

Folate and vitamin B₁₂

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The folates are made up of a pteridine ring attached to a *p*-aminobenzoate and a polyglutamyl chain. The active form is tetrahydrofolate which can have C₁ units enzymically attached. These C₁ units (as a formyl group) are passed on to enzymes in the purine pathway that insert the C-2 and C-8 into the purine ring. A methylene group (-CH₂-) attached to tetrahydrofolate is used to convert the uracil-type pyrimidine base found in RNA into the thymine base found in DNA. A further folate cofactor, i.e. 5-methyltetrahydrofolate, is involved in the remethylation of the homocysteine produced in the methylation cycle back to methionine. After activation to *S*-adenosylmethionine this acts as a methyl donor for the dozens of different methyltransferases present in all cells. Folate deficiency results in reduction of purine and pyrimidine biosynthesis and consequently DNA biosynthesis and cell division. This process is most easily seen in a reduction of erythrocytes causing anaemia. Reduction in the methylation cycle has multiple effects less easy to identify. One such effect is certainly on the nerve cells, because interruption of the methylation cycle causing neuropathy can also happen in vitamin B₁₂ deficiency due to reduced activity of the vitamin B₁₂-dependent enzyme methionine synthase (*EC* 2.1.1.13). In vitamin B₁₂ deficiency, blocking of the methylation cycle causes the folate cofactors in the cell to become trapped as 5-methyltetrahydrofolate. This process in turn produces a pseudo folate deficiency in such cells, preventing cell division and giving rise to an anaemia identical to that seen in folate deficiency.

Folate: Tetrahydrofolate: Methylation cycle: Vitamin B₁₂: Neural-tube defect

Folic acid and folate

Summary of chemistry, absorption and biochemical functions

The folates function in cells as their reduced form conjugated to a polyglutamate chain. They have a single type of function in that they can accept so-called C₁ units from various donors and pass them on in various biosynthetic reactions (Scott & Weir, 1994). Thus, in cells folates will be a mixture of polyglutamyl tetrahydrofolates and various C₁ forms of tetrahydrofolate (e.g. 10-formyl-, 5,10-methylene- and 5-methyltetrahydrofolate), depending on which C group is attached to them (Fig. 1).

Cells and consequently food contain a mixture of reduced folate polyglutamates. These reduced forms of the vitamin, particularly the unsubstituted dihydro- and tetrahydro-forms, are extremely unstable chemically (Blakley, 1969). They are very easily cleaved between the C-9 and N-10 bond, splitting apart to give a pteridine and *p*-aminobenzoic acid, and thus losing their biological activity

(Fig. 2). While substituting the N-5 or N-10 with a C group decreases the tendency to cleave, the substituted forms are also susceptible to oxidative chemical rearrangements and consequently loss of activity (Blakley, 1969).

The chemical lability of all naturally-occurring folates causes very significant loss of activity during harvesting, storage, processing and preparation. Actually half or even three-quarters of initial folate activity may be lost during these procedures. This chemical instability of the natural reduced folates is to be contrasted with that of the synthetic form, folic acid. In the synthetic form the pteridine ring is not reduced, which renders this form very resistant to chemical oxidation. While ultimately the synthetic form will be reduced in cells by the enzyme dihydrofolate reductase (*EC* 1.5.1.3) to the dihydro- and tetrahydro- forms, this reduction takes place during passage through the intestinal mucosal cells where what emerges in the plasma is 5-methyltetrahydrofolate (Chanarin, 1979). The consequence of this chemical instability and enzymic reduction is that what is delivered to the body will depend on whether the

