SIGNIFICANCE OF COBALAMIN INACTIVATION IN NORMAL AND MALIGNANT HEMATOPOIESIS

BETEKENIS VAN COBALAMINE-INACTIVATIE VOOR DE NORMALE EN KWAADAARDIGE BLOEDCELVORMING

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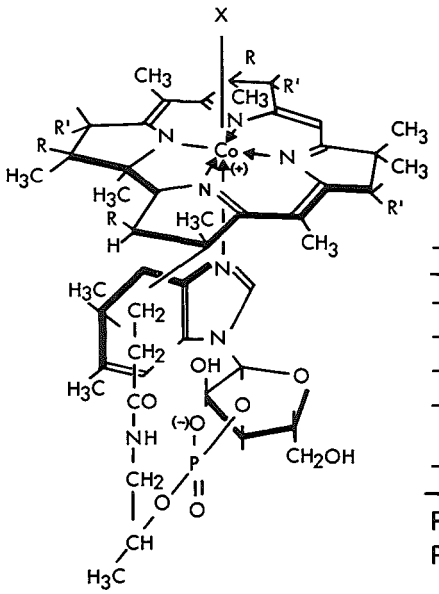
Chapter 1

Introduction

1.1. Folate metabolism.

1.1.1. Biochemistry of cobalamin and folates.

Cobalamin. Cobalamin, or vitamin B_{12}, is a dietary factor which plays an indispensable role in the proliferation of hematopoietic cells. Since the discovery by Minot and Murphy in 1926 that a fatal form of anemia (pernicious anemia) could be treated by excessive consumption of liver (1), the structure and function of this so-called "extrinsic factor" (2) have been a challenge for scientists. The specific compound was purified from liver and isolated in crystalline form in 1948 (3,4) and called vitamin B_{12}. Its complex three-dimensional structure was elucidated by Dorothy Hodgkin in 1956 (5) (see figure 1.1).



- X	name	
- CN	cyanocobalamin	(CN-Cbl)
- OH	hydroxocobalamin	(OH-Cbl)
- H_2O	aquocobalamin	(HOH-Cbl)
- deoxy-adenosyl	coenzyme B_{12}	(AdoCbl)
- CH_3	methylcobalamin	(MeCbl)

R = CH_2CONH_2
R^1 = $CH_2CH_2CONH_2$

Figure 1.1: Structure of the cobalamin molecule and the (an)organic anionic ligands that can be attached to it at "X".

The core of the molecule consists of a corrin ring with a central cobalt atom which clarifies its present name: cobalamin (6). The corrin ring, like a porphyrin, has four pyrrole units. Two of them are directly bonded to each other, the others are joined by methene bridges. The side chains on the pyrrole rings are methyl, propionamide or acetamine groups, as is shown in figure 1.1. A cobalt atom is bonded to the four pyrrole nitrogens. Below the corrin plane a fifth bond is always occupied by a derivative of dimethylbenzamidazole, containing ribose-3-phosphate and amino-isopropanol. The aminogroup of the latter is also linked with a side chain of

the corrin ring. Finally a sixth position, located above the corrin plane, can be either -CN, -CH3, -OH or deoxyadenosyl (see figure 1.1). The oxidation state of the cobalt atom in the cobalamin molecule can be either cob(I)-, cob(II)-, cob(III)alamin.

In cyano- and hydroxycobalamin, the cobalt atom is trivalent. However, the biosynthesis of adenosyl- or methylcobalamin requires the reduction to cob(I)alamin before these organic anionic ligands can be attached to the molecule. Cobalamins can only be synthetized by bacteria and are not present in plants. A number of cobalamin-dependent bacterial enzymes has been described for both methylcobalamin and adenosylcobalamin. However, in man only one coenzyme function for each cobalamin form has been extensively documented. Methylcobalamin is required as a cofactor in the methylation of homocysteine to methionine by methionine synthase (7-9) (E.C.:2.1.1.13), which is an important step in the folate metabolism. Adenosylcobalamin is the coenzyme of the mitochondrial enzyme methylmalonyl CoA mutase which catalyzes the intramolecular isomerisation of methylmalonyl CoA to succinyl CoA (10,11). Methylmalonyl CoA is formed from isoleucine and valine and also from odd-chain fatty acids and therefore this cobalamin-dependent pathway forms a link between the lipid catabolism and the citric acid cycle.

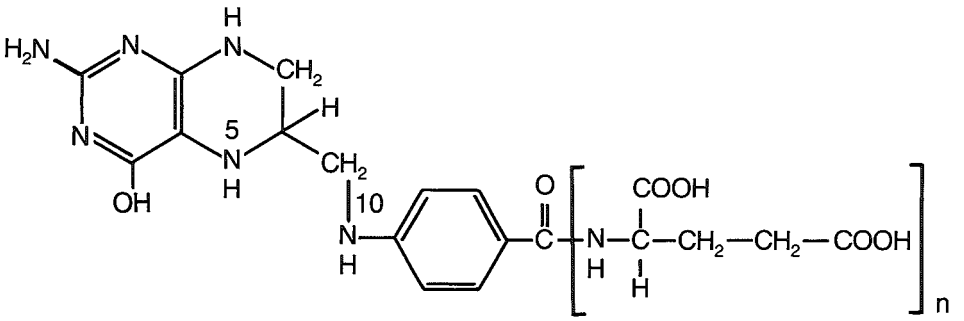
In the literature, the impaired activity of either cobalamin-dependent enzyme is proposed to explain the neuropsychiatric abnormalities that can be found during cobalamin deficiency (12). Decreased methionine synthase activity would cause reduced methylation of the "myelin basic protein" (13). On the other hand it has been suggested that impaired activity of methylmalonylCoA mutase causes deranged biosynthesis of myelin (14).

Folates. Folate compounds are a group of derivatives essentially consisting of a substituted pteridine, p-aminobenzoate and glutamate (15) (see figure 1.2) and were first isolated from plant leaves (16). The parent compound pteroylglutamic acid, also called folic acid, is normally not found in foods or in the human body. It is biochemically not active but becomes so after reduction, with the attachment of additional hydrogens on position 5,6,7 and 8 of the pteridine ring. This reduced form, which is called tetrahydrofolate (THF), can be further modified by covalent binding of a one carbon unit at nitrogen atom 5 and/or 10. This unit can exist at several oxidation states (see figure 1.2) which are interconvertable. Besides reduction of the pteridine ring and addition of one carbon unit, folates can also be changed by the additional coupling of glutamic acid residues through peptide bonds with the glutamate already present (17,18). The so formed folylpolyglutamates mainly contain 3-6 glutamate residues and possess properties significantly different from the parent folatemonoglutamate (see also 1.2).

1.1.2. Folate metabolism in mammalian cells.

The folates present in food are predominantly polyglutamates (15) which are completely converted to monoglutamates by specific conjugases during their transit through the intestinal mucosa (19). Moreover, all folates are converted in 5-methyl-THF, the plasma folate form, before entering the circulation (20). Plasma 5-methylTHF enters the cell through a membrane carrier of which the activity is

Structure of folates



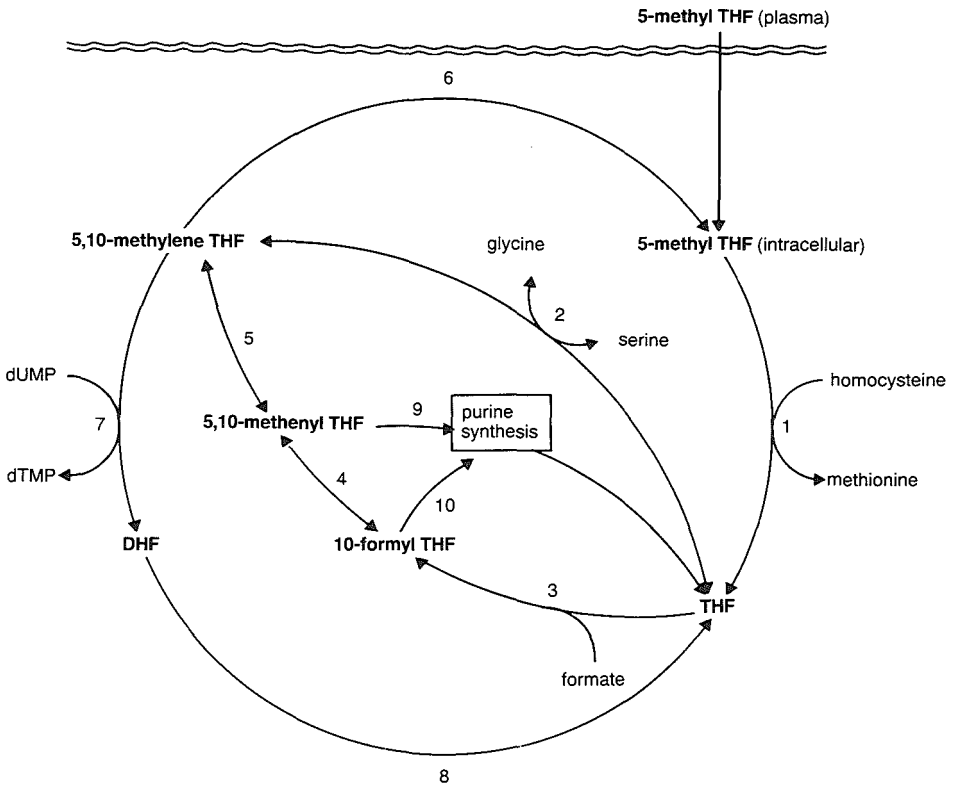
tetrahydrofolate (THF)

Substitutions:	at N-5:	at N-10:
5-methyl-THF	$-CH_3$	- H
5-formyl-THF	- CHO	- H
10-formyl-THF	- H	- CHO
5-formimino-THF	- CHNH	- H
	between N-5 and N-10 :	
5,10-methylene-THF	$-CH_2-$	
5,10-methenyl-THF	= CH-	

Figure 1.2: Structure of tetrahydrofolate molecule and the various substitutions that can be attached to nitrogen 5 and/or nitrogen 10.

probably regulated by the intracellular level of folate (21-24). The exact mechanism through wich the folate uptake proceeds is still subject to dispute. Internalized 5-methylTHF enters the pool of active folate coenzymes after conversion to THF by methionine synthase (enzyme 1, figure 1.3), which concomitantly methylates homocysteine to methionine (7-9). This reaction is dependent on the intact function of the methylcob(I)alamin coenzyme. In addition, S-adenosylmethionine (SAM) is catalytically active in this reaction (9,25,26). THF is involved in several pathways (27-29). Serine hydroxymethyltransferase (enzyme 2, figure 1.3) catalyses the conversion of serine to glycine, while forming 5,10-methyleneTHF. In mammalians, this pathway predominantly provides the one carbon units for the folate metabolism. Another metabolic route, which also leads to the synthesis of 5,10-methyleneTHF is via 10-formylTHF and 5,10-methenylTHF. These consecutive steps are performed by

3 enzymatic activities present on one polypeptide (30), which is therefore called the "trifunctional enzyme" (enzyme 3,4,5, figure 1.3). The enzyme 5,10-methyleneTHF reductase (enzyme 6, figure 1.3) catalyses the formation of 5-methylTHF in which the one carbon unit exists in its most reduced state. This reaction is unidirectional (31,32) and under allosteric regulation by SAM (33,34). The folate coenzyme 5,10-methylene-THF is also very important as it is involved in the thymidylate (dTMP) synthesis from uridylate (dUMP) by dTMP synthase (enzyme 7, figure 1.3). This reaction provides the only pathway for *de novo* synthesis of dTMP necessary for DNA synthesis. The reaction product DHF is rapidly converted to THF by DHF reductase (enzyme 8, figure 1.3). The importance of folate-dependent dTMP synthesis has resulted in the chemotherapeutic use of several antimetabolites directed towards the 2 enzymes just



1: methionine synthase
2: serine hydroxymethyltransferase
3: 10-formyl THF-synthetase
4: 5,10-methenyl THF-cyclohydrolase
5: 5,10-methylene THF-dehydrogenase
6: 5,10-methylene THF-reductase
7: thymidylate synthetase
8: DHF-reductase
9: glycinamideribotide formyltransferase
10: aminoimidazolecarboxamideribotide formyltransferase

Figure 1.3: Scheme of the most important folate-dependent pathways and the enzymes involved.

mentioned. Notably 5-fluorouracil (inhibiting dTMP synthase) and methotrexate (inhibiting DHF reductase) have achieved widespread application in the treatment of neoplastic diseases (35,36).

Two enzymes involved in the *de novo* synthesis of purines also use folate coenzymes. Formylation of glycinamide ribotide (GAR) by 5,10-methenylTHF provides carbon no. 8 of the purine molecule and is catalysed by GAR transformylase (enzyme 9, figure 1.3). The second reaction involves formylation of aminoimidazole carboxamide ribotide by 10-formylTHF to add carbon no. 2, which closes the purine ring to form inosinic acid (AICAR transformylase, enzyme 10, figure 1.3).

The levels of the different folate cofactors in tissues and the activity of the related enzymes is very dependent on the function and proliferative state of the studied cells. Moreover a distinct compartimentalisation of the folate coenzyme pools has been observed in liver cells (37). In resting cells 5-methylTHF is the predominant folate form present (38-42) while in rapidly dividing tissues equal levels of 5-methylTHF and 10-formylTHF exist (40,43-45). Moreover, nutrional deficiencies of either cobalamin (46-49), or methionine (47,50-60) exert direct effect on the distribution of intracellular folates among the various coenzyme pools.

An increasing body of evidence indicates that retention and concentration of folates in mammalian cells requires the conversion of folate to polyglutamate derivatives by polyglutamate synthetase (17,18). The observed end-product inhibition of long-chain folylpolyglutamates on this particular enzyme probably effects steady-state intracellular folate concentrations (61,62). Not surprisingly, folylpolyglutamate synthetase is the most active in rapidly proliferating cells, presumably because of low intracellular folate levels after cell division.

The level of polyglutamation of the various folate coenzyme forms plays an essential role in the regulation of the folate metabolism as the affinity of folate coenzymes for several enzymes can depend on the level of polyglutamation (63,64). Notably monoglutamate forms have proven to possess the lowest affinity towards folate-dependent enzymes (18). Moreover, 5-methylTHF has shown to be a poor substrate for polyglutamation (65-71) and therefore requires the cobalamin-dependent conversion to THF before glutamate residues can be added, by which its intracellular retention increases (65,72). This particular property of 5-methylTHF further emphasizes the key role of methionine synthase in cellular folate metabolism.

1.1.3. Cobalamin, folate and methionine metabolism.

The cobalamin-dependent enzyme methionine synthase plays an important role in the methionine metabolism as it provides the most common pathway for the regeneration of this amino acid from homocysteine (7-9). Its significance is evidenced by the elevated levels of plasma homocysteine in patients with inborn errors of 5-methylTHF (73,74) or methylcobalamin metabolism (75-77). Moreover, perturbation of other pathways of the folate metabolism (78) or folate and cobalamin deficiency (79) also result in raised levels of circulating homocysteine.

The synthesis of methionine through methionine synthase is regulated in several ways. First of all methionine itself exerts some negative feedback on the activity of the enzyme through end product inhibition (9,50,80). Secondly, methionine is

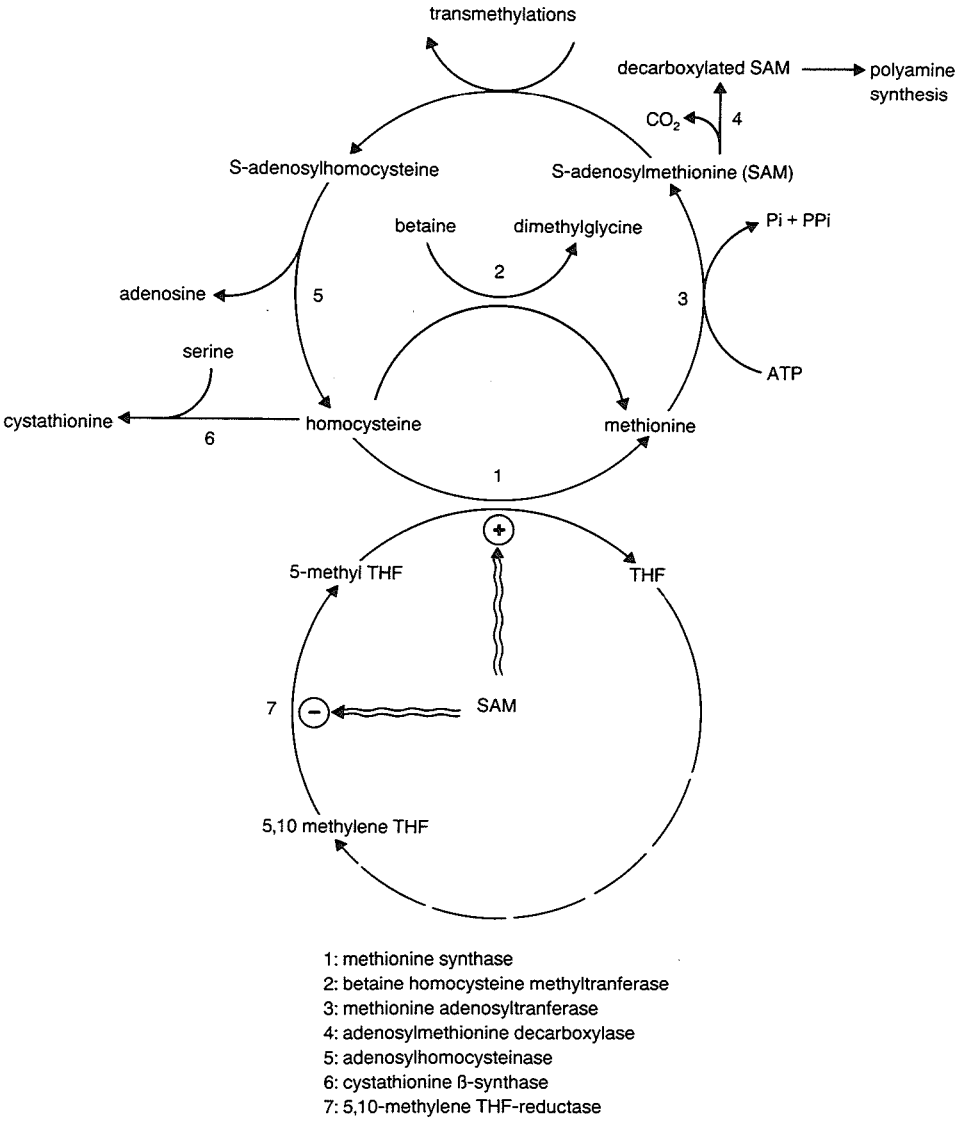


Figure 1.4: Scheme of the most important pathways of the methionine metabolism.

converted to S-adenosylmethionine (SAM, see figure 1.4). This high energy compound is considered to have a regulating effect on folate-dependent methionine synthase through strong allosteric inhibition of 5,10-methyleneTHF reductase (33,34), by which the amount of 5-methylTHF available for methionine synthesis is reduced. Moreover,

it has been shown that SAM is catalytically active in the actual methionine synthase reaction (9,25,26). Thus, low methionine and low SAM concentrations, as in methionine deficiency, lead to accumulation of 5-methylTHF at the expense of other reduced folates. On the other hand, high concentrations of SAM result in reduced 5-methylTHF levels. However, most research on this subject has been conducted in liver tissues (47,49-58), and its significance for other cell types has yet to be elucidated as some controversial results have been published (81,82). For instance, addition of low doses methionine to cobalamin deficient bone marrow cells are able to correct disturbed folatedependent dTMP synthesis while high doses aggrevate the disturbances of the folate metabolism (83).

