Megaloblastic anaemia, cobalamin, and folate

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SUMMARY Developments relating to cobalamin and folate are reviewed. Current work on the relations between these two coenzymes are discussed, particularly those that have emerged in studies using nitrous oxide, which inactivates cobalamin.

During the 1960s many ideas were developed about the megaloblastic anaemias. Cobalamin assays on human sera, initially at the Royal Postgraduate Medical School, had been consolidated, and patients with megaloblastic anaemia could be grouped into those with low B_{12} concentrations and those with normal concentrations.[1] Isotopically labelled B_{12} became available and B_{12} absorption tests became part of clinical practice.[2] A method for the assay of serum folate was described[3] and was quickly extended to the more useful red cell folate assay.[4] By the end of 1963 an assay for the intrinsic factor content of gastric juice[5][6] and for the detection of antibodies in serum to intrinsic factor had been introduced as well as methods for detecting gastric parietal cell antibodies.

The use of urinary formiminoglutamic acid (Figlu) as a test for folate deficiency reared its ugly head.[7] And, finally, two groups put forward a hypothesis to account for the strange interrelation between B_{12} and folate that came to be known as the methylfolate trap hypothesis.[8][9]

This review covers the developments of the past quarter century and how the ideas of 25 years ago have stood the test of time.

Automated cell counters and mean corpuscular volume

The introduction into haematology laboratories of accurate and sophisticated blood counting machines is the major technical development of the past 25 years. It has highlighted the value of red cell size (mean corpuscular volume, MCV) in diagnosis and emphasised the raised MCV in many disorders that are unrelated to impairment of B_{12} and folate metabolism. Normoblastic macrocytosis occurs in chronic alcoholism, hypothyroidism, normal pregnancy, healthy neonates, marrow failure, chronic haemolytic states with raised reticulocyte values, many pre-leukaemic (myelodysplastic) disorders, and treatment with many cytotoxic drugs including, chlorambucil, mephalan, methotrexate, azathioprine etc. Finally,

there is a common macrocytosis occurring in premenopausal women whose only complaint is lassitude, which remains unexplained.

Not all patients with megaloblastic anaemia will have an increased MCV. Some who have thalassaemia trait, anaemia of chronic disorders, or concurrent iron deficiency may have a frankly megaloblastic marrow with a normal MCV.[10] When treated with B_{12} or folate, or both, the MCV falls to that associated with the underlying disorder below the normal range of 80 to about 94 fl.

Finally, there is no agreement as to what constitutes the normal MCV: quoted ranges vary from 76 at the lower end to 108 fl at the upper end. This is because there is no absolute way to obtain this value and the machine methods relate to the manner in which the settings are manipulated.

The serum cobalamin (B_{12}) concentration

The early assays for B_{12} were all microbiological assay procedures, and in essence these gave similar results. The assays were tedious to perform and often assays were rejected because of loss of purity of the organism, introduction of extraneous B_{12} in the assay system, and poor growth of the assay organism. For these reasons the development of an alternative method based on dilution of isotopically labelled B_{12} by the native B_{12} in a serum sample (saturation analysis) was widely welcomed. These assays lent themselves to the preparation of commercial kits that have become the mainstay of laboratory assays.

It soon became apparent that often a lot more B_{12} was being measured by the isotope methods than microbiological methods.[11][–][13] Thus the lower limit of the normal range could be 300 pg/ml with an isotope assay but was about 170 pg/ml by microbiological assay. A value of 250 pg/ml was low by isotope assay but plumb normal when assayed microbiologically.

