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COBALAMIN DEFICIENCY AND THE PATHOGENESIS OF NERVOUS SYSTEM DISEASE

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INTRODUCTION

Severe untreated cobalamin (Cbl) deficiency in humans is associated with crippling neurological disease, ataxia, and death. According to Chanarin (12), the earliest suggestion of neuropathy associated with cobalamin (Cbl) deficiency is contained in the account of Osler & Gardner (49) in 1877 of anemic patients with numbness of the fingers, hands, and forearms. The association between Cbl deficiency and spinal cord lesions was recognized in the same year (41), and the features of the characteristic subacute combined degeneration of the spinal cord were documented in 1900 (53). The demyelination lesion begins as swelling of the myelin sheaths, is followed by breakdown of myelin and disruption of the axon, and results in a spongy vacuolated appearance of the cord. The process is most severe in the posterior columns of the thoracic region, but the lateral columns are also affected in due course. Changes in the brain consist of foci of demyelination in the white matter. In the peripheral nerves, the number of myelin sheaths may be reduced.

Although the features of the Cbl neuropathy were described more than a century ago, the underlying pathogenic mechanism of the lesions remains poorly understood. This long period with relatively little progress can be largely attributed to the lack of availability of suitable animal models of the Cbl neuropathy. Only in the last decade, with the use of nitrous oxide (N_2O) to induce Cbl deficiency, have suitable models been introduced, and most of the recent advances in knowledge in this area have been derived from studies with these models. This review focuses on the data that have become available in the last decade.

The induction of Cbl deficiency by dietary manipulation alone is difficult. The small daily requirement for Cbl, coupled with the relatively large body stores, necessitates prolonged dietary deprivation, often longer than a year, before severe deficiency can occur. In addition, as feces are a rich source of the vitamin, rigid exclusion of coprophagy is necessary for deficiency to develop.

Before 1975, the only known experimental animal in which Cbl neuropathy could be induced was the monkey (34). The long period (2 to 3 years) required to induce dietary deficiency, and the cost of maintaining these relatively large laboratory animals, limited their use to one or two laboratories. The observation that in the fruit bat (*Rousettus aegyptiacus*) severe Cbl deficiency accompanied by neuropathy similar to that of Cbl-deficient humans could be induced by minimal manipulation of the diet provided the first small animal model of the Cbl neuropathy (30, 47). However, a prolonged period (9–12 months) of dietary restriction was still necessary to produce the

neuropathy, which limited the usefulness of the model. The introduction of the anesthetic gas N_2O to induce Cbl deficiency represented a major advance in that its use shortened to weeks the time period required to render animals Cbl deficient. Repeated exposure of monkeys (18), fruit bats (61), and pigs to N_2O (70) leads to the development of typical Cbl neuropathy in these animals.

BIOCHEMICAL REACTIONS REQUIRING COBALAMIN, AND THE ACTION OF NITROUS OXIDE

To date, Cbl is known to be required in two biochemical reactions in humans (Figure 1). The coenzyme forms of Cbl differ in the two reactions. (*a*) Cbl in the form of adenosylcobalamin (AdoCbl) is required as cofactor for methylmalonyl CoA mutase (MMCoM) (EC 5.4.99.2), which is essential for the isomerization of methylmalonyl CoA (MMCoA) to succinyl CoA, the final step in the metabolism of propionate to succinyl CoA. (*b*) Cbl in the form of the methylcobalamin (MeCbl) is required as cofactor for the enzyme methionine synthetase (MS) (5-methyltetrahydrofolate homocysteine methyltransferase: EC 2.1.1.13), which catalyzes the recycling of homocysteine to methionine. Folate, in the form of methyltetrahydrofolate, is also required in this reaction (Figure 1).

Nitrous oxide oxidizes active reduced cob(I)alamin to inactive cob(III)alamin (4). As Cbl in the form of reduced MeCbl is required as cofactor for MS, exposure to N_2O causes rapid inactivation of this enzyme (15). The inactive Cbl is excreted, so that repeated exposure to N_2O results in depletion of body Cbl stores, with reduced AdoCbl, so that the activity of the AdoCbl-dependent enzyme MMCoAM is also compromised (15).

ANIMAL MODELS OF THE COBALAMIN NEUROPATHY

To be acceptable, experimental animal models of the Cbl neuropathy must show pathological changes in the spinal cord similar to those described in humans with Cbl deficiency. This type of neuropathy can be induced in the monkey (18, 28, 57), the fruit bat (20, 30), and the pig (70) by dietary deprivation of Cbl, or by repeated exposure to N_2O, or by a combination of the two.

