Occasional articles

Cobalamin and folate: Recent developments

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Introduction

New concepts in this field continue to develop at the clinical level, within the laboratory and in the realm of basic research. The appreciation that the anaesthetic gas nitrous oxide (N_2O) specifically inactivates the cobalamin dependent enzyme, methionine synthetase, and produces megaloblastic anaemia in man, has made it possible to perform extensive animal studies on cobalamin-folate interrelations, and to test the methylfolate trap hypothesis in a meaningful way. This has led to the development of alternative ideas as to how cobalamin regulates folate metabolism, based on a large body of data obtained from study of animals in which cobalamin was inactivated. Cobalamin neuropathy, too, has been explored in greater depth than has been possible hitherto. On the diagnostic side, considerable claims have been made for the measurement of serum methylmalonic acid as an indicator of cobalamin deficiency.

Serum methylmalonic acid and cobalamin neuropathy

An increased urinary excretion of methylmalonic acid (MMA) is well established as a specific test for cobalamin deficiency. Although the test seems to be specific, it is not an early sign, and some of the least anaemic patients, usually with higher though reduced serum cobalamin concentrations, have normal MMA excretion.¹ Treatment with cobalamin is followed by a rapid restoration of a normal output of MMA in three to five days. A method for assay of MMA in serum² has allowed MMA concentrations to be measured in the same samples sent for cobalamin assay and it has been combined with measurement of the serum homocysteine concentration.

Plasma homocysteine is raised in most patients with megaloblastic anaemia due to either cobalamin or folate deficiency as impairment of the conversion of homocysteine to methionine is common to both deficiencies.³

Of 196 consecutive serum samples with cobalamin concentrations below 170 pg/ml, 33% had both raised MMA and homocysteine concentrations, 45% had normal concentrations of both metabolites, and 22% an increase in only one of these substances.⁴ All but two of the patients with an increase in either MMA or homocysteine had evidence of cobalamin or folate deficiency, although whether bone marrow aspirations, dU suppression tests, or cobalamin absorption tests were done, is not stated.

Lindenbaum *et al*⁵ reported a high incidence

of cobalamin neuropathy in New York in the absence of anaemia or macrocytosis. However, the same incidence of neuropathy (68%) was found in a London study of consecutive patients with macrocytosis, megaloblastic marrows, and low serum cobalamin concentrations.⁶ Routine serum cobalamin assays in patients presenting with neuropathy did not reveal additional cases with cobalamin deficiency. Thus, although the same prevalence of cobalamin neuropathy was found in these two studies, in the United Kingdom all the patients were identified by a routine blood count, but in the USA only a proportion seem to have been identified by this means. Such patients were identified by a raised serum MMA concentration. In a second report of 145 patients with serum cobalamin concentrations below 200 pg/ml Stabler *et al*⁷ found that 86 responded to treatment with cobalamin, and 94% of these had raised serum MMA concentrations as did 14% of patients who did not respond to cobalamin. One third of these patients were haematologically normal. This group extended their studies to patients with serum cobalamin concentrations up to 300 pg/ml and found significant numbers with neuropathy, raised serum MMA concentrations, and response of the neuropathy to treatment with cobalamin (Communication to the FASEB Research Conference, Saxon's River, USA, August, 1990).

Attempts to identify undiagnosed pernicious anaemia, including neuropathy, in large population groups have been made by a variety of means⁸ and none has indicated a pool of missed patients. Thus claims to having discovered a significant group of patients in whom cobalamin deficiency has been overlooked, need to be looked at critically and demand more solid proof than has been offered.

The ability to confirm cobalamin deficiency from a serum sample with a low cobalamin concentration by raised MMA concentration is a potentially welcome advance. Apart from the difficulty of the assay, which will preclude its use by most laboratories, there are a number of loose ends that need to be tied.

The specificity of the assay has to be addressed. Some eight of 59 (14%) of patients not responding to cobalamin also had increased MMA concentrations.⁷ Some several months, rather than days, elapsed before serum MMA concentrations declined with cobalamin treatment (FASEB Conference, 1990). The decline in urinary MMA after oral valine or isoleucine represents the return to normal of the metabolism not of MMA, but of MMA-coenzyme A.

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MMA, given orally or intravenously, is excreted into urine in a similar way in both controls and patients with cobalamin deficiency.¹ Assay of serum MMA may therefore not have the specificity or sensitivity of urinary MMA excreted after a dose of a physiological precursor.

Fourteen of the cobalamin responders reported by Stabler *et al*⁷ had normal Schilling tests, and some of the abnormal Schilling tests, as is often the case, could have been due to incomplete urine collection. What was the clinical diagnosis in the apparently cobalamin deficient patients who absorbed cobalamin normally?

The major evidence offered—that the raised serum MMA concentration signified true cobalamin deficiency—was the improvement of neuropathy in response to treatment with cobalamin. While some might regard it as unethical to carry out a double blind trial with cobalamin *v* placebo, at least the assessment of response should be done blind by a neurologist not familiar with the treatment that had been given. Biased assessment of minor neurological signs in the elderly can be misleading.

Evaluation of the place of serum MMA in diagnosis requires assessment in fully characterised case material. The clinical importance of a raised serum MMA needs to be assessed in relation to established criteria, including urinary MMA excretion after oral valine, marrow morphology, and the dU suppression test, as well as other criteria normally used in the investigation of patients with megaloblastic anaemia.