When performing the isotope assay, a standard amount of $[^{57}Co]B_{12}$ is added to an extract of serum

containing a varying amount of native B_{12}. The next step is to take out an aliquot of the B_{12} in the mixture: this is done by adding a B_{12} binding protein. The greater the amount of native B_{12} the greater the dilution of $[^{57}Co]B_{12}$, and hence less $[^{57}Co]B_{12}$ will attach to the binder (fig 1). The explanation of the discrepancy between isotope and microbiological assay lay in the nature of the B_{12} binding protein used in the assay. When purified gastric intrinsic factor was used as the B_{12} binding protein the results were similar to those obtained by microbiological assay. When other B_{12} binders, such as those present in serum or in saliva—collectively termed R-binders—were used the result was always higher than that obtained with microbiological assay. Kolhouse *et al*[14] suggested that the R-binders were picking up forms of B_{12} in serum not picked up by intrinsic factor. This suggestion was shown to be correct by cross absorption studies.[15] By attaching intrinsic factor to polyacrylamide beads and adding these to serum extracts, all B_{12} analogues detectable both by microbiological assay and an isotope method with intrinsic factor were removed. The serum extract, however, still had B_{12} activity detected by an isotope assay with an R-binder. It now seems that microbiological assays measure about half the B_{12} present in serum. The same analogues are measured by microbiological assay and an intrinsic factor isotope assay.[16]

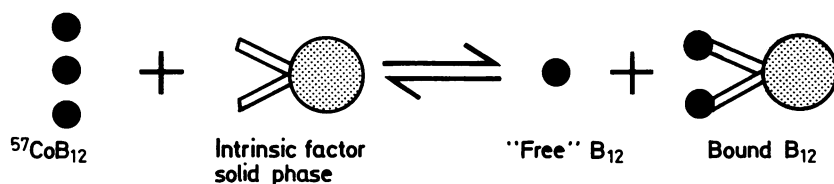The identity of the B_{12} analogues undetected by microbiological assay remains unknown and its origin is equally conjectural. In practice, an isotope assay for B_{12} using purified intrinsic factor is preferred because it gives a marginally better distinction between normal and B_{12} deficient sera,[17] but claims that large numbers of patients deficient in B_{12} were missed because R-binder assays were used, are exaggerated. Failure to detect low B_{12} concentrations is more likely to be due to a badly designed and badly performed assay than to the nature of the binder.

Finally, it is widely believed that a low serum B_{12} concentration is synonymous with B_{12} deficiency. This is not the case.[10] B_{12} deficiency is the commonest cause of low B_{12} concentration, but there are other causes. One third of patients with megaloblastic anaemia due to folate deficiency have low B_{12} concentrations, which return to normal within days of treatment with folic acid.[18] Low B_{12} concentrations are common near term in normal pregnancy, in patients with iron deficiency, after partial gastrectomy as well as in treated epileptics. Low B_{12} concentrations are often found in otherwise perfectly healthy people. Except in vegetarians with nutritional B_{12} deficiency, all patients with clinically important low B_{12} concentrations also have impaired B_{12} absorption. Where B_{12} absorption is normal and the patient is taking a mixed diet, a low B_{12} concentration is of no clinical importance.

B_{12} ABSORPTION

Tests for B_{12} absorption have become important and

^{57}Co-B$_{12}$ and intrinsic factor only

^{57}CoB$_{12}$ Intrinsic factor solid phase   "Free" B$_{12}$ Bound B$_{12}$

^{57}Co-B$_{12}$ with test sample and intrinsic factor

^{57}CoB$_{12}$ B$_{12}$ in test sample   Intrinsic factor solid phase   "Free" ^{57}CoB$_{12}$ increased   Bound ^{57}CoB$_{12}$ reduced
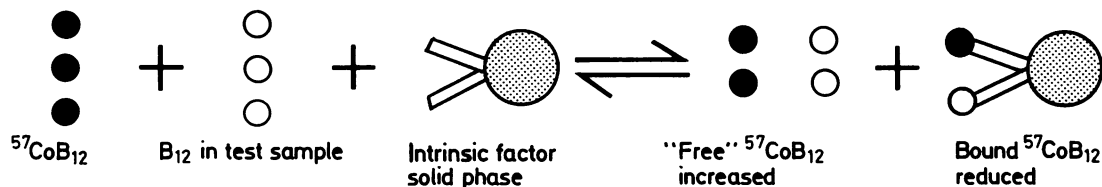
Fig 1   *When $[^{57}Co]B_{12}$ is mixed with intrinsic factor (in this example intrinsic factor is attached to a solid matrix), some B_{12} attaches to intrinsic factor and, when B_{12} is present in excess, some remains free. When, in addition to $[^{57}Co]B_{12}$, B_{12} is also present from the serum sample under test, the $[^{57}Co]B_{12}$ is diluted and hence the B_{12} binding to intrinsic factor is a mixture of $[^{57}Co]B_{12}$ and native B_{12} from serum. Thus the more native B_{12} present the greater the dilution of the $[^{57}Co]B_{12}$, and this is the basis on which the assay is performed.*

