Subject Review

The Biochemical Basis of Cobalamin Deficiency

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• Objective: In this report, our goal was to summarize the current knowledge of the biochemical basis for the impaired DNA synthesis and neuropathy associated with vitamin B₁₂ deficiency.

• Material and Methods: We reviewed the pertinent literature and our clinical experience with cobalamin deficiency.

• Results: Studies have established that the megaloblastic hematopoiesis associated with vitamin B₁₂ and folate deficiency is secondary to impaired DNA synthesis. Two mechanisms of impairment of DNA synthesis have been proposed: the “methylfolate trap hypothesis” and the “formate starvation hypothesis.” One possibility is that both hypotheses may be contributory—that is, incoming dietary folate may be inaccessible for polyglutamation in accordance with the methylfolate trap hypothesis, whereas the formate starvation hypothesis may explain the failure to use already polyglutamated forms of folate.

• Conclusion: Although the pathophysiologic mechanisms of vitamin B₁₂ and folate deficiency are not completely understood, nutritional anemias offer suitable models for the study of the biochemical basis of disease.

The megaloblastic anemias result from partially impaired DNA synthesis during hematopoiesis, which eventuates in morphologically evident larger-than-normal precursor cells with delayed nuclear maturation. The impaired DNA synthesis is attributable to a perturbation of the enzymatic DNA repair or synthetic pathways. Ineffective enzymatic activity may be caused by either a deficiency or an inhibition of a cofactor activity. The latter mechanism is being exploited in cancer chemotherapy. Two of the cofactors necessary for DNA synthesis are vitamin B₁₂ and folate. A knowledge of their interdependent metabolic pathways is essential for understanding the pathophysiologic aspects of megaloblastic anemia caused by vitamin B₁₂ or folate deficiency.

VITAMIN B₁₂

Vitamin B₁₂ was first isolated in 1948 and synthesized in 1973. It is structurally classified as a corrinoid. The corrinoids are a family of compounds with a corrin ring (Fig. 1). The corrin ring consists of four reduced pyrrole subrings joined in a macrocyclic ring linked by the \( \alpha \) positions of the pyrrole subrings in a planar configuration (corrin nucleus). The pyrrole subrings are linked to a central cobalt atom. The term “corrin” was initially proposed because this structure forms the core of the vitamin B₁₂ molecule, not because it contains cobalt.

Subfamilies of the corrinoids are formed by the addition of various substituents on the cobalt atom, above and below the plane of the corrin nucleus. For example, the cobalamins (Cbls) are corrinoids in which 5,6-dimethylbenzimidazole (a nucleotide) is linked to the cobalt atom below the plane of the corrin nucleus (Fig. 1). Furthermore, the addition of various anionic group ligands, linked to the cobalt atom above the plane of the corrin nucleus, yields the various forms of the Cbls (Fig. 1). Accordingly, the addition of cyanide (CN) gives rise to cyano-Cbl (CN-Cbl), methyl (Me) to Me-Cbl, adenosyl (Ado) to Ado-Cbl, and hydroxyl (OH) to OH-Cbl.

Animal products are the primary dietary source of Cbls. Meat contains OH-Cbl and Ado-Cbl, and dairy products contain OH-Cbl and Me-Cbl. Crystalline vitamin B₁₂, which is used for treating Cbl deficiency, is CN-Cbl. For the rest of our discussion, vitamin B₁₂ will be referred to as CN-Cbl. The coenzymatically active forms are Me-Cbl and Ado-Cbl—the respective cofactors for methionine synthase (Fig. 2 A) and methylmalonyl-coenzyme A (MeMaCoA) mutase (Fig. 2 B). (These cofactors will be discussed in more detail subsequently in this report.) OH-Cbl and CN-Cbl are converted into the coenzymatically active forms (Me-Cbl and Ado-Cbl) in human tissue.
Cbl in food is bound to protein and must undergo peptic digestion in the acidic environment of the stomach to be released. The freed Cbl initially binds to R-binder (a cobalophilin with a rapid electrophoretic mobility [hence the "R"], found in saliva and gastric juice), which is later degraded by pancreatic enzymes in the duodenum; this process releases, once again, free Cbl. Thereafter, the Cbl binds to parietal cell-derived intrinsic factor (IF). This relationship is a prerequisite for absorption of Cbl at the terminal ileum by means of IF receptors (Fig. 3).

Absorbed Cbl is transported from the enterocyte to Cbl-requiring tissues by transcobalamin (TC) II. Approximately 80% of plasma Cbl, however, is bound to other cobalophilins (TC I and the granulocyte-derived TC III)—which, unlike TC II, also bind Cbl analogues and transport them to the liver. The holotranscobalamin complex (TC II-Cbl complex) binds to specific cellular receptors and is internalized (Fig. 3).