Cobalamin neuropathy and methylation

A substantial proportion of methionine in mammalian cells is converted into S-adenosylmethionine, the principal donor of methyl groups in transmethylation reactions. The impairment of methionine synthesis in cobalamin and folate deficiency has raised the possibility that lack of methionine may in turn result in lack of S-adenosylmethionine and hence impairment of methylation. Substance was given to this view by the observation of impaired choline synthesis in rats with cobalamin deficiency.⁹ Scott *et al*¹⁰ found that cobalamin neuropathy in monkeys, induced by inactivation of cobalamin by nitrous oxide (N₂O), was improved by feeding them methionine. They suggested that impaired transmethylation in nerve tissue was the explanation.

Further indirect evidence came from the neuropathy that developed in pigs exposed to N₂O. When S-adenosylmethionine donates its methyl group it becomes S-adenosylhomocysteine. Normally the adenosine is removed and the remaining homocysteine is recycled back to methionine by accepting a methyl group from either methylfolate or from betaine. In normal brain only the cobalamin dependent pathway using methylfolate as methyl donor, is present. In cobalamin deficient brain neither pathway is present. Thus there is no mechanism for recycling the homocysteine other than by transporting it to the liver where betaine methyltransferase is available. S-adenosyl-

homocysteine is a potent inhibitor of transmethylation reactions and its persistence in the central nervous system suggests that it could interfere with methylation pathways. Weir *et al*¹¹ found a significant increase in S-adenosylhomocysteine concentrations in cobalamin deficient pig brain and this lent further support to their hypothesis that transmethylation was impaired. Direct assessment of methylation was not attempted.

There are, however, many data which militate against the hypothesis of an impaired transmethylation. The concentration of S-adenosylmethionine in cobalamin deficient brain remains normal or even raised.^{12,13} There was no impairment of labelled methyl group incorporation into brain phospholipids in fruit bats dying of cobalamin neuropathy, and no changes in synaptosomal and myelin lipid methylation.¹⁴ Methylation of myelin basic protein in cobalamin deficient fruit bats was no different from that in controls.¹⁵

Viera-Makings *et al*¹⁶ repeated the porcine studies of Weir *et al* in fruit bats, a species that has been widely used to study cobalamin neuropathy. In cobalamin deficient fruit bats fatal neuropathy ensued but, unlike the case in pigs, S-adenosylmethionine and S-adenosylhomocysteine concentrations in brain remained unchanged from those in controls. Thus the weight of evidence is that impaired methylation is not the cause of cobalamin neuropathy. The observations of Weir *et al* remain of great interest.

Clinical implications of nitrous oxide anaesthesia

Although nitrous oxide has been in general use as an anaesthetic agent for over 100 years and its safety is well established, the observation by Lassen *et al*¹⁷ that N₂O inhalation given for five to six days to control spasms in tetanus produced severe, even fatal, megaloblastic anaemia was unexpected. Banks *et al*¹⁸ showed that N₂O reacted with transition-metal complexes of which cobalamin in vivo was the prime example. N₂O reacts with cob[I]alamin in methionine synthetase; the N₂O is cleaved and the active oxygen species produced oxidises both the cobalt of cobalamin as well as the apoenzyme. Recovery after N₂O requires not only new cobalamin but further synthesis of apoenzyme, taking three to four days. The mutase enzyme, which does not have reduced cobalamin, is not affected by N₂O until cobalamin stores fall, and there is no increase in urinary MMA excretion.

Amess and his colleagues¹⁹ noted that the development of megaloblastic haemopoiesis and an abnormal dU suppression test were improved by the addition of cobalamin, in patients given N₂O after surgery, although even exposure to the gas over 24 hours did not produce significant clinical problems. Megaloblasts persist in the marrow for a further three days and giant granulocyte precursors are still present up to five to six days later after which hypersegmented neutrophils are first seen in the marrow and then in the peripheral blood.²⁰

Intermittent N_2O exposure is usually the result of N_2O abuse by dentists, operating theatre technicians, and others. It leads to classic cobalamin neuropathy.²¹ It can occur when N_2O is given regularly even for as little as 15 minutes twice daily, to allow painful procedures to be done.²²

Probably the most important effect of N_2O exposure may be when it is used as an anaesthetic agent in patients with cobalamin malabsorption (pre-pernicious anaemia, ileal disease, etc) when residual cobalamin stores are destroyed. Impaired cobalamin absorption prevents the replenishment of these cobalamin stores from dietary cobalamin postoperatively, and megaloblastic anaemia or neuropathy develops several months later.²³

The effects of prolonged or repeated N_2O exposure can largely be prevented by 30 mg folinic acid given six hourly.²⁴

Effect of cobalamin deficiency on folate metabolism

The diagnosis and treatment of patients with disorders of cobalamin or folate metabolism generally poses few problems. The interrelation of these two trace vitamins continues to be the subject of debate.

Folates are required for the transfer of single carbon (1-C) units in the synthesis of three of the four bases of DNA (guanine, adenine, and thymine), for methionine synthesis, as well as for synthesis of other compounds.²⁵ These 1-C units are formate ($-\text{CHO}$) required for purine synthesis, methylene ($-\text{CH}_2-$) for thymidine synthesis, and the methyl ($-\text{CH}_3$) for methionine synthesis.

