with a high malignant potential (RR: 1.67; 95% CI: 1.00, 2.80), along with a >2-fold increased risk of having ≥3 adenomas (RR: 2.32; 95% CI: 1.23, 4.35). A possible explanation for this result is that folic acid might have promoted the progression of already existing, undiagnosed preneoplastic lesions (eg, aberrant crypt foci or microscopic adenomas) or adenomas missed on initial colonoscopy in these patients at a high risk of developing colorectal cancer. Another unexpected secondary finding from this trial was that the risk of cancers other than colorectal cancer was significantly increased in the folic acid–supplemented group ($P = 0.02$); this was largely due to an excess of prostate cancer ($P = 0.01$) (32). The mean age of the study participants was 57 y (64% were men); therefore, it is highly likely that some of the male participants harbored precursor lesions in the prostate, which progressed more rapidly with folic acid supplementation. Thus, overall, the randomized clinical trials of folic acid tend to show an increase in incident cancer and preneoplastic lesions.

What is the effect of the dramatically increased folate status resulting from mandatory folic acid fortification and supplementation on cancer incidence in the United States and Canada? To address this important public health concern, Mason et al (34) examined a temporal trend of colorectal cancer incidence in the United States and Canada after fortification using 2 data sets from these countries: the Surveillance, Epidemiology and End Result registry and Canadian Cancer Statistics, respectively. Their analysis demonstrates that, concurrent with folic acid fortification, the United States and Canada experienced abrupt reversals of the downward trend in colorectal cancer incidence that the 2 countries had enjoyed in the preceding decades. Absolute rates of colorectal cancer began to increase in 1996 (United States) and in 1998 (Canada) and reached a maximum in 1998 (United States) and in 2000 (Canada), and rates have continued to exceed the pre-1996/1997 trends by 4 to 6 additional cases per 100 000 individuals, ie, some 15 000 extra cases per year. These investigators hypothesized that the institution of folic acid fortification may have been wholly or partly responsible for the observed changes in colorectal cancer rates in the late 1990s. Changes in the rate of colorectal cancer screening endoscopic procedures do not seem to account for this increase in colorectal cancer incidence.

However, because there was no control group and because it was impossible to completely control for all potential confounders inherent in the 2 data sets, these observations do not prove a causal link between folic acid fortification and increased rates of colorectal cancer in North America in the late 1990s.

We suggest that the results reviewed above are consistent with the concept that folates play a dual role in human carcinogenesis (33, 73, 75), as illustrated in Figure 2, but additional explanations for the apparent contradictions are also possible. For example, it is biologically plausible that any effect of folate on carcinogenesis will interact with a large number of other risk factors and that the patterns of these risk factors will differ between individuals. Observational studies have identified many factors, apart from age and sex, that might interact with folate in cancer risk, including vitamin B-12 (95, 96), alcohol (35), smoking (97), and polymorphisms in genes coding for enzymes related to one-carbon metabolism (98–100). The genotype can change a protective effect of folate into a harmful effect. For example, Ulrich et al (101) reported that a folate intake >400 μg/d was associated with a reduced risk of colorectal adenomas in those homozygous for the 3-repeat polymorphism in the promoter region of the gene for thymidylate synthase. In contrast, the same folate intake was associated with an increased risk in those homozygous for the 2-repeat polymorphism. A striking example of the interaction of risk factors comes from a Norwegian study of the occurrence of preneoplastic high-risk adenomas and hyperplastic polyps in the colorectum (102). In the entire cohort, the risk of adenomas was inversely related to the red blood cell folate concentration, with an OR of 3.05 (95% CI: 1.34, 6.96) for the bottom tertile versus the top tertile, consistent with a protective effect of folate status on colorectal cancer. Overall, there was no association between the *MTHFR* 677C→T genotype and adenomas, but 2 distinct high-risk groups were found when the cohort was divided into those with folate intakes below and above the median and into smokers and nonsmokers. In smokers with low folate, those carrying the *T* allele had an increased risk (OR: 8.21; 95% CI: 2.4, 28.1), whereas in smokers with high folate the *T* allele was no longer a risk. In contrast, smokers with high folate status and with the *CC* genotype had a greatly increased risk (OR: 11.85; 95% CI: 2.86, 49.1). Another study has shown that sex and the presence of a truncating mutation in the tumor suppressor gene adenomatous polyposis coli (APC) interacts with folate intake in the risk of colon cancer (103). Higher folate intake was protective against colon cancer in men without the mutation (APC^-), but increased the risk in men with the mutation (APC^+): those in the top tertile of folate intake had an RR of 2.77 (95% CI: 1.29, 5.95) compared with those in the bottom tertile. No such associations were found for women. These findings are important, first because they could account for some of the discrepancies between different studies of folate and colorectal cancer. Second, these studies show that it is not justified to assume that the finding of a protective effect of high folate in a whole population necessarily applies to all people within that population.