The alternative route for methionine synthesis leads via betaine homocysteine methyltransferase but only exists in liver and probably kidney. The enzyme appears to adopt its activity to the supply of methionine, betaine and choline (a precursor of betaine), but is considered to be of minor importance (84-86).

Besides its regulatory function in the folate metabolism SAM is important as the major donor of methylgroups in a wide variety of transmethylation reactions and also the precursor of decarboxylated SAM, necessary for polyamine synthesis (see figure 1.4). The co-product of these transmethylations, S-adenosylhomocysteine, is hydrolysed to yield homocysteine which can be remethylated to methionine or condensed with serine to form cystathionine (84,86,87).

1.2. The importance of cobalamin-dependent folate metabolism in hematopoiesis.

1.2.1. Megaloblastic anemia

The direct importance of cobalamin for the maintenance of hematopoiesis is evident from the severe anemia which may develop in case of cobalamin deficiency. Already in 1849 Addison described a fatal form of anemia characterized by the presence of abnormal large cells in the bone marrow which were named megaloblasts by Ehrlich (88). In 1926 it was found that this particular syndrome (pernicious anemia) could be effectively treated with an extract from liver (1), which finally resulted in the discovery of vitamin B_{12} or cobalamin. The underlaying cause of anemia turned out to beimpaired absorption of cobalamin through the absence of Castle's intrinsic factor (2), which on its turn is caused by a proces of autoimmune atrophic gastritis (89,90).

In 1931, Lucy Wills isolated from yeast a second factor, which was capable to correct megaloblastic anemia of pregnancy and tropical sprue (91). Later on, this compound was also isolated from plant leaves and therefore called folic acid (92). These clinical observations resulted in the recognition of folate deficiency as a separate cause of megaloblastic anemia (93). The clinical similarity of either cobalamin or folate deficiency led to intensive research on the possible functional relationship between these two vitamins and more specifically their actual role in the proliferation of hematopoietic progenitor cells. Up till now, the only known metabolic pathway common to both folate and cobalamin is the reaction catalysed by methionine synthase (see figure 1.5), in which homocysteine is methylated to methionine (7-9). Based on this knowledge and some clinical observations in patients with cobalamin deficiency, Noronha & Silverman (46) and Herbert & Zalusky (94)

advanced the "methylfolate trap" hypothesis to explain the effect of cobalamin deficiency on hematopoietic proliferation.

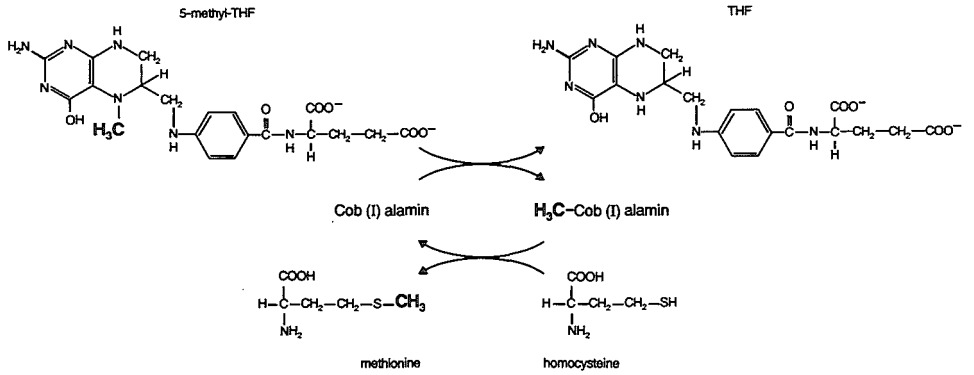


Figure 1.5: Outline of the enzymatic transfer of the methyl group of 5-methylTHF to homocysteine through cob(I)alamin.

1.2.2. The methylfolate trap hypothesis

Based on the role of cobalamin in the methionine synthase reaction and the elevated serum folate levels in patients with cobalamin deficiency, the "methylfolate trap" states that under conditions of cobalamin deficiency methionine synthase activity is diminished (95) and folate is "trapped" as 5-methylTHF. Since reoxidation via the 5,10-methyleneTHF reductase pathway is impossible under physiological conditions (31,32), a functional deficiency of all other reduced folate forms ensues, causing impairment of folate-dependent reactions. The subsequent reduction of synthesis of dTMP and purines decreases DNA replication and results in reduced proliferation of the hematopoietic progenitor cells.

The main implications of this "methylfolate trap" are a relative accumulation of 5-methylTHF in cobalamin-deficient tissues and the correction of cellular proliferation by all reduced folates with the exception of 5-methylTHF. Both consequences have been thoroughly studied and have been demonstrated to be correct (resp. 46-49,96 and 97-105), although the actual demonstration of increased tissue levels of 5-methylTHF has been difficult because of the low intracellular folate content and the instability of the various folate coenzyme forms.Twenty-eight years after postulation of the "methylfolate trap" as an explanation for the cobalamin deficiency-induced megaloblastic anemia, the quintessence of this hypothesis still stands, although some modifications appeared necessary.

First of all, ameliorating effects of methionine on some features of disturbed folate metabolism (e.g.: increase of deranged folate-dependent histidine degradation (51,54,57) and intracellular folate repletion (47,50,56,58) had to be accounted for by

the inhibiting effect of SAM on 5,10-methyleneTHF reductase (33,34) and the catalytic action of SAM in the methionine synthase reaction (9,50,80) (see figure 1.4). The consequences of this regulation mechanism are both reduced synthesis and accumulation of 5-methylTHF during methionine supplementation of cobalamin-deficient subjects.

Secondly the "methylfolate trap" had to be expanded to explain the lowered tissue folate levels in cobalamin-deficient animals. Both the recognition that polyglutamation of folates is essential for their intracellular retention (65), and the observation that 5-methylTHF is a poor substrate for folylpolyglutamatesynthase (65-71) have provided the key to this problem. Since internalized 5-methylTHF cannot be converted to other reduced folate forms in case of cobalamin deficiency, insufficient polyglutamation occurs, causing decreased uptake and retention of folates in tissues.

Based on the "methylfolate trap" Killman et al. (105) developed a functional test which could discriminate between cobalamin and folate deficiency as the primary cause of megaloblastic anemia. In marrow cells from megaloblastic patients folate-dependent dUMP methylation to dTMP is very low. Incubation of marrow cells with deoxyuridine (dU) prior to [^{3}H]thymidine incubation hardly reduces the incorporation of the latter in DNA. However, preincubation of these cells with cobalamin or folic acid will restore the folate-dependent dTMP synthesis and decrease the cellular [^{3}H] thymidine incorporation in DNA. The specific correction pattern by cobalamin and/or folic acid is indicative for either deficiency (102-104,106).

Although a vast amount of experimental evidence exists, which is in agreement with the "methylfolate trap" hypothesis, several other theories have been presented for the interrelationship between folate, cobalamin and methionine metabolism. Most importantly, Chanarin et al. proposed the "formate starvation" hypothesis as alternative explanation for the phenomena which can be observed during cobalamin deficiency.

1.2.3. The formate starvation hypothesis

Essentially, this explanatory theory by Chanarin and coworkers emphasizes the importance of the reduced methionine synthesis during cobalamin deficiency (107-110). They observed that, compared with THF, 5-formylTHF was superior in correcting disturbed folate-dependent dTMP synthesis in bone marrow cells and therefore concluded that the THF production by methionine synthase is of little importance for cellular folate metabolism (111-114). Instead they proposed that decrease of methionine supply is the actual cause of disturbed folate metabolism.The metabolic link between those two is based on the conversion of methionine to "active formate" via a 5-steps pathway (see figure 1.6). In addition they found that the formate precursors methionine and SAM could restore the impaired folylpolyglutamate synthesis (115-117). Low methionine concentrations would cause reduced formate levels and therefore impair synthesis of formylTHF which is considered to be the optimal substrate for folylpolyglutamate synthesis (114). Over the years Chanarin et al. have published a vast amount of interesting studies, the results of which were always interpreted in favor of the formate starvation theory.

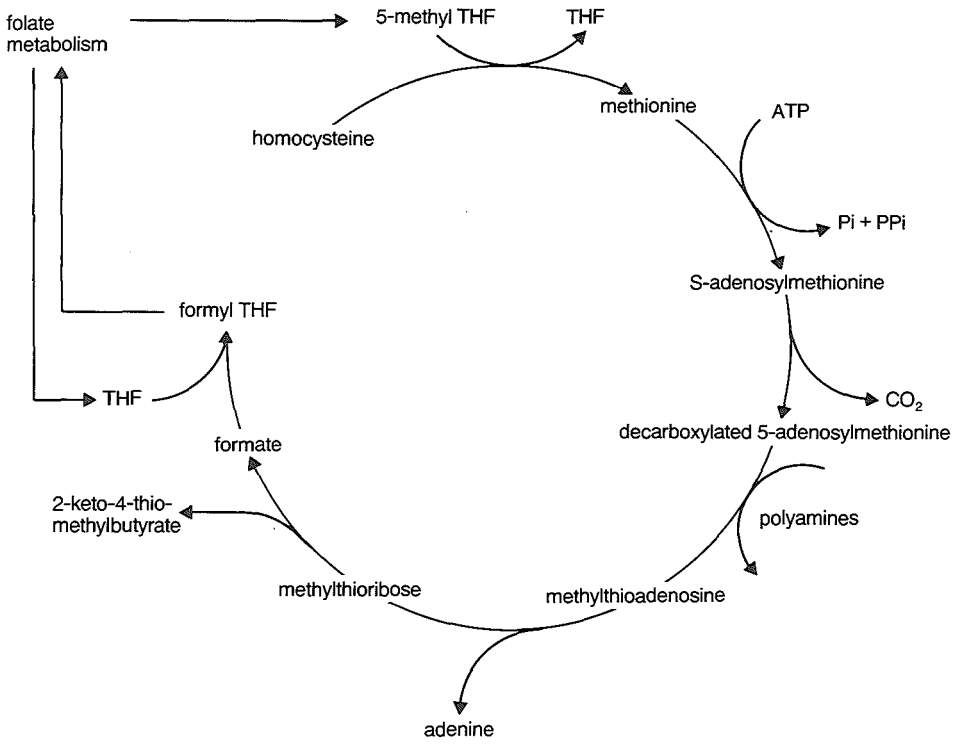


Figure 1.6: Pathway of formate synthesis from methionine as postulated by Chanarin et al..

Still, it seems rather unlikely that "formate starvation" is the primary cause of impaired hematopoiesis during cobalamin deficiency. The proposed pathway for formate production seems rather complicated and direct administration of sodium formate to rats with a functional cobalamin deficiency does not result in restoration of the intracellular folate homeostasis (117). Moreover, the observation by Chanarin et al that 5-formylTHF is the preferable substrate for polyglutamate synthesis has not been confirmed by others (67,71). The demonstrated superiority of 5-formylTHF over THF in correcting disturbed deoxyuridine suppression tests from cobalamin-deficient bone marrow cells may well be attributed to its greater stability and its more effective uptake by cells (28).

1.2.4. Cobalamin-dependent folate metabolism in malignant hematopoiesis

The folate metabolism has been an important target for cancer chemotherapy ever since Faber demonstrated the effectiveness of folic acid analogues in leukemia treatment (118). Notably methotrexate is succesfully used in the management of various malignancies and currently incorporated in most treatment protocols for acute

lymphoid leukemia (36). Recently, antileukemic effect of 5-fluorouracil (119) and CB3717 (120), both inhibitors of the folate-dependent dTMP synthase has also been demonstrated in the Brown Norway model for myeloid leukemia (BNML).

Clinical resistance to antifolates especially methotrexate has been observed in several malignancies (36). It can be caused by increased DHFR levels, changes in the kinetic properties of DHFR or reduced uptake and/or polyglutamation of the antifolate. The occurence of drug resistance stimulated the development of new folate derivatives directed against one of the various folate-dependent enzymes directly or indirectly involved in dTMP or purine synthesis (121). For instance CB3717 (N^{10}-propargyl-5,8-dideazafolic acid) acts primarily on dTMP synthase and DDATHF (5,10-dideazatetrahydrofolic acid is an inhibitor of the enzyme glycinamide ribotide transformylase. Until now, however, only little progress has been made on the inhibition of the cobalamin-dependent methionine synthase reaction as a possible chemotherapeutic target. It is very probable that this particular enzyme plays a vital role in the proliferation of leukemic and other malignant cells as it does in normal hematopoietic and other tissue proliferation. In two patients acceleration of leukemic growth has been observed after cobalamin supplementation, administered for the treatment of a megaloblastic anemia (122,123). Levels of both cobalamin and folate are elevated in leukemic cells as compared with normal bone marrow cells (124-127) and the activity of methionine synthase is also increased, especially in lymphoid leukemia (128-131). Kroes et al. demonstrated the antileukemic effect of impaired methionine synthase activity in the BNML using nitrous oxide to inactivate the cobalamin coenzyme (132). Moreover they demonstrated enhanced chemotherapeutic activity of several drugs that interfere with the folate metabolism if combined with cobalamin inactivation (133-136, see also 1.3.4.: Effects of nitrous oxide on malignant hematopoiesis). Regarding the importance of folate metabolism for malignant proliferation, the specific exploration of cobalamin-dependent methionine synthase as a potential target in cancer chemotherapy seems justified (137).

1.3. The effect of nitrous oxide on cobalamin.

1.3.1. History.

Nitrous oxide is a colorless, odorless gas, which already has been applied in anesthesia for more than a century. Although it is not a very potent anesthetic, it has gained general application as an adjuvant to other anesthetics. Presently, a gas mixture of 70 % nitrous oxide/30 % oxygen is commonly used to ventilate anesthetized patients. During almost a century the use of nitrous oxide had been considered to haveno significant side-effects. However, clinical observations in tetanus patients in the fifties revealed that prolonged inhalation of nitrous oxide could result in megaloblastic and aplastic bone marrow depression (138-140). Later studies in rodents demonstrated a striking leukopenia during nitrous oxide exposure which, however, was not accompanied by megaloblastic changes in the bone marrow (141-145).

Until 1978 the observed hematological toxicity of nitrous oxide remained unexplained but this changed when Amess et al. (146) and Deacon et al. (147)

correlated the occurrence of megaloblastic anemia in nitrous oxide-treated patients with the chemical reaction between cobalt (I) complexes, like reduced cobalamin, and nitrous oxide. This specific reaction between transition-metal complexes and nitrous oxide, already described in the chemistry literature in 1968 (148,149), results in the oxidation of cob(I)alamin to cob(II)alamin. Since this explanation of nitrous oxide-induced megaloblastic anemia, further research, has been aimed at the elucidation of the mechanism of cobalamin inactivation by nitrous oxide and its consequences for mammalian folate metabolism. Moreover, the phenomenon provided experimenters with a easy tool to further investigate the metabolic effects of cobalamin deficiency, which otherwise is rather difficult to induce in laboratory animals.