Cobalamin, with folate, is necessary for methionine synthesis, but cobalamin is not directly involved in the synthesis of any of the bases needed for DNA. Yet in cobalamin deficiency synthesis of all these bases is severely impaired.²⁶ This is the result of a role cobalamin has in the availability of 1-C units.

THE METHYLFOLATE TRAP

Methylfolate arises by reduction of the $-\text{CH}_2-$ group on $\text{CH}_2\text{-H}_4\text{folate}$ to $\text{CH}_3\text{H}_4\text{folate}$. The enzyme involved is methylene H_4folate reductase. In vitro studies show that the reaction strongly favours methylfolate synthesis²⁷ and that the reaction does not go in the reverse direction (methylfolate to methylenefolate) to any great extent. This forms the basis for the methylfolate trap hypothesis put forward in 1962 by Herbert and Zalusky²⁸ and by Noronha and Silverman.²⁹

The hypothesis proposes that, as in cobalamin deficiency, the methyl group of methyl H_4folate cannot be passed on to homocysteine to form methionine and, secondly, the methyl group cannot be oxidised back to methylene to form methylene H_4folate : the H_4folate portion is immobilised or trapped. In time an overall lack of free H_4folate will interfere with other 1-C unit transfers and hence depress thymidine and purine synthesis. This hypothesis was intellectually appealing and was

widely accepted despite the absence of studies to test the hypothesis which proved difficult to devise. Many observations were made that were compatible with such a hypothesis but they were equally compatible with any other hypothesis that postulated interference of folate function as a result of cobalamin deficiency. Nevertheless, belief in the hypothesis was such that Dr J Bertino was able to tell an international meeting on pteridines and folates at La Jolla, California, USA, that the methylfolate trap was not hypothesis; it was fact.

The proof required to validate a methylfolate trap is: (1) evidence that in vivo (as well as in vitro) methylene reductase operates only in one direction; (2) that methylfolate is not metabolised in cobalamin deficiency; and (3) its sequestration, if this is the case, is such as to curtail 1-C unit transfer.

A few observations appeared that were at odds with the hypothesis. Methylfolate was given intravenously to patients with cobalamin deficiency in the expectation that, as it could not be used, it would be cleared from plasma at a slower rate than normal.³⁰ In fact, the opposite occurred. The removal of methyl H_4folate was more rapid than normal and was most rapid in the most anaemic patients.

Methylfolate was found to be a methyl donor in the methylation of biogenic amines such as dopamine. The mechanism proved to be oxidation of the methyl group of methylfolate to formaldehyde (methylene) and this was transferred to the biogenic amine, the methylene again being reduced to methyl. Three groups isolated the enzyme responsible for oxidising the methyl group of methylfolate and agreed that it was methylene reductase.³¹⁻³³ Methylene reductase can be readily made to go in the forbidden direction by provision of an electron acceptor and, indeed, the standard assay for methylene reductase is in the direction of methyl into methylene.

Finally, Thorndike and Beck³⁴ reported that the methyl group of methylfolate was oxidised in an essentially similar manner by lymphocytes from normoblastic subjects and by the lymphocytes from a patient with untreated pernicious anaemia. The likely reason (see below) is the provision of methionine in the suspending medium which promotes prompt methyl group oxidation.

Accumulation of methyl H_4folate in cobalamin deficiency

Inactivation of cobalamin by N_2O leads to an initial rise in the concentration of methylfolate polyglutamate in tissues due to cessation of transfer of the methyl group to homocysteine. After 12 hours, however, the methylfolate concentration starts to fall to well below normal. After five days 80% of folate, mainly methylfolate, has disappeared from the liver and other tissues. The fall is the result of considerable loss of methylfolate into the urine.³⁵ Although there is no accumulation of methylfolate, the data neither support nor contradict a biochemical folate trap. Both H_4folate and smaller amounts of formyl-

Table 1 Utilisation of [14 C] formate by bone marrow cells from normal and cobalamin deficient rats

End product of single carbon unit metabolism	Marrow cells from:	
	Controls: Mean (SD)	Cobalamin deficient: Mean (SD)
Guanine—RNA	0.97 (0.52) (n = 7)†	0.27 (0.09) (n = 3)
—DNA	0.52 (0.28) (n = 6)	Trace* (n = 3)
Adenine—RNA	1.51 (0.75) (n = 7)	0.97 (0.29) (n = 3)
—DNA	0.27 (0.12) (n = 6)	Trace* (n = 3)
Thymine—DNA	2.60 (0.61) (n = 6)	0.72* (0.14) (n = 3)
Methionine	21.2 (11.2) (n = 5)	0* (n = 3)
Serine	44.6 (8.3) (n = 4)	3.3* (0.1) (n = 2)
Choline	59.2 (28.2) (n = 5)	14.1 (2.1) (n = 3)
Protein†	735 (265) (n = 4)	228* (76) (n = 2)
CO ₂ †	0.54 (0.15) (n = 4)	0.07 (0.03) (n = 3)
MethylH ₄ folate	0 (n = 3)	0 (n = 3)
FormylH ₄ folate	0 (n = 3)	0 (n = 3)

*Significantly different from control value.