Overall, the evidence reviewed above provides cause for concern that increasing folate levels in an entire population may, in some people, increase the risk of cancer. More research is needed to identify the genetic and nongenetic factors that interact with folate in the prevention and promotion of carcinogenesis.

WILL INCREASING THE CONCENTRATION OF BLOOD FOLATES MODIFY THE EFFECTS OF ANTIFOLATE DRUGS?

Drugs designed to interfere with enzymes in the metabolic pathways of folates are widely used in medicine (104), mainly for the treatment of cancer, rheumatoid arthritis, bacterial infections, malaria, psoriasis, and ectopic pregnancy.

From basic principles of pharmacology, it is clear that folates should antagonize the effects of most antifolates (39), and, indeed, a folate derivative (folinic acid) is often given as an antidote. However, rather little research has been directed at the question of whether raising the folate status in patients will modify the efficacy of antifolate drugs. In a review about antifolates in cancer chemotherapy, Robien (105) pointed out that no human studies have evaluated dietary folate intake as an effect modifier, and she expressed concern that folate may interfere with the effectiveness of the antifolate treatment and possibly support cancer growth. Clearly, more research is needed on this question, particularly because antifolates are widely used: for example, there are >10 million prescriptions for methotrexate alone each year in the United Kingdom. Methotrexate is particularly important in the treatment of childhood leukemias, of which there are nearly 500 new cases each year in the United Kingdom.

There are indications from noncancer chemotherapy that a patient's folate status may influence the response to methotrexate. Methotrexate is the most widely used disease-modifying drug for the treatment of rheumatoid arthritis, which affects ≈500 000 people in the United Kingdom (41). A post hoc analysis of 2 randomized trials found that patients who were taking 1–2 mg folic acid/d had a poorer clinical response to methotrexate (42), and it has been reported that patients with higher concentrations of red blood cell folates showed a poorer response to methotrexate (106). Supplementation with 5 mg folic acid/d reduced the effectiveness of methotrexate in the treatment of psoriasis in a randomized trial (44). Finally, a study of 50 women with ectopic pregnancies treated with methotrexate found that those with serum folate concentrations >20.7 ng/mL had a higher failure rate and needed more methotrexate (107). Although studies with lower doses of folic acid are needed to see whether the concentrations attained after fortification might antagonize the effects of methotrexate, it is noteworthy that in 2 of the reports, the poorer efficacy was related to higher blood concentrations of folates.

To date, there are few, if any, studies on how folate status affects the incidence of conditions such as psoriasis and rheumatoid arthritis, including the seriousness or frequency of an attack, or if treatment choice and drug efficacy differs according to folate concentrations. Such studies should be performed and could provide critical data about whether folic acid fortification is safe to use in sections of the population that are likely to use antifolate drugs.

Many antimalarial drugs are antifolates, and there is evidence of the reduced efficacy of these drugs after high-dose (2.5–5 mg/d) folic acid treatment (108). This is one of the possible reasons why iron plus folic acid supplementation in children in a population at high risk of malaria (eg, Tanzania), where malaria was treated with antifolates, was associated with an increased risk of severe illness and death, whereas no such increased risk was found in children in Nepal, where there is little malaria and the children were not treated with antifolates (109). It is not

known whether lower doses of folic acid have any effect on the efficacy of antimalarial drugs. A perhaps equally important question is whether improved folate status renders a subject at higher risk of getting malaria and, if already infected, influences the seriousness or frequency of an attack. We therefore suggest that these issues should be investigated in regions with malaria before folic acid fortification is introduced.

An important problem with the use of antifolates in chemotherapy is the development of drug resistance (110). Resistance can occur at many stages, from drug transport to increased expression of target enzymes or by metabolism of the drug. Increased folic acid levels could theoretically facilitate drug resistance at many of these sites (110). In vitro studies have shown that the cellular folate concentration is a determining factor in the sensitivity of cells to antifolates (40). Folic acid, being in the oxidized state, has to be reduced by DHFR before it can act as a cofactor (Figure 1). Indeed, many antifolate drugs are inhibitors of DHFR; therefore, folic acid will compete with them for the active site of the enzyme. Furthermore, increased concentrations of folates may lead to up-regulation of DHFR activity and thus to drug resistance. Another mechanism of resistance to antifolates is mediated by proteins of the multidrug resistance family; these transporters are up-regulated by exposure to elevated concentrations of folates and thus facilitate transport of antifolates out of the cell (111). This finding led Hooijberg et al (112) to state the following: "The existence of multi-drug resistance transporters implies that folate supplementation is a double-edged sword, which should be handled with care." A schematic representation of their hypothesis is shown in **Figure 3**.