1.3.2. Effect of cobalamin inactivation on folate metabolism.

Effect on methionine synthase. The inactivation of the cobalamin coenzyme of methionine synthase by nitrous oxide has been studied thoroughly both *in vitro* and *in vivo*.Recently, the gene encoding for the enzyme has been cloned (150) and the resulting availability of large amounts of pure and active enzyme will surely facilitate research into the specific mechanism of the methionine synthase-catalysed reaction and its inactivation by nitrous oxide. The proposed mechanism of oxidation of the cobalamin coenzyme is as follows (150,151):

$$\text{Cob(I)alamin} + N_2O + H^+ \text{-----> Cob(II)alamin} + N_2 + OH^\cdot$$

The generation of a hydroxyl radical at the active site of the enzyme is responsible for the irreversible loss of catalytic activity and it also causes partial destruction of the cobalamin prosthetic group. The actual *in vivo* inactivation of methionine synthase by nitrous oxide has now been demonstrated in a variety of species and tissues (147,151-162). Studies by Royston et al. (163) have shown that rat methionine synthase is inactivated at a higher rate (t_{half}: 5 min.) than the human enzyme (t_{half}: 45 min.). The latter implicates that effects of nitrous oxide exposure on human folate metabolism occur only after several hours of exposure. Indeed some studies revealed that it takes at least 5-6 hours before the deoxyuridine suppression tests in bone marrow cells becomes abnormal (164).

During exposure to nitrous oxide no recovery of the enzyme activity (less than 10 % of control values) occurs (155,160). Withdrawal of the gas results in slow recovery of methionine synthase activity within several days.

Effects on intracellular folate metabolism. The primary effect of cobalamin inactivation is the disturbed methylation of homocysteine by 5-methylTHF. Secondary to this event a cascade of biochemical changes in the folate-dependent metabolism occurs which altogether seems to concur with the methylfolate trap hypothesis. Tissue folate levels decrease (165-167) because of the impaired metabolisation of internalized 5-methylTHF and because the relative increase of this "trapped" folate form (166-168) reduces folylpolyglutamation (68,116,117,166,169). Subsequently, rates of purine (162,170-172) and dTMP synthesis, both dependent on folate coenzymes, decrease.

The latter has been thoroughly studied, using the deoxyuridine suppression test as described before (111,164,173-179).

The defective methionine synthesis also causes a drop in tissue levels of methionine as well as SAM (179-181), and a decrease of plasma methionine (159,178,182,183). Noteworthy a rise of plasma homocysteine has only been shown in rats (184). After several days of nitrous oxide exposure some replenishment of methionine levels in rat liver occurs, probably by induction of the alternative homocysteine-betaine methyltransferase pathway (179). Finally, nitrous oxide exposure disturbs several catabolic pathways, notably the oxidation of formate, produced during methanol poisoning, (168,180,185, 186), and the degradation of histidine (180,187), which require THF and become impaired by cobalamin inactivation.

Cobalamin-folate homeostasis during nitrous oxide exposure. Only a few studies have been performed on the actual effect of nitrous oxide on the tissue cobalamin levels. A slow decrease was found in rats and fruit bats (154,188,189). Moreover, the formation and excretion of cobalamin analogues has been reported (189), which concurs with the suggested damage to the cobalamin molecule by nitrous oxide (150,151).

Tissue folate levels decrease during nitrous oxide exposure and this is accompanied by an increased loss of folates with the urine (190). The recovery of tissue folate levels requires the dietary intake of new folate (191).

1.3.3.Effect of nitrous oxide on normal hematopoiesis: implications of its clinical use.

The disturbance of folate metabolism by nitrous oxide results in hampering of impaired cellular proliferation because of reduced nucleotide synthesis. Just like in cobalamin deficiency, the dependence of hematopoiesis on the intact function of the cobalamin coenzyme of methionine synthase becomes evident by exposure to nitrous oxide.Before the interaction between nitrous oxide and cobalamin was recognized, Lassen et al. had described several cases of severe bone marrow depression after prolonged exposure to nitrous oxide (138). Since the rediscovery of this toxic effect by Amess et al. (146), several studies have been conducted in order to determine the risks of bone marrow depression during nitrous oxide anesthesia. Normaly it takes 5-6 hours of exposure before dTMP synthesis becomes depressed (164), and 12-24 hours before macrocytic/megaloblastic changes can be seen in the bone marrow (146,177, 178).

Presently, in most cases anesthesia is too short to cause significant hematological toxicity. Moreover, a temporary reduction of hematopoiesis may well be dampened by the vast pool of mature blood elements in the blood and bone marrow. However, in case of multiple nitrous oxide exposures (192) (e.g. trauma patients), seriously ill patients (193) or subjects with a marginal or manifest cobalamin and/or folate deficiency (194), bone marrow depression may already develop within 2 hours. A special situation occurs when nitrous oxide anesthesia is given prior to the administration of a folate antagonist. Recently, several reports have drawn attention to the interaction between nitrous oxide and methotrexate causing unexpected gastrointestinal toxicity and myelosuppression in cancer patients (195-197).

Despite the apparent hematological side effects of nitrous oxide it has maintained a

remarkable record of safety for over a century. Considering the possible toxicity of other anesthetics it may be concluded that nitrous oxide is still an extremely useful analgesic/anesthetic agent but one must remain aware of hematological side effects in some patients. Noteworthy, supplementation with folinic acid (5-formylTHF) has proven to provide adequate protection against the myelosuppressive effects of this anesthetic gas (198,199).

1.3.4. Effects of nitrous oxide on malignant hematopoiesis: possible applications.

Already 3 years after their publication of the nitrous oxide-induced bone marrow depression, Lassen and coworkers reported on the succesful application of nitrous oxide in the treatment of 2 patients with chronic myelogenous leukemia (200). They observed a remarkable drop in the leukocyte counts but this effect was always quickly reversed upon withdrawal of the nitrous oxide. A few years later, Eastwood et al. used nitrous oxide in the treatment of 2 cases of acute myeloid leukemia with comparable results (201) and recently Ikeda et al. described effective reduction of peripheral leukocytes and size of the spleen in a case of chronic myelogenous leukemia (202).

Besides these few clinical reports no data are available on the effect of nitrous oxide on human leukemia. *In vitro* however, a specific sensitivity of hematological cell lines for nitrous oxide has been observed in the deoxyuridine suppression test and in reduced cell growth (203). Moreover a differentiation inducing effect of nitrous oxide on the promyelocytic HL-60 cell line has been described, which is probably related to the reduced methionine synthesis during cobalamin inactivation (204). Studies in mice have also revealed antitumor activity of nitrous oxide in fibrosarcoma (205), Ehrlichs acites tumor (206) and lymphoma (207).

Recently, an extensive study by Kroes et al. (132) demonstrated that nitrous oxide has antiproliferative properties in a rat model for myeloid leukemia (BNML) causing reduction of the leukemia-induced leukocytosis and hepatosplenomegaly. Moreover it was shown that the antiproliferative effect of nitrous oxide can be enhanced by combination with several drugs interfering with other folate-dependent pathways. Especially the antileukemic effects of cycloleucine (inhibitor of methionine adenosyltransferase), methotrexate (inhibitor of dihydrofolate reductase) and 5-fluorouracil (inhibitor of thymidylate synthase) could be strongly potentiated by preexposing rats to nitrous oxide (133, 134-135). Administration of 25 mg/kg 5-fluorouracil after three days nitrous oxide exposure, repeated every five days, resulted in almost 200 % extended survival time of the leukemic rats. Cobalamin inactivation by nitrous oxide combined with methylglyoxalbis(guanylhydrazone), an inhibitor of SAM decarboxylase, also reduced polyamine synthesis, which is considered a potential target in cancer chemotherapy. However, antiproliferative activity of the induced polyamine depletion in the BNML is rather limited (136).

1.4. Introduction to the experimental work.

This thesis deals with several aspects of the effects of cobalamin inactivation by nitrous oxide on cellular folate metabolism and the consequences on normal and

malignant hematopoiesis. Kroes et al. demonstrated the antileukemic effects of nitrous oxide either or not in combination with other drugs interfering with the folate metabolism (132-136). Especially the potentiation of methotrexate activity in the employed rat model for myeloid leukemia (BNML) after preexposure to nitrous oxide was striking (134).

Based on those findings part of the work presented in this thesis focuses on the *in vivo* interaction between nitrous oxide and methotrexate. In order to establish the cytotoxicity of the nitrous oxide-methotrexate combination for normal cells, we conducted an extensive toxicological study in healthy rats in which we concentrated on the effects on the rapidly dividing cells of bone marrow and gut mucosa (Chapter 3). Moreover, effects of nitrous oxide and/or methotrexate on folate metabolism and folate-depedent dTMP synthesis of fresh human leukemic cells were studied in order to clarify the actual working mechanism (Chapter 4,5).

Kroes et al. came to the conclusion that cobalamin inactivation by nitrous oxide appeared applicable in the treatment of human leukemia. However, several questions still have to be answered before nitrous oxide can be used for this particular purpose. For instance, no information on a differential sensitivity of normal hematopoietic and leukemic cells for cobalamin inactivation is available. We therefore investigated the effects of nitrous oxide on both normal and malignant hematopoiesis in the BNML model (Chapter 2) employing *in vitro* bone marrow culture techniques. In this particular type of bioassay, bone marrow cells are cultured *ex vivo* in the presence of growth factors supporting the proliferation of the hematopoietic progenitor cells. These cells are very important for the maintenance of hematopoiesis and their *in vitro* quantification therefore enables assesment of normal bone marrow functioning. Similar *in vitro* bone marrow culture techniques were used to determine the effects of cobalamin inactivation on human bone marrow function in hematologically normal patients, receiving nitrous oxide anesthesia (Chapter 6). The sensitivity of the various forms of human leukemia for cobalamin inactivation was studied *in vitro* by means of the deoxyuridine suppression test and of measurement of the intracellular folate coenzyme levels (Chapter 4,5).

Finally, on the basis of a panel of biochemical parameters in blood and urine of hematologically normal patients receiving nitrous oxide anesthesia the velocity of nitrous oxide-induced cobalamin inactivation and the recovery period is monitored *in vivo* (Chapter 7).

In chapter 8, the presented work will be discussed in view of the present knowlegde on the role of the cobalamin-dependent folate metabolism in both normal and malignant hematopoiesis.

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Chapter 2

Nitrous oxide selectively reduces the proliferation of the malignant cells in experimental rat leukemia

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2.1. Summary.

A consideral reduction of hepatosplenomegaly and leucocytosis in leukemic rats of the Brown Norway Myeloid Leukemia (BNML) can be achieved by exposure to 50% nitrous oxide /50% oxygen. In this study the differential antiproliferative effect of nitrous oxide, inactivating vitamin B_{12}, on normal and leukemic hemopoiesis was investigated in this rat model. Rats injected with leukemic cells and exposed to nitrous oxide for ten days showed 30% reduction of hepatosplenomegaly and 50% reduction of leukocytosis. Similarly treated healthy rats showed no signs of impaired hemopoiesis as measured by peripheral blood parameters. Clonogenic assays of erythroid and myeloid progenitors from both healthy and leukemic rats revealed that exposure to nitrous oxide did not suppress normal bone marrow functioning. On the contrary, the reduction of leukemic proliferation by nitrous oxide retarded the leukemic infiltration of the bone marrow compartment.

2.2. Introduction.

Exposure to the anesthetic gas nitrous oxide inactivates the cobalamin coenzyme of methionine synthetase (1). This enzyme plays an essential role in the cellular retention and metabolism of folate coenzymes. Nitrous oxide induced inactivation of cobalamin (Vitamin B_{12}) therefore leads to secondary impairment of the folate-dependent synthesis of thymidylate (dTMP), which subsequently affects DNA synthesis of proliferating cells (2). The continuous production of mature peripheral blood cells requires the rapid selfrenewal, proliferation and maturation of hemopoietic progenitor cells in the bone marrow. These cells have shown to be particularly dependent on the functional cobalamin coenzyme. For instance in humans, exposure to nitrous oxide for several days results in overt megaloblastic anemia (1,3). In rats a reversible leukopenia without other clear signs of bone marrow depression can be induced by prolonged exposure to the anesthetic (4,5).

The importance of reduced folate metabolism for proliferating cells makes it a potential target for chemotherapy of malignancies as is illustrated by the frequent use of the antifolate methotrexate. Interference with folate metabolism by inactivation of methionine synthetase is only possible through exposure to nitrous oxide. In early studies the use of nitrous oxide in the treatment of chronic and acute leukemia has shown remarkable regression of leukemia (6,7). However further trials concerning this antineoplastic effect of nitrous oxide have not appeared.

In our laboratory the antiproliferative effect of cobalamin inactivation was investigated in a rat leukemia model, the Brown Norway Myeloid Leukemia (BNML). This transplantable acute leukemia has been described in detail before (8) and is considered to be a reliable model for chemotherapeutic studies (9). The antileukemic effect of nitrous oxide alone or in combination with several other agents has been documented before (10-14). However, the applicability of an anticancer drug is determined by its selectivity towards the neoplastic cells. Nowadays hemopoietic progenitors can be cultured *in vitro*. The addition of specific growth factors to these bone marrow cultures results in the proliferation of either myeloid or erytroid progenitor cells. This technique therefore provides a way to asses the normal

hemopoietic proliferation capacity of the bone marrow. The purpose of the present study was to investigate the differential effect of nitrous oxide on normal and malignant hemopoiesis in the BNML using the *in vitro* techniques decribed above.

2.3. Materials and methods.

Animals. Male rats of the Brown Norway inbred strain were used (body weight ± 200 g). Food and water were supplied *ad libitum* during the experiments.
Brown Norway Myeloid Leukemia. Properties of the BNML have been decribed before. Briefly, rats were injected i.v. with 10^7 leukemic cells, which after progressive infiltration of bone marrow, liver and spleen leads to death in 20-24 days. In the present study rats were evaluated 18 days after transfer of leukemia.
Exposure to nitrous oxide. Exposure to nitrous oxide was carried out in a 40 L flow chamber into which a mixture of 50 % nitrous oxide and 50 % oxygen was blown at a rate of 500 ml per min. Rats not exposed to nitrous oxide, were kept in air but otherwise treated identically.
Evaluation of leukemic growth. Two groups of 5 rats were injected with leukemic cells on day 0. At day 7 of leukemia one group was transferred to the flow chamber and exposed to nitrous oxide for 10 days. At day 18 of leukemia all rats were killed by exsanguination. Liver and spleen were carefully removed and weighed. Leukocytes and thrombocytes were counted electronically and hemoglobin concentration was measured by the hemoglobin cyanide spectrophotometrical assay.
Bone marrow morphology. Femurs of 2 groups of 5 healthy rats (one group exposed to nitrous oxide for 10 days) were removed and cut into 2 pieces. Bone marrow was aspirated from the open shaft with a fine needle and used for smears. After staining with May-Grunwald Giemsa, one smear of each rat was used for a differential count of 500 cells.
Bone marrow cultures. To quantify the hemopoietic progenitors in bone marrow of healthy and leukemic rats, either exposed to air or to nitrous oxide, *in vitro* culture techiques have been applied as described by Kimura et al (14). Briefly, bone marrow cells were flushed out of the femur with sterile Alpha DMEM medium and dispersed into a single cell suspension by aspiration through a 22-gauge needle. After being washed once, 5.10^5 cells were plated in triplicate in 35 mm culture dishes in 1 ml Alpha medium containing 0,9 % methylcellulose, 10 % fetal calf serum, 3.10^{-5} M lecthine, 1% (w/v) bovine serum albumine, transferinne 3.10^{-6} M and 2.10^{-5} M mercaptoethanol. Growth of myeloid progenitors was supported by the addition of 10 % rat spleen-conditioned medium as a source of growth factors (for preparation see also ref. 14). Erythroid precursors were stimulated by the addition of 0,5 U sheep erythropoietin (Step III, Connaught, Willowdale, Canada) per ml. Dishes were incubated at 37 °C for 6 days. Clusters (10-50 cells) and colonies (more than 50 cells) were counted with the help of an inverted microscope.

2.4. Results.

Table 2.1 summarizes the results of 2 experiments with either normal, healthy or leukemic animals exposed to air or to 50 % nitrous oxide and 50 % oxygen for 10

Table 1.

Effect of nitrous oxide on hematological parameters of normal and leukemic brown Norway rats (mean ± sem, n = 5)

	normal rats		leukemic rats	
parameters	air	10d.N_2O	air	10d.N_2O
Leukocytes (10^9/L)	6,5 ± 0,5	5,7 ± 0,6	18,1 ± 4,0	9,3 ± 2,1**
Thrombocytes (10^{11}/L)	790 ± 34	810 ± 30	89 ± 13	85 ± 4
Hemoglobine (mM)	9,3 ± 0,5	8,2 ± 0,5	8,8 ± 0,4	9,0 ± 0,2

days. Exposure to nitrous oxide at this concentration did not influence the behaviour or activity of the rats nor their nutritional condition. In healthy rats nitrous oxide had no statistical significant effect on the peripheral blood parameters during the observation period. Also differential counts of bone marrow smears did not show any substantial difference between the 2 treatment groups (see figure 2.1), except for a significant reduction of mature granulocytes (in figure 2.1 indicated as "polysegmented") after nitrous oxide exposure.