†Expressed as nmol formate/mg protein.

H₄folate are always detectable in cobalamin deficient tissues. Disappearance of H₄folate, postulated in the methylfolate trap, cannot be demonstrated.

Deacon *et al*²⁶ incubated bone marrow cells from control and cobalamin deficient rats with [14 C]formate for one and a half hours and measured the incorporation of this 1-C unit into purines, thymine, methionine, etc, by isolating these end products by high pressure liquid chromatography. The utilisation of formate requires its uptake by H₄folate to form formylH₄folate and thereafter its transfer in synthetic pathways (table 1). All the values with cobalamin deficient cells were lower than those with control cells, indicating an overall impairment of 1-C unit transfer. There was no demonstrable labelled formylH₄folate, indicating that the transfer of formate was rapid. Furthermore, there is no detectable label as methylH₄folate. The absence of [14 C]methyl-H₄folate accumulation indicates that the trapping of methylfolate cannot be the explanation for the overall failure of 1-C unit metabolism with cobalamin deficient marrow cells (table 1). This study is one of the few direct tests for methylfolate trapping and none was found.

Metabolism of MethylH₄folate in cobalamin deficiency

Noronha and Silverman²⁹ found that when methionine was added to a rat diet in both control and cobalamin deficient animals, there was a fall in liver methylfolate and a rise in the concentrations of formylfolate and H₄folate. This has been confirmed many times, usually giving methionine by injection. A parenteral dose of methionine in an amount well within the daily intake produces disappearance of 90% of the methylfolate from liver within 15 minutes and a rise in formylH₄folate (fig 1).³⁶⁻³⁸ This occurs in both control and cobalamin deficient animals. Methylfolate only reaccumulates in liver when the methionine concentrations have fallen to base line. Thus there is a profound difference between *in vitro* and *in vivo* data. In intact animals methylene reductase readily works in both directions reducing methylene to methyl or oxidising methyl to methylene. Methionine is a toxic aminoacid in excess and its concentration is rigidly controlled. Excess methionine can only arise from

dietary intake or possibly excessive protein catabolism, and in such circumstances synthetic pathways are shut down and methionine precursors including methylH₄folate removed.

In intact animals taking water only the half-life of the methyl group on methylH₄folate in rat liver is two hours.³⁹ In cobalamin deficiency the half-life is much longer and the mechanism of disposal of the methyl group is different. Intact fasting control animals not receiving methionine pass on the methyl group of methylfolate to homocysteine. Fasting cobalamin deficient animals use the methylfolate as a substrate for adding on glutamic acid residues (forming folate polyglutamate, the active coenzyme). When six glutamic acid residues are present the methyl group is oxidised, releasing H₄folate. Thus after giving methylH₄folate labelled in the methyl group to cobalamin deficient rats, labelled methyl-H₄folate pentaglutamate is detected in liver but not methylH₄folate hexaglutamate.³⁹

The available data do not lend any support to a methylfolate trap hypothesis. In addition there is a considerable body of data that cannot be explained by a methylfolate trap (see below).

FORMATE STARVATION HYPOTHESIS

This view indicates that the role of cobalamin is in the supply of formylH₄folate and, in particular, in promoting the attachment of formate to tetrahydrofolate. In the absence of cobalamin, formate accumulates in tissues and is excreted in the urine. "Active" formate normally arises in the course of intermediary metabolism, particularly of methionine, and this formate is readily linked to H₄folate in both control and cobalamin deficient cells. The formate starvation hypothesis is based on a considerable body of data accumulated largely by study of cobalamin deficient rats exposed to N₂O, and provides a satisfactory explanation for virtually all the observations made in relation to the effects of Cobalamin deficiency.

Figure 1 Rats were given 100 μ mol methionine intraperitoneally and the folate analogues in the liver isolated by high pressure liquid chromatography and assayed microbiologically. The injection of methionine was followed by a pronounced fall in the concentration of methyl-H₄folate and, at the same time, a rise in formyl-H₄folate and unsubstituted H₄folate. The data indicate that methionine led to oxidation of the methyl group on methyl-H₄folate to formyl and CO₂.

Table 2 Synthesis of folate-polyglutamate in livers of rats given folate-monoglutamate

Folate analogue	Percentage of folate analogue in rat liver converted into folate-polyglutamate	
	Controls	Cobalamin deficient
Folate	51	0
H ₄ folate	55	0
Methyl-H ₄ folate	42	0
10-formyl-H ₄ folate	52	46
5,10-methenyl-H ₄ folate	55	59
5-formyl-H ₄ folate	52	49

Reversal of cobalamin deficiency by formylH₄folate (but not H₄folate)

There are two sets of observations indicating that the effects of cobalamin deficiency are reversed by supplying folate carrying a 1-C unit at the formate level of oxidation. This is available as folinic acid (5, formyl-H₄folate). All active folates are reduced—that is, unlike pteroylglutamic acid used pharmacologically, they have four additional hydrogens and are called tetrahydrofolates or H₄folates. H₄folate is unstable unless protected by reducing agents such as ascorbate. Its relative instability is one of the criticisms that has been made in relation to these studies.⁴⁰ Thus H₄folate (Sigma) has always been freshly reconstituted from the dry state in 1% ascorbate and used immediately. Its identity has been confirmed by its spectrophotometric absorption. It is very unlikely that the oxidation of H₄folate was a factor in any of these results.