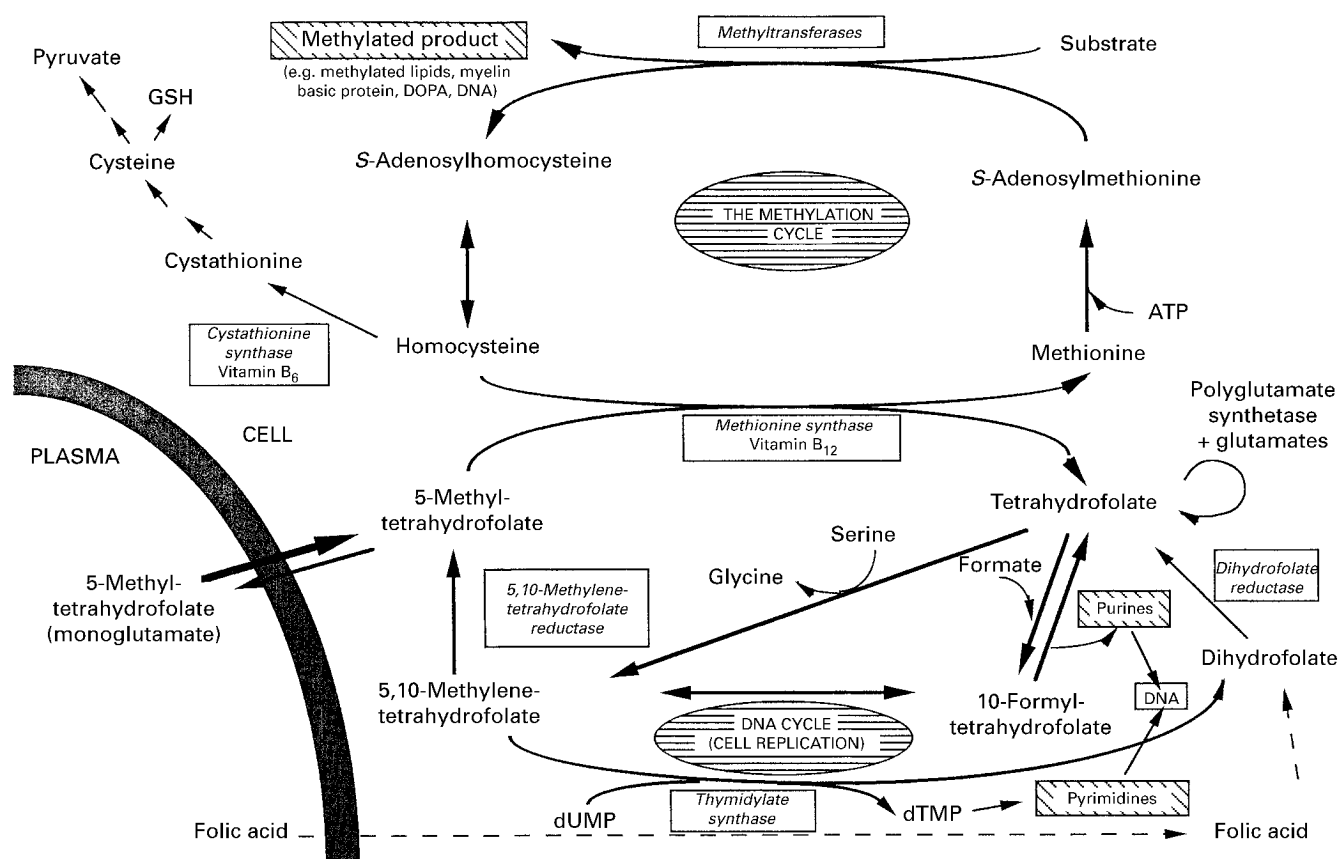


Fig. 1. The biochemical pathways involving the folates and vitamin B₁₂. DOPA, 3,4-dihydroxyphenylalanine.

dietary source of the vitamin is the synthetic form folic acid or the natural form folate. While natural folates rapidly lose activity in foods over periods of days or weeks, folic acid in, for example, fortified foods is almost completely stable for months or even years. In addition, the natural folates in cells are all conjugated to a polyglutamyl chain of different numbers of glutamic acids depending on the type of cells making up the food. This polyglutamyl chain is removed in the brush border of the mucosal cells by the enzyme conjugase (*EC* 3.4.19.9), resulting in a single glutamic acid remaining, with this reduced folate monoglutamate being absorbed. If it is not already in the 5-methyltetrahydrofolate form, which is true for most food folates, it will be converted to this form during transit through the intestinal mucosa. Thus, usually the only form entering the human circulation from the intestinal cells is 5-methyltetrahydrofolate monoglutamate. Folate in human plasma is almost exclusively in this form, with a small percentage of other reduced forms being reported in some studies (see Chanarin, 1979). As mentioned previously, folic acid is also reduced to the tetrahydro- form and then converted to 5-methyltetrahydrofolate monoglutamate during its transit through the gut mucosa and before its release into the portal vein. However, this process is limited in capacity, and if enough folic acid is given orally, unaltered folic acid appears in the circulation (Kelly *et al.* 1997). This folic acid can then be taken up by

other cells, and as long as those cells have the enzyme dihydrofolate reductase they can reduce it and use it. The process of removing the polyglutamate chain from natural folates by the intestinal conjugase is not apparently total. There is a resultant reduction in the bioavailability of natural folates of perhaps as much as a one-quarter to half. Synthetic folic acid by contrast appears to have a bioavailability of approximately 100 %. The poor bioavailability, but more importantly the poor chemical stability, of the natural folates has a profound influence when their recommended dietary allowances or dietary reference values are being considered. This is particularly true if some of the dietary intake is to be contributed by the much more stable and bioavailable synthetic form folic acid, if it has been added to the diet by way of food fortification of breakfast cereals, flour etc.