In contrast, in leukemic rats 10 days exposure to nitrous oxide reduced the leucocyte count to about 50 %. It was accompanied by a reduction of liver weight

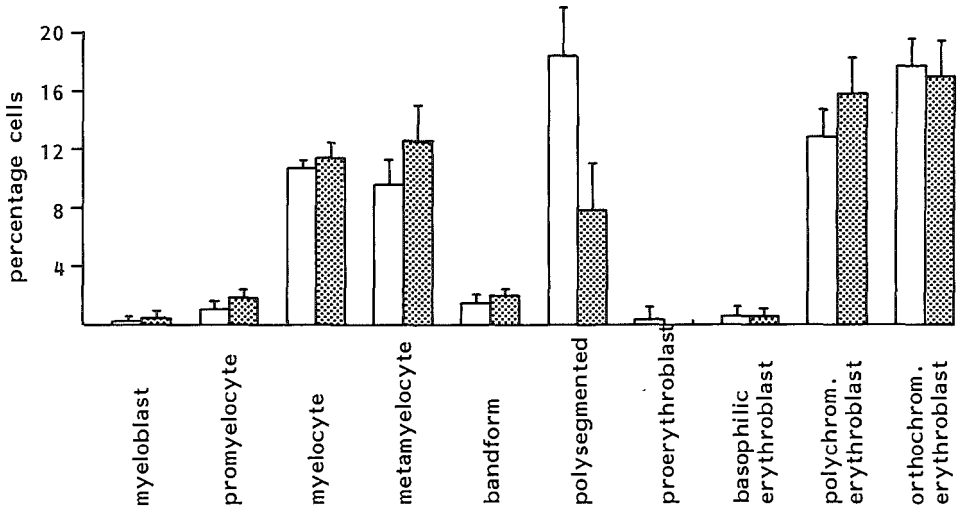

Figure 2.1: Effect of nitrous oxide on morphologic profile of normal rat bone marrow. (mean ±s.e.m. of 4 rats)

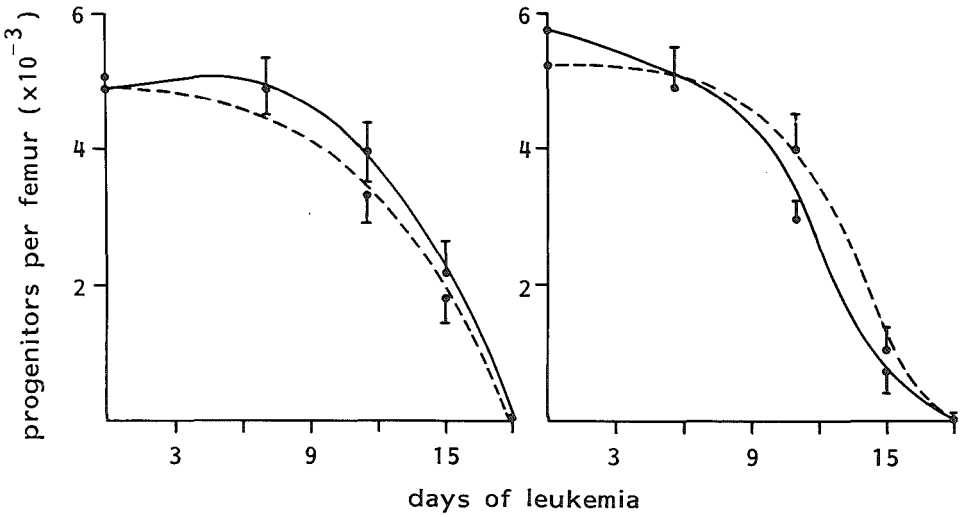

Figure 2.2:In vitro colony formation of erythroid (left) and granulocyte/macrophage (right) progenitors from rat bone marrow during development of leukemia. (—: colonies, --: clusters, mean ±s.e.m. of 4 rats)

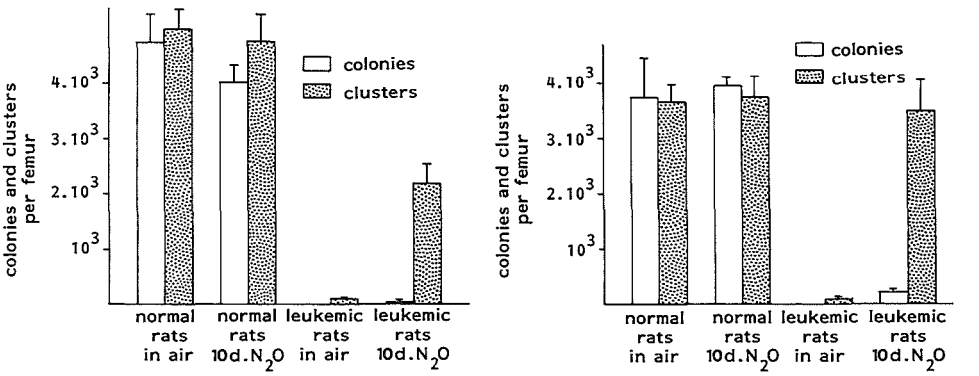

Figure 2.3: Effect of nitrous oxide on erythroid (left) and granulocyte/ macrophage (right) progenitors in normal and leukemic bone marrow. (mean ±s.e.m. of 4 rats)

(from 18.7±1,3 g to 16.6±1.9 g) and spleen weight (from 4.2±0.4 g to 2.8±0.1 g) as has been described before (10). Platelet counts in these rats, which had fallen during the development of leukemia, did not show any adverse effect of the treatment with nitrous oxide.

However, evaluation of treatment effects on normal hemopoiesis by solely studying peripheral blood parameters of the BN rat can be deceptive as lymfocytes make up

about 95 % of the circulating leukocytes. Moreover, effects on erythropoiesis are masked because of the long life span of erythrocytes. Therefore clonogenic assays were performed to assess the effect of nitrous oxide on the number of both erythroid and granulocyte/macrophage progenitors in rat bone marrow. First, groups of 4 leukemic rats were sacrificed on day 0,7,11,15 and 18 of leukemia and assayed for both types of progenitors. Figure 2.2 shows that from day 11 on, a time dependent decrease of progenitor cells was observed, resulting in almost complete disappearance of normal hemopoiesis om day 18.

In the next experiment a group of healthy rats and a group of leukemic rats (day 7) were exposed to nitrous oxide. Concomitantly, 2 similar groups were kept in air. After 10 days all rats were sacrificed and bone marow was assayed for hemopoietic progenitors. Figures 2.3A and 2.3B show that there was no effect of nitrous oxide on the cluster and colony forming cells in healthy rats. Leukemic rats in air showed only residual cluster forming cells as in the experiment above. However in bone marrow of leukemic rats exposed to nitrous oxide a substantial number of both erythroid and granulocyte/macrophage cluster-forming cells was present. Moreover colony forming cells of the latter were still detectable after exposure to the anesthetic.

2.5. Discussion.

Nitrous oxide has proven to reduce the growth of the BNML in a dose dependent way (10). Moreover its combination with other drugs interfering with the folate metabolism (e.g. methotrexate and 5-fluorouracil) has shown to be very effective in the treatment of the applicated rat leukemia model (11-14).

However, little attention has been paid to the treatment effects on normal hemopoiesis. As bone marrow depression is the most important dose limiting toxicity in leukemia treatment, we investigated this phenomenon in the BNML during treatment with nitrous oxide.

Several studies on the effects of nitrous oxide on rat hematopoiesis revealed a dose dependent development of leukopenia and slight morphological changes in bone marrow (4,5). However, quantification of hemopoietic progenitors by clonogenic assays after nitrous oxide exposure has never been performed before. The method for the culture of clonogenic cells in normal rat bone marrow agreed with that of Kimura et al (14) except that fetal calf serum was added to the culture of erythroid precursors instead of rat plasma. Prins et al. (15) have shown that leukemic cells of the BNML particularly lodge near the endosteal sites in femoral bone marrow. As this region is also preferred by normal hematopoietic progenitors, a gradual replacement of hematopoiesis during development of leukemia occurs. This explains the observed decrease in platelet counts in leukemic rats. Suppression of erythropoiesis leads only very slowly to a reduction of the hemoglobin level because of the lifespan of erythrocytes (>100 days) and is therefore still undetectable on day 18 of leukemia. Nitrous oxide is not capable of reversing the process described above but the significant reduction of leukemic growth also results in reduced leukemic infiltration of the bone marrow. Especially the more mature precursors, only capable of *in vitro* cluster formation, are spared as they are situated towards the centre of the femur (15). From all peripheral blood and bone marrow parameters studied it can be

concluded that nitrous oxide selectively decreases leukemic proliferation with little suppression of normal hemopoiesis in the BN rat.

Possibly BN rats are by chance relatively unsensitive towards the hematological effects of nitrous oxide, but a marked strain dependency in rats has been described (16). Still, the BNML has proven to be a good model for the chemotherapeutic response of human acute myeloid leukemia. As human hematopoiesis is more vulnerable to cobalamin inactivation than rat hematopoiesis (17) this might also imply a greater sensitivity of human leukemia for the effects of nitrous oxide. *In vitro* studies (18) have shown that the folate-dependent dTMP synthesis in cells of allmost all types of human leukemia can be disturbed by exposure to nitrous oxide. However only nitrous oxide exposure of leukemia patients will provide the clinical evidence for a possible differential effect on normal and and malignant hemopoiesis in humans. In anesthesia nitrous oxide is considered to be a very safe agent without any other known side effects. Since the preliminary reports on leukemia treatment with this gas (6,7), the biochemical knowledge concerning the interference of nitrous oxide and cellular proliferation has extended enormously. Nowadays the effects of nitrous oxide-induced cobalamin inactivation on hemopoiesis can be monitored easily (3). Moreover, eventually induced bone marrow depression can be reversed by the treatment with folinic acid (19), a folate form which is capable to replenish intracellular reduced folate pools independently of methionine synthetase activity. The clinical application of nitrous oxide in the treatment of hematological malignancies therefore deserves renewed attention.

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Chapter 3

Toxicity of methotrexate in rats preexposed to nitrous oxide

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3.1. Summary.

Several chemotherapeutic protocols for the treatment of malignancies include administration of methotrexate (MTX) during or shortly after total anesthesia. Clinical observations in patients treated for breast carcinoma or childhood cancer have shown unexpected myelosuppression and mucosal damage. This phenomenon may be attributed to the synergistic effects of nitrous oxide (N_2O), inactivating the cobalamin coenzyme of methionine synthase, and MTX, inhibiting dihydrofolate reductase, on folate metabolism. However, no quantitative data on dose/effect relationships are available regarding the combined toxicity of MTX and N_2O. We investigated the effect of exposure to N_2O on the toxicity of MTX. Groups of male Wistar rats were exposed to either 50% N_2O/50% O_2 or air for 12 - 48 hours. Subsequently, a single i.p. injection of 10, 20, 40 or 80 mg MTX per kg body weight was given. Gastrointestinal toxicity resulted in diarrhoea and weight loss in all groups during 5 days after MTX administration. Concomitantly, bone marrow depression with leucocytopenia and thrombocytopenia occurred. Exposure to N_2O did not alter the plasma clearance of MTX. No substantial liver or kidney toxicity could be detected. Still, the LD_{50} for MTX was reduced from 60 mg/kg to 10 mg/kg if rats had been exposed to N_2O for 48 hours, the main causes of death being dehydration and bleedings. The administration of 5-formyltetrahydrofolate (4x10 mg,i.p.) but not 5-methyltetrahydrofolate protected against the lethal effect of the drug combination. Altogether, cytotoxic effects of MTX on proliferating cells are potentiated by N_2O. Therefore, the use of this anesthetic shortly before or during MTX administration has to be avoided.

4.2. Introduction.

The folate antagonist methotrexate (MTX) has become a widely used antimetabolite in cancer chemotherapy (1,2). The drug is a potent inhibitor of dihydrofolate reductase. It is subject to intracellular polyglutamation which results in increased cellular retention and higher affinity for other folatedependent enzymes (3). Subsequent severe disturbance of folate metabolism, accompanied by a decrease of cellular reduced folate compounds which are required for thymidylate (dTMP) and purine biosynthesis, is considered to be the backbone of MTX interference with DNA replication.

Not surprisingly common side effects of MTX treatment are myelosuppression and gastrointestinal toxicity. Renal and liver damage may occur as well, in particular after high dose and/or chronic administration of MTX (1,2). Combination of MTX with several other drugs has proven to increase the therapeutic efficiency and/or the toxicity *in vitro* and *in vivo* (1,2,4). The clinical significance of many of these interactions remains to be determined (4).

Observations in patients treated for metastatic breast carcinoma (5,6) or childhood leukemia (7) have shown that ordinary MTX administration during or shortly after N_2O anesthesia may provoke severe symptoms of which bone marrow depression and gastrointestinal toxicity are the most pronounced. This may be explained by the fact that the anaesthetic gas N_2O inactivates the cobalamin coenzyme of methyl-

tetrahydrofolate homocysteine methyltransferase or methionine synthase (E.C.2.1.1.13). Since the discovery of this effect of N_2O on cobalamin, many studies have been performed to elucidate the consequences of prolonged exposure to N_2O on cellular folate metabolism. These data were recently reviewed (8,9). Methionine synthase appears to be essential for the cellular retention of reduced folates because methyltetrahydrofolate (5-methylTHF), the major extracellular folate, has to be demethylated to THF, before it can be polyglutamated and converted to the other active coenzyme forms. Prolonged exposure to N_2O therefore leads to depletion of cellular folates by loss of 5-methylTHF and severe disturbance of folate-dependent methylation reactions such as the *de novo* synthesis of dTMP. Regarding the specific biochemical lesions of MTX and N_2O it is not surprising that both agents, through inhibition of different metabolic pathways of folate mutually potentiate their effects on the DNA synthesis of proliferating cells. *In vitro* studies on human normal bone marrow have proven a synergistic effect of MTX and N_2O on nucleotide synthesis (10). Both in lymphoblast cell lines (11) and in a rat model for myeloid leukemia (12), exposure to N_2O increased the sensitivity of proliferating leukemic cells for MTX. Still, no experimental data exist on the toxicological consequences of the N_2O-MTX interaction. In the present study we investigated the potentiation of MTX by N_2O and the impact of this combination on the tissues which are most vulnerable for MTX toxicity.

4.3. Materials and methods.

Animals. Male rats of the Wistar inbred strain were used, at the age of 12 - 16 weeks (200-250 g). Food and water were supplied *ad libitum* during all experiments.
Materials. Both sodium Methotrexate and calcium 5-formylTHF were obtained from Lederle (Etten-Leur, The Netherlands). N_2O used in the studies was obtained from Hoekloos (Schiedam, The Netherlands). 5-MethylTHF was obtained from Sigma (St Louis, USA).
Exposure of rats to N_2O. Rats were placed in a 40 L flow chamber through which a gas mixture of 50% N_2O/50% oxygen was blown at a rate of 800 ml/min. Moreover, excess carbon dioxide, water and contaminating volatile compounds were eliminated in a cleaning circuit (13). Oxygen concentration was monitored with an oxygen analyser (Teledyne Analytical Instruments). In one experiment various amounts of N_2O (0%-50%), replenished by nitrogen (50%-0%) and 50 % oxygen were used to study the effect of the inhaled N_2O concentration on MTX toxicity. Rats not exposed to N_2O were kept in air but otherwise treated identically.
LD_{50} study. Groups of 25 rats were exposed to N_2O for 12, 24 or 48 hours or to air. Directly thereafter each group was divided in 5 clusters of 5 rats which received an i.p. injection of 1 ml MTX solution in 0,9% NaCl, resulting in a dose of 0 (controls), 10, 20, 40 or 80 mg MTX per kg body weight. Subsequently, they were kept in normal housing conditions for 3 weeks and body weight and number of deaths were checked daily.
Persistence of N_2O induced effects on MTX toxicity. For this experiment 25 rats were exposed to N_2O for 48 hrs. Subsequently groups of 5 rats received 40 mg MTX/ kg 0, 6, 12, 24, and 48 hrs after termination of N_2O exposure. Rats were observed for 3

weeks.

Clearance of MTX. The plasma MTX clearance following i.p. injection of 20 mg MTX per kg body weight was monitored in 2 groups of 5 rats, one of which had been exposed to N_2O for 48 hours. Rats were anesthetized mildly by ether inhalation when blood (0,8 ml) was sampled by orbita plexus puncture to determine plasma concentrations of MTX and 7-hydroxymethotrexate (7-OH- MTX) as described before (14).

Evaluation of gastrointestinal toxicity. Gastrointestinal tract toxicity was assessed on the basis of body weight and the occurrence of diarrhoea in rats used for determination of LD_{50} values. The gastrointestinal tract of rats sacrificed for evaluation of hematological, liver and kidney toxicity, was also inspected macroscopically.

Evaluation of hematological toxicity. In the first experiment 2 groups of 5 rats, of which one had been exposed to N_2O for 48 hrs., were i.p. injected with 10 mg MTX/kg body weight. At day 1, 4, 7, 13 and 21 after MTX, 0,8 ml blood was collected by orbita plexus puncture. Leucocyte count and hemoglobin concentration were determined with a Sysmex Cellcounter cc-120. Thrombocytes were counted with the Platelet Analyser 800 (Baker). Bone marrow toxicity was investigated in a second experiment. Rats were exsanguinated directly or 48 hrs or 96 hrs after (N_2O-)MTX administration (10 mg per kg body weight). The femurs were flushed with 10 ml Alpha medium containing 0,1% fetal calf serum. The collected bone marrow cells were washed, and 10^5 cells were plated in triplicate in methylcellulose cultures for assessment of Colony Forming Unit-Fibroblast (CFU-F) (15) and Colony Forming Unit-Granulocyte/Macrophage (CFU-GM) (16).