The Monkey

Prolonged (33–45 months) dietary deprivation of Cbl leads to severe clinical neuropathy with spastic paralysis of the hind limbs, accompanied by neuro-

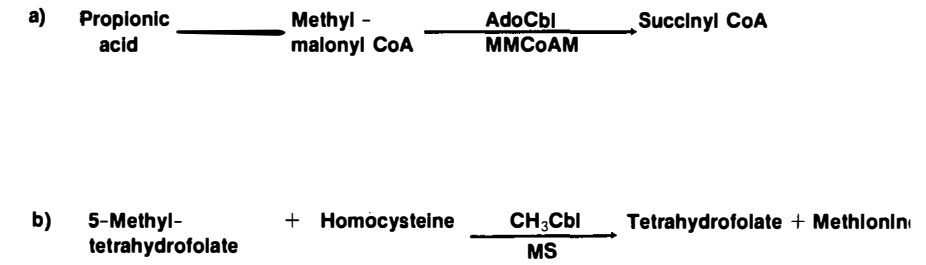

Figure 1 The two cobalamin-dependent biochemical reactions in humans. Abbreviations: AdoCbl, adenosylcobalamin; MMCoAM, methylmalonyl CoA mutase; CH_3Cbl, methylcobalamin; MS, methionine synthetase.

pathological changes in the spinal cord indistinguishable from those of human subacute combined degeneration (1, 2). These changes include separation and vacuolation of myelin lamellae, with eventual complete destruction of myelin sheaths. Repeated exposure to N_2O leads to the onset of clinical neuropathy within 9–12 weeks, followed by ataxia 2–3 weeks later (18). The neuropathy is accompanied by degeneration of myelin sheaths and axis cylinders in the posterior columns of the spinal cord, and accumulation of lipid-laden macrophages. Although the degree of clinical neuropathy in N_2O- and dietary-induced models is similar, the neuropathologic changes are less severe in the N_2O-induced model (18, 57).

The Fruit Bat

As fruit bats normally subsist on an all fruit diet, and fruit contains no Cbl, these animals presumably obtain their Cbl from contaminated water or from pests infesting the fruit. Thus, dietary Cbl deficiency can be induced by simply feeding pest-free, washed fruit and uncontaminated water. Such a diet leads to severe Cbl deficiency within about nine months (30). Neuropathy is manifested clinically by loss of ability to fly, progressing to ataxia, spastic paralysis, and death. When exposure to N_2O is combined with dietary deprivation, the onset of neuropathy is after about nine weeks. The neuropathological changes in the posterior columns of the spinal cord include vacuolation of the myelin, which leads to marked distention and separation of the lamellae, with thinning or frank loss of myelin sheaths (20).

The Pig

Repeated exposure to N_2O leads to progressive ataxia within nine weeks. Neuropathological changes in the spinal cord comprise multiple foci of

myelin degeneration. The lesions are less severe than those accompanying N_2O-induced Cbl neuropathy in the monkey.

Other small animals such as the rabbit, the mouse, and the rat do not develop neuropathy when Cbl deficiency is induced. The rat has been used extensively as an animal model for Cbl deficiency, and neuropathy has not been induced by dietary means or with N_2O. However, a recent report describes spongy degeneration throughout the white matter of all the spinal cord segments in rats rendered Cbl deficient by total gastrectomy (55). The changes occurred as rapidly as two months after the operation. It is difficult to assess the significance of this report, as the Cbl-deficient rats apparently did not show clinical signs of neuropathy, and histological changes were found in sham-operated rats, but to a lesser degree. Why Cbl deficiency in the rat after total gastrectomy is followed by neuropathological changes, when similar changes do not occur with deficiency induced by dietary means or exposure to N_2O, is not clear. Confirmation of these findings would provide an additional small animal model of the Cbl neuropathy.

THE ADENOSYLCOBALAMIN-DEPENDENT METHYLMALONYL CoA MUTASE AND THE COBALAMIN NEUROPATHY

Cbl is required for the normal functioning of two enzymes, MMCoAm and MS. Consequently, the lesion underlying the Cbl neuropathy has been sought in the reactions catalyzed by these enzymes. Neuropathy is a frequent complication of severe, prolonged Cbl deficiency in humans, but it is not usually associated with folate deficiency. For years, therefore, attention was focused on the AdoCbl-requiring MMCoAM reaction in propionic acid catabolism, where folate plays no role, rather than on the MeCbl-dependent MS reaction in which both Cbl and folate are involved.

Changes in Odd-Chain and Branched-Chain Fatty Acids

An understanding of the mechanism whereby deficiency of AdoCbl leads to the Cbl neuropathy is based on the hypothesis that impairment of the AdoCbl-dependent MMCoAM reaction with subsequent intracellular accumulation of the intermediates of propionic acid metabolism, propionyl CoA and methymalonyl CoA (MMCoA), leads to the formation of abnormal fatty acids (5, 8, 9, 23–25, 35) (Figure 2). Substitution of propionyl CoA for acetyl CoA and of MMCoA for malonyl CoA would lead respectively to the formation of