The folates get their C₁ groups from either the C-3 of serine or from formate (Fig. 1) These C₁ substituted forms are most obviously important since as 10-formyltetrahydrofolate they are required twice during the *de novo* biosynthesis of the purine ring, i.e. the enzymes on the purine biosynthetic pathway that insert the C-2 and the C-8 into the purine ring must have these C presented attached to tetrahydrofolate. Likewise, the conversion of the uracil-type base found in RNA into the thymine-type base found in DNA is brought about by the enzyme thymidylate synthase

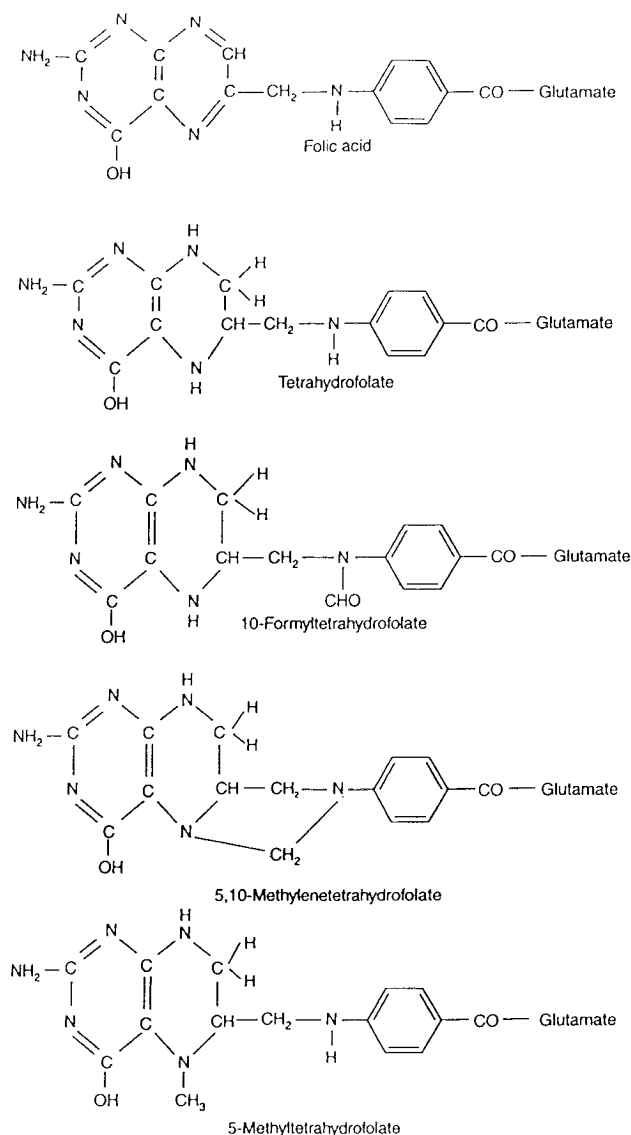


Fig. 2. The chemical formulae of synthetic folic acid and some of the naturally-occurring reduced folates.

(EC 2.1.1.45) which uses the folate cofactor 5,10-methylenetetrahydrofolate as its C₁ donor. Thus, folate in its reduced form tetrahydrofolate is essential for the DNA biosynthesis cycle shown in Fig. 1.

Alternatively the form of folate (5,10-methylenetetrahydrofolate) used for thymidylate synthase can be channelled up to the 'methylation cycle'. This cycle performs two functions. It ensures that the cell always has an adequate supply of *S*-adenosylmethionine, which is an activated form of methionine acting as a methyl donor to a whole range of methyltransferases. These enzymes methylate (add a CH₃ group) to a wide range of substrates as varied as lipids, hormones, DNA and proteins. One such important methylation is that of myelin basic protein. This protein makes up about one-third of myelin, the insulation cover on nerves. When the methylation cycle is interrupted, as it is during vitamin B₁₂ deficiency, one of the clinical conse-

quences is the demyelination of nerves, resulting in a neuropathy which leads to ataxia, paralysis and, if untreated, ultimately death. Other methyltransferases down-regulate DNA and suppress cell division, methylate 3,4-dihydroxyphenylalanine and lipids etc.

In the liver the methylation cycle also serves the function of degrading methionine (Fig. 1). Methionine is an essential amino acid in human subjects and comes exclusively from the diet. It is present in the usual diet in about 60 % excess over requirement for protein synthesis and other uses of methionine. This excess methionine is degraded via the methylation cycle to homocysteine. At this point homocysteine can either be catabolized (degraded) to sulfate and pyruvate, with the latter being used for energy, or alternatively can be remethylated back to methionine. Whether homocysteine is degraded or conserved by its remethylation back to methionine depends on how well the cycle is maintaining intracellular *S*-adenosylmethionine levels.