Evaluation of hepatotoxicity and renal toxicity. EDTA plasma, already sampled for other purposes, was also used for determination of alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) on an ACP 5040 (Eppendorf), of fibrinogen, according to Claus et al. (17) on a coagulometer (Amelung), of plasma total protein by the biuret assay and of creatinine and urea on a R.A.1000 (Technicon).

Rescue study. Three groups of rats were exposed to N_2O for 48 hrs and injected with 40 mg MTX/kg. One group received no further treatment, a second group received 4 x 10 mg 5-methylTHF/kg every 12 hrs starting 24 hrs after MTX, and a third group received 4 x 10 mg 5-formylTHF in a similar schedule. Toxic deaths were recorded during 3 weeks thereafter.

Statistical analysis. The Mann-Whitney U-test was used to evaluate significance of observed differences among treatment groups.

4.4. Results.

LD_{50} study. In order to quantify the interaction of N_2O with MTX, a matrix of combinations of both agents was administered to groups of 5 rats. Table 3.1 shows that 12 hours of N_2O exposure induced lethal toxicity when combined with 40 mg MTX per kg body weight. N_2O preexposure of 24 or 48 hrs resulted in an increased lethality of all MTX dosages. Animals usually died between 6 and 10 days after MTX administration. Interpolation of combined data resulted in a LD_{50} for i.p. MTX of 60 mg per kg body weight. In combination with prior exposure to N_2O for 48 hrs the LD_{50} of MTX became approximately 10 mg/per kg body weight.

Table 3.1:

Toxicity of i.p. MTX after N_2O exposure in rats

		N_2O preexposure (in hrs)			
		0	12	24	48
MTX (in mg/kg)	0	0	0	0	0
	10	0	0	0	2
	20	0	0	1	3
	40	0	1	2	4
	80	4	5	N.D.	N.D.

Clusters of 5 rats were exposed to N_2O for various periods prior to i.p. MTX (0-80 mg/kg). The number of toxic deaths in each cluster are shown.

Table 3.2:

Effect of various N_2O concentrations on MTX toxicity.

Composition of inhaled gas mixture (% v)			n	toxic deaths
N_2O	O_2	N_2		
0	20	80	5	0
0	50	50	5	0
5	50	45	5	0
10	50	40	5	1
25	50	25	5	1
50	50	0	5	3

Rats were exposed to gas mixtures containing 0 - 50 % N_2O for 48 hrs. Subsequently they received i.p. 20 MTX/kg. The number toxic deaths are shown.

Effect of various N_2O concentrations on MTX toxicity. Toxicity 20 mg MTX/kg in combination with 48 hrs exposure to several N_2O concentrations was studied. Table 3.2 shows that already 10 % N_2O increases the lethal toxicity of MTX. Table 3.2 also reveals that 50% oxygen by itself (in combination with 50% nitrogen) does not enhance MTX toxicity.

Exposure to N_2O after MTX administration. Table 3.3 demonstrates that MTX toxicity is substantially less when N_2O exposure follows than when it precedes MTX (see also table 3.1).

Table 3.3:

Toxicity of i.p. MTX followed by N_2O exposure for 48 hours.

Treatment	n	toxic deaths
10 mg MTX	4	0
10 mg MTX + 2 days N_2O	5	0
20 mg MTX	4	0
20 mg MTX + 2 days N_2O	5	1

Table 3.4:

Toxicity of i.p. MTX (40 mg per kg) administrated directly, 6, 12, 24 of 48 hrs after 48 hrs N_2O exposure.

Interval between N_2O exposure and MTX administration	n	toxic deaths
0	5	5
6 hrs	5	5
12 hrs	5	3
24 hrs	5	2
48 hrs	5	0

Persistence of N_2O induced effect on MTX toxicity. Table 3.4 demonstrates that exposure to N_2O for 48 hours prior to i.p. 40 mg MTX/kg, leading to 100 percent morbidity when MTX is given directly after N_2O exposure, becomes less toxic when the interval between N_2O and MTX is prolonged to more than 6 hours.

MTX clearance. The plasma clearance of MTX was studied after 48 hrs exposure to N_2O. Figure 3.1 Tabl
reveals that elimination of the drug from the systemic circulation does not change by exposure to N_2O. Only trace amounts of 7- OH-MTX, the major metabolite of MTX, were detected in plasma of both treatment groups.

Gastrointestinal toxicity. The physical condition of all rats used for the LD_{50} determinations was checked daily. Usually the first sign of toxicity was loss of body weight followed by diarrhoea and bleeding from the nose. If the weight loss had not been regained within 5 days after MTX administration, death usually followed within a few days. In figure 3.2 the average body weight per group on day 5 after MTX are expressed as the percentage of the body weight on the day of MTX administration.

As the rats were only 12 weeks old, absence of toxicity resulted in increased body weight (approximately 15% in 5 days). Macroscopical dissection of rats after death

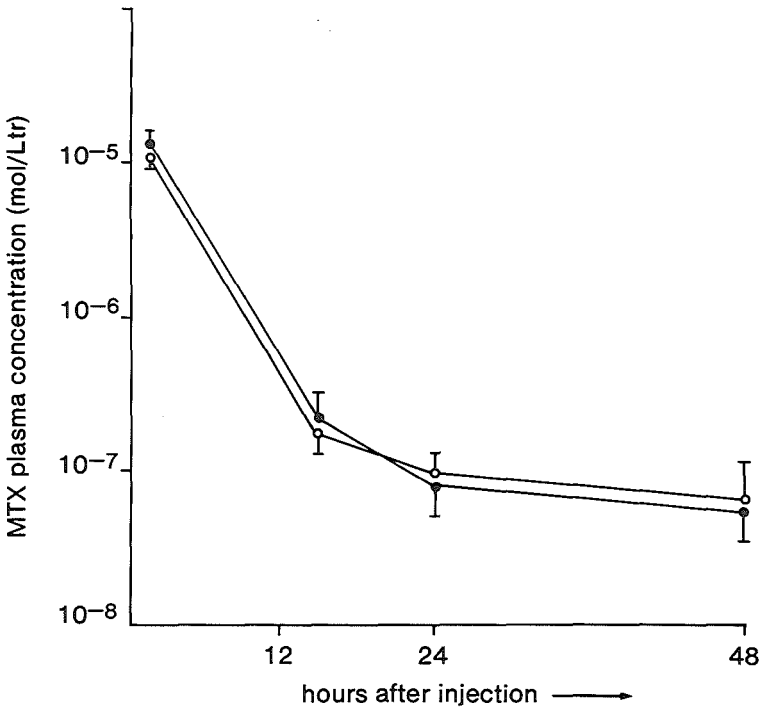

Figure 3.1: Plasma clearance of MTX (20 mg/kg i.p.) in control rats (o) and in rats exposed to N_2O for 48 hrs (•). Values are the mean ± s.d. of 5 rats.

revealed extensive bleeding from ulcerations throughout the gastrointestinal tract of rats suffering from diarrhoea. N_2O alone had no noticeable effect on body weight (data not shown).

Hematological toxicity. In figure 3.3 the effect of 10 mg MTX/kg with or without exposure to N_2O (48 hrs) on the number of peripheral leucocytes and platelets are presented. Both numbers are suppressed when MTX is preceded by N_2O and reached a nadir approximately 7 days after MTX administration. MTX alone also induced a decrease in the leucocyte count, but 7 days later. The hemoglobin concentration of peripheral blood in the combination treatment group had increased 15% on day 4 after MTX but returned to 10% below normal afterwards (data not shown). In the N_2O-MTX group 2 rats died at day 8 and 9 respectively, so data from day 14 and 21 are the average of 3 rats.

As granulocytes and monocytes make up only 10 percent of peripheral leucocytes in rats, clonogenic assays were performed to investigate the impact of N_2O-MTX on myelopoiesis. Stromal integrity was measured by culturing fibroblast colony forming cells (CFU-F). Figure 3.4B reveals that both MTX and N_2O-MTX cause a reduction of CFU-GM in bone marrow. However, recovery after MTX alone is much faster than after preexposure to N_2O as is clear from total marrow cellularity (figure 3.4A)

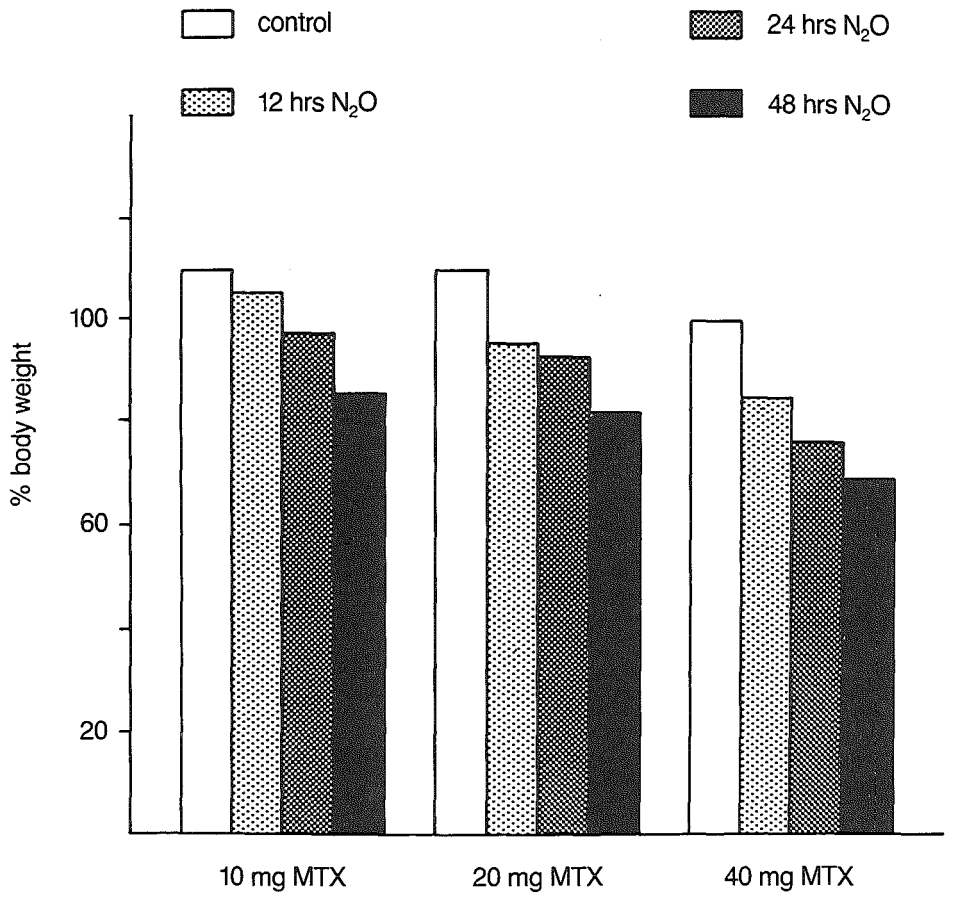

Figure 3.2: Effect of exposure to N_2O prior to i.p. MTX on body weight of clusters of 5 rats, 5 days after MTX administration. N_2O exposure time and MTX dosage were variable. Mean body weight of each cluster is expressed as a percentage of the weight just before MTX administration. All bars < 100% were statistically different from the control bars ($p<0.05$).

and the amount CFU-GM present in bone marrow at day 4. Cytotoxic effect of N_2O-MTX on CFU-F (figure 3.4C) is maximal after 4 days, but by this time bone marrow stroma in MTX treated rats has already recovered.

Exposure to N_2O for 48 hrs alone causes no changes in the studied parameters afterwards (data not shown).

Hepatotoxicity and renal toxicity. Table 3.5 presents a panel of plasma factors determined to assess hepatic and renal toxicity, 96 hrs after MTX administration when the animals are suffering maximally from induced toxicity. There was no evidence of specific damage to liver or kidneys. Exposure to N_2O for 48 hrs alone causes no changes in the studied parameters afterwards (data not shown).

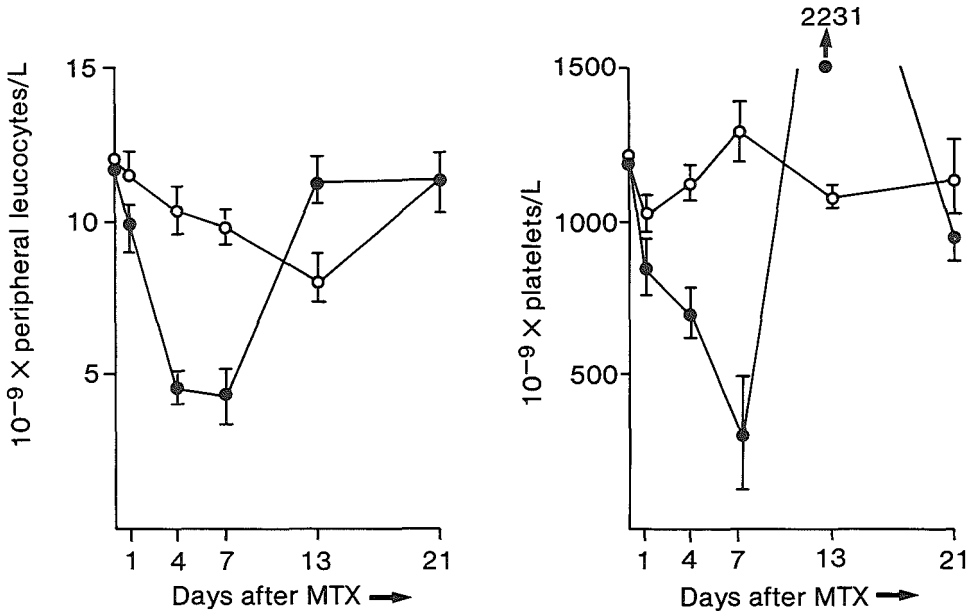

Figure 3.3: Peripheral leucocyte and platelet counts in MTX treated rats (10 mg/kg) with (•) or without (o) 48 hrs preexposure to N_2O. Values are means ± s.d. of 5 rats.

Table 3.5:

Effect of N_2O-MTX on several parameters, indicative for hepatic and renal toxicity.

Treatment	n	ASAT (u/l)	ALAT (u/L)	Fibrinogen (g/L)	Plasma total protein (g/L)	Plasma urea (mmol/L)	Plasma creatine (µmol/L)
Controls	5	43 ± 5	33 ± 2	1.6 ± 0.1	64 ± 3	7.8 ± 0.7	53 ± 3
N_2O	5	40 ± 2	43 ± 12	1.6 ± 0.1	64 ± 2	7.1 ± 1.1	47 ± 3
10 mg MTX/kg	4	57 ± 10	39 ± 3 *	1.7 ± 0.1	71 ± 2*	8.7 ± 0.9	69 ± 10*
N_2O + 10 mg MTX/kg	5	47 ± 5	25 ± 5 *	1.6 ± 0.2	61 ± 4	6.0 ± 0.7**	42 ± 12

Rats, either or not exposed to 48 hrs N_2O, received 10 mg MTX/kg i.p.. After 4 days rats were sacrificed and plasma collected for determination of listed parameters. Values are means ± s.d., *: $p < 0.05$, **: $p < 0.01$.

Rescue study. Table 3.6 reveals that 5-formylTHF administered 4 times over a period of 48 hrs is able to prevent the lethal toxicity of 48 hours N_2O in combination with 40 mg MTX/kg. However with 5-methylTHF, administered in a similar schedule, only 2 of the 4 animals survived.

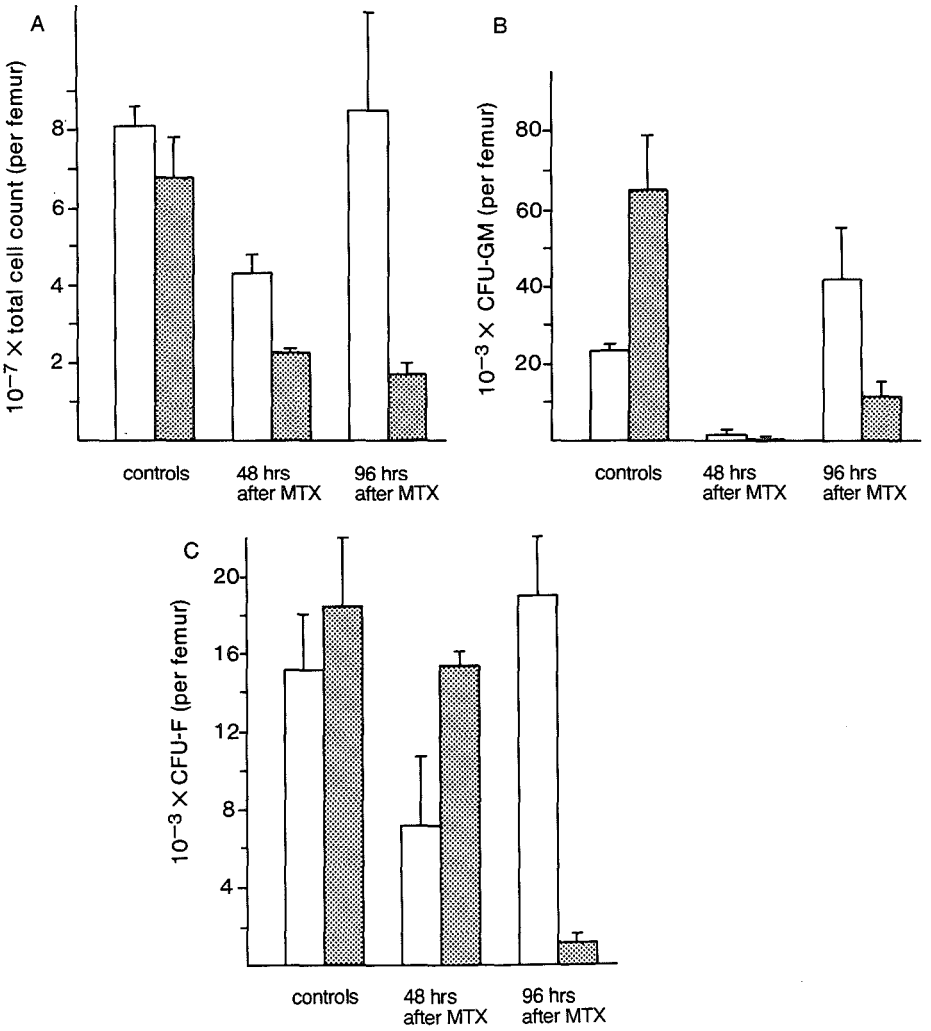

Figure 3.4: Effect of MTX (10 mg/kg) on bone marrow of rats either or not pretreated with 48 hrs N_2O. Values for total femoral cell count, myeloid progenitors (CFU-GM) and fibroblast progenitors (CFU-F) (mean ± s.d.). Open bars: controls, spickled bars: N_2O exposed. All differences between controls and N_2O treated groups at 48 hrs and 96 hrs are statistically significant ($p<0.05$).