reliable indicators of ileal function and of availability of gastric intrinsic factor. It is less widely appreciated how often misleading results are recorded because of incomplete urine collection.[19] Assessment of the result by measuring the radioactivity of the labelled B_{12} in plasma collected after eight to 12 hours is as reliable an indicator of the result as a complete urine collection; it should be mandatory to insure against loss of urine, which occurs in between 25 to 50% of all tests, and in most tests carried out in the elderly, by combining the standard Schilling test with measurement of plasma radioactivity.

In addition to the known permutations of the B_{12} absorption test by giving B_{12} with intrinsic factor, performing the test after antibiotics etc, it has been suggested that additional information can be obtained by "binding" the labelled B_{12} to protein. Some have bound the labelled B_{12} to chick serum,[20] others have cooked the B_{12} with egg and served up an aliquot of this material.[21] The test thus requires digestive processes to release B_{12} from this unpromising material so that it can bind to intrinsic factor, which in turn will enable B_{12} to be absorbed. Even normal subjects are unable to achieve normal B_{12} absorption with this method, the mean urinary excretion with egg white being between 2·7 to 4·0% of a 1·68 μg oral dose and 5·1 to 6·0 with egg yolk. By contrast, the mean result in the standard Schilling test was 23·6%. Normal has been defined as the B_{12} absorption of aqueous B_{12} or absorption of food B_{12} as natural B_{12} in lamb.[22] Not surprisingly, achlorhydric patients and patients after gastric surgery fare even worse than controls with protein bound B_{12}. This is presumably because they cannot activate pepsinogen to aid proteolytic digestion of the binding proteins. The test has been interpreted, however, as detecting a lack of intrinsic factor at a stage when the standard B_{12} absorption test is still normal. There are no available data to suggest that this is likely; nor is there any evidence that the test is of any clinical value.

Dietary B_{12} is present either as part of methionine synthetase or as part of methylmalonyl CoA-mutase. Small amounts are linked to R-binders. Once B_{12} is released from the holoenzyme, much of it links to R-binder mainly from saliva, although R-binder is also present in gastric and intestinal juice. Pancreatic enzymes serve to release B_{12} from its R-binder link and permit its uptake by intrinsic factor. In pancreatitis B_{12} absorption is impaired because of failure to degrade the R-binder. Brugge et al[23] devised a suitable B_{12} absorption test in which the absorption of B_{12} bound to R-binder is compared with absorption of B_{12} bound to intrinsic factor. With controls, the Schilling test result with B_{12} and intrinsic factor was 21·2%, and with R-binder, 16·2%. With pancreatic disease the absorption of B_{12} with intrinsic factor was

16·3% and with R-binder only 1·0%. The combination is clearly of value in the diagnosis of chronic pancreatic disease.

There remain unresolved problems in relation to B_{12} absorption in patients with atrophic gastritis and after partial gastrectomy, where there is no clear correlation between the serum B_{12} concentration and the results of standard B_{12} absorption tests.