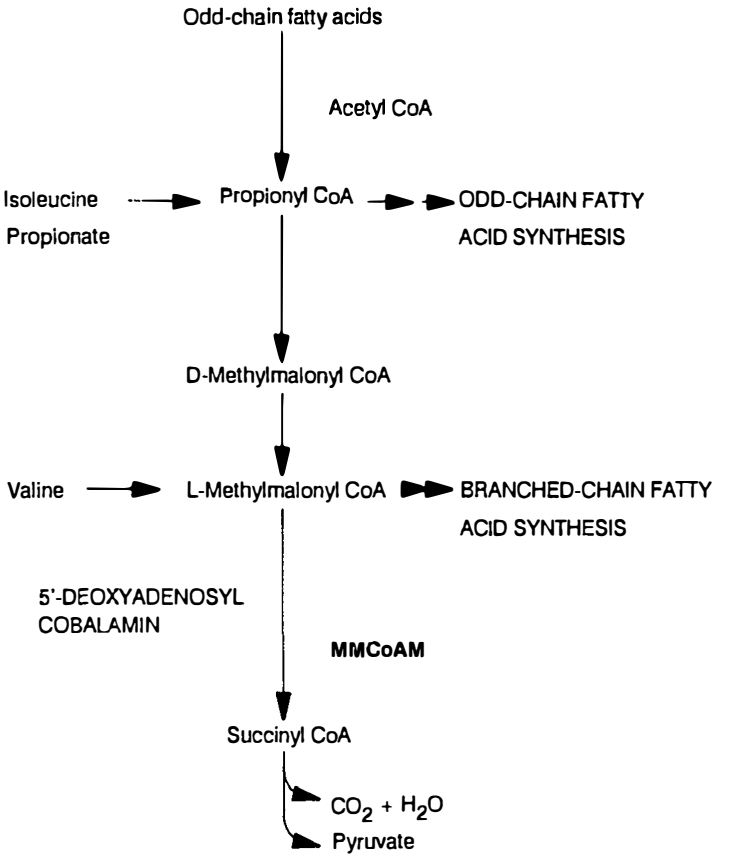

Figure 2 The propionic acid pathway in cobalamin deficiency. (Reproduced with permission from J. Metz and J. van der Westhuyzen, 1987, *Comp. Biochem. Physiol.* 88A:171–77.)

increased odd-chain and branched-chain fatty acids (9). Excessive odd-chain and branched-chain fatty acids would accumulate in membrane lipids of nervous tissue. As the synthesis of normal myelin is dependent on the availability of specific fatty acids, synthesis of abnormal in place of normal fatty acids could result in altered myelin integrity and in demyelination, thus leading to impaired nervous system functioning (25).

The evidence in support of this hypothesis has been derived from earlier in vitro studies and observations on humans with Cbl deficiency and on animals with experimentally induced deficiency. Patients with Cbl neuropathy were shown to excrete in the urine excessive amounts of methylmalonic acid (MMA), presumably derived from the accumulation of MMCoA (71). The intermediates of propionic acid metabolism, propionyl CoA and MMCoA,

have been shown to inhibit in vitro fatty acid synthesis (8, 23, 25), and accumulation of propionyl CoA may result in a relative increased biosynthesis of odd-chain fatty acids (5, 24, 35).

An increase in odd-chain and branched-chain fatty acids in nervous tissues has been reported from earlier studies on humans with Cbl deficiency and on experimental animals with dietary-induced deficiency. In a young child who died with MMA accumulation owing to an inherited inability to convert Cbl to the AdoCbl coenzyme form, odd-chain fatty acids (15:0, 17:0) were significantly increased in the glycerolipids of the spinal cord (35, 40). Branched-chain fatty acids of C17 were detected in the brain and spinal cord while control tissue contained only trace amounts or none. A patient with an inherited deficiency of both the AdoCbl and MeCbl coenzymes showed a relative increase in odd-chain fatty acids (15:0, 15:1, 17:0, 17:1) in myelin lipids, when compared with controls (52). Methyl branched C17 fatty acids were also identified. Humans with Cbl-deficient pernicious anemia showed a decrease in net synthesis of fatty acids but an increase in the amounts of odd-chain fatty acids (15:0, 17:0) present in biopsies of peripheral nerve (24). Labelled propionate was shown to be incorporated into these odd-chain fatty acids.

Accumulation of odd-chain fatty acids 15:0 and 17:0 in the cerebral lipids of rats with dietary-induced Cbl deficiency has been reported from a number of studies (3, 21, 50, 51). In addition, diminished amounts of fatty acids 20:4 and 22:5 were found in one study (50); as malonyl CoA is required for the elongation of 18:2 to form 20:4 and 22:5, it was suggested that the accumulation of MMCoA in Cbl deficiency interfered with such elongation reactions. Some studies failed to demonstrate increased synthesis of odd-chain fatty acids in the Cbl-deficient rats. In one study in which propionate was administered, contrary to expectation, C15 and C17 fatty acids were present in liver triglycerides in smaller amounts than in control animals (22).

These earlier studies do not make clear what relationship, if any, the changes demonstrated in nervous system fatty acids bear to the development of the Cbl neuropathy, and whether small changes in membrane fatty acid composition could affect membrane stability and renewal to a degree that could cause demyelination. There was in fact no evidence of a correlation between the relative differences in odd chain and branched-chain fatty acids in the lipids of neural tissues and the functional changes in these tissues of humans or rats. Evidence of an etiological relationship would be the consistent finding of excess odd-chain and branched-chain fatty acids in the neural tissue of experimental animals with Cbl neuropathy and a correlation between the severity of the neuropathy and the abundance of these fatty acids. Acceleration or retardation of the neuropathy by dietary manipulation should be reflected by corresponding changes in the levels of fatty acid accumulation.

A series of studies of neural tissue of the fruit bat with Cbl neuropathy has failed to provide this evidence. The Cbl neuropathy in this animal is accompanied by decreased activity of MMCoAM and accumulation of MMA in serum and urine (67). The brains of bats deprived of dietary Cbl have a marginally higher percentage of 18:3 fatty acid in phosphotidylcholine and a slightly higher percentage of 15:0 fatty acid in sphingomyelin, with no other significant changes in odd-chain fatty acids (59). In spinal cord myelin the concentrations of odd-chain fatty acids 15:0, 15:1, 17:1, and 19:0 are all higher in Cbl-deficient bats (60). In fruit bats with Cbl neuropathy induced by a combination of dietary deprivation and exposure to N_2O, no significant differences in the concentration of odd-chain fatty acids in brain lipids were demonstrable, compared with levels in control animals, and branched-chain fatty acids were not present in detectable amounts in either group (63).