The DNA and methylation cycles both regenerate tetrahydrofolate, and as such the vitamin is not used up. However, as well as loss of folate through a small amount of urinary excretion, and through skin and bile, there is a considerable amount of catabolism (or breakdown) of folate (McPartlin *et al.* 1993). Thus, there is a need to replenish the body's folate content by uptake from the diet. If there is underprovision of dietary folate to meet requirements, this will cause a reduction in both the DNA cycle and the methylation cycle. A decrease in the former will in turn reduce DNA biosynthesis, and with this cell division. While DNA biosynthesis is seen in all dividing cells, deficiency will be most obvious in those cell types that are rapidly dividing. Thus, folate deficiency is most obviously seen as a reduction in erythrocytes, thereby producing anaemia. However, there is also a reduction in other bone-marrow-derived cells leading to leucopenia and thrombocytopenia. There is also a reduction in the methylation cycle. The most obvious expression of this reduction is an elevation in plasma homocysteine, which presumably has arisen from a reduction in the activity of the methylation cycle due to an under-provision of new methyl groups necessary for the remethylation of plasma homocysteine. Previously it was considered that a rise in plasma homocysteine was just a good back-up biochemical marker of possible folate deficiency. However, there is ever-increasing evidence, many would say conclusive evidence, that an elevation in plasma homocysteine causes cardiovascular disease or stroke (Scott & Weir, 1996). Furthermore, even moderate elevation of plasma homocysteine to levels seen in subjects with a folate status hitherto not considered as being deficient may put subjects at increased long-term risk of cardiovascular disease or stroke (Wald *et al.* 1998). Thus, interruption of the methylation cycle due to impaired folate status (or decreased vitamin B₁₂ or vitamin B₆ status: see p. 446) may have serious long-term risk. Interruption of the methylation cycle due to folic deficiency is currently thought to also have associated with it impaired synthesis of myelin basic protein. Such interruption (as seen for example in conditions where there is vitamin B₁₂ deficiency such as pernicious anaemia) does indeed cause a very characteristic demyelination and neuropathy, known as sub-acute combined degeneration of the spinal cord and peripheral

nerves. If untreated this condition leads to ataxia, paralysis and ultimately death. This form of neuropathy is not usually associated with folate deficiency, but is seen if folate deficiency is very severe and very prolonged (Manzoor & Rucie, 1976). The explanation may lie in the well-established ability of nerve tissue to concentrate folate to a level of about five times that in the plasma. This accumulation of folate may ensure that nerve tissue has a worthwhile level when folate being provided to the rapidly-dividing cells of the bone marrow has been severely compromised for a prolonged period. Thus, the resultant anaemia will inevitably present earlier than the neuropathy, usually resulting in treatment.

Vitamin B₁₂

Summary of chemistry, dietary sources, absorption and metabolic function

The more correct name for vitamin B₁₂ used in most biochemical and chemical texts, is cobalamin. The nutritional literature still tends to use the term vitamin B₁₂. Vitamin B₁₂ is the largest of the B complex of vitamins, with a molecular weight of over 1000. It consists of a porphyrin ring made up of four pyrroles, just as in haem or the cytochromes, except that instead of Fe in the centre of the ring there is Co.

There are several vitamin B₁₂-dependent enzymes in bacteria and algae. There are none in plants; in fact plants without exception do not have the necessary enzymes to make the vitamin and thus are completely devoid of it, a factor that has implications when one considers the sources of vitamin B₁₂ in the diet. In mammalian cells there are only two vitamin B₁₂-dependent enzymes. One enzyme, methionine synthase (*EC* 2.1.1.13), uses the chemical form methylcobalamin where the Co has a methyl group attached to it. The other enzyme, methylmalonyl-CoA mutase (*EC* 5.4.99.2), uses 5'-deoxy-5'-adenosylcobalamin. In nature there are two other forms that arise from the two active forms, i.e. hydroxycobalamin and aqueocobalamin, where respectively hydroxyl and water groups are attached to the Co. These two forms are enzymically activated to methylcobalamin or 5'-deoxy-5'-adenosylcobalamin in all mammalian cells. Finally, the synthetic form found in supplements and fortified foods is cyanocobalamin with a cyanide group attached to the Co, which is again enzymically activated in all cells to the two active forms.

Dietary sources of vitamin B₁₂

All vitamin B₁₂ arises initially from synthesis in micro-organisms. All types of bacteria and algae, with a few exceptions, synthesize the vitamin, which enters the human food chain by being incorporated into food of animal origin. Incorporation occurs mainly in herbivorous animals in which fermentation in the gastrointestinal tract supports the growth of a wide range of micro-organisms that synthesize various forms of vitamin B₁₂ which are absorbed and incorporated into the tissues of the animals. These tissues are then a rich dietary source of vitamin B₁₂, particularly liver where it is stored in large concentrations. Alternatively, milk produced by these herbivorous animals represents one of the

most important dietary sources of the vitamin. Omnivores or carnivores, including man, derive their dietary vitamin B₁₂ from the tissues or products from these animals, e.g. milk, butter, cheese and eggs. It appears that human subjects do not derive a significant amount of their vitamin B₁₂ requirement from their own microflora, in contrast to herbivores. If vitamin B₁₂ does arise in a free state in the large intestine, it is simply not absorbed by the active process (see pp. 444–445).