In relation to proposals to fortify food with folic acid in the United Kingdom, we can carry out a "thought experiment," as follows. The prevalence of psoriasis is 1.5% of the population (≈0.9 million) and of rheumatoid arthritis is 0.8% (≈0.5 million) (41). Thus, ≈1.4 million people in the United Kingdom have psoriasis or rheumatoid arthritis or are at high risk of developing these conditions. A rate of only 1 in 100 of those with psoriasis or rheumatoid arthritis adversely affected by increased folate status corresponds to 14 000, or 230 per million UK subjects. Put simply, is it acceptable that for every NTD prevented, ≈200 people might suffer from an increased severity of their psoriasis or rheumatoid arthritis?

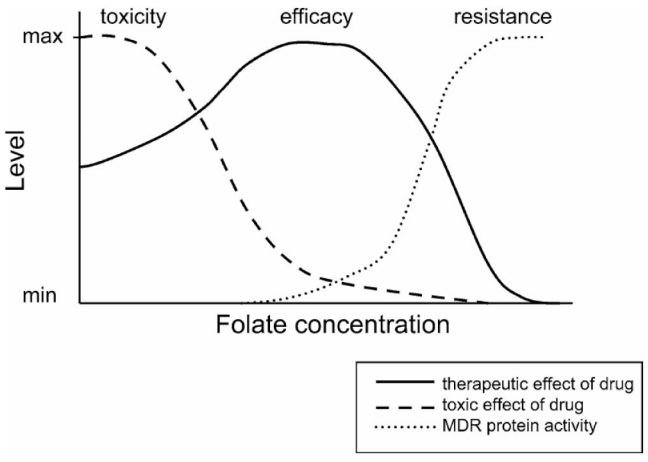

FIGURE 3. Therapeutic window for folate supplementation. With increasing concentration of folates administered during chemotherapy, the efficacy of single drugs or drug combinations may be improved; simultaneously, drug toxicity decreases. Overdose of folates, however, can induce multiple drug resistance, which decreases drug efficacy. Reprinted with permission from Hooijberg et al (112).

We conclude that there is some evidence that folates might modify the response to therapy by antifolate drugs, but that more research is needed to see whether the expected blood concentrations of folate after fortification have any influence on the response to antifolates in humans. Furthermore, we raise the question of whether increasing folate levels will influence the incidence or natural history of the many diseases that are sensitive to antifolates, ie, could persons with conditions such as rheumatoid arthritis or psoriasis become dependent on using a toxic drug such as methotrexate after folic acid fortification? We also raise the possibility that a low folate status may provide natural resistance to malaria and that fortification, therefore, could increase the risk of infection and the resistance to commonly used antimalarial drugs.

DOES FOLIC ACID HAVE ANY GENETIC OR EPIGENETIC EFFECTS?

One of the main functions of folate is as a cofactor that delivers one-carbon units for purine and thymidine synthesis, and adequate folate status is essential for nucleic acid synthesis and cell division. Low folate status in humans is associated with the misincorporation of uracil instead of thymine into DNA and with an increase in DNA strand breaks (7). Another key role of folate is in the provision of methyl groups for the conversion of homocysteine to methionine, which is incorporated into proteins and also has other important functions related to methylation reactions (Figure 1). The initiation of mitochondrial protein synthesis requires *N*-formylmethionyl-tRNA, and folate is necessary for the synthesis of the methionyl and formyl residues. Methionine can be converted to the methyl-group donor *S*-adenosylmethionine (SAM), a molecule with many functions (113–115), including methylation of cytosine residues in DNA (116) and of arginine and lysine residues in histones (117, 118), both of which are involved in regulating gene expression (119).

DNA methylation

In the mammalian genome, methylation only occurs on cytosine residues that occur 5' to a guanosine residue in a CpG dinucleotide. CpG dinucleotides are enriched in "CpG islands," which are found proximal to the promoter regions of about half the genes in the genome, but these are predominantly unmethylated. The other CpG dinucleotides are globally distributed and are normally heavily methylated. Methylation of the promoter-related CpG islands can suppress gene expression by causing chromatin condensation, whereas methylation of isolated CpG dinucleotides in coding regions can lead to mutations because methylcytosine residues are prone to hydrolytic deamination. DNA methylation is important as an epigenetic determinant of gene expression, in the maintenance of DNA integrity, in chromatin organization and in the development of mutations (120). Errors in normal epigenetic processes have been called epimutations, defined as epigenetic silencing of a gene that is not normally silenced or epigenetic activation of a gene that is normally silent (121). It is now believed that epimutations might underlie several human diseases. One cause of such mutations is the aberrant methylation of DNA and histones (122–125).