In the most recently reported study of Cbl neuropathy in the fruit bat (67), MMA metabolism was examined in relation to fatty acid concentrations in the nervous system. The Cbl-deficient bats had low hepatic MMCoAM activity, with elevated plasma and urinary MMA levels, yet no significant changes in the concentration of odd-chain or branched-chain fatty acids in the spinal cord or brain could be demonstrated. Branched-chain and odd-chain fatty acids were detectable in only trace amounts in both spinal cord and brain, and no significant increase in branched-chain or odd-chain fatty acids in phosphatylethanolamine, phosphatylcholine, or sphingomyelin was observed.

Thus the studies of fatty acids in spinal cord and brain in fruit bats with severe Cbl neuropathy have produced variable results with little or no changes in individual branched-chain or odd chain fatty acids, despite impaired MMCoAM activity and accumulation of MMA. It seems unlikely that the essentially small and inconsistent changes in fatty acids play a significant role in the pathogenesis of the fatal neuropathy in the Cbl-deficient fruit bat.

Effect of Supplementation with Amino Acids

If the Cbl neuropathy is related to the block in propionic acid catabolism and accumulation of MMA, one would expect a significant reduction in the MMA levels in animals where dietary supplementation delayed the onset of neuropathy. Supplementation of the diet with methionine delays the onset of the N_2O-induced Cbl neuropathy in the pig, but does not lead to a concomitant reduction in plasma MMA levels (70). Methionine supplementation has a similar ameliorating effect on the Cbl neuropathy in the fruit bat, but again fails to maintain MMCoAM activity, and there is persistent methylmalonyl acidemia (67). Thus it is unlikely that the effect of methionine in delaying the onset of the Cbl neuropathy is mediated by an effect on the propionic acid pathway.

Studies of dietary supplementation with valine and isoleucine have likewise failed to provide evidence that a defect in propionic acid metabolism is the

cause of the Cbl neuropathy. These two amino acids are catabolized exclusively via the propionic acid pathway. If the underlying defect is in the propionic acid pathway, increasing the dietary intake of these amino acids would be expected to aggravate the Cbl neuropathy by providing additional substrate to be metabolized by the already compromised MMCoAM. However, no such effect of these amino acids was noted on the Cbl neuropathy in the fruit bat (69). In fact, the opposite effect was noted, whereby supplementation with valine and isoleucine delayed the onset of neuropathy. Failure of valine and isoleucine loading to aggravate the Cbl neuropathy is not consistent with the hypothesis that a defect in propionic acid metabolism is the underlying basis for the Cbl neuropathy.

Additional Evidence Against the Adenosylcobalamin Hypothesis

Further evidence against the AdoCbl hypothesis comes from observations on children with inherited disorders associated with accumulation of MMA. Inherited mutations of the apomutase or of AdoCbl synthesis (Cbl A and Cbl B mutants) are accompanied by marked methylmalonylacidemia and methylmalonylaciduria (14, 43). Children with these defects may show muscular hypotonia and mental retardation, but they do not develop the Cbl neuropathy. Thus, accumulation of large amounts of MMA from defects other than Cbl deficiency does not produce neuropathy.

Chronic intermittent exposure to N_2O in humans leads to a syndrome similar to Cbl neuropathy (6, 39, 54). That the inhibitory effect of N_2O on cobalamin metabolism is primarily on the MeCbl-dependent MS reaction rather than on the AdoCbl MMCoAM reaction, suggests that the Cbl neuropathy is not related to AdoCbl deficiency. This interpretation is probably correct, but during long-term exposure to N_2O, MMCoAM is affected as body deficiency of Cbl develops, so that the eventual N_2O effect is not limited to the MS reaction.

Thus a large body of evidence can now be marshalled against the hypothesis that decreased function of AdoCbl is the mechanism of the Cbl neuropathy. Although this hypothesis was dominant for about two decades, it seems no longer tenable when considered in conjunction with the results derived from more recent studies of experimental Cbl neuropathy.

DEFECTIVE METHYLATION AND THE COBALAMIN NEUROPATHY

Adenosylmethionine Concentration in the Nervous System

The first suggestion that the Cbl neuropathy might be related to defective methylation in the nervous system came from observations on a patient with an inherited mutation in Cbl (52) and from studies of cycloleucine-treated

mice (26, 33). In the patient with inherited deficiency of both AdoCbl and MeCbl, the amount of basic protein in myelin was found to be markedly reduced. This prompted the suggestion that lack of normal methylation of basic protein, due to the impairment of the MS reaction in Cbl deficiency, may have rendered the myelin basic protein unstable (52). Cycloleucine, an analogue of methionine, is a competitive inhibitor of the enzyme methionine adenosyltransferase (EC 2.5.1.6), which converts methionine (Met) to adenosylmethionine (AdoMet). Cycloleucine administered to mice caused a neurological condition similar to the Cbl neuropathy (26, 33). AdoMet is an important donor of methyl groups for transmethylation reactions, and possibly cycloleucine produced the neurologic lesion through defective methylation of myelin lipid.