Table 3.6:

Rescue efficiency of 5-formylTHF and 5-methylTHF for N_2O-MTX induced toxicity.

Treatment	n	toxic deaths
40 mg MTX	5	0
2 days N_2O + 40 mg MTX	5	4
2 days N_2O + 40 mg MTX + 5-FORMYLTHF	4	0
2 days N_2O + 40 mg MTX + 5-METHYLTHF	4	2

Rats were treated with 48 hrs N_2O and 40 mg MTX/kg. After 24 hrs one group received 4 x 10 mg/kg leucovorin and another group received 4 x 10 mg/kg 5-methylTHF. Rats were observed and toxic deaths recorded.

3.5. Discussion.

Possible mechanism. The present study provides experimental evidence that N_2O potentiates the toxic effects MTX on normal proliferating cells *in vivo* whereas the plasma clearance of MTX remains unchanged. Exposure to N_2O before administration of MTX is a prerequisite for synergism because N_2O treatment after injection of the antifolate does not cause increased toxicity. The possibility that the increase in lethal toxicity of MTX is caused by the elevated oxygen concentration (50%) can be excluded on both experimental (see table 3.2) and literature data (18,19).

The potentiation of MTX toxicity by the various periods of N_2O exposure closely follows the intracellular changes occurring during cobalamin inactivation. 12 Hrs of N_2O exposure increases lethal toxicity of MTX. Studies in rats have shown that after N_2O exposures of this duration, methionine synthase activity is completely suppressed and the conversion of 5-methylTHF to other reduced folates, necessary for various coenzym functions in thymidylate and purine synthesis, also is impaired (8,9). Prolonging the N_2O exposure to 48 hrs results in further increase of lethality from MTX. These observations agree with those of Chanarin et al. (8,9) who demonstrated a massive depletion of rat tissue folates within 48 hours N_2O (thereby increasing the sensitivity of proliferating cells for MTX). Moreover, increased activity of folylpolyglutamate synthase (9,20) as a consequence of the induced folate depletion, may result in enhanced polyglutamation of the drug (20,21), which enhances its cytotoxicity.

Already 10 % N_2O in the inhaled gas mixture is sufficient to increase the lethal toxicity of MTX. This is in agreement with Kondo et al.(22) who showed that inhibition of methionine synthase already occurs during exposure to 2% N_2O.

Although complete recovery of methionine synthase activity from N_2O exposure and subsequent repletion of tissue folates require several days (22, 23), the mortality from 48 hrs N_2O plus 40 mg MTX/kg decreases rapidly if MTX administration is postponed. This suggests that partial restoration of methionine synthase activity is already sufficient to prevent the synergistic interaction between N_2O and MTX.

Specific organ toxicity. The effect of N_2O-MTX consists of cytotoxic action towards the rapidly dividing cells of mucosa and bone marrow. Little is known about the effect of N_2O exposure alone on the mucosa although inactivation of methionine synthase and reduced uptake of dietary folates (without diarrhoea) have been demonstrated (24). Combination of non-lethal dosages N_2O and MTX causes lifethreatening loss of body weight by diarrhoea induced dehydration and reduced food intake by the sick animals. Effects of N_2O-MTX on hematopoiesis also show synergistic features. Bone marrow depression and pancytopenia in N_2O pretreated rats is more pronounced and the recovery of myelopoiesis is delayed. N_2O-MTX effects on stromal elements, which are considered to be essential for hematopoiesis, increases up to 4 days suggesting cytotoxic activity towards pre-CFU-F cells and may contribute to the sustained bone marrow depression. N_2O alone induces an increase of the myeloid progenitors. This agrees with earlier observations in rats in which N_2O induced accumulation of the myeloid precursors in the bone marrow (25).

It has been suggested that the hepatotoxicity of chronic MTX administration may be related to a reduction of folate-dependent methionine synthesis as this causes hepatic depletion of choline, the necessary precursor for the alternative, betaine-dependent methionine synthesis (26,27). However, N_2O-induced inhibition of methionine synthase does not provoke acute liver toxicity from MTX treatment as is clear from the data presented in table 3.5. This is probably related to the minor importance of the thymidylate synthase/dihydrofolatereductase pathway for the restoration of THF pools in the non-dividing cells of the liver (28).

Clinical implications and perspectives. For this study rats were chosen as test animal as substantial knowledge is available on both N_2O and MTX effects on folate metabolism in rats. Moreover the biochemical events of N_2O exposure in the rat closely resemble those in man (9) although the latter is known to be more vulnerable towards N_2O effects on cell proliferation (29). Comparison of the presented data and reported clinical observations in breast cancer patients (5,6) reveals some striking similarities. The incidence of unpredictable side effects after postoperative chemotherapy (including MTX) was inversely correlated with the period between N_2O anesthesia and MTX administration. Toxicity could be alleviated with 5-formylTHF, altbough the effect of this rescue therapy on the efficiency of the employed chemotherapeutic protocol is not yet known.

In our study, 5-formylTHF was also efficient as rescue agent. However, the exact mechanism by which this drug restores the intracellular reduced folate pool is still subject of dispute. *In vivo* this rescue agent is rapidly converted to 5-methylTHF (30) and several studies have revealed that the latter may also play an important role in reversal of MTX toxicity (31,32). In N_2O treated subjects however, only unmetabolised 5-formylTHF is capable of entering the cellular reduced folate pools (11,33). As conversion of 5-formylTHF to 5-methylTHF differs considerably among the various routes of administration (34), differences in rescue efficiency in case of N_2O-MTX toxicity may be expected.

Recently it has been suggested that N_2O reduces the antineoplastic effects of MTX as a consequence of induced toxicity (7). However, Kroes et al (12) have already demonstrated the chemotherapeutic benefit of MTX when combined with N_2O exposure in a rat model for myeloid leukemia. Moreover, it is shown that the

deleterious effects of MTX on folate metabolism of fresh human leukemic cells could be significantly enhanced by N_2O (35) implicating a possible antineoplastic synergism of both drugs as has been suggested before (10).
Conclusion. Exposure to N_2O prior to MTX administration results in a dosedependent synergistic cytotoxicity towards the proliferating cells of mucosa and bone marrow. The biochemical mechanism through which the synergism between N_2O and MTX occurs is not yet fully understood but is related to the inhibition of 2 important enzymes in the formation of reduced folates, necessary for both purine and dTMP synthesis. Effective alleviation from resulting toxicity can be achieved by intravenous 5- formylTHF administration. However the demonstrated synergistic action of the anesthetic gas N_2O and MTX on dividing cells merits further investigation of its possible applicability in the treatment of neoplastic diseases.

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Chapter 4

Effect of cobalamin inactivation on folate metabolism of leukemic cells

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4.1. Summary.

Exposure to nitrous oxide inactivates the cobalamin coenzyme of methionine synthetase, an essential enzyme in folate metabolism. Hemopoietic cells are especially dependent on the function of cobalamin for the folate-dependent synthesis of thymidilate (dTMP). Inhibition of methionine synthetase may therefore be of potential value in the treatment of hematological malignancies. In the present study we investigated the effect of nitrous oxide induced cobalamin inactivation on folate metabolism of fresh leukemic cells and the human myelomonocytic cell line U937. Cells were exposed to nitrous oxide for 20 hours. Subsequently they were subjected to the deoxyuridine suppression test (dU test), which measures the disturbance of folate-dependent dTMP synthesis. In all bone marrow samples, cobalamin inactivation resulted in a 200% increase of the dU test value, implicating a decreased *de novo* synthesis of dTMP. Incubation of leukemic cells with methotrexate, 5-fluorouracil or cycloleucine induced similar increases of the dU test values which could be further raised to 400% with the addition of N_2O exposure. Prolonged experiments with U937 cells revealed that the disturbance of folate metabolism aggravated up to 48 hours of nitrous oxide exposure. It can be concluded that cobalamin inactivation in human leukemic cells results in disturbed folate-dependent dTMP synthesis. Moreover, effects of several drugs interfering with folate metabolism can be enhanced.

4.2. Introduction.

Cobalamin, or vitamin B_{12}, is a nutritional factor indispensable for the maintenance of normal hematopoiesis. This is explained by its role as a coenzyme in methionine synthetase, or 5-methyltetrahydrofolate homocysteine methyltransferase, the enzyme which is essential for the intracellular conversion of reduced folates into forms that can be retained in the cell. Several steps in the biosynthesis of nucleotides require the presence of folate coenzymes. In particular the *de novo* synthesis of thymidylate (dTMP) from uridylate (dUMP) appears to be sensitive to reduced availability of the necessary coenzyme 5,10- methylene tetrahydrofolate (See figure 4.1). As dTMP is an indispensable precursor for DNA synthesis, this provides the link between cobalamin deficiency and a disturbance of hematopoietic proliferation.

This immediate importance to proliferating cells makes the metabolism of reduced folates a potential target in the chemotherapy of malignant tumors. Structural analogues of reduced folates, notably methotrexate (MTX), have demonstrated the validity of this approach. Attempts have been made to find similar antagonists of the essential coenzyme function of cobalamin in methionine synthethase. However early efforts, mainly with structural analogues, were largely unsuccessful (1-3). No effective antagonist of cobalamin was known until the recognition of a peculiar side effect of the anesthetic gas nitrous oxide (N_2O) in 1978 (4), which already was known to cause megaloblastic hematopoiesis on prolonged exposure (5). N_2O inactivates the cobalamin coenzyme of methionine synthetase (7) by oxidation of its cobalt(I) moiety. This particular reaction results in a virtually complete and irreversible inhibition of this enzyme (6). The availability of a genuine cobalamin antagonist has revived the interest in possible applications in chemotherapy.

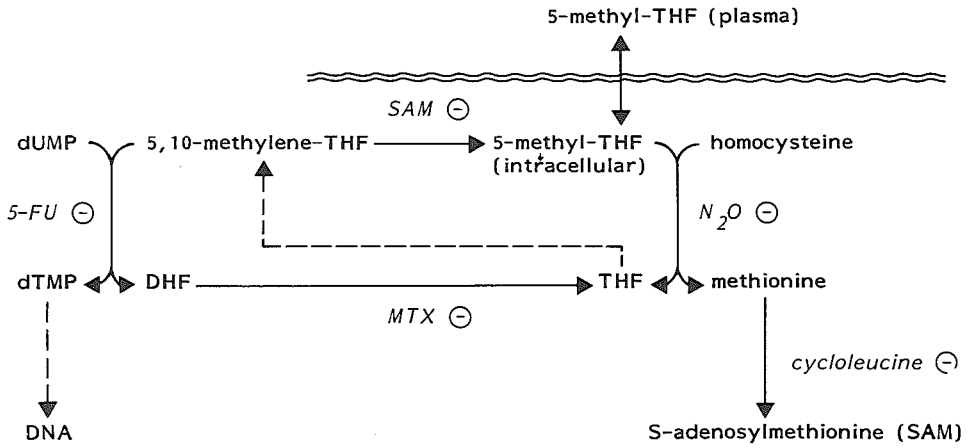

Figure 4.1: Outline of the most important intracellular interconversions of reduced folates and the principal biochemical targets of the drugs used in this study.

In addition, the demonstration of the cobalamin dependence of malignant proliferation is of more general interest. We have shown previously that nitrous oxide reduces the growth of leukemia in rats (8), and renders the leukemic rats more susceptible to subsequent therapy of several drugs interfering with other pathways of folate metabolism (see figure 4.1) (9,10,11). Kano et al. (12) have demonstrated that N_2O inhibits nucleotide synthesis and proliferation in a number of human hematopoietic cell lines.

In this study, we have investigated the *in vitro* effects of N_2O on human leukemic cells derived from patients with different types of acute leukemia and the myelomonoblastic cell line U937 (13). Moreover combination of cobalamin inactivation with MTX, 5-FU and cycloleucine, an effective inhibitor of methionine adenosyltransferase (14), was studied. The assessment of the sensitivity of human leukemia to N_2O should be considered an essential step towards the application of cobalamin inactivation in clinical chemotherapy. The deoxyuridine suppression test, being an established method to demonstrate the metabolic consequences of cobalamin deficiency or inactivation, was used to evaluate the effects of nitrous oxide.

4.3. Materials and methods.

Leukemic bone marrow and blood cells. Bone marrow aspirates and peripheral blood samples were obtained from consenting patients with a diagnosis of acute leukemia. The patients were untreated, or, in some cases, in early relapse of their disease. Each marrow aspirate, usually 1-3 ml, was collected in a sterile bottle containing 0.4 ml (2000 IU) heparin. Peripheral blood was collected in heparinized tubes. Each case of acute myeloid leukemia (AML) was morphologically and cytochemically classified according to the FAB clas sification (15,16), and cases of acute lymphoid leukemia

(ALL) were classified according to immunological phenotype. The percentage of leukemic blasts in each bone marrow sample was determined in a differential count of 500 cells.

Isolation of nucleated cells from marrow and blood. Bone marrow aspirates and peripheral blood samples were diluted with an equal volume of Hanks' balanced salt solution and gently admixed with 0,5 ml methylcellulose solution (2%w/v) to promote rouleaux formation. After 10 minutes, the red cells were sedimented and nucleated cells could be isolated from the supernatant. The cells were washed in Hanks' balanced salt solution, counted and diluted to a concentration of 10^6/ml with McCoy's medium 5A with 10% Fetal Calf Serum. Folic acid (pteroylglutamic acid) was replaced by 400 nmol/l methyltetrahydrofolate (Sigma, St.Louis, USA), and methionine was limited to 25 mmol/l, as these constituents have to be carefully controlled in studies on cobalamin and folic acid metabolism. Viability of the cells was checked by trypan blue exclusion.

Culture of the cell line U937. The myelomonoblastic cell line was cultured in suspension, in specially prepared McCoy's medium 5A similar to the one descibed above. Experimental conditions were as described by Kano et al., with 200 nmol/l of 5-methyltetrahydrofolate (10) and were carried out with cells in a logarithmic growth phase. Exposure to nitrous oxide and subsequent incubations were performed as described for bone marrow cells.

Exposure of cells to nitrous oxide. Cell suspensions (5 ml in flasks of 50 ml) were exposed to nitrous oxide by gassing the culture flasks during 30 min with a filtered mixture of 50% nitrous oxide, 20% oxygen, 25% nitrogen and 5% carbon dioxide, released from a premixed cylinder (Hoekloos, Schiedam, The Netherlands). After this period, the flasks were closed and incubated overnight for about 20 h at 37 °C. Control suspensions were exposed to a mixture of air with 5% carbon dioxide, and otherwise treated identically. After incubation, viability was checked again by trypan blue exclusion and the cells were directly subjected to the dU test.

Addition of drugs. If enough cells were available, MTX (final concentration 5.10^{-8} mol/L, Ledertrexate), 5-FU (final concentration 5.10^{-6} mol/L, Hoffmann LaRoche) or cycloleucine (final concentration 10^{-3} mol/L, Sigma) were added to the cell suspensions at the start of the exposure to the gas mixtures and remained there during the whole incubation period.

Deoxyuridine suppression test. This test was used to evaluate the impairment of the *de novo* synthesis of thymidylate as a consequence of the disturbance of folate metabolism. [^{3}H]-Thymidine incorporation into DNA is measured with and without the pre-incubation of cells with deoxyuridine. Deoxyuridine suppresses the incorporation of [^{3}H]-thymidine if it can be converted to thymidylate by folate-dependent methylation. If this conversion is impaired, the suppression by deoxyuridine will be reduced. The results of this assay are expressed as the percentage ratio of the [^{3}H]-thymidine incorporation with and without deoxyuridine in similar cell suspensions. The test was carried out essentially as described by Metz et al. (17), with some modifications. Briefly 10^6 cells in 1 ml Hanks' balanced salt solution with or without 0,1 mmol/L dU were incubated for 2 hours at 37 °C, followed by another 2 hour incubation in the presence of 0,3 μCi [^{3}H]-thymidine (specific activity: 25 Ci/mmol, Amersham, UK). All results were the mean of triple

incubations.
Statistical Analysis. Data were statistically evaluated with the Wilcoxon's signed- ranks test for 2 groups.