Low folate status is often associated with impairment of DNA methylation (126), but sometimes it leads to hypermethylation and thus could affect gene expression in complex ways (77, 122, 127, 128). It is not known whether an excess of folate might have any adverse effects on these functions. Folates often show strong substrate inhibition of the enzymes that make use of them, and modeling studies suggest that high folate levels could, under certain circumstances, have the same functional effect as low folate status (4). Other modeling studies imply that DNA methylation may be relatively protected from changes in folate status (11). More experimental evidence is needed on this point.

The influence of folate status on DNA methylation in animals and humans is likely to be tissue-, site-, and gene-specific (77, 128). Changing the folate status in humans has been shown to influence DNA methylation, but, despite much research, it is not yet established whether alterations in DNA methylation after changes in folate status are harmful in humans, for example, by regulating the expression of oncogenes or tumor-suppressor genes (77, 128–130). Often, both CpG island hypermethylation and genome-wide hypomethylation are found in the same tumor cells (128, 131). Hypermethylation of CpG islands in promoter regions of tumor-suppressor genes, which leads to gene silencing (epimutation), is as common in cancer as are mutations in these genes (120). In human colonic mucosa cells, methylation of a CpG island in the putative tumor suppression gene *ESR1*, which encodes estrogen receptor-α, occurs in an allele-specific manner. This is consistent with a role of hypermethylation in aberrant gene silencing, which could initiate neoplasia when both alleles are silenced (132). Notably, in the mouse, CpG island DNA methylation of *ESR1* in colonic cells can be increased in an age-related and dose-dependent manner by increasing levels of dietary folic acid (132). It is, of course, too simplistic to assume that elevated folate status will inevitably lead to hypermethylation of tumor-suppressor gene promoters because the control of methylation is complex, involving several different DNA-methyltransferases, histones, and other regulatory proteins (133–135). The key question is as follows: Does elevated folate status increase the probability of such hypermethylation?

An epigenetic effect of maternal diets rich in folic acid has been elegantly shown in the *agouti* mouse (136, 137), which displays marked phenotypic variation due to variable cytosine methylation in the transposon promoter region of the gene. When the promoter is fully active, ectopic *agouti* expression occurs in all tissues and results in mice with a yellow coat that have a tendency for obesity, cancer, diabetes, and a short life. Feeding the dams a methylation-rich diet that includes 2.5-fold more folic acid (and also extra vitamin B-12) results in progeny that are darkly mottled (**Figure 4**), leaner, and healthier and that have a normal life span. These changes are paralleled by increasing methylation of the promoter: methylated CpG sites occurred in 7% of the cells in the *agouti* mice, whereas methylated promoter CpG sites were found in 80% of the cells in the mice with the darkest coat color, who were born to dams fed the high-folate diet. There were strong correlations between dietary supplementation and the degree of methylation and between methylation and coat color (136, 137). Thus, merely supplementing a mother's already nutritionally adequate diet with extra folic acid, vitamin B-12, choline, and betaine can permanently affect the offspring's DNA methylation at an epigenetically susceptible locus and has a consequential impact on the phenotype.

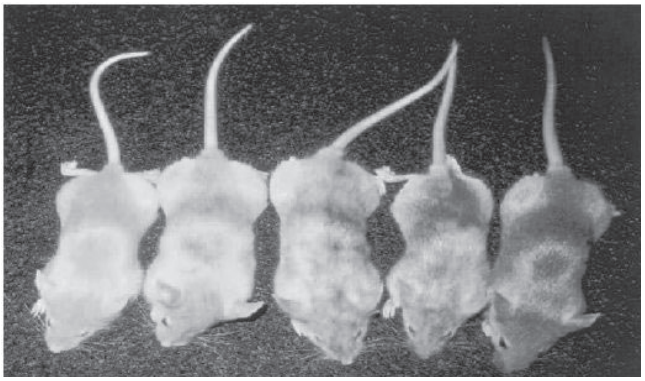

FIGURE 4. Genetically identical mice whose phenotype is determined by methylation of cytosine residues in the transposon promoter that regulates expression of the *agouti* gene. The yellow mouse on the left received a normal diet, whereas the mottled mice received supplements containing extra folic acid, vitamin B-12, choline, and betaine. The increase in methylation at the *agouti* promoter locus was graded in the mice from left to right. Reprinted with permission from Waterland and Jirtle (137).