The active folate coenzyme in vivo is not H₄folate but H₄folate polyglutamate. Thus intracellularly glutamic acid residues are added to H₄folate or to one of its analogues so that the number increases from one up to seven. Table 2 shows the amount of folate polyglutamate

formed in liver from six folate analogues labelled with either [¹⁴C] or [³H] by control and cobalamin deficient rats. The control rats used all six folate analogues equally with about half the folate taken up by liver being converted into polyglutamate. The cobalamin deficient animals were not able to use the first three analogues at all, including H₄folate itself, but used the last three normally.⁴¹ These last three folate analogues all had a 1-C unit at the formate level of oxidation. The enzyme which adds glutamic acid residues to folate, folate polyglutamate synthetase, is induced in cobalamin deficient rats.⁴²

Thus the effect of cobalamin deficiency was bypassed by providing formylH₄folate. Furthermore, the inability of cobalamin deficient animals to use H₄folate, or folate itself, which is completely stable, is at odds with the methyl-folate trap hypothesis because these analogues are outside the “trap” and should be used normally.

The second set of circumstances in which formylH₄folate but not H₄folate bypasses the effects of cobalamin deficiency is in the synthesis of thymidine. In this pathway a 1-C unit (methylene or -CH₂-) from methylene-H₄folate is added to deoxyuridine to form thymidine. Using marrow cells from patients with untreated pernicious anaemia,⁴³ as well as marrow from cobalamin deficient rats,⁴⁴ formylH₄folate restored thymidine synthesis completely but H₄folate was relatively ineffective at the same dose.

The effectiveness of formylH₄folate as opposed to the ineffectiveness of H₄folate as a substrate suggests that cobalamin is concerned with either the supply to or utilisation of formate by the folate coenzyme.

Reversal of cobalamin deficiency by provision of formate by methionine

The synthesis of methionine requires both cobalamin and folate. Over many years Stokstad and his colleagues⁴⁵ have shown that methionine reverses the effects of cobalamin deficiency induced by diet in rats. This has proved to be the case in cobalamin deficiency caused by N₂O as well. Thus methionine restored folate polyglutamate synthesis in cobalamin deficient rats⁴¹; restored impaired thymidine synthesis⁴⁶; prevented the development of cobalamin neuropathy in cobalamin deficient monkeys,⁴⁷ in cobalamin deficient fruit bats,⁴⁸ and in cobalamin deficient pigs¹¹; restored formylation of H₄folate in small gut segments from cobalamin deficient rats⁴⁹; and reduced formiminoglutamic acid excretion in cobalamin deficient rats⁵⁰ and man.^{51 52}

There are two ways in which methionine provides “active” formate that bypasses the effects of cobalamin deficiency.

Methylthioadenosine: In man between 0.5–1 mmol of methionine is metabolised each day along the polyamine pathway via S-adenosyl-methionine in the synthesis of spermidine and putrescine (fig 2). The synthesis of these compounds uses three of the carbons of methionine, leaving behind a methylthio- (CH₃SH =) unit attached to ribose. The latter is recycled

Figure 2 The pathway by which methionine yields an active formate (CHO) unit. Ado = adenine.

into methionine using the $\text{CH}_3\text{SH}=\text{}$ of the starting methionine, the remaining carbons coming from ribose. A 1-C unit as formate is released for each mole of methionine formed.⁵³ Methylthioadenosine was significantly more effective than methionine itself in restoring folate polyglutamate synthesis in the livers of cobalamin deficient rats⁴¹ and restored normal formylation of H_4folate in cobalamin deficient gut segments.⁴⁹

Methylthioadenosine labelled with [^{14}C] in the carbon converted to formate is a potent donor of 1-C units in purine synthesis by marrow cells from both control and cobalamin deficient rats.⁵⁴ The formate released from methionine via the polyamine pathway is sufficient to meet man's formate requirements (S Harvey-Mudd, personal communication).

Serine is a potent donor of 1-C units in *in vitro* systems, but Deacon (unpublished observations) was unable to demonstrate use of the β carbon of serine for purine or thymidine synthesis in intact animals. It may be that *in vivo* serine is so rapidly metabolised along other pathways that little is available to donate 1-C units. On the other hand, labelled formate was readily used as a 1-C unit *in vivo*. Formate via methionine may be the major source of 1-C units *in vivo*.

Oxidation of methyl H_4folate : A rise in the methionine concentration leads to rapid oxidation of the methyl group of methyl H_4folate to methylene and formate and hence makes formate available (fig 1). Thus methionine makes formate available by the release of "active" formate in the course of the synthesis of polyamines from methionine, as well as promoting the oxidation of the methyl group of methylfolate to methylene and formate. This seems to be the explanation for the effect of methionine in reversing cobalamin deficiency.

Accumulation of formate in tissues in cobalamin deficiency

Direct measurement of endogenous formate concentrations in liver, blood,²⁶ and brain (unpublished observations) show a striking accumulation of formate in all these tissues in cobalamin deficient rats (table 3). Increased formate in the urine of cobalamin deficient rats has been reported.^{55,56} The accumulation of formate in brain indicates that the same biochemical defect that is present in liver is present in the central nervous system. Thus it seems likely that the same biochemical lesion is present in all tissues in cobalamin deficiency.