Scott and co-workers (19, 57) produced the first experimental evidence that the Cbl neuropathy was related to the Cbl-dependent MS reaction by demonstrating that monkeys exposed to N_2O developed neurological changes resembling subacute combined degeneration found in humans. They hypothesized that the impaired MS activity attendant upon N_2O exposure resulted in decreased Met synthesis, which in turn led to decreased amounts of AdoMet for methylation reactions in myelin (Figure 3). In this way, methyl group deficiency would result in demyelination and the clinical neuropathy. In a subsequent study, supplementation of the diet with Met prevented the onset of clinical neuropathy and partially prevented demyelination in the spinal cord. Measurement of Met and AdoMet concentration in neural tissue was not performed as part of these studies. The induction of Cbl neuropathy by exposure to N_2O, and the action of dietary Met supplementation in delaying the onset of neurological signs and neuropathological changes in the spinal cord, was confirmed in the fruit bat (61) and in the pig (70).

The postulate that Cbl neuropathy was related to deficient synthesis of AdoMet was tested by direct measurement of this compound in the tissues of fruit bats with N_2O-induced Cbl neuropathy (27, 65). A small reduction of about 10% in the mean concentration of AdoMet was observed when livers of test animals were compared with those of controls. In the brain, however, contrary to expectation, the mean AdoMet concentration of animals with the Cbl neuropathy was significantly greater than that of controls. In the same experiment, groups of animals received dietary supplements of either Met or pteroylglutamic acid (PGA). As had been demonstrated previously, supplementation of the diet with Met delayed the onset of the neuropathy. Supplementation of the diet with PGA accelerated the onset of the neuropathy. The AdoMet concentrations in brains of animals receiving dietary supplements of Met or PGA were not significantly different from those of animals exposed to N_2O without supplementation. This study thus failed to reveal any reduction in AdoMet levels in brains of animals with N_2O neuro-

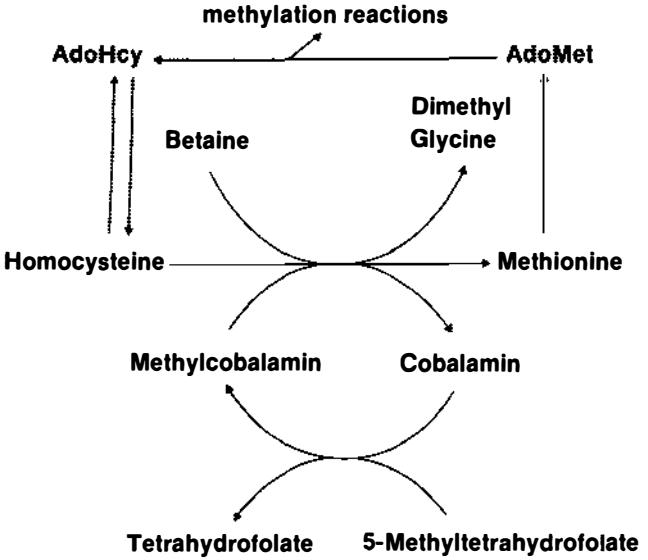

Figure 3 Synthesis of methionine, adenosylmethionine (AdoMet) and adenosylhomocysteine (AdoHcy).

pathy; furthermore, the effects of PGA in aggravating and Met in delaying the onset of progression of the neuropathy were not reflected by changes in the AdoMet concentration in the brain. Thus in the fruit bat model, these findings did not provide support for the hypothesis that the Cbl neuropathy was related to defective methylation caused by reduced concentration of AdoMet in the brain.

Similar findings were reported in the rat (42) and in the pig (70). In rats rendered Cbl deficient by repeated exposure to N_2O, the Met and AdoMet levels in brain were not reduced, compared with levels found in control animals, despite a fall in MS activity in the brain. It was suggested that the brain was able to maintain its Met levels in the absence of normal Cbl-dependent MS activity owing to increased uptake of Met from plasma or via a yet undiscovered other pathway of Met synthesis in the brain. In the pig with N_2O-induced Cbl neuropathy, the concentration of AdoMet in the nervous system was not reduced (70).

Further evidence that the Cbl neuropathy was not related to nervous system Met levels came from studies of the effect of dietary supplementation with betaine on the Cbl neuropathy in the fruit bat (66). Met is synthesized from homocysteine not only through the MS reaction but also through the reaction catalyzed by the enzyme betaine-homocysteine methyltransferase (EC 2.1.1.5) (58). This reaction is independent of Cbl or folate, and the methyl

donor is betaine. Supplementation of the diet with betaine delayed the onset of the Cbl neuropathy in fruit bats exposed to N_2O, but betaine was less effective than Met. The Met levels in brains of betaine-supplemented animals were lower than those of the N_2O-exposed animals without betaine supplementation, despite the protective effect of betaine on the development of neurological impairment. The results of the betaine experiments confirm the importance of adequate Met synthesis in the prevention of the Cbl neuropathy, but indicate that the neuropathy is not related directly to the concentration of Met in neural tissue.