The absorption of food and synthetic forms of vitamin B₁₂ in human subjects is complex. Vitamin B₁₂ in food is bound to proteins. In the stomach the bound vitamin is released from these proteins by the action of the high concentration of HCl present, giving the free forms of the vitamin. The free forms are immediately passed to a mixture of glycoproteins secreted by the stomach and salivary glands. These glycoproteins, the R binders (or haptocorrins), protect vitamin B₁₂ from chemical denaturation in the stomach. The HCl secreted by the stomach is produced by the parietal cells which also secrete a specific glycoprotein, intrinsic factor. The function of intrinsic factor is to bind vitamin B₁₂ and ultimately facilitate its active absorption. While the formation of the vitamin B₁₂ – intrinsic factor complex was initially thought to occur in the stomach, it is now clear that this is not the case. At acidic pH the affinity of vitamin B₁₂ for intrinsic factor is low while its affinity for the R binders is high. When the contents of the stomach enter the duodenum the R binders become partly digested by the proteases secreted by the pancreas. This process results in the release of the bound vitamin B₁₂, which quickly attaches to intrinsic factor which has a high binding affinity at the more neutral pH found in the duodenum. The vitamin B₁₂–intrinsic factor complex then proceeds to the lower end of the small intestine where it is absorbed by phagocytosis by specific ileal receptors (Weir & Scott, 1998).

Malabsorption of vitamin B₁₂ can occur at several points during this process. By far the most important is the autoimmune disease pernicious anaemia (PA; see Chanarin, 1979). In most cases of PA autoantibodies are produced against the parietal cells causing them to atrophy. The cells then lose their ability to produce intrinsic factor and also to secrete HCl. In some forms of PA where the parietal cells remain intact, autoantibodies are produced against intrinsic factor forming a complex which thus prevent the intrinsic factor binding with vitamin B₁₂. There is a further less-common form of PA where the antibodies allow vitamin B₁₂ to bind with intrinsic factor but prevent absorption of the intrinsic factor – vitamin B₁₂ complex by the ileal receptors. Like most autoimmune diseases the incidence of PA increases markedly with age. Thus, in most ethnic groups it is virtually unknown to occur before 50 years of age, with a progressive rise thereafter. Afro-Americans are known to have an earlier age of presentation. PA causes not only malabsorption of dietary vitamin B₁₂, but it also results in an inability to reabsorb the vitamin B₁₂ that is secreted daily in the bile (usually calculated to be between 0.3 and 0.5 µg/d). Interruption of this so-called enterohepatic circulation of vitamin B₁₂ causes the body to go into a significant negative balance for the vitamin (see Chanarin, 1979).

While vitamin B₁₂ is the best stored of all vitamins, with enough stores to last 3–5 years in a normal replete subject,

once PA has been established the subject not only fails to absorb new vitamin B₁₂, but also starts losing vitamin B₁₂ rapidly because of this negative balance. Once the stores have been depleted the final stages of deficiency are often quite rapid, resulting in death in a period of months in the final stages if left untreated. This rapid final stage was why PA was so named when it was first recognized. Historically PA was considered to be the major cause of vitamin B₁₂ malabsorption, but it was a fairly rare condition, perhaps affecting approximately 1 % of even very elderly subjects. More recently it has been suggested that a far more common problem is that of achlorhydria, where there is a progressive reduction with age in the ability of the parietal cells to secrete HCl. It is claimed that perhaps up to one-quarter of elderly subjects could have achlorhydria to varying degrees (Carmel, 1995). This absence of acid, it is suggested, prevents the release of vitamin B₁₂ from food, but does not interfere with the absorption of free vitamin B₁₂ such as would be found in fortified foods or in supplements. Furthermore, achlorhydria would not prevent the reabsorption of biliary vitamin B₁₂ and, therefore, does not produce the negative balance seen in PA. However, it is agreed that with time a reduction in the amount of vitamin B₁₂ being absorbed from the diet will eventually deplete even the usually adequate vitamin B₁₂ stores, resulting in overt deficiency. The different handling of free and food-bound vitamin B₁₂ becomes important when assessing the recommended dietary allowances for the elderly, because assessment requires consideration of absorption of vitamin B₁₂ from sources such as fortified foods or supplements as compared with dietary vitamin B₁₂. A further consideration is that the intrinsic factor-mediated system absorbs all free vitamin B₁₂ at intakes of less than 1.5–2.0 µg. However, absorption of food-bound vitamin B₁₂ has been reported to vary from 9 to 60 % depending on the study and the source of the vitamin, perhaps related to its incomplete release from food (Weir & Scott, 1998). This factor has led to allowances of up to 50 % to correct for bioavailability of absorption from food.

While vitamin B₁₂ is a cofactor for a wide variety of enzymes in micro-organisms, apart from methionine synthase there is only one other vitamin B₁₂-dependent enzyme in human subjects, methylmalonyl-CoA mutase (EC 5.4.99.2). This enzyme is involved in the interconversion of methylmalonyl-CoA to succinyl-CoA, and lies on the pathway for the degradation of the last C₃ of odd-chain fatty acids together with the degradation of certain amino acids such as valine. This enzyme is extremely important in herbivorous animals because one of the principal products of the fermentation of cellulose in the rumen is propionate. In fact, conversion of propionate via methylmalonyl-CoA into the citric acid cycle at succinyl-CoA is the principal source of carbohydrate used for energy in these animals, i.e. the alternative to glucose in most other mammals. Methylmalonyl-CoA mutase is also important in human subjects; in its absence, or indeed in vitamin B₁₂ deficiency, methylmalonyl-CoA accumulates and gets degraded to methylmalonic acid (MMA). It is not clear if this accumulation is itself toxic or if it gives rise to some other metabolite that is toxic. Alternatively, it is not clear if the inability to degrade some metabolite through the vitamin B₁₂-dependent methyl-