A similar effect of a high-methylation maternal diet has been shown on the expression of the *AxinFused* epiallele in mice: progeny of mothers fed this diet had a lower probability of having a kinked tail than did progeny of mothers fed a standard diet. The suppression of the kinked tail was paralleled by increased CpG methylation in the promoter (138). It was recently shown that these diet-induced epigenetic changes can be transmitted to future generations (139, 140), which led the authors to speculate that "in light of the roughly 20-year generation time of humans, our results suggest that current dietary habits may have an influence on grandchildren who will be born decades from now, independent of the diets that their parents consume" (139).

Other animal studies have shown that the intake of folate by the mother can influence the development and health of her progeny. Mice carrying teratogenic mutant genes can be protected by supplying the mother with extra folic acid, even when their diet is folate-replete (141). There is a long history of animal studies showing that maternal nutrition can influence the physical and cognitive health of the progeny (142, 143), but relatively few studies have specifically looked at folic acid status. A widely studied model is dietary restriction of protein in the pregnant rat (144), whose offspring, for example, have a higher blood pressure than do offspring of mothers fed a normal diet. Folic acid supplementation of the low-protein diet of pregnant rats prevented the elevated blood pressure in the offspring and also prevented some of the adverse vascular changes (145, 146). However, some undesirable consequences of folic acid supplementation to pregnant rats have been found in their offspring: when additional folic acid was fed to mothers on a normal-protein diet, the blood pressure of the offspring was higher than that in the offspring of mothers whose diet was not supplemented (145). When folic acid was fed to rats on a low-protein diet, the male offspring showed a drop in growth rate after 7 mo, a higher blood glucose and corticosterone concentrations, and 40% lower brain concentrations of docosahexaenoic acid than offspring of mothers on a control diet (147, 148). Persistent changes in the phenotype of the offspring imply changes in gene expression caused by epimutations. Evidence consistent with this idea is that decreases in the degree of CpG methylation in the promoters of

genes for peroxisome proliferators–activated receptor α and for the glucocorticoid receptor occurred in the livers of offspring of mothers who had been on a low-protein diet during pregnancy (149). Reduced expression of DNA methyltransferase-1 was also found (150). The changes in DNA methylation were accompanied by increases in the expression of both genes, as assessed by mRNA concentrations. The addition of folic acid to the low-protein diet prevented both the decreased CpG methylation and the increased gene expression.

The animal studies reviewed here establish the principle that varying the folate content of the diet of the pregnant mother can influence the development and health of their offspring. Increasing the folate content of the mother's diet is usually for the good, but may not always be. It is clearly important to establish whether dietary supplementation with folic acid can have similar epigenetic effects in humans.

Clues might come from studies on imprinting in humans. Imprinted genes are an example of an epigenetic phenomenon in which an allele from one parent is preferentially expressed. Animal studies have led to the concept that the "marking" of these genes in the sperm or ova can occur by specific methylation of certain CpGs (151). These imprints control the epigenetic changes that ensure monoallelic expression in the developing animal. Loss of imprinting leads to biallelic expression, whereas silencing of both alleles results in complete loss of gene function.

More than 40 human genes that are subject to imprinting have been identified (152). Disregulation of imprinted genes can influence placental and embryonic development and later phenotype. Thus, it is important to identify environmental factors that might influence imprinting. Numerous animal studies have established that the preimplantation embryo is sensitive to environmental conditions, both in relation to maternal diet and to in vitro culture conditions (153). Changing the composition of culture media for mouse embryos can alter allelic methylation and the expression of imprinted genes in the embryo and fetus (154, 155). Manipulation of human embryos in vitro also appears to induce imprinting changes, as shown by the increased risk of Beckwith-Wiedemann syndrome in children born after in vitro fertilization (156). This syndrome is accompanied by epigenetic alterations in 2 imprinted genes, *LIT1* and *H19* (157). It is not known what components of the media might influence imprinting, but, because of its role in DNA methylation, folate is one of the likely candidates. More research is needed on which factors used in assisted reproductive technologies can lead to these effects.

Loss of imprinting, which leads to biallelic expression of *IGF2* in lymphocytes, has been found in $\approx$10% of the normal population and was associated with hypomethylation of the differentially methylated region of *IGF2* (158). A similar loss of imprinting for *IGF2*, *H19*, and *SYBL1* in blood cells has been found in patients with hyperhomocysteinemia due to renal failure (159). Hyperhomocysteinemia leads to inhibition of methylation reactions due to an accumulation of *S*-adenosylhomocysteine, a powerful inhibitor of DNA methyltransferases (160). In these patients the abnormal biallelic expression could be converted to monoallelic expression by administration of a high dose (15 mg/d) of 5-methyl-THF for 8 wk, which was accompanied by increased methylation of the promoter regions of these genes. Whether more usual concentrations of folate can influence epigenetic phenomena in humans is not known, but a recent report from India is suggestive (65). These authors found that the 6-y-old children of mothers who had high folate status and a low vitamin B-12 status in pregnancy were more obese and had a higher risk of insulin resistance than did children of mothers with normal folate levels. The authors suggested that this "nutritional programming" of childhood features might be related to epigenetic regulation mediated by DNA methylation in a manner similar to the folate-sensitive nutritional programming in animals discussed above (149).