Although Met and AdoMet levels in the nervous system are not reduced in the fruit bat or pig models of Cbl neuropathy, evidence from one study in humans suggests that reduced Met and AdoMet levels may be associated with neurological changes similar to those of Cbl deficiency. In patients with deficiency of 5,10-methylenetetrahydrofolate (CH_2THF) reductase, formation of methyltetrahydrofolate (CH_3THF) from CH_2THF is reduced. CH_3THF is the donor of the methyl group for the conversion of homocysteine to methionine via the MS reaction. Patients with 5,10-CH_2THF reductase deficiency have reduced concentration of both Met and AdoMet in the cerebrospinal fluid (CSF) and show demyelination in the brain and subacute combined degeneration of the spinal cord (32). Methionine is effective in the treatment of only some of these patients; betaine, however, prevented the progress of the neurologic symptoms in all patients in whom it was tried, and betaine restored CSF AdoMet concentration to normal.

Although reduced levels of Met and AdoMet in neural tissue have not been demonstrated in patients with CH_2THF reductase deficiency, the finding of low levels in CSF does indicate that nervous system deficiency of Met and Adomet may occur in association with a defect in the MS reaction. Furthermore, the progression of the neurological disease in some of the children was halted by activation of the alternative betaine-dependent methionine synthesis reaction, and this was associated with restoration of the CSF AdoMet levels to normal. The relevance of these findings, in which the MS reaction is impaired owing to lack of the CH_3THF cofactor, to the Cbl neuropathy in which the impairment is due to lack of MeCbl is uncertain. In particular, the relative effects of Met and betaine supplementation are different. In experimental Cbl-deficient neuropathy, Met produces a greater and more certain protective effect on the neuropathy than does betaine (66), while the opposite is the case in children with neuropathy associated with CH_2THF reductase deficiency.

Adenosylhomocysteine Toxicity

Inability to demonstrate reduced AdoMet concentrations in the neural tissue of the Cbl-deficient fruit bat (27, 65), rat (42), and pig (70) suggested that undermethylation was not the underlying cause for the Cbl neuropathy.

Attention was then focused on the AdoMet/Adenosylhomocysteine (AdoHcy) ratio as an index of methylation. An altered methylation ratio, due to either a decrease in AdoMet or an increase in AdoHcy, would be expected to inhibit all transmethylation reactions (56). In pigs with N_2O-induced Cbl neuropathy, AdoHcy levels in the spinal cord were significantly increased while the AdoMet concentration was not elevated, thus resulting in an inversion of the AdoMet/AdoHcy ratio from a control value of 15.0 to 0.8. (70) The accumulation of AdoHcy was presumably the result of a sequence of impaired reactions in Cbl deficiency (Figure 3). AdoMet, in transferring its methyl group in transmethylation reactions, is converted to AdoHcy, which is subsequently degraded to adenine and homocysteine. As the activity of the MS reaction is impaired in Cbl deficiency, the homocysteine formed from AdoHcy cannot be remethylated to Met. Accumulation of homocysteine leads to accumulation of AdoHcy and the altered AdoMet/AdoHcy ratio. Liver behaved differently from neural tissue, in that the values for AdoMet, AdoHcy, and the AdoMet/AdoHcy ratio were not significantly different in animals with N_2O-induced neuropathy compared with control animals.

In pigs whose diet was supplemented with Met, the severity of the Cbl neuropathy was greatly reduced. In these animals, the AdoHcy levels in spinal cord were elevated, but the AdoMet levels were significantly higher, so that there was not the same degree of fall in the AdoMet/AdoHcy ratio as in the animals exposed to N_2O but without dietary supplementation with Met. The added Met was presumed to exert its protective action by increasing the tissue levels of AdoMet in neural tissue, thereby compensating for the elevated AdoHcy levels and thus maintaining a near normal methylation ratio. In the same study, exposure of rats to N_2O produced only a moderate fall in AdoMet/AdoHcy ratio, despite a comparable degree of inhibition of MS in neural tissue. This relatively modest fall in the methylation ratio might explain the absence of neuropathy in the Cbl-deficient rat.

In a further experiment, weanling pigs exposed to N_2O for seven days showed a marked increase in AdoHcy concentrations in all tissues except liver (48). The elevated AdoHcy levels resulted in a fall in the AdoMet/AdoHcy ratio, particularly in neural tissues.

The hypothesis that the Cbl neuropathy is the result of AdoHcy toxicity could not be confirmed in the fruit bat model (68). In bats with severe Cbl neuropathy induced by a combination of dietary deprivation and exposure to N_2O, no significant differences in AdoMet and AdoHcy levels in spinal cord and brain tissue were found, compared with levels in control animals, nor were there any differences in the AdoMet/AdoHcy ratios. In the liver there was a small but significant rise in AdoHcy levels but no change in AdoMet levels. Supplementation of the diet with Met resulted in a rise in AdoMet concentration in the liver, a much smaller increase in the spinal cord and brain cortex, and an insignificant fall in the AdoMet/AdoHcy ratio.

Direct Measurement of Methylation Reactions

Studies of methylation reactions (17, 46) and myelin protein (44, 45) in the tissues of Cbl-deficient fruit bats failed to provide evidence of defective methylation. When bats rendered Cbl deficient by dietary deprivation and exposure to N_2O were compared to control animals, no differences in [^{14}C]ethanolamine incorporation into liver and brain phospholipids could be detected (46). Using synaptosomes and myelin as substrates for the incorporation of methyl groups into membrane lipids, no abnormality in lipid methylation could be demonstrated in Cbl-deficient bats (64). The rate of synaptosomal and myelin protein methylation was similar in Cbl-deficient and normal bats. Thus the Cbl-deficient neuropathy in the fruit bat is not associated with changes in the rate of protein or lipid methylation. Furthermore, no differences in protein profile of the myelin membrane in Cbl-deficient bats compared to that of control bats could be demonstrated (7).