Intrinsic factor, its assay, and its antibody

Intrinsic factor, necessary for the intestinal absorption of B_{12}, is secreted by the gastric parietal cell. An assay of intrinsic factor was introduced simultaneously by Ardeman et al[5] and Abels et al in 1963.[6] The problem in developing such an assay is that gastric juice has at least three B_{12} binding proteins: intrinsic factor, partially digested intrinsic factor, and R-binder. When labelled B_{12} is added to gastric juice some binds to all three components, hence the binding of B_{12} alone cannot be used as a measure of intrinsic factor. Each of these binding proteins, however, can be prevented from taking up B_{12}. An intrinsic factor antibody present in serum in more than half the patients with pernicious anaemia, and when added to gastric juice, will react with intrinsic factor and prevent B_{12} uptake by intrinsic factor. Thus B_{12} binding is now due only to R-binder, and when this value is subtracted from the total B_{12} binding of gastric juice, it is a measure of intrinsic factor content. One unit of intrinsic factor is the amount binding 1 ng of B_{12}.

Cobinamide is a B_{12} analogue that lacks the benzimidazole moiety. It binds firmly to R-binder but not at all to intrinsic factor. Addition of cobinamide to gastric juice will block the R-binder, so that subsequent addition of labelled B_{12} will bind only to intrinsic factor.[24]

Assay of intrinsic factor has confirmed its virtual disappearance in pernicious anaemia; there is a vast excess present in healthy subjects. Only 1% of the normal daily output is required for normal B_{12} absorption.[10] Finally, abnormal forms of intrinsic factor have been shown in megaloblastic anaemia in infancy—that is, these have bound labelled B_{12} but have failed to potentiate its intestinal absorption.[25]

Detection of antibody to intrinsic factor is a useful aid in diagnosis of pernicious anaemia but, rarely, this antibody can be present in patients with Graves' disease, atrophic gastritis etc in the absence of pernicious anaemia.[26] More than 40% of patients with pernicious anaemia, however, do not have such an antibody in serum but may have it in the gastric secretion, when it is likely to be an IgA immunoglobulin rather than a IgG as in serum. Even those who lack humoral antibody against intrinsic factor generally show evidence of cell mediated immunity against intrinsic fac-

tor.[27] The importance of cell mediated immunity is shown by the high incidence of pernicious anaemia in patients with acquired hypogammaglobulinaemia, 30% of whom develop the disease.[10] These patients are unable to form humoral antibodies. These intrinsic factor antibodies are able to neutralise residual intrinsic factor secretion, or damage intrinsic factor secreting cells and convert a just adequate B_{12} absorption (present in simple atrophic gastritis) to an inadequate one so that a strong negative B_{12} balance develops. This is soon followed by clinically overt pernicious anaemia.

B_{12} transport proteins

R-protein (transcobalamins I and III) is present in all body fluids and most cells. A physiological role for these glycoproteins has not been identified. TC III in plasma is largely shed from leucocytes.

Transcobalamin II is the prime transport protein for B_{12}. It has a molecular weight of 35 000 daltons and is produced by the liver, macrophages, and ileal enterocytes. It is essential for moving B_{12} out of the ileal enterocyte to plasma during B_{12} absorption and for uptake of B_{12} by cells. There are specific receptors for TC II, and TC II is internalised into cells by endocytosis. In the absence of TC II a severe B_{12} deficient megaloblastic anaemia develops, usually within six weeks of birth, which has to be treated with very large doses of B_{12} (1000 μg two to three times a week) so that some enters cells by passive diffusion. Even B_{12} present in serum on R-binder in TC II deficiency cannot be used because of lack of the transport protein, so that paradoxically normal serum B_{12} concentration occurs concomitantly with severe tissue depletion of B_{12}.[10]

Folates

Assay of folate concentration with *Lactobacillus casei* in serum and whole blood has provided data that are similar to those resulting from B_{12} assay. A low serum folate concentration probably represents a state of negative folate balance and may be found in one third of all hospital patients. Red cells, on the other hand, contain some 30 times more folate than serum. Folate is incorporated into the red cell in the marrow by the developing erythroblast, and there is no folate turnover by mature red cells. Thus folate is locked in the red cell for the duration of its life span. Change in red cell folate comes from release of red cells from the marrow of different folate content. A low red cell folate represents established folate deficiency, irrespective of whether the subject is normoblastic or megaloblastic. Unfortunately, isotope methods used to assay folate have concentrated on the trivial serum folate assay and not on the clinically important red cell assay.