The accumulation of formate indicates that

Table 3 Endogenous formate concentrations in rat blood, liver, and brain in cobalamin deficiency (nitrous-oxide exposure)

Days of N_2O exposure	Formate		
	Blood ($\mu\text{g}/\text{ml}$)	Liver ($\mu\text{g}/\text{g}$)	Brain ($\mu\text{g}/\text{g}$)
0	23 (3)*	29 (8)	0
1	80 (18)	83 (18)	14 (14)
2	73 (20)	75 (17)	26 (14)
5	72 (12)	66 (8)	

*1 SD.

the biochemical lesion in cobalamin deficiency is a failure to link formate to H_4folate . By contrast, formate produced in other pathways, such as a formate arising from the metabolism of methionine along the polyamine path, is used normally by cobalamin deficient cells. The term "active" formate has been used for this form of formate. In biochemical terms the difference between formate and "active" formate is not clear. The data suggest that cobalamin may have a role in the formation of "active" formate but the necessary investigations to explore this possibility have yet to be done.

The enzyme linking formate to H_4folate is formyl H_4folate synthetase. The enzyme is induced in cobalamin deficiency^{57,58} and the increased enzyme activity returns to normal only after cobalamin deficiency has been corrected—for example, by returning animals breathing N_2O to an air environment.

Folate in the prevention of neural tube defects

Reports that folate (alone or with other vitamins) significantly reduced the incidence of neural tube defects when taken before or very early in pregnancy^{59,60} stimulated the setting up of a large trial. A total of 1817 women who had had a previous infant affected with a neural tube defect were randomly placed in one of four categories.⁶¹ Twenty seven infants were born with neural tube defects, six in the group taking 4 mg folic acid daily, and 21 in the groups not receiving folic acid. Folate was given before conception until the twelfth week of pregnancy. The difference was highly significant. Other vitamins had no protective effect.

Earlier data⁶¹ had suggested that the women having infants with neural tube defects did not have evidence of folate deficiency. The effect on the embryo may be an example of localised folate deficiency, the supply of folate to the embryo being limited even in women with apparently adequate folate nutrition, and it results in impaired cell division at this crucial time in the development of the embryo. An increase in folate concentrations in tissue fluids may overcome this failure of local folate supply.

- 1 Chanarin I, England JM, Mollin C, Perry J. Methylmalonic acid excretion studies. *Br J Haematol* 1973;25:45-53.
- 2 Stabler SP, Marcell PD, Podell ER, Allen RH, Lindenbaum J. Assay of methylmalonic acid in the serum of patients with cobalamin deficiency using capillary gas chromatography-mass spectrometry. *J Clin Invest* 1986;77:1686-12.
- 3 Stabler SP, Marcell PD, Podell ER, Allen RH, Savage DG, Lindenbaum J. Elevation of total homocysteine in the serum of patients with cobalamin or folate deficiency detected by capillary gas chromatography-mass spectrometry. *J Clin Invest* 1988;81:466-74.
- 4 Green R, Gatautis V, Jacobsen DW. Serum methylmalonic acid (MMA) and homocysteine (Hcy) are more specific tests than serum vitamin B_{12} for identifying true cobalamin (CBL) deficiency. *Blood* 1990;76(Suppl 1):33a.
- 5 Lindenbaum J, Heaton EB, Savage DG, et al. Neuropsychiatric disorders caused by cobalamin deficiency in the absence of anemia or macrocytosis. *N Engl J Med* 1988;318:1720-8.
- 6 Shorvon SD, Carney MWP, Chanarin I, Reynolds EH. The neuropsychiatry of megaloblastic anaemia. *Br Med J* 1980;281:1036-8.
- 7 Stabler SP, Allen RH, Savage DG, Lindenbaum J. Clinical