Cobalamin Mutants Affecting Methylcobalamin

Observations on patients with inherited disorders of Cbl metabolism affecting MetCbl have provided further evidence focusing on the MS reaction rather than on the mutase reaction in the pathogenesis of the Cbl neuropathy. The Cbl mutants CblC and CblD are associated with failure to form both AdoCbl and MetCbl. In contrast to those mutants (CblA and CblB) in which only AdoCbl is affected, patients with the CblC and CblD mutants may show prominent neurological disorders with spasticity and cerebral atrophy in older patients (14, 31).

Patients with the CblE and CblG mutations, which are associated with MetCbl deficiency only, have been the subject of a recent review (31). In these infants reduced activity of the MS enzyme, elevated levels of homocysteine in plasma and urine, and reduced plasma methionine have been observed. Examination of the brain by CT scans and magnetic resonance imaging revealed atrophy and hypoplasia of the brain, with delayed myelination. Although these findings provide significant additional evidence of the importance of MetCbl in the Cbl neuropathy, data derived from infants with an inherited disorder in Cbl metabolism are not necessarily valid for Cbl deficiency in general (31).

One patient with the CblG mutant presented at age 21 with clinical neuropathy closely resembling subacute combined degeneration, thus providing further evidence implicating the MetCbl-dependent MS in the pathogenesis of the Cbl neuropathy (11).

The Methionine Synthetase Reaction and the Cobalamin Neuropathy

A large body of data now identifies the impairment of the MeCbl-dependent MS reaction, rather than the AdoCbl-dependent mutase reaction, as the

underlying biochemical lesion in the Cbl neuropathy, and indicates that Met plays a central role in the development of the lesion. It is uncertain how the impairment in MS activity, with the attendent changes in homocysteine and Met metabolism, results in neuropathy. The hypomethylation hypothesis, which is based on the the accumulation of homocysteine and AdoHcy, with a fall in the AdoMet/AdoHcy ratio and resultant inhibition of methyltransferases involved in myelin and other brain proteins, can be applied to the Cbl neuropathy in the pig only. Cbl neuropathy in the pig is associated with a fall in the AdoMet/AdoHcy ratio. A lesser, but still significant fall in the ratio occurs in the Cbl-deficient rat, but without the development of neuropathy. By contrast the fruit bat develops severe neuropathy without a significant change in the ratio in neural tissue. In view of the lack of consistent correlation between the development of Cbl neuropathy and the changes in the methylation ratio in neural tissue in various experimental animals, defective methylation cannot be accepted at present as the universal basis for the Cbl neuropathy.

These variant findings in the experimental animals could be ascribed to species differences. However, in all four species in which Cbl neuropathy occurs, namely humans, monkeys, fruit bats, and pigs, the neurological and neuropathological changes are quite similar. Furthermore, the effect of supplementation with Met in ameliorating the neuropathy is consistent for the monkey, the fruit bat, and the pig. In humans, for obvious ethical reasons, the possible effect of Met on the Cbl neuropathy will probably never be known. In view of these similarities, it is tempting to postulate a common pathogenesis for the Cbl neuropathy that is applicable to all four species in which the neuropathy occurs.

The results of a number of studies have confirmed the central role of Met in the pathogenesis of the Cbl neuropathy in the fruit bat. In contrast with the pig, it has not been possible to show a defect in methylation reactions associated with the Cbl deficiency. This would suggest that the Met effect could be mediated via its role in formate metabolism (13, 16) or by some as yet unidentified pathway. Metabolic pathways showing the metabolism of Met and AdoMet are illustrated in Figure 4.

COBALAMIN ANALOGUE TOXICITY

In addition to the biologically active forms of Cbl, some Cbl analogues are apparently physiologically inactive. A number of such analogues have been identified in mammalian tissues and in human plasma (36, 37). We do not know to what extent, if any, Cbl analogues, when present, could substitute for active Cbl in humans and in this way exacerbate the effect of true Cbl deficiency and possibly play a role in the pathogenesis of the Cbl neuropathy.