There is thus strong evidence that DNA methylation in dividing cells and during development of the fetus is a dynamic process that can be influenced by the folate or methylation status in the body. But what about DNA methylation in postmitotic cells? The dogma has been that DNA methylation state in such tissues is laid down during embryonic development and is stable (161). However, there is now evidence that this is not always the case. An early report showed that DNA methylation in anterior pituitary cells in vivo is a dynamic process that is related to gestation and lactation (162). The adult brain contains very high concentrations of DNA methyltransferase (163), whose expression can be influenced by centrally acting drugs (164). Furthermore, ischemia can alter the rate of incorporation of methyl groups into rat brain DNA (165). A diet low in folic acid induced hyperhomocysteinemia in rats and was paradoxically associated with increased CpG methylation of the differentially methylated domain of the *H19* gene in brain (166). In neither of the 2 latter studies is it known whether changes in DNA methylation occurred in postmitotic neurons or in glial cells, which can divide. There is, however, more direct evidence that DNA methylation can occur in adult neurons. A cell culture study has shown that depolarization of neurons, which leads to increased synthesis of brain-derived neurotrophic factor, is accompanied by a reduction in the level of CpG methylation in the regulatory region of the *Bdnf* gene (167). Furthermore, the degree of methylation of CpG islands in the promoter region of the gene for reelin from the frontal cortex can be increased by daily administration of methionine to rats, and this was accompanied by a decreased expression of the gene as shown by a fall in the mRNA concentrations (168). Because reelin is expressed exclusively in GABAergic interneurons in the frontal cortex, this result is strong evidence that DNA methylation is a dynamic process in vivo in postmitotic cells. Such a conclusion is supported by the finding that DNA methyltransferase is expressed in cortical GABAergic neurons (169) and that inhibition of DNA methyltransferase leads to decreased methylation of specific CpG islands in the promoter regions of the genes for reelin and for brain-derived neurotrophic factor in mouse hippocampus (170). Notably, the degree of methylation of the reelin gene is elevated in patients with schizophrenia (171–173).

Striking evidence of the dynamic nature of DNA methylation in neurons in vivo is provided by a series of studies on the mechanism by which maternal behavior in the rat can influence the expression of the glucocorticoid receptor in the hippocampus of the offspring (174–176). It was found that a highly specific 5'CpG site in the promoter of the gene for the glucocorticoid receptor became demethylated in 1-wk-old pups of mothers who displayed strong licking and grooming behaviors. The demethylation persisted into adulthood and was associated with increased gene expression and with a reduced response to stress in adulthood. Remarkably, these biological effects could be reversed by central infusion of methionine in the mature animal, an

effect that was associated with increased methylation of the CpG site (177, 178). The authors concluded that "DNA methylation patterns are dynamic and potentially reversible even in adult neurons" (175). This work was followed up by similar studies on the estrogen receptor in the hypothalamus, in which maternal grooming led to demethylation of the promoter in the pups that was associated with increased expression of the estrogen receptor. This change persisted into adulthood, and the female progeny also displayed the same kind of maternal grooming behavior as their mothers had given them (179). The authors proposed that "epigenomic changes serve as an intermediate process that imprints dynamic environmental experiences, such as variations in parental care, on the fixed genome resulting in stable alterations in phenotype" (176).

It is not just maternal diet and behavior that can influence the offspring: it has been known for some time that the diet of the neonatal animal or human can also influence its future health and well-being (142). It is thus of interest that Waterland et al (180) have shown that the composition of the postweaning diet in the rat can influence the degree of methylation of CpG sites in the paternal allele of the *Igf2* gene in the kidney, which leads to loss of imprinting. These authors speculated that "persistent differences observed between formula-fed and human milk-fed individuals (142) are the result of epigenetic alterations induced by subtle nutritional differences between human milk and infant formula" (180).