Urinary Figlu excretion is too sensitive for diagnosing folate deficiency for routine clinical practice. Like the serum folate concentration, abnormal results are found in one third of hospital patients, and it is no longer used.

The nature and function of intracellular folates have been clarified to a large extent. The folate analogue that is absorbed from food[28] and which circulates in body fluids is 5-methyltetrahydrofolate. There is some suggestive evidence for a membrane associated transport protein that facilitates its entry into cells.[29] Once in the cell, additional glutamic acid residues are added to give a form with 5-glutamic acid residues, although analogues with 4, 6, and 7 residues are present. In this form the folate (now termed folate polyglutamate) is retained in the cell and serves as the active coenzyme in transfer of single carbon units for purine, pyrimidine, and methionine synthesis. Cells that have lost the ability to make polyglutamates cannot grow unless the end products of folate metabolism such as purines are supplied.[30]

The folate antagonist methotrexate is also taken into the cell and is converted into a polyglutamate in which form it persists in the cell over a long period. Not all intracellular folate is polyglutamate and about one third has one glutamic acid residue.

The deoxyuridine (dU) suppression test

This test, which was modified by Metz *et al*[31] from the original account by Killman,[32] assesses the manner in which marrow cells convert deoxyuridine into deoxythymidine. The single carbon unit required in this step (methylene or -CH_2-) is supplied by 5, 10-methylene tetrahydrofolate. An aliquot of marrow cells is incubated with deoxyuridine. Normal cells will meet about 95% of their thymidine requirements by synthesis from deoxyuridine. The results of the test are assessed by adding preformed [^{3}H]thymidine. The remaining 5% requirement for thymidine is met by using this labelled compound.

Marrow cells from megaloblastic patients are unable to methylate deoxyuridine as well as normal cells, and less than 90% is used and more than 10% [^{3}H]thymidine is taken up into DNA. If B_{12} is added to a further aliquot of marrow cells there is some correction of the impairment in B_{12} deficient marrows but no correction with those deficient in folate. Formyltetrahydrofolate (such as folinic acid) will correct the impairment in both B_{12} and folate deficiency.

The dU suppression test is a test for "megaloblastosis" and provides an indication of the nature of the deficiency when it is combined with addition of B_{12} or folate to the incubation mixtures. It is, how-

ever, time consuming, the manipulations taking the better part of a day.

Cobalamin-folate interrelations

The methylfolate trap hypothesis to explain B_{12} folate interrelations was proposed just over 25 years ago.[8][9] Both B_{12} and folate are required for the synthesis of methionine (fig 2). The methyl group from methylfolate is passed on to homocysteine to form methionine, and B_{12} is an intermediary in this transfer. In the absence of B_{12} this pathway is interrupted. Two further observations led to the formulation of the methylfolate trap hypothesis.

1 The thermodynamics of the reaction by which methylfolate is formed from methylenefolate (5, 10, CH_2 folate———→5-CH_3 folate) is strongly shifted to the right—that is, on the basis of in vitro studies it was concluded that once methylfolate was formed it was improbable that it could be oxidised back to methylenefolate.

2 In untreated pernicious anaemia the serum folate (largely methylfolate) is increased. It was therefore deduced that in B_{12} deficiency methylfolate could not be used, it could not be oxidised back to methylenefolate, and as more folate accumulated in the methyl form a shortage of other folate analogues emerged. It was consequently supposed that there was impaired transfer of formyl for purine synthesis and methylene transfer for thymidine synthesis and that this led to interference with DNA synthesis and megaloblastosis. The raised serum folate was cited as supporting methylfolate trapping.