**FOLATE**

Unlike the Cbls, the dietary sources of folate are both animal products and leafy vegetables. Structurally, folic acid (pteroylglutamic acid [PGA]) consists of a pteridine moiety linked to a para-aminobenzoic acid residue attached to the glutamate side chain (Fig. 4). Although the synthetic vita-
mediary metabolic pathways leads to effective DNA synthesis. Although these pathways have not been precisely characterized, several prior investigations have provided a framework on which reasonable postulates can be constructed and discussed.

The primary intracellular function of folates is to transfer one-carbon (1-C) units, at the oxidation levels of methyl (-CH₃), methylene (-CH₂-), or formyl (HCO), to facilitate DNA synthesis. To achieve this coenzymatic activity, the folate analogues must be in both a reduced form (THF) and the polyglutamated form (THFn).12

The primary function of Cbl is to provide coenzymatic activity for the synthesis of methionine and succinyl-coenzyme A (CoA) (Fig. 2). For this function, the Cbl derivatives must be converted to Me- or Ado-Cbl, and the Cbl molecule must be in a reduced state for optimal enzyme binding.13

In DNA synthesis, the formation of methionine is the “central” reaction (Fig. 6). Both Cbl and folate are necessary for this reaction. Me-Cbl is the cofactor for methionine synthase in the conversion (by methylation) of homocysteine to methionine (Fig. 2 A and 6). The original methyl donor for the reaction is Me-THF in either the monoglutamate form (Me-THF₁, obtained primarily from the diet) or the polyglutamate form (Me-THFₙ, obtained primarily from intracellular recycling of folate intermediates).13 The methyl group is first transferred from Me-THF to the enzyme-bound Cbl to form Me-Cbl, which then transfers the methyl group onto homocysteine to generate methionine (Fig. 6). This central reaction provides two products essential for DNA synthesis—THFn for 1-C unit transport and methionine, which, although controversial, may facilitate the availability of the appropriate folate intermediary in the process.

**FORMATION OF INTERMEDIATE FOLATE ANALOGUES**

Polyglutamation (by means of folypolyglutamate synthase) of folate analogues is necessary for cellular retention12 and use in 1-C transfer pathways during DNA synthesis. Furthermore, Me-THF must be demethylated before it can be polyglutamated.12 This sequence of events offers a partial explanation for the shortage of THF during Cbl deficiency. Of note, the poor substrate activity for polyglutamation is restricted to Me-THF₁ and does not involve other monoglutamate forms, including methylene-THF₁ and formyl-THF₁.14

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**Fig. 3.** Enteric processing and absorption of cobalamin (Cbl). IF = intrinsic factor; R-binder = a cobalophilin with a rapid (compared with IF) electrophoretic mobility; TCn = transcobalamin II.

**Fig. 4.** Chemical structure of folic acid.
A methylene group is added to THF, by serine-glycine hydroxymethyltransferase (SGMT) during the conversion of serine to glycine (Fig. 6). This is a reversible reaction in that methylene-THF can be converted back to THF. Alternatively, certain data suggest that, in vivo, methylene-THF may be produced by the oxidation of Me-THF, a reaction catalyzed by methylene reductase (Fig. 6). Pertinent to a later discussion herein, the reduction of methylene-THF to Me-THF (by the same enzyme) is inhibited by methionine derivatives.

Methylene-THF is a key intermediate and may be used in one of three ways: (1) it can provide the methylene group to convert deoxyuridylate into thymidylate, (2) it can be oxidized to formyl-THF, which provides 1-C fragments in purine synthesis, and (3) it can be reduced back to Me-THF to provide a methyl group during methionine synthesis (Fig. 6).

Formyl-THF is the other folate analogue that is directly involved with DNA synthesis (purine synthesis). Formyl-THF may be produced by oxidation of methylene-THF and vice versa (Fig. 6). Alternatively, formyl-THF may be produced by direct formate transfer (through formyl-THF synthase) from THF (Fig. 6). For the latter reaction, the sources of formate include methionine, which may independently facilitate the formate transfer without being the formate donor.

The foregoing discussions support one point of view, which maintains that methionine may be important in promoting the availability of the appropriate folate analogues (methylene-THF and formyl-THF), which are essential for DNA synthesis. Methionine inhibits the reduction of methylene-THF to Me-THF and facilitates formate transfer in the formation of formyl-THF, from THF. Therefore, when the production of methionine is directly curtailed by Cbl deficiency, DNA synthesis is indirectly affected.

MECHANISM OF IMPAIRED DNA SYNTHESIS IN Cbl DEFICIENCY

The "Methylfolate Trap Hypothesis."—As an extension of the foregoing material, methylene- and formyl-THF can be considered directly involved in thymidylate and purine synthesis, respectively. At least two sources of methylene-THF are available, one of which is the circulating Me-THF. As discussed previously, Me-THF cannot be demethylated in the absence of Cbl and thus is inaccessible for polyglutamation. The second source involves a recycling of THF as a by-product of the 1-C transfer reactions. Some of the methylene-THF is reduced to Me-THF (a reaction that is normally inhibited by a methionine derivative [S-adenosylmethionine or SAM]; see Figure 6), which could be "trapped" at the methionine synthase reaction stage—the basis for the methylfolate trap hypothesis.