4.4. Results.

Patient characteristics. 18 patients were involved in this study and their characteristics are summarized in table 4.1. The mean blast count of the blood samples was 63 ± 22%, the bone marrow cell fractions contained 70 ± 25% leukemic blasts.

Table 4.1:

Characteristics of leukemia patient samples used for in vitro cobalamin inactivation.

Number of patients involved	18
Male	11
Female	7
Diagnosis	
Acute myeloid leukemia	12
Acute lymphoid leukemia	4
Leukemic transformed M.D.S.	2
Studied material	
Bone marrow aspirates	16
Peripheral blood	9

Effect of cobalamin inactivation on fresh leukemic samples. The results of deoxyuridine suppression tests after nitrous oxide exposure of leukemic marrow cells, compared to the same cells exposed to air, are shown in table 4.2. In all cases, the exposure to nitrous oxide caused a marked increase of the test value, indicating an impaired ability to use dUMP as a substrate in the *de novo* synthesis of dTMP. In normal healthy bone marrow the percentage suppression by dU varies but usually the upper level of the normal range is considered to be 10%. The variation of suppression values after exposure to air, from 3 to 15% in our study, with a mean of 9%, is probably related to the heterogeneity of cell types in different forms of leukemia and the patients physical condition. DU tests of leukemic bone marrow performed directly after the aspiration show a similar degree of variation (data not shown). After nitrous oxide exposure of cells derived from the same bone marrow samples, the test values range from 8 to 57%, with a mean of 21% (significance: $p<0.01$). Results obtained with blood cells from 9 leukemia patients, are also presented in table 4.2. The increase in mean suppression values, from 8 to 14%, is comparable to the result obtained with the marrow cells. However this time the pattern is somewhat less consistent as 2 of the 9 samples were found to be unresponsive to nitrous oxide, both from cases of ALL.
Effect of N_2O with concomitant exposure to other drugs. Methotrexate was added, prior to the incubation, to 6 of the leukemic marrow cell suspensions and 2 peripheral

Table 4.2:

Effect of N_2O exposure (20h) on the dU suppression value of leukemic cells derived from bone marrow and peripheral blood (mean ± s.d.).

Origin of cells	n	dU test values	
		air	N_2O
Bone marrow	16	9,0 ± 3,8 %	21,4 ± 13,2 %
Peripheral blood	9	7,5 ± 4,5 %	13,4 ± 8,1 %

Table 4.3:

Effect of several drugs and the concomitant exposure to N_2O (20 h) on the dU suppression value of leukemic bone marrow cells (mean ± s.d.).

n	air	N_2O	MTX (5.10^{-8}M)	N_2O + MTX
6	10,5 ± 3,0 %	22,0 ± 10,1 %	38,9 ± 17,2 %	49,5 ± 12,2 %
n	air	N_2O	5-FU (5.10^{-6} M)	N_2O + 5-FU
7	7,4 ± 4,2	21,1 ± 15,8 %	34,7 ± 22,1 %	46,3 ± 22,6 %
n	air	N_2O	cycloleucine (10^{-3} M)	N_2O + cycloleucine
5	7,9 ± 3,7 %	20,5 ± 4,7 %	9,5 ± 2,1 %	27,9 ± 4,2 %

blood cell suspensions. The results are shown in table 4.3 and 4.4 respectively. The addition of methotrexate alone to the marrow cells increased the suppression value in all cases, from a mean of 11% to a mean value of 39%. If the cells were, in addition, exposed to N_2O, this resulted in a significant, further increase of the mean suppression value to 50% ($p<0.05$). In some cases, however, in which methotrexate was already very effective, no substantial further increase was observed; this applies to values of 60% and higher. It should be emphasized that these values indicate a rather extreme disturbance of the folate metabolism. Therefore it is possible that aggrevation of this disturbance by cobalamin inactivation is not detectable anymore. The two peripheral samples, demonstrated a similar enhanced effect of methotrexate after concomitant exposure to N_2O.

A comparable pattern was obtained with the combination of N_2O with 5- FU (see table 4.3 and 4.4). Both blood samples and 6 of the 7 marrow samples showed an appreciable effect of 5-FU on the dU test value (mean 35%), which could be

Table 4.4:

Effect of several drugs and the concomitant exposure to N_2O on the dU suppression value of individual leukemic cell samples derived from peripheral blood.

air	N_2O	MTX	N_2O + MTX
8,6	8,5	24,4	29,5
15,5	25,2	52,5	68,5
air	**N_2O**	**5-FU**	**N_2O + 5-FU**
6,2	13,4	15,7	29,6
13,5	21,5	25,2	36,4
air	**N_2O**	**cycloleucine**	**N_2O + cyclo**
7,1	13,6	5,5	27,4
9,2	11,0	9,2	15,1

Table 4.5:

Effect of MTX or 5-FU and the concomitant exposure to N_2O for two days on the dU suppression value of U937 cells.

treatment	day 0	day 1	day 2
air	12,1	14,2	12,6
N_2O	12,1	16,3	24,2
MTX (5.10^{-8} M)	12,1	18,4	50,5
N_2O + MTX	12,1	24,7	77,4
air	5,8	13,7	12,6
N_2O	5,8	12,1	11,1
5-FU (5.10-6 M)	5,8	23,2	34,7
N_2O + 5-FU	5,8	28,4	51,6

signifcantly aggrevated by N_2O (mean 46%, $p<0,05$).

Cycloleucine by itself did not affect the dU suppression value in most of the tested cases (mean 10%). In combination with N_2O however a sharp increase was noted (mean 27%) in all bone marrow samples ($p<0,05$) and the two peripheral blood samples (see table 4.3 and 4.4).

Effects on the cell line U937. Finally, results obtained with the cell line U937, with culture conditions as described by Kano et al. (12), are presented in table 4.5. Prolonged exposure to N_2O with or without 5-FU or MTX up to 2 days resulted in a

time dependent increase of the dU suppression values. Consistently the cells exposed to the drugs in combination with N_2O showed the largest increase of the dU test value.

4.5. Discussion.

At present, there is only limited evidence for the cobalamin- dependence of malignant hematopoiesis in human leukemia, which is an essential requirement for the possible application of cobalamin antagonists in chemotherapy. N_2O, the only available effective antagonist of the cobalamin coenzyme in methionine synthetase, has been shown to inhibit rat leukemia *in vivo* (8), and human leukemic cell lines *in vitro* (12). There are also two early observations of a distinct antileukemic effect of nitrous oxide exposure in patients (18,19), dating from the time before this agent was known to inactivate cobalamin. Moreover, two cases have been described of patients with leukemia, in which the administration of cobalamin was considered to have enhanced leukemic proliferation (20,21).

A disturbance of the deoxyuridine suppression test in normal bone marrow cells after N_2O exposure of about 24 hours is well established (22). The results of the present study provide evidence that in nearly all cases of acute leukemia the folate metabolism can also be disturbed by cobalamin inactivation, as is apparent from elevated values of the deoxyuridine suppression test. This *in vitro* metabolic effect of cobalamin inactivation on malignant blood cells is fully comparable to the consequences of cobalamin deficiency on normal marrow cells. It may therefore be expected that the application of nitrous oxide will result in the same inhibition of cellular proliferation as is observed in cobalamin deficient normal marrow cells.

Combination of N_2O with other drugs resulted in an enhanced disturbance of the folate metabolism. These observations are in accordance with a study in which the effect of N_2O with MTX on normal bone marrow cells was examined (23). More interestingly our observations agree well with studies of the antileukemic activity of N_2O combined with respectively cycloleucine, MTX, and 5-FU in the BNML. In this rat leukemia, which is considered to be a reliable model for human AML, the effective antiproliferative action of the three combinations mentioned above, was also reflected by severely disturbed dU test values (8-11).

As nearly all cases of leukemia were responsive to cobalamin inactivation, it is difficult to draw conclusions on the differential sensitivity of particular types. It may be relevant, however, that all samples that were found to be unresponsive to N_2O were cases of ALL.

With regard to the results obtained with the leukemic cell line U937, it can be concluded that the effects of cobalamin inactivation in combination with other drugs increase over substantial periods of time. This may possibly reflect the gradual changes in folate coenzyme pools induced by N_2O (24,25), 5-FU (26) or MTX (27).

In conclusion, the results of this study demonstrate that the metabolic consequences of cobalamin inactivation by nitrous oxide can be observed in malignant hematopoietic cells, as in normal marrow cells. The deoxyuridine suppression test may well be predictive for the clinical sensitivity of acute leukemias to N_2O, as results obtained with N_2O *in vitro* are in agreement with those obtained by treating rat

leukemia *in vivo*. Moreover, these findings encourage further investigations, eventually directed at the administration of N_2O to selected patients with leukemia.

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Chapter 5

Effect of nitrous oxide and methotrexate on folate coenzyme pools of blast cells from leukemia patients

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Submitted.

5.1. Summary.

The effects of methotrexate (inhibiting dihydrofolate reductase) and nitrous oxide (inactivating methionine synthase) on intracellular folate coenzyme levels of leukemic cells were studied. Blast cells from 10 cases of acute myeloid leukemia (AML) and 5 cases of acute lymphoid leukemia (ALL) were incubated with $5x10^{-8}$ M [^{3}H] 5-formyltetrahydrofolate (5-formylTHF) for 18 hours (h) to label intracellular folate pools, which were subsequently quantitated by high performance liquid chromatography (HPLC). In AML 5-methylTHF made up 53% of the total folate pool followed by 10-formylTHF (26%), 5-formylTHF (10%), THF (9%) and DHF (1%). Cells from ALL differed from AML ($p<0,05$) with respect to 10-formylTHF (17%) and DHF (10%).

Exposure to nitrous oxide (8 h) caused an equal decrease of 10-formylTHF and 5-formylTHF in both AML (30%) and ALL (45%), whereas 5-methylTHF increased (130%). Methotrexate (4 h, 10^{-6}M) caused an accumulation of DHF and a decrease of 5-methylTHF in both AML (32%) and ALL (12%). A specific reduction of the 10-formylTHF (50%) and 5-formylTHF (25%) pools was noticed in ALL. Exposure to nitrous oxide prior to methotrexate treatment aggravated the reduction of 10-formylTHF and 5-formylTHF presumably by impaired replenishment from the 5-methylTHF pool. In conclusion, this study demonstrates a significant difference in folate coenzyme distribution between cells from AML and ALL. Moreover it is shown that nitrous oxide and methotrexate treatment of leukemic cells cause an accumulation of 5-methylTHF and DHF respectively at the expense of other folate forms. The presence of substantial amounts of DHF in cells from ALL together with the specific reduction of 10-formylTHF (necessary for purine synthesis) during MTX treatment may in part explain the efficacy of methotrexate in the treatment of ALL.

5.2. Introduction.

Folate coenzymes play an important role in the biosynthesis of purines and thymidylate (dTMP) (1), precursors of RNA and DNA. This immediate importance to cellular proliferation makes the metabolism of reduced folates a potential target in the chemotherapeutic treatment of malignancies. Structural analogues of folate, especially methotrexate (MTX), have demonstrated the success of this approach (2). Most research on the folate metabolism and interfering drugs has been performed in experimental animals and cell lines, as studies on fresh human material (*ex vivo*) have been hampered by the instability of the various folate coenzymes and even more by their low intracellular concentration (3,4,5,6). Recently, Baram et al. (7) demonstrated the stable radioactive labeling of these coenzymes pools in fresh human myeloid precursor cells, allowing extensive investigations on the folate metabolism with a minor amount of biological material.

In the following study this particular method has been used to determine the folate coenzyme distribution in blast cells from patients with acute leukemia. In order to further elucidate the significance of the various folate cofactor pools, we studied the response of leukemic cells to 2 drugs interfering with the folate metabolism: nitrous oxide and methotrexate (MTX).

Nitrous oxide inactivates the cobalamin coenzyme of methionine synthase (methyltetrahydrofolate homocysteine methyltransferase, E.C.2.1.1.13) (8). This enzyme plays an essential role in the cellular retention of reduced folates by demethylating new intracellular 5- methylTHF to THF, thereby facilitating polyglutamation and conversion to other less exchangeable folate forms. Cobalamin inactivation by nitrous oxide therefore leads to accumulation of 5-methylTHF at the expense of other reduced folates which forms the quintessence of the "methylfolate trap" hypothesis of cobalamin deficiency (1,9). The final results of this disturbance in the folate metabolism are the impaired *de novo* synthesis of dTMP and purines as a consequence of depleted folate coenzyme pools.

Methotrexate (MTX) is a strong inhibitor of dihydrofolate reductase (DHFR) (2). The induced accumulation of DHF also causes disturbed *de novo* purine and dTMP synthesis by depletion of the required folate coenzymes. Moreover intracellular formation of DHF and MTX polyglutamates during MTX treatment causes direct inhibition of both AICAR transformylase, an important enzyme of purine synthesis and dTMP synthase (7,10,11).

In man, both normal and malignant hemopoiesis have proven to be vulnerable to the effects of nitrous oxide (8,12,13,14) and MTX (2) on folate metabolism. In the following study, the specific redistribution of folate coenzymes in fresh leukemic cells during exposure to both drugs is investigated.

5.3. Materials and methods.

Chemicals. Methotrexate and 5-formylTHF were obtained from Lederle (Etten-Leur, The Netherlands). The premixed gas cylinder containing 50% nitrous oxide, 20% oxygen, 25% nitrogen and 5% carbon dioxide was purchased from Hoekloos (Schiedam, The Netherlands). [^{3}H] (6S) 5-formylTHF (Specific activity: 40 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA, U.S.A.). Bovine Serum Albumin (B.S.A.) was obtained from Sigma (St. Louis, MO, U.S.A.), Lymphoprep from Mycomed (Oslo, Norway) and Fetal Calf Serum (F.C.S.) from Flow (Rickmansnorth, England). Hog kidney hydrolase was prepared according to Mc Martin et al. (15), pure human transcobalamin II was purified from Cohn's fraction III according to Van Kapel et al. (16). RPMI 1640 medium and Hanks Balanced Salt Solution (HBSS) were prepared in the laboratory.

Patient material. Bone marrow aspirates and peripheral blood samples were obtained after informed consent from healthy volunteers and leukemia patients at presentation. Acute myeloid leukemias were classified according to the FAB classification on both morphological and cytochemical criteria (17). Cases of acute lymphoid leukemia were classified according to immunological phenotype. The percentage blast cells in each sample was determined in a differential count of 500 cells.

Radioactive labeling of intracellular folate pools. Bone marrow aspirates and peripheral blood were collected in EDTA containing tubes. The mononuclear cells were isolated by lymphoprep centrifugation and washed with HBSS, 0,2% BSA. Monocytes and macrophages were removed by adherence to a large (150 cm^2) culture flask during a one hour incubation of the cells in HBSS with 10% FCS at 37 °C. The percentage blasts in the final leukemic cell suspensions always exceeded 80%. Cells were labelled

with [^{3}H] 5-formylTHF according to Baram et al. (7). A suspension of 2.10^6 cells per ml in RPMI 1640 (without folic acid or cobalamin), containing 10% dialysed F.C.S., 50 nM [^{3}H] 5- formylTHF and 40 pM transcobalamin II was prepared. After incubation at 37 °C, 5% CO_2 for 18 hours, cells were washed with HBSS, 0,2% BSA and resuspended (at 2.10^6 cells/ml) in RPMI 1640 without folic acid or cobalamin, containing 10% dialysed F.C.S.

Exposure of leukemic cells to nitrous oxide and/or MTX. After resuspension, 6.10^6 cells were used for direct determination of total intracellular [^{3}H] folate content and HPLC separation of the folate coenzymes (see below). If available, remaining leukemic cells were divided over 4 culture flasks (6.10^6 cells in 5 ml RPMI 1640 per flask). Two flasks were exposed to nitrous oxide by flushing during 15 minutes with a filtered mixture released from a premixed cylinder. Subsequently, the culture flasks were closed and incubated at 37 °C for 8 hrs. The 2 other flasks were flushed with a mixture of air and 5% carbon dioxide for 15 minutes and also incubated for 8 hours at 37 °C. MTX in a negligible volume was added for just the last 4 hrs of incubation (concentration: 10^{-6}M) to one flask flushed with nitrous oxide and one with air. Before and after incubation, cell viability (trypan blue exclusion) and cell concentrations were checked. Total cell loss never exceeded 10 %.

Quantitation of folate coenzyme pools. After incubation, cells were washed with HBSS, 0,2% BSA and counted, a sample was drawn from each flask for measurement of total intracellular [^{3}H] folate content. A standard mixture of unlabeled folates was added to the remaining cells. Subsequently, folates were extracted according to Baram et al.(7), using a 90 s boil of the cell pellet in 2 ml 2% mercaptoethanol, 2% ascorbic acid, pH 6.0. After treatment for 1 h with hog kidney hydrolase in order to break down folate polyglutamates, again 2 ml of the extraction solution was added prior to an additional 90 s boil. Folates were further purified and concentrated on C_{18} SEP-PAK cartridges, and than separated by reverse phase HPLC essentialy as described by Allegra et al. (10), with the following modifications. The prepared folate extracts were dissolved in 400 ul 2% mercaptoethanol/2% vitamin C pH 6.0. From this extract 200 ul was injected onto a chromsphere C18 column, 200 x 3 mm (Chrompack Inc., Bridgewater, NJ, USA). Elution conditions: 0-15 minutes: isocratic with 10 mM ammoniumphosphate, 5mM Pic A, 12,5% methanol, pH 5.5. 15-49 minutes: linear gradient from 12,5% to 22% methanol in same buffer. The flow was 0.4 ml/minute. Retention times of folates were as follows: 10-formylTHF: 21.2 min., THF: 24.2 min., 5-formylTHF: 28.9 min., DHF: 31.9 min., 5-methylTHF: 37.9 min. Samples were collected at 30 s intervals. The amount of radioactive folates was quantitated by liquid scintillation counting. Unlabeled folate standards were detected at 290 nm with a spectrophotometer (Spectroflow 757, Kratos) and used for identification of the radioactive folate peaks. Background counts in the HPLC eluate were ± 100 dpm, fractions of folate peaks contained 100-2000 dpm above the background. Before and after analysis of radioactive extracts, unlabeled standard folate mixtures (the same as mentioned above) were injected onto the HPLC system. Calculation of intracellular radioactive folate pools was based on the recovery of the unlabeled folate standards in the cell extract, and the amount of radioactivity in the peaks. Although present in small amounts, 5,10-methyleneTHF could not be quantitated accurately in the present study.