These views have been difficult to test as patients with untreated pernicious anaemia do not readily lend themselves to the sort of study required.

The situation was transformed by the observation that the anaesthetic gas nitrous oxide (N_2O) is activated by organometallic complexes of which B_{12} is the prime example.[33] N_2O is cleaved and at the same time B_{12} is oxidised to an irreversibly inert form. N_2O, in fact, specifically inactivates the enzyme methionine synthetase, of which B_{12} is a coenzyme.[34] Exposure to N_2O rapidly produces "B_{12} deficiency." In man this can lead to megaloblastic anaemia, neuropathy, and even death. Other species do not become megaloblastic, but some like the monkey and probably fruit bats develop a neuropathy. All, however, develop very similar biochemical changes, and these have now been studied in some depth. It is now possible to assess the validity of the premises on which the methylfolate trap hypothesis was formulated.

1 DOES METHYLFOLATE TRAPPING ACCOUNT FOR THE KNOWN DEFECTS IN B_{12} DEFICIENCY?
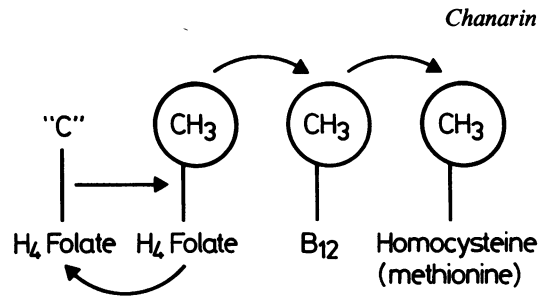
As the methyl group of methylfolate cannot be trans-

Fig 2 *A single carbon unit ("C") taken up by folate is reduced if necessary, to —CH=, —CH_2—, and ultimately —CH_3 (methyl). The methyl group is passed on to homocysteine to form methionine and B_{12} is a coenzyme in this transfer.*

ferred to homocysteine in B_{12} deficiency there is impaired use of methylfolate. Thus in the dU suppression test methylfolate did not correct the defect in B_{12} deficient marrow cells. Neither in marrow cells from pernicious anaemia nor from rats treated with N_2O, however, was tetrahydrofolate itself able to correct the defect in thymidine synthesis, although formylfolate was fully active.[35] This is quite an unexpected result from the point of view of the methylfolate trap hypothesis as tetrahydrofolate is outside the trap.

The same pattern of results emerged when the form of folate used by liver for making folate polyglutamate was studied. Normal rat liver used all folate analogues equally for making the polyglutamate. When B_{12} was inactivated by N_2O, however, no polyglutamate at all was made from methylfolate and tetrahydrofolate, but normal amounts from formylfolate.[36]

Failure to use tetrahydrofolate and the normal use of formyltetrahydrofolate are not predicted by methylfolate trapping. Rather they suggest a role for B_{12} in the formylation of folate.

2 IS THE IN VITRO PREDICTION THAT METHYLFOLATE CANNOT BE OXIDISED BACK TO METHYLENE AND FORMYLFOLATE CORRECT IN IN VIVO?

One of the two papers in 1961 that formulated the methylfolate trap theory also contained the seeds for its destruction.[8] Noronha and Silverman found that 1% methionine added to the diet and fed for 24 hours to rats before sacrifice resulted in a considerable decline in the methylfolate concentrations in liver while increasing the formylfolate and tetrahydrofolate content. This occurred in both control and B_{12} deficient rats. This implies oxidation of the methyl group to formyl and to carbon dioxide. This has been confirmed many times since. Brody *et al*[37] showed that this occurred within 30 minutes of an injection of

methionine and that the methyl group oxidation occurred on folate hexaglutamate.

The amount of methionine needed to promote oxidation of the methyl group of methylfolate was as little as $0.5 \mu mol$. S-adenosylmethionine was less active than methionine and seemed to be active only through its further metabolism to methionine. Thus the hepatic concentration of methylfolate is regulated by that of methionine in both normal and B_{12} deficient animals.