Observations that contradict the methylfolate trap hypothesis include the reversal of the defect by methionine, the possibility that Me-THF can be directly oxidized to methylene-THF, and the inability of demethylated THF to correct the defect. These observations have been used as evidence to support an alternative hypothesis (see subsequent section) that considers the availability and utilization of formate the main defect in Cbl deficiency.
Although the methylfolate trap hypothesis can explain both plasma and tissue accumulations of Me-THF in Cbl deficiency, alternative explanations also exist. Plasma Me-THF accumulations in Cbl deficiency can result from reduced cellular uptake rather than leakage of trapped cellular Me-THF. Similarly, the lack of methionine and its derivative (SAM) in Cbl deficiency results in excess Me-THF because of lack of inhibition, by SAM, of methyl-THF reductase, which converts methylene-THF to Me-THF (Fig. 6).

The “Formate Starvation Hypothesis.”—An alternative hypothesis, the formate starvation hypothesis, considers the lack of methionine (as a result of Cbl deficiency) the most important causative factor in impaired DNA synthesis. This theory is based on several observations, including the substantial but incomplete reversal of Cbl deficiency states with methionine and the lack of formyl-THF generation and increased formate accumulation during Cbl deficiency, emphasizing the importance of methionine in formate transfer. The incomplete reversal of clinical features with methionine may reflect the failure of methionine to improve cellular uptake of folates rather than suboptimal coenzymatic activity in formate delivery and utilization.

In addition, methionine may be adenosylated (SAM) and may be used as a source of methyl and formate groups (Fig. 6). With methionine deficiency, concentrations of SAM decline, a situation that promotes methylene-THF reduction to Me-THF (SAM inhibits the forward conversion of methylene-THF to Me-THF; see Figure 6). Furthermore, an effort to conserve SAM for essential methylation might result in decreased SGMT activity and lead to reduced generation of methylene-THF (SGMT adds a methylene group to THF during conversion of serine to glycine; see Figure 6).

Moreover, the inability of THF, but not formyl-THF, to correct folate-dependent reactions in Cbl deficiency has been cited as further evidence in support of the formate starvation hypothesis. A recent study, however, has demonstrated that thymidylate synthesis in Cbl deficiency can be corrected by THF, although with less efficiency than formyl-THF.

Could Both Hypotheses Be Contributory?—One possibility is that circulating methylfolate (Me-THF) may be handled differently than recycled cellular polyglutamated methylfolate (Me-THF). Thus, incoming dietary folate (Me-THF) may be inaccessible for polyglutamation in accordance with the methylfolate trap hypothesis, whereas the formate starvation hypothesis may explain the failure to use already polyglutamated forms of folate. Nevertheless, neither hypothesis can satisfactorily explain the reversal of megaloblastic hematopoiesis in some patients treated with pharmacologic doses of folic acid.
**BIOCHEMICAL BASIS OF NEUROPATHY IN Cbl DEFICIENCY**

The absence of neuropathy in patients with folate deficiency suggested that methionine synthesis may not be causally related to Cbl-associated neuropathy, and attention initially focused on the other Cbl-dependent reaction, which is the conversion of MeMaCoA to succinyl-CoA catalyzed by MeMaCoA mutase (Fig. 2 B). For this reaction, Ado-Cbl is the cofactor, and the oxidative state and intracellular site of the Cbl molecule differ from those of Me-Cbl, factors that may explain the initial absence of methylmalonic aciduria in N₂O poisoning.

Earlier studies showed that excess MeMaCoA inhibited long-chain fatty acid synthesis by being incorporated into terminal positions and resulting in the formation of abnormal branched-chain fatty acids. Similarly, an excess of propionyl-CoA, the immediate precursor of MeMaCoA, results in odd-chained fatty acid synthesis. Therefore, investigators have presumed that these abnormal fatty acids participated in abnormal myelin formation.

Recent observations, however, do not support the aforementioned hypothesis. First, hereditary MeMaCoA mutase deficiency and defects in Ado-Cbl synthesis do not cause Cbl neuropathy, whereas N₂O poisoning, which inactivates methionine synthase, does not appreciably affect MeMaCoA mutase and yet causes neuropathy. Second, hereditary defects of the methionine synthase reaction cause Cbl neuropathy, and the administration of methionine to Cbl-deficient animals ameliorates the neuropathy.

Methionine is adenosylated into SAM, which along with its derivatives is needed in transmethylation reactions and polyamine synthesis, including those that occur in the central nervous system. Although defective amine turnover in the central nervous system is thus a possibility, the exact mechanism by which reduced methionine causes demyelination remains to be elucidated. A recent communication suggested that cultured human glial cells may be suitable for study of the biochemical basis of Cbl neuropathy.

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