malonyl-CoA mutase is a problem due to its earlier accumulation. What is clear from observations of children with a mutation of methylmalonyl-CoA mutase, where the enzyme is present at very restricted levels, is that such children have serious developmental dysfunctions. This impairment could be due to some function of the mutase in embryonic development, or accumulation of products mentioned previously in early life. However, crucially, children with inborn errors of the mutase do not get demyelination or the neuropathy seen in adults with vitamin B₁₂ deficiency even though they sometimes have profound accumulation of MMA. This finding adds weight to the experimental evidence that it is impaired functioning of methionine synthase that causes the demyelination and neuropathy.

As discussed in the section on folic acid and folate (see pp. 441–444), one of the vitamin B₁₂-dependent enzymes (methionine synthase) functions in one of the two folate cycles, i.e. the methylation cycle (Fig. 1). As explained previously, this cycle is necessary to maintain the supply of the methyl donor *S*-adenosylmethionine, and if it is interrupted it produces a reduction in a wide range of methylated products. One such important methylation process is that of myelin basic protein; its reduction, as seen in PA and other causes of vitamin B₁₂ deficiency, produces sub-acute combined degeneration. This neuropathy is one of the main presenting conditions in PA. The other principal presenting condition in PA is a megaloblastic anaemia morphologically identical to that seen in folate deficiency. At first sight it is hard to see why impairment of the methylation cycle should cause a lack of DNA biosynthesis and anaemia. It is suggested that this impairment can be explained by the 'the methyl trap hypothesis' (Scott & Weir, 1994). This hypothesis suggests that once the folate cofactor 5-methyltetrahydrofolate is formed, the enzyme 5,10-methylene-tetrahydrofolate reductase (EC 1.7.99.5) that forms the cofactor cannot use it in the back reaction *in vivo*. Thus, the only way for this folate cofactor to be recycled to tetrahydrofolate, and thus participate in DNA biosynthesis and allow cell division, is through the vitamin B₁₂-dependent enzyme methionine synthase (Fig. 1). When the activity of this enzyme is compromised, as it would be in PA, the cellular folates will become progressively trapped as 5-methyltetrahydrofolate. As a result the cell will suffer a sort of pseudo folate deficiency; it will have adequate folate but it will be trapped in a form that cannot be used for DNA biosynthesis. The consequence will be an anaemia identical to that seen in true folate deficiency. Thus, in PA there will be a neuropathy and/or an anaemia (Scott *et al.* 1981). Treatment with vitamin B₁₂, if given intramuscularly, will reactivate methionine synthase allowing myelination to restart. The trapped folate will also be released, thus allowing DNA synthesis and generation of erythrocytes, redressing the anaemia. It is known that treatment with high concentrations of folic acid will also treat the anaemia but not the neuropathy in PA, because when the synthetic form of folate (folic acid) enters the cell is first converted via dihydrofolate to tetrahydrofolate (Fig. 1). This process initially occurs outside the methyl trap and can thus initiate DNA biosynthesis, so treating the anaemia. While such folic acid is ultimately trapped as 5-methyltetrahydrofolate, if it is given continuously and in amounts high enough to exceed

the capacity of the intestinal mucosal cells to convert it to 5-methyltetrahydrofolate, the folic acid will keep DNA biosynthesis going and thus appear to have treated the anaemia. However, administration of folic acid cannot do anything to restart the methylation cycle, blocked as it is at the vitamin B₁₂-dependent enzyme methionine synthase. Thus, the neuropathy will continue, only to be diagnosed later when treatment with vitamin B₁₂ may only partly reverse the nerve damage. It should be stressed that so-called 'masking' of the anaemia of PA is not thought to occur at concentrations of folate found in food, or at intakes of the synthetic form of folic acid found at the usual recommended dietary allowance levels of 200 or 400 µg/d (Kelly *et al.* 1997). However, 'masking' definitely occurs at high concentrations of folic acid (greater than 1000 µg/d). The latter becomes a concern when it is proposed to add synthetic folic acid to the diet by way of fortification of a dietary staple such as flour. The concern is that if folic acid is added to have the desirable effect of reducing neural-tube defects (NTD) and/or lowering plasma homocysteine, in order to ensure that those individuals on low flour intakes get enough to be effective, those individuals on high flour intakes could inadvertently become exposed to very high levels of folic acid. This situation would also occur in the elderly and, it is suggested, could prevent the timely diagnosis of PA via presentation as the anaemia. This delay in diagnosis could allow the neuropathy to progress to such an extent that when it is eventually diagnosed it is partly or largely irreversible.

In addition to causing neuropathy and anaemia, even a modest reduction in vitamin B₁₂ status will cause elevation of plasma homocysteine, because one of the principal routes of homocysteine metabolism is to recycle it to methionine via methionine synthase (Fig. 1).