Other observations also point to in vivo oxidation of the methyl moiety of methylfolate. Methylfolate can serve as the methyl donor in the methylation of biogenic amines.[38] The mechanism entails the oxidation of the methyl group to formate, which in turn is transferred to the biogenic amine. The enzyme responsible for the oxidation of the methyl group was the same one concerned with its formation—namely, methylenetetrahydrofolate reductase.[38]

Furthermore, Thorndike and Beck[39] reported that the methyl group of methylfolate was oxidised in an essentially similar manner by lymphocytes from both normoblastic controls and one patient with pernicious anaemia.

3 WHAT IS THE PATHWAY FOR OXIDATION OF THE METHYL GROUP OF METHYLFOLATE IN B_{12} DEFICIENCY?

Lumb *et al*[40] showed that in B_{12} inactivated rats where the methyl group could not be transferred to homocysteine, methyltetrahydrofolate itself was used as the substrate for forming methylfolate-polyglutamate. When methylfolate labelled with [^{14}C] in the methyl group was used [^{14}C]-labelled polyglutamates containing 3, 4, and 5 glutamic acid residues were identified. As no polyglutamate with the [^{14}C] label having 6 glutamic acid was present it was concluded that the methyl group was oxidised from the hexaglutamate.

Methionine and cobalamin-folate function

Not only are both folate and cobalamin required for methionine synthesis, but many of the effects of B_{12} deficiency in both man and rat are overcome to a greater or lesser degree by the provision of methionine.

In man methionine given to patients with pernicious anaemia in relapse will abolish an abnormal urinary formiminoglutamic acid excretion and, used in appropriate dose, will correct the abnormal dU suppression test.[41]

In fruit bats treated with N_2O[42] and in monkeys[43] methionine will considerably improve neuropathy. In B_{12} inactivated rats methionine will restore to normal folatepolyglutamate synthesis.[36]

Even more effective than methionine, is a derivative of methionine via S-adenosylmethionine—namely, 5-methylthioadenosine.[36] This compound is further metabolised to methionine and formate. Methionine can also yield formate by a second pathway—namely, oxidation of its methyl group.

How B_{12} deficiency affects folate metabolism

Methionine is the pivotal compound in B_{12} folate metabolism. Lack of B_{12} produces a shortage of methionine that is only partially alleviated by induction of another pathway to methylate homocysteine—namely, betaine methyltransferase whereby betaine supplies the methyl group to methylate homocysteine instead of methylfolate. Lack of methionine has two consequences:

1 Lack of single carbon units at the formate level of oxidation, which arises from both oxidation of the methyl group of methionine and via S-adenosylmethionine → decarboxylated S-adenosylmethionine → 5-methylthioadenosine → 5-methylthioribose → methionine plus formate. Formate is required for purine synthesis and for formation of formyltetrahydrofolate, which seems to be the preferred substrate for folate polyglutamate synthesis, and by further reduction into methylene for pyrimidine synthesis.

2 The lack of S-adenosylmethionine, as indicated, may restrict supply of formate. It may interfere with general transmethylation reactions, although a role for transmethylation in haemopoiesis has still to be shown. There is no fall in S-adenosylmethionine values in the brain in fruit bats treated with N_2O and hence impaired transmethylation in brains is unlikely.

Serine has been widely regarded as the main source of single carbon units largely because it is readily available by synthesis from glucose. B_{12} inactivation does not affect the pathways by which serine can donate a single carbon unit. Nevertheless, large doses of serine do not in any way ameliorate the effect of B_{12} inactivation. This must cast doubt on the importance of serine as a major single carbon unit donor.

The hypothesis that has been advanced to explain B_{12}-folate interrelations—the formate-starvation hypothesis—postulates that B_{12} deficiency leads to shortage of methionine, and this in turn, to a shortage of active-formate derived from methionine.

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