Protein methylation

Methylation of carboxy-, histidine-, lysine-, and arginine-residues in proteins, for which SAM is the methyl donor, has wide-ranging effects on protein repair, protein targeting, signal transduction, modulation of enzyme activity, RNA metabolism, and transcription regulation (114, 118). Methylation of lysine and arginine residues in histones plays multiple and complex roles in the regulation of gene expression and also in epigenetic silencing by promoting the formation of heterochromatin (117–119, 181). A striking recent finding is that environmental factors can influence histone methylation. A mouse model of depression is the social avoidance that follows exposure to chronic defeat stress for a few days: in such animals the expression of *Bdnf* was down-regulated and this was correlated specifically with a 4-fold increase in the dimethylation of lysine 27 in histone-3 in the hippocampus (182). This methylation persisted for $\geq$1 mo, which led the authors to suggest that chronic stress can mark a repressive state that cannot easily be reversed and that "hypermethylation may represent a stable stress-induced scar in the hippocampus."

Although it was originally thought that histone methylation is effectively irreversible (183), recent work has shown that specific enzymes exist that can remove the methyl residues (118, 184, 185). The dynamic state of histone methylation raises the question of whether it can be influenced by the methylation status in the cell, particularly by the folate status. In contrast with DNA methylation, there do not appear to be any studies that have addressed this question for histones. However, in theory, folate status could affect histone methylation by influencing both the availability of SAM and the level of the product, *S*-adenosylhomocysteine, which shows strong product inhibition of many methyltransferases (114). Further research is needed to test this idea.

Thus, the experimental evidence shows that epigenetic effects mediated by changes in the methylation status of DNA or histones can occur in postmitotic cells in animals and can be caused by simple environmental exposures. These data are of great concern if applicable to humans because folate concentrations can influence methylation. Changes in folate status may thus have the potential to cause long-lasting changes in the functioning of critical organs, such as the brain.

Does folate status in the mother influence the child's genotype?

MTHFR is a key enzyme in the folate cycle (Figure 1) because it converts 5,10-methylene-THF, a precursor for nucleic acid synthesis, into 5-methyl-THF, the substrate that provides the methyl group for the conversion of homocysteine to methionine. The common *MTHFR* (677C→T) polymorphism is associated with instability and reduced enzyme activity, and *T* allele carriers show a shift in folate metabolism away from methyl group synthesis and in favor of thymidylate synthesis (186). The instability of the enzyme in *T* allele carriers can be largely overcome by high folate status, which protects the enzyme from loss of its cofactor flavin adenine dinucleotide (187, 188). In Spain, the prevalence of the *TT* genotype has reportedly approximately doubled in the population since the introduction in 1982 of folic acid supplements for women in early pregnancy (189, 190). The authors speculated that infants with the *T* allele normally have a greater chance of spontaneous abortion (191) because of elevated homocysteine concentrations in the mother (192) and that folate supplementation stabilizes the MTHFR enzyme, lowers the homocysteine concentration, and reduces the risk of abortion, thus leading to an increased proportion of children born with the *T* allele. Although it has been suggested that the result may have arisen from sampling bias (193), the finding is potentially important and needs to be replicated. It is important because carriers of the *T* allele are at increased risk of stroke (194), and it has been suggested of other diseases, such as depression (195, 196), schizophrenia (196, 197), bipolar disorder (196), male infertility (198–200), neural tube defects (201), some but not all cancers (202, 203), and possibly Down syndrome (204, 205). These considerations led Lucock and Yates (206) to suggest that folic acid fortification and supplement use might be "a genetic time bomb." The first premise of this dramatic claim, that folic acid use increases the proportion of children born with the *T* allele of *MTHFR*, is as yet poorly documented and is clearly in urgent need of further study. Studies of the *MTHFR* genotype frequencies in children before and after fortification should be carried out in countries planning fortification of food with folic acid.

Thus, saving fetuses that have a genetic constitution that favors abortion or nonsurvival could lead to children being born with genotypes that favor increased disease during life. This important question needs more research, but it is also an ethical issue for which there is no easy answer.

Folic acid in pregnancy and twins

There has been much debate about whether or not the periconceptual use of folic acid supplements increases the likelihood of twin pregnancies. The consensus appears to be that some of the earlier reports did not take into account those pregnancies that followed assisted reproduction methods and that, for normal pregnancies, there is no increase in twins (207, 208). It is therefore of considerable interest that a study of women undergoing in

vitro fertilization has found a positive relation between blood folate concentrations and the incidence of twin births (209). The authors estimated that if folic acid fortification is introduced into the United Kingdom at the same level in food as in the United States, then an additional 600 twin births would occur in pregnancies resulting from assisted reproduction methods. Notably, although twin births often are considered a positive event, twin pregnancies are associated with an increased risk for both the mother and the child. Twin pregnancies have a higher risk of complications, including pregnancy-induced hypertension, anemia, antepartum and postpartum hemorrhage, and maternal mortality. In addition, twin infants are more likely to deliver preterm, have low birth weight, and to have greater perinatal mortality rates (210). Thus, even a modest change in twinning, confined to special populations such as those undergoing in vitro fertilization, may counterbalance the beneficial effect of folic acid fortification on NTDs.