Conventional view of folate and vitamin B₁₂ deficiency

In terms of the nutritional status of folate or vitamin B₁₂, up to a decade ago it was thought that there were certain well-defined clinical consequences resulting from deficiency. It would have been recognized that there were also biochemically-detectable consequences, but these were considered only to be useful as a marker indicating a subject had become deficient, and not to pose any risk in themselves. Finally, the concept that there was any chronic risk for a subject whose status was not deficient was unknown. The latter is now known to exist definitively for NTD, probably for cardiovascular disease and stroke, and possibly for colorectal cancer. This area will be discussed under three headings: clinical deficiency, biochemical deficiency, and suboptimal status.

Clinical deficiency

As mentioned previously, clinical deficiency of either folate or vitamin B₁₂ is synonymous with a very characteristic megaloblastic anaemia in both instances. In vitamin B₁₂ deficiency there may or may not be sub-acute combined degeneration. In severe prolonged folate deficiency there is also evidence of a neuropathy (Manzoor & Runcie, 1976). Milder deficiency of either nutrient would also have associ-

ated clinically-detectable changes in the blood. There would be a detectable increase in the mean corpuscular volume above normal and also an increased lobe count in granulocytes (Chanarin, 1979).

Biochemical deficiency

It has long been recognized that at a folate status where there was no overt clinical signs or symptoms and even no changes in the peripheral blood, there would be biochemically-detectable changes. The principal change would be an elevation of the circulating level of total plasma homocysteine. This increased level would frequently be $>2SD$ above the normal mean i.e. $>14 \mu\text{mol/l}$. A similar increase in total plasma homocysteine would also occur when vitamin B₁₂ status was reduced. Again, high total plasma homocysteine levels would occur not only during overt deficiency but would be above the normal mean or perhaps above the normal range of $14 \mu\text{mol/l}$, even in the complete absence of clinical or morphological changes in the blood. A further biochemical change was also characteristic of reduced vitamin B₁₂ status, i.e. an increase in plasma and urinary MMA due to reduced activity of methylmalonyl-CoA mutase, the second vitamin B₁₂-dependent enzyme in human subjects (Scott & Weir, 1994). There are other reasons why MMA accumulates in the plasma, principally due to decreased renal function. However, it was the usual experience that impaired vitamin B₁₂ status was always associated with raised MMA. This situation has been questioned by Carmel (1995) and Lindenbaum *et al.* (1990), who reported a very small number of cases of people with apparently normal plasma vitamin B₁₂ levels having raised MMA that was reduced by vitamin B₁₂ therapy. However, it is not clear if this finding was due to their use of radiometric assays perhaps detecting vitamin B₁₂ analogues. Certainly the usual experience with the microbiological assay is that it always detects low plasma vitamin B₁₂ levels that are invariably accompanied by raised MMA, making MMA a very reliable secondary marker for reduced vitamin B₁₂ status.

Suboptimal status

The conventional view that the only considerations were clinical deficiency or biochemical evidence of deficiency was challenged for the first time with respect to the risk of a mother having a baby affected by spina bifida or another type of NTD. It has become clear from various case-control studies and intervention trials that three-quarters of NTD could be prevented by the periconceptual ingestion of folic acid (Scott *et al.* 1994). What was unclear initially was whether this effect was due to the additional folic acid simply treating maternal folate deficiency or whether the additional folic acid was overcoming some sort of metabolic block in folate metabolism. In a prospective study our own group collected over 50 000 blood samples from pregnant women attending their first maternity booking clinic (Kirke *et al.* 1993). It was subsequently determined that approximately eighty-four of these pregnancies had been affected by an NTD. The plasma and erythrocyte folate levels together with the plasma vitamin B₁₂ level were compared for these eighty-four cases and 247 randomly-selected

controls. The NTD group had significantly reduced levels of erythrocyte folate compared with the controls, but only twelve of the cases had erythrocyte folate levels below 150 µg/l (the value that is conventionally considered to diagnose either deficiency or tendency towards deficiency; Kirke *et al.* 1993). Thus, the prevention of NTD by giving additional folic acid did not appear to be treating maternal folate deficiency in this the index pregnancy, at least by the conventional definition of folate deficiency (Scott *et al.* 1994). A further analysis of the same data gave an even greater insight into what was happening. We analysed the risk per 1000 births of a pregnancy being associated with an NTD with respect to the different levels of erythrocyte folate found in NTD cases compared with the controls (Fig. 3). This analysis showed that over what would be considered the normal range there was a tenfold decrease in risk when going from low normal status to high normal status. The risk of an NTD-affected birth at 150 µg/l was seven per 1000 births whereas the risk at 400 µg/l was 0.7 per 1000 births. Thus, even though folate status was normal in these women and they had no signs of deficiency, their risk of having an NTD-affected birth was strongly reduced as status improved. The possibility that extra folic acid could act in preventing NTD by overcoming some sort of metabolic block within normal range was reinforced by the demonstration of differences between cases and controls with respect to homocysteine metabolism (Mills *et al.* 1995). There seemed to be difficulty in the folate-dependent metabolism of homocysteine in the NTD cases compared with the controls. In addition, comparing plasma vitamin B₁₂ levels for the NTD cases and controls showed that vitamin B₁₂ status was a risk factor for NTD-affected births independent of folate status (Daly *et al.* 1995). This finding suggests that an event involved in the closure of the neural tube was responsive independently to both folate status and vitamin B₁₂,