Cbl analogues have been demonstrated in the serum of human patients with

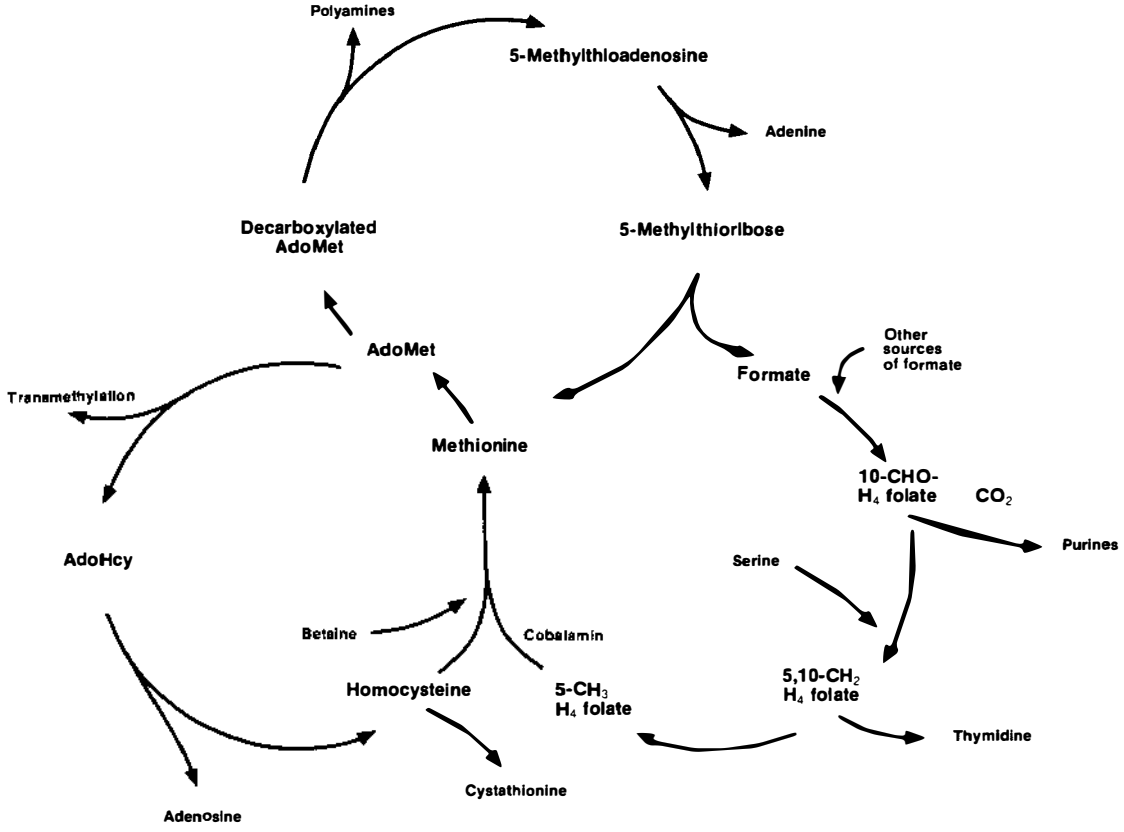

Figure 4 The central role of methionine in the metabolism of cobalamin, folate, and adenosylmethionine (AdoMet). (Reproduced with permission from E. Vieira-Makings, J. Metz, J. van der Westhuyzen, T. Bottiglieri, and I. Chanarin, 1990, *Biochem. J.* 266:707–11.)

Cbl deficiency (36). In one study, patients with Cbl deficiency and primarily neurological symptoms had significantly higher analogue levels than Cbl-deficient patients with primarily hematological abnormalities (10). On the basis of these findings, it was suggested that the retention or acquisition of analogues in Cbl deficiency may affect the nervous system preferentially.

The results of studies of Cbl analogues in animals with experimental Cbl deficiency have failed to provide evidence of a role for Cbl analogues in the development of the Cbl neuropathy. Exposure of rats to N_2O is associated with the conversion of Cbl to Cbl analogues in the liver, brain, and serum, but the animals do not develop neurological changes (38). No accumulation of Cbl analogues could be demonstrated in fruit bats with Cbl neuropathy induced by dietary deprivation (29) or by exposure to N_2O (62).

The evidence for a role of Cbl analogues in the pathogenesis of the Cbl neuropathy is thus limited. If the proposed toxicity of the analogues is due to substitution for active Cbl and aggravation of the degree of deficiency, it is difficult to see why this effect is exerted preferentially on the nervous system complications of Cbl deficiency. Furthermore, any hypothesis of the pathogenesis of the Cbl neuropathy should include an explanation of the mode of action of Met in ameliorating the neuropathy. It is not clear how Met might retard the formation of analogue or attenuate any effect analogues may have in compromising nervous system function.

SUMMARY

Neuropathy commonly complicates cobalamin (Cbl) deficiency in humans, monkeys, fruit bats, and pigs. The neuropathy is characterized by demyelination of the posterolateral columns of the spinal cord (subacute combined degeneration). The lesion was thought to arise primarily from impairment of the adenosylcobalamin-dependent methylmalonyl CoA mutase reaction, leading to the formation of abnormal odd-chain and branched-chain fatty acids and their incorporation into myelin with resultant demyelination. Data from recently developed animal models of the Cbl neuropathy induced by exposure to nitrous oxide do not substantiate this hypothesis, but rather identify impairment of the methylcobalamin-dependent methionine synthetase reaction as the more important basic defect. The key evidence for this hypothesis is the ability of methionine to delay the onset of Cbl neuropathy in experimental Cbl deficiency. In the Cbl-deficient pig, adenosylhomocysteine accumulates in neural tissue, presumably owing to the inability to recycle homocysteine via the defective methionine synthetase reaction. Accumulation of adenosylhomocysteine results in a fall in the adenosylmethionine:adenosylhomocysteine methylation ratio, and this change is believed to cause defective methylation and demyelination in the nervous system. However, in the Cbl neuropathy in

the fruit bat, adenosylhomocysteine does not accumulate in the nervous system, the methylation ratio does not change, and no defect can be demonstrated in the methylation of myelin lipid or basic protein. Although a central role for methionine in the pathogenesis of the Cbl neuropathy has been established, defective methylation attendant upon impairment of the methionine synthetase reaction may not be the universal defect underlying the Cbl neuropathy. This would suggest that the methionine effect could be mediated via its role in formate metabolism or polyamine synthesis, or by some as yet unidentified pathway.

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