- spectrum and diagnosis of cobalamin deficiency. *Blood* 1990;76:871-81.
- 8 Chanarin I. *The megaloblastic anaemias*. 2nd ed. Oxford: Blackwell Scientific, 1979.
 - 9 Arnstein HRV, Neuberger A. The effect of cobalamin in the quantitative utilization of serine, glycine and formate for the synthesis of choline and methyl groups of methionine. *Biochem J* 1953;55:259-71.
 - 10 Scott JM, Dinn JJ, Wilson P, Weir DG. Pathogenesis of subacute combined degeneration: A result of methyl group deficiency. *Lancet* 1981;ii:334-7.
 - 11 Weir DG, Keating S, Molloy A, et al. Methylation deficiency causes vitamin B₁₂-associated neuropathy in the pig. *J Neurochem* 1988;51:1949-52.
 - 12 Lumb M, Sharer N, Deacon R, Jennings P, Purkiss P, Perry J, Chanarin I. Effects of nitrous oxide-induced inactivation of cobalamin on methionine and S-adenosylmethionine metabolism in the rat. *Biochim Biophys Acta* 1983;756:354-9.
 - 13 Van der Westhuyzen J, Metz J. Tissue S-adenosylmethionine levels in fruit bats (*Rousettus aegyptiacus*) with nitrous oxide-induced neuropathy. *Br J Nutr* 1983;50:325-30.
 - 14 McLoughlin JL, Cantrill RC. Nitrous oxide induced vitamin B₁₂ deficiency: Measurement of methylation reactions in the fruit bat (*Rousettus aegyptiacus*). *Int J Biochem* 1986;18:199-202.
 - 15 Deacon R, Purkiss P, Green R, Lumb M, Perry J, Chanarin I. Vitamin B₁₂ neuropathy is not due to failure to methylate myelin basic protein. *J Neurol Sci* 1986;72:113-7.
 - 16 Viera-Makings E, Metz J, Van der Westhuyzen J, Bottiglieri T, Chanarin I. Cobalamin neuropathy. Is S-adenosylhomocysteine toxicity a factor? *Biochem J* 1990;266:707-11.
 - 17 Lassen HCA, Henriksen E, Neukirch F, Kristensen AS. Treatment of tetanus. Severe bone marrow depression after prolonged nitrous-oxide anaesthesia. *Lancet* 1956;ii:527-30.
 - 18 Banks RGS, Henderson RJ, Pratt JM. Some reactions of nitrous oxide with transition-metal complexes. *J Chem Soc* 1968;2886-9.
 - 19 Amess JAL, Burman JF, Rees GM, Nancekivell DG, Mollin DL. Megaloblastic haemopoiesis in patients receiving nitrous oxide. *Lancet* 1978;iii:339-42.
 - 20 Skacel PO, Hewlett AM, Lewis JD, Lumb M, Nunn JF, Chanarin I. Studies on the haemopoietic toxicity of nitrous oxide in man. *Br J Haematol* 1983;53:189-200.
 - 21 Layzer RB. Myeloneuropathy after prolonged exposure to nitrous oxide. *Lancet* 1978;ii:1227-30.
 - 22 Nunn JF, Sharer NM, Gorchein A, Jones JA, Wickramasinghe SN. Megaloblastic haemopoiesis after short-term exposure to nitrous oxide. *Lancet* 1982;ii:1379-81.
 - 23 Schilling RF. Is nitrous oxide a dangerous anaesthetic for vitamin B₁₂-deficient subjects? *JAMA* 1986;255:1605-6.
 - 24 Amos RJ, Amess JAL, Nancekivell DG, Rees GM. Prevention of nitrous oxide induced megaloblastic change in bone marrow using folic acid. *Br J Anaesth* 1984;56:103-7.
 - 25 Blakley RL. *The biochemistry of folic acid and related pteridines*. Amsterdam:North Holland, 1969.
 - 26 Deacon R, Perry J, Lumb M, Chanarin I. Formate metabolism in the cobalamin-inactivated rat. *Br J Haematol* 1990;74:354-9.
 - 27 Green JM, Ballou DP, Matthews RG. Examination of the role of methylenetetrahydrofolate reductase in the incorporation of methyltetrahydrofolate into cellular metabolism. *FASEB J* 1988;2:42-7.
 - 28 Herbert V, Zalusky R. Interrelation of vitamin B₁₂ and folic acid metabolism: folic acid clearance studies. *J Clin Invest* 1962;41:1263-76.
 - 29 Noronha JM, Silverman M. On folic acid, vitamin B₁₂, methionine and formiminoglutamic acid metabolism. In: Heinrich HC, ed. *Vitamin B₁₂ and intrinsic factor*. 2 Europaisches Symposium. Stuttgart: Enke, 1962:728-36.
 - 30 Chanarin I, Perry J. Metabolism of 5-methyltetrahydrofolate in pernicious anaemia. *Br J Haematol* 1968;14:297-301.
 - 31 Pearson AGM, Turner AJ. Folate dependent 1-carbon transfer to biogenic amines mediated by methylenetetrahydrofolate reductase. *Nature* 1975;258:173-4.
 - 32 Taylor RT, Hanna ML. 5-methyltetrahydrofolate aromatic alkylamine N-methyltransferase: an artefact of 5, 10-methylenetetrahydrofolate reductase activity. *Life Sci* 1975;17:111-20.
 - 33 Stebbins RD, Meller E, Rosengarten H, Friedhoff A, Silber R. Identification of N5-N10 methylene tetrahydrofolate reductase as the enzyme involved in the 5-methyltetrahydrofolate-dependent formation of a beta-carbonine derivative of 5-hydroxytryptamine in human platelets. *Arch Biochem Biophys* 1976;173:673-9.
 - 34 Thorndike J, Beck WS. Production of formaldehyde from N5-methyltetrahydrofolate by normal and leukemic leukocytes. *Cancer Res* 1977;37:1125-32.
 - 35 Lumb M, Perry J, Deacon R, Chanarin I. Urinary folate loss following inactivation of vitamin B₁₂ by nitrous oxide in rats. *Br J Haematol* 1982;51:235-42.