WILL BLOOD FOLATE CONCENTRATIONS AFTER FORTIFICATION BE ABOVE THE THRESHOLD TO CAUSE HARM?

Although a safe upper limit of folic acid intake of 1 mg/d for adults and 300–800 μg/d for children, depending on age, has been proposed by the Institute of Medicine in the United States (27), there is no consensus about what blood concentrations of folate might cause harm. The limited evidence available has been reviewed above and suggests that plasma concentrations of folate >59 nmol/L might be associated with harm in a subset of the elderly (52). However, this value was arbitrarily defined and represents the beginning of the top quintile of folate concentrations in the population being studied. A detailed study of >7000 people of different ages and races has shown that the effect of folic acid fortification on blood folate concentrations in the United States is quite marked (26). The shift in folate concentrations after fortification in 1998 is shown in **Figure 5**. As can be seen, there is skewness toward higher concentrations of serum folate, which might in part reflect particular dietary habits among children and the elderly, who consume large amounts of bread and/or use folic acid–containing supplements. The use of dietary supplements in the United States is very common; surveys suggest that from 33% to 67% of the population take them regularly (17, 18, 52, 60, 81, 211).

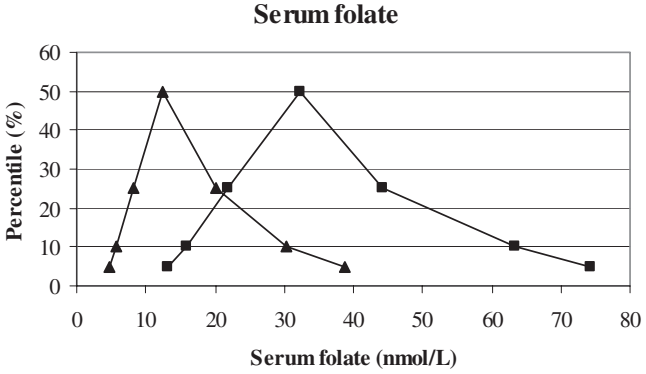

FIGURE 5. Frequency distribution of serum folate for persons aged ≥3 y in 1999–2000 (■) and for persons aged ≥4 y in 1988–1994 (▲) in the United States. Reprinted with permission from Pfeiffer et al (26).

The data reported by Pfeiffer et al (26) show that the section of the US population with the highest blood concentrations of folate after fortification is children aged ≤5 y, 43% of whom had serum folate concentrations >45.3 nmol/L. Ten percent of these children had concentrations >77.3 nmol/L. We can estimate the intake of folic acid equivalents needed to achieve these concentrations from the formula provided by Quinlivan and Gregory (25): 43% of children aged <5 y are consuming the equivalent of >780 μg folic acid/d, ie, double the proposed tolerable upper limit (300–400 μg/d) for children of that age. It is striking that 10% are consuming >1320 μg folic acid/d, which is well above the tolerable upper limit of 1000 μg/d for adults. The next highest blood concentrations were found in children aged 6–11 y; the third highest concentrations occurred in those aged ≥60y, of whom 38% had >45.3 nmol/L. We simply do not know whether these concentrations may cause harm, but it must be of concern that such high concentrations occur, particularly in children at a rapid stage of development, when it likely that epigenetic changes are occurring in many tissues. A recent study in mice showed that varying the methyl donor status of the postweaning diet in mice could influence the methylation status and the expression of imprinted genes (180). Could the same thing happen in young children?

CONCLUSION

The question of whether the higher folate concentrations that occur in significant sections of the population after fortification can cause harm needs much further research (51), as does the question of whether the presence of unmetabolized folic acid in the blood (18, 19) could interfere with folate-dependent metabolism. The studies reviewed above show that, in animals, many crucial biological processes depend on methylation reactions. Furthermore, dietary intake of methyl donors and/or the folate status of the diet can influence these processes. In addition, increasing evidence points to the harmful effect of an imbalance between folate and vitamin B-12 status, something that is likely to occur in vegetarians, certain ethnic minorities, and in the elderly with vitamin B-12 malabsorption. Fortification was introduced specifically to prevent NTDs, and we all believe that improved folate status achieved by increasing folic acid intakes influences biological processes related to the neural tube. However, we have to ask the following question: What other biological processes are these concentrations of folate capable of influencing, and are the effects always beneficial or could they sometimes be harmful?

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