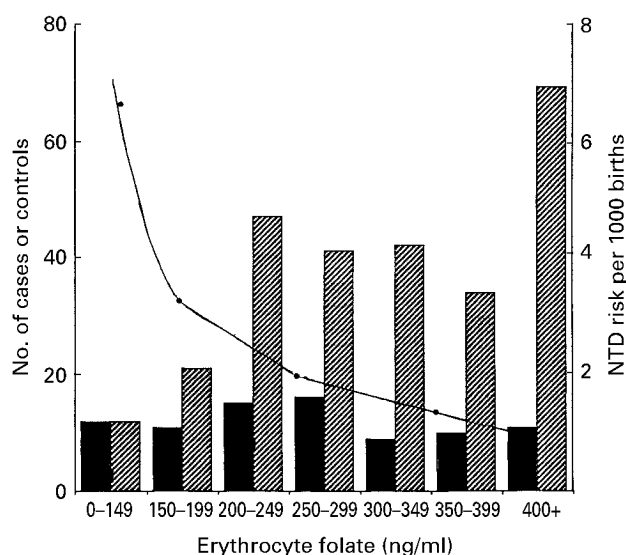


Fig. 3. The maternal erythrocyte folate status of eight-four cases of neural-tube defects (NTD; ■) compared with that of 247 randomly-selected controls (▨), showing alteration in risk of an affected birth per 1000 births (●-●).

but at levels of both nutrients well within the conventional normal range.

A further area where risk rather than deficiency and overt clinical signs and symptoms was also emerging was with respect to homocysteine and cardiovascular disease and stroke. Plasma homocysteine is a normal constituent of human plasma. It is derived exclusively from the degradation of dietary methionine (Fig. 1). At the point where homocysteine is formed in the methylation cycle it can either be degraded via cystathionine to cysteine and ultimately pyruvate and sulfate, or it can be recycled to methionine. The supply or level of *S*-adenosylmethionine (which is needed by all cells to keep the forty or so methyltransferases supplied with this methyl donor) determines which process occurs in the cells. Three enzymes are involved in controlling the plasma homocysteine level, cystathionine synthase (*EC* 4.2.1.22), methionine synthase and indirectly 5,10-methyltetrahydrofolate reductase. Cystathionine synthase requires vitamin B₆ for its activity, while the other two enzymes require folate. Methionine synthase also requires vitamin B₁₂. Reduction in the levels of any of these three nutrients is known to elevate plasma homocysteine, and this factor has been used as a sensitive biochemical marker for such reduced status. What began to emerge, however, was that plasma homocysteine was itself an independent risk factor or risk marker for cardiovascular disease and stroke (Scott & Weir, 1994). What was critical was that this increased risk occurs at a folate status or vitamin B₁₂ status which conventionally would be considered to be well within the normal range. In addition, it has been shown recently that the risk associated with plasma homocysteine does not have a plateau (Wald *et al.* 1998). Furthermore, there is evidence that there is an association between folate status in what is usually considered the normal range and increased risk of colo-rectal cancer (Mason, 1995). There is also an apparent increase in mental disorders associated with reduced folate status (Bottiglieri *et al.* 1995). It is difficult to determine whether this reduced status is a cause of the increased incidence of mental disorders or just occurs as a result of reduced intake associated with such diseases. Finally, there is some evidence that folate status is a risk factor for the development of orofacial clefts (Tolarova & Harris, 1995) and other birth defects (Czeizel, 1996).

In summary, it is necessary to consider not only the folate or vitamin B₁₂ status that causes signs and symptoms of deficiency but also the concept of increased risk of NTD-affected births, cardiovascular disease and stroke, colo-rectal cancer, mental illness, and orofacial clefts and other birth defects. It is now clear that these increased risks occur at a folate status that would not conventionally be considered to be deficient or even at risk of becoming deficient. This concept of risk will require a radical rethink when setting new reference values for food intake (recommended dietary allowances, dietary reference values, and population reference intakes etc.). In this context it is also noteworthy that one variant of a folate-dependent enzyme (5,10-methylenetetrahydrofolate reductase) has been identified that for the first time shows a genetically-carried impairment in the ability to assimilate a nutrient (Molloy *et al.* 1997). This variant is very common, having a heterozygous prevalence of approximately 50 % and a homozygous prevalence of

about 10 % in most populations (Whitehead *et al.* 1995). Under the usual guidelines for setting reference intakes only requirements for 'normal' individuals are considered. If one in ten individuals have an increased requirement for just a single variant it is probable that other variants also determine the status of nutrients such as folate. Thus, there may be no such thing as a normal individual, thus the method of setting reference intakes must be re-evaluated.

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