 - 36 Brody T, Watson JE, Stokstad ELR. Folate pentaglutamate and folate hexaglutamate mediated one-carbon metabolism. *Biochemistry* 1982;21:276-82.
 - 37 Lumb M, Chanarin I, Deacon R, Perry J. In vivo oxidation of the methyl group of hepatic 5-methyltetrahydrofolate. *J Clin Pathol* 1988;41:1158-62.
 - 38 Lumb M, Bottiglieri T, Deacon R, Perry J, Chanarin I. Regulation of 5-methyltetrahydrofolate synthesis. *Biochem J* 1989;258:611-2.
 - 39 Lumb M, Chanarin I, Perry J, Deacon R. Turnover of the methyl moiety of 5-methyltetrahydropteroylglutamic acid in the cobalamin-inactivated rat. *Blood* 1985;66:1171-5.
 - 40 Babior BM. Erythrocyte disorders: anemias related to disturbances of DNA synthesis (megaloblastic anemias). In: Williams WJ, Beutler E, Erslev AJ, Lichtman MA, eds. *Hematology* 4th ed. New York: McGraw-Hill, 1990: 453-81.
 - 41 Perry J, Chanarin I, Deacon R, Lumb M. Chronic cobalamin inactivation impairs folate polyglutamate synthesis in the rat. *J Clin Invest* 1983;71:1183-90.
 - 42 Perry J, Chanarin I, Deacon R, Lumb M. Folate polyglutamate synthetase activity in the cobalamin-inactivated rat. *Biochem J* 1985;227:73-7.
 - 43 Deacon R, Chanarin I, Perry J, Lumb M. A comparison of tetrahydrofolate and 5-formyltetrahydrofolate in correcting the impairment of thymidine synthesis in pernicious anaemia. *Scand J Haematol* 1982;28:289-92.
 - 44 Deacon R, Chanarin I, Perry J, Lumb M. The effect of folate analogues on thymidine utilization by human and rat marrow cells and the effect on the deoxyuridine suppression test. *Postgrad Med J* 1981;57:611-6.
 - 45 Shin YS, Buehring KU, Stokstad ELR. The relationships between vitamin B₁₂ and folic acid and the effects of methionine on folate metabolism. *Mol Cell Biochem* 1975;9:97-108.
 - 46 Sourial NA, Brown A. Regulation of cobalamin and folate metabolism by methionine in human bone marrow cultures. *Scand J Haematol* 1983;31:413-23.
 - 47 Dinn JJ, McCann S, Wilson P, Reed B, Weir D, Scott J. Animal model for subacute combined degeneration. *Lancet* 1978;ii:1154.
 - 48 Van der Westhuyzen J, Fernandez-Costa F, Metz J. Cobalamin inactivation by nitrous oxide produces severe neurological impairment in fruit bats: protection by methionine and aggravation by folates. *Life Sci* 1982;31:2001-10.
 - 49 Perry J, Deacon R, Lumb M, Chanarin I. Impaired formylation and uptake of tetrahydrofolate by rat small gut following cobalamin inactivation. *Biochim Biophys Acta* 1987;923:286-90.
 - 50 Deacon R, Perry J, Lumb M, Chanarin I. Increased urinary excretion of formiminoglutamic acid in nitrous oxide-treated rats and its reduction by methionine. *Eur J Biochem* 1983;129:627-8.
 - 51 Chanarin I. Urocanic acid and formiminoglutamic acid excretion in megaloblastic anaemia and other conditions: the effect of specific therapy. *Br J Haematol* 1963;9: 141-57.
 - 52 Herbert V, Sullivan LW. Formiminoglutamic aciduria in humans with megaloblastic anemia: diminution by methionine or glycine. *Proc Soc Exp Biol Med* 1963;112:304-5.
 - 53 Trackman PC, Abeles RH. The metabolism of 1-phospho-5-methyl-ribose. *Biochem Biophys Res Commun* 1981;103:1238-44.
 - 54 Deacon R, Bottiglieri T, Chanarin I, Lumb M, Perry J. Methylthioadenosine serves as a single carbon source to the folate coenzyme pool in rat bone marrow cells. *Biochim Biophys Acta* 1990;1034:342-6.
 - 55 Stokstad ELR, Webb RE, Shah E. Effect of vitamin B₁₂ and folic acid on the metabolism of formiminoglutamate, formate, and propionate in the rat. *J Nutr* 1966;88:225-32.
 - 56 Brothers V, Rowley BO, Gerritson T. Oxidation of compounds metabolised through folate coenzyme pathways in vitamin B₁₂ deficient rats. *Arch Biochem Biophys* 1975;116:475-82.
 - 57 Perry J, Deacon R, Lumb M, Chanarin I. The effect of nitrous oxide-induced inactivation of vitamin B₁₂ on the activity of formyl-methenyl-methylenetetrahydrofolate synthetase, methylenetetrahydrofolate reductase and formiminotetrahydrofolate transferase. *Biochem Biophys Res Commun* 1980;97:1329-33.
 - 58 Deacon J, Perry J, Lumb M, Chanarin I. Cobalamin inactivation induces formyltetrahydrofolate synthetase. *Fed Eur Biochem Soc* 1990;263:303-4.
 - 59 Smithell RW, Shepherd S, Schorah CJ, et al. Possible prevention of neural-tube defects by periconceptional vitamin supplementation. *Lancet* 1980;ii:339-40.
 - 60 Laurence KM, James N, Miller MH, Tennant GB, Campbell H. Double-blind randomized controlled trial of folate treatment before conception to prevent recurrence of neural-tube defects. *Br Med J* 1981;282:1509-11.
 - 61 MRC Vitamin Study Research Group. Prevention of neural tube defects: results of the medical research council vitamin study. *Lancet* 1991;ii:131-7.