Statistical evaluation. Observed differences between folate cofactor pools of AML and ALL were statistically tested with the Mann-Whitney U Test. Treatment effects of MTX and/or nitrous oxide were tested against the control values with the Wilcoxon signed rank test for paired observations.

5.4. Results.

Intracellular folate coenzyme distribution. Folate coenzyme levels were analysed in normal bone marrow (n=5) and leukemic cells after 18 hours of incubation with 5.10^{-8} M [^{3}H] 5-formylTHF. Samples of 10 cases of AML and 5 cases of ALL were investigated (table 5.1). Total intracellular [^{3}H] folate was highest in cells from AML followed by ALL and normal bone marrow cells. Overall, there was a large variety in the specific folate coenzyme levels among the studied leukemia samples. 5-MethylTHF was the major cofactor in all cells, representing 52% , 55% and 60% of the total [^{3}H] folate in respectively AML, ALL and normal bone marrow (table 5.1). A significant difference ($p<0,05$) between AML and ALL was noticed in the intracellular levels of 10-formylTHF (resp. 26% and 17%) and DHF (resp. 1% and 10%). Interestingly, the fraction of intracellular [^{3}H] folate present as 5- methylTHF was increased while 10-formylTHF was decreased in the cells from cases of more differentiated AML and ALL (patient 8,9,10 and 15). The folate distribution of normal bone marrow cells was almost identical to the pattern observed in myeloid leukemias.

Effect of drug treatment on total [^{3}H] folate content. From the samples of AML patients 1,3,4,5,7 and of all patients with ALL enough cells could be isolated to perform experiments with nitrous oxide and/or MTX. After incubation for 8 hrs in folate free RPMI 1640, untreated AML and ALL cells had lost 17% and 25% of their initial [^{3}H] folate content (table 5.2). However, no change in the folate coenzyme distibution was noticed. Exposure of the cells to nitrous oxide (8 hrs) and/or MTX (10^{-6} M, the last 4 hrs) did not affect the total [^{3}H] folate content of the AML cells but 12 % loss occurred in ALL cells only during combined nitrous oxide/MTX treatment.

Effects of nitrous oxide on folate pools. Exposure of the leukemic cells to nitrous oxide for 8 hrs caused an increase of 5-methylTHF to 130% and an equivalent decrease of 10- formylTHF and 5-formylTHF of 30% in AML and 45% in ALL (figure 5.1). Moreover, a 35% decrease of THF was noticed in AML cells and a 45% reduction of DHF in ALL cells.

Effect of MTX on folate pools. Incubation of the leukemic cells with 10^{-6} M MTX for 4 hrs largely increased the DHF pools in both AML and ALL cells (figure 5.1). In AML cells this rise of DHF was accompanied by a 33% decrease of 5-methylTHF. ALL cells predominantly compensated the accumulation of DHF by a decrease of 10-formylTHF (50%) and 5-formylTHF (25%) and just a small reduction of the 5-methylTHF level. For 10-formylTHF this difference between AML and ALL cells was statistically significant ($p<0,01$). Studies of Baram et al. (18) have shown that during MTX treatment of cells, substantial amounts of 10-formylDHF are produced. With the HPLC separation of folates employed for this study, THF and 10-formylDHF co- elute and cannot be distinguished from each other. The indicated

Table 5.1:

Intracellular [^{3}H] folate coenzyme levels (fmol per 10^6 cells) in bone marrow from healthy persons and leukemia patients.

No.	Diagnose	Source	Total	N^{10}Formyl	N^5Formyl	DHF	THF	MTHF
1	M1	B.M.	531	186	53	0	42	260
2	M1	P.B.	641	288	103	0	90	160
3	M2	B.M.	652	174	67	17	67	328
4	M4	P.B.	554	131	27	0	60	326
5	M4	B.M.	453	122	41	0	23	267
6	M4	P.B.	277	75	22	11	19	147
7	M4	B.M.	448	99	31	13	31	278
8	M5B	B.M.	745	66	37	0	67	574
9	M5B	B.M.	485	53	49	0	44	340
10	M5B	B.M.	517	88	16	0	0	414
Mean ± s.e.m.:			530 ± 40	128 ± 21	45 ± 8	4 ± 2	44 ± 8	299 ± 42
% of total uptake:			100 %	26 %	10 %	1 %	9 %	53 %
11	cALL	B.M.	326	66	29	49	49	137
12	cALL	P.B.	653	39	7	20	7	574
13	cALL	B.M.	465	107	32	60	32	233
14	cALL	B.M.	425	123	77	85	77	72
15	TALL	P.B.	171	12	0	0	0	137
Mean ± s.e.m.			408 ± 72	69 ± 19	29 ± 12	43 ± 13	36 ± 8	230 ± 81
% of total uptake:			100 %	17 %	7 %	10 %	10 %	55 %
Normal bone marrow: (mean ± s.e.m., n = 5)			313 ± 55	* 74 ± 9	22 ± 3	7 ± 3	22 ± 5	188 ± 40
% of total uptake:			100 %	24 %	7 %	2 %	7 %	60 %

Intracellular folate pools of bone marrow cells were labeled by 18 hrs incubation with 50 nM [^{3}H] 5-formylTHF prior to quantitation of the folate cofactor pools by HPLC analysis. B.M.: bone marrow, P.B.: peripheral blood.

Figure 5.1 (opposite page): Effect of nitrous oxide and/or MTX on intracellular [H^3] folate levels in cells from AML (n=5, top panel) and ALL (n=5, bottom panel). Intracellular folate pools of blast cells from leukemia patients were labeled by 18 hrs incubation with 50 nM [^{3}H] 5-formylTHF. Subsequently cells were exposed to air (8 hrs), nitrous oxide (8 hrs), air (8 hrs) and MTX (10^{-6} M, last 4 hrs) or nitrous oxide (8 hrs) and MTX (10^{-6} M, last 4 hrs), prior to quantitation of the folate cofactor pools by HPLC analysis. See Material and Methods section for specific incubation conditions. N^{10}: 10-formylTHF, N^5: 5-formylTHF, DHF: dihydrofolate, THF: tetrahydrofolate, FDHF: 10-formyldihydrofolate, MTHF: 5-methylTHF. (*: statistically different from controls, $p<0,05$)

Erratum:"top panel" and "bottom panel" are "left panel" and "right panel".

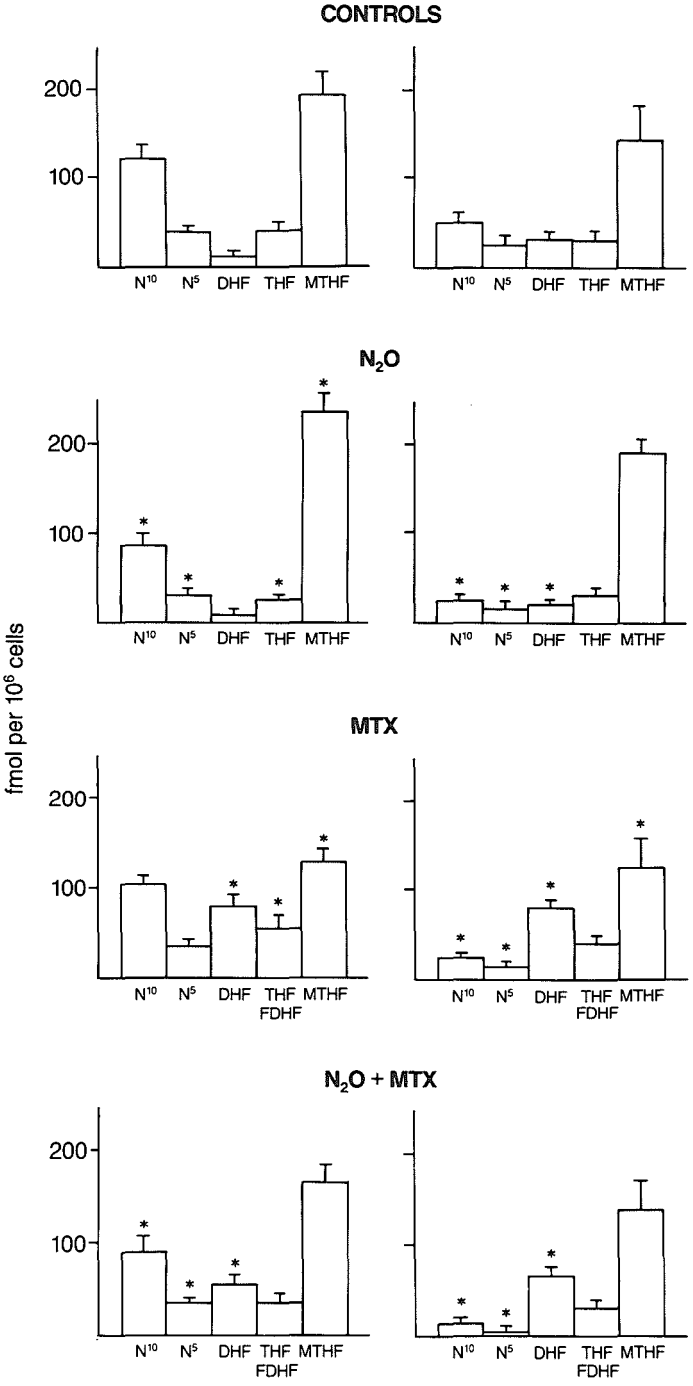
CONTROLS
200
100
N10 N5 DHF THF MTHF
N2O
MTX
N2O + MTX
fmol per 10^6 cells
N10 N5 DHF THF MTHF FDHF

Table 5.2:

Total intracellular [^{3}H] folate content (fmol per 10^6 cells) of blast cells derived from AML and ALL patients.

Diagnose	n	Preincubation	Control	N_2O	MTX	N_2O + MTX
AML	5	498 ± 65	412 ± 30	400 ± 30	411 ± 39	400 ± 31
ALL	5	408 ± 72	300 ± 41	276 ± 29	285 ± 42	249 ± 36

Intracellular folate pools were labeled by 18 hrs incubation with 50 nM [^{3}H] 5-formylTHF prior to exposure to air (8 hrs), nitrous oxide (8 hrs), air (8 hrs) and MTX (10^{-6} M, last 4 hrs) or nitrous oxide (8 hrs) and MTX (10^{-6} M, last 4 hrs). Subsequently samples were drawn form the cell suspensions for determination of total [^{3}H] folate content.

increase of THF during MTX treatment is therefore most likely caused by the presence of 10-formylDHF.

Effect of nitrous oxide and MTX on folate pools. The exposure to nitrous oxide for 8 hrs combined with the 10^{-6} M MTX incubation during the last 4 hrs resulted in a substantial increase of DHF at the expense of 10-formylTHF and 5-formylTHF in both types of acute leukemia cells (figure 5.1). However the response of cells from ALL to the nitrous oxide/MTX treatment was more profound, causing a 70% depletion of both formylated reduced folates versus 25% in AML cells. This difference between AML and ALL was significant ($p<0,01$). Both 5- methylTHF and the THF/10-formylDHF pools remained unchanged.

5.5. Discussion.

Studies on nitrous oxide exposure or MTX treatment on the intracellular folate metabolism have predominantly been carried out in respectively animals (1,3) and cell lines (10,11,18-20). The present paper, for the first time, provides information on the folate coenzyme levels of fresh human leukemic cells and the effects of nitrous oxide and/or MTX and compared with untreated normal bone marrow cells. The results of the latter are in accordance with literature data on myeloid precursor cells (7), which favors the reliability of the employed methods.

Although there is a wide variation in the concentrations of the various folate forms in leukemic cells after incubation with [^{3}H] 5- formylTHF, several conclusions can be drawn. In both AML and ALL cells, 5-methylTHF was the predominant folate form accounting for more than 50% of the total folate pool. In purified myeloid precursor cells from normal bone marrow 5-methylTHF and 10-formylTHF appeared to be present in about equal concentrations (7). In contrast with this, we found that 10-formylTHF accounted for only 26% of the total [^{3}H] folate pool in AML cells and 17% in ALL cells. This difference between AML and ALL is difficult to explain as additional data on proliferation index and rate of purine synthesis are not available. Cells from several subtypes of AML have been used. The data in table 5.1 suggest that a higher differentiation level of myeloid leukemic cells is accompanied by an

increase in 5-methylTHF (from 38% to 70% of total [^{3}H] folate) and a decrease of 10-formylTHF (from 40% to 9% of total [^{3}H] folate). This observation concurs with the general assumption that high levels of 5-methylTHF are indicative for well-differentiated tissues (3,4,5,6). Probably this also explains why the fraction of 5-methylTHF in purified myeloid precursors (7), appeared to be smaller than in the normal bone marrow samples in this study which still contain differentiated cells.

DHF was scarcely present in AML blast cells nor in normal bone marrow cells. However substantial levels of DHF were noticed in ALL cells, which is in contrast with folate distributions reported in animal tissues (3,4,5,6) and cell lines (19,20). Kamen et al. (21) showed the activity of dihydrofolate reductase (DHFR) in *ex vivo* ALL cells to be very low compared to cell lines and animal tissues. As the enzyme is very stable (21) these data suggest that the presence of DHF in ALL cells may result from a restricted activity of dihydrofolate reductase.

Eight hours after incubation with [^{3}H] 5-formylTHF, some loss of [^{3}H] folate from the leukemic cells has occurred however without change of the folate coenzyme distribution. This demonstrates the stability of the equilibrium, achieved by the 18 hrs incubation with [^{3}H] 5-formylTHF. The [^{3}H] folate content was higher in AML than in ALL cells which agrees with earlier investigations which showed a higher total folate content of cells from AML compared with ALL (22,23).

Based on previous studies by Boss (24) and Baram et al. (7), 8 hrs of nitrous oxide exposure and/or 4 hrs incubation with 10^{-6} M MTX were used to interfere effectively with the folate metabolism of the leukemic cells. In our study the inactivation of methionine synthase by nitrous oxide did not reduce the total intracellular [^{3}H] folate content (3), which may require a longer nitrous oxide exposure, but it did induce a significant redistribution of the folate coenzymes. In both AML and ALL cells the 5-methylTHF pool rose at the expense of the other folate coenzymes, which supports the "methyl trap hypothesis" for cobalamin deficiency (3,9). The substantial depletion of 10-formylTHF during nitrous oxide exposure may imply an important reduction of purine synthesis during cobalamin inactivation, as has been suggested before (24).

The effects of MTX on folate coenzyme pools differs significantly between blast cells from AML and ALL. In both types of acute leukemia, the inhibition of DHFR resulted in a steep rise of the DHF pool and probably also of the 10-formylDHF pool. In AML cells this accumulation of oxidized folates went mainly at the expense of 5-methylTHF, as has been decribed before for purified myeloid blasts from normal bone marrow (7). However, MTX treatment of ALL cells caused substantial depletion of 5-formylTHF and more importantly of 10-formylTHF. This reduction of 10-formylTHF may have a negative effect on the activity of AICAR and GAR transformylase and suggests an relatively high sensitivity of the purine metabolism in ALL cells for MTX.

Prior inactivation of methionine synthase by nitrous oxide exposure aggrevates the effect of MTX because the partial replenishment of the other decreased folate coenzymes levels by conversion of 5-methylTHF to THF has been made impossible. Especially in ALL cells this results in severe depletion of both 10-formylTHF and 5-formylTHF. The increased toxicity of MTX on bone marrow after nitrous oxide anesthesia, (25, 26) may at least partially be ascribed to this mechanism.

In conclusion, the dissimilarities in folate coenzyme pools between ALL and AML

cells suggest an intrinsic difference in the quantitative importance of several folate-dependent pathways. Especially the presence of DHF in ALL cells, suggesting low DHFR activity, may be clinically relevant. Moreover, the more pronounced depletion of 10-formylTHF in ALL cells during inhibition of the normal folate metabolism is also in support of a higher MTX sensitivity of ALL cells than AML cells, which may be reflected by the fact that MTX has been retained in many ALL but not in AML treatment protocols.

5.6. References.

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Chapter 6

Effect of nitrous oxide anesthesia on the number of hematopoietic progenitors in bone marrow, determined by clonogenic assays

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6.1. Summary.

Nitrous oxide inactivates the cobalamin coenzyme of methionine synthase. This enzyme plays an essential role in the cellular retention of reduced folates. Earlier studies have shown that prolonged exposure (>24 h) to nitrous oxide can result in macrocytic/megaloblastic bone marrow depression as a consequence of impaired folate-dependent synthesis of thymidylate (dTMP). In the present study the effect of shorter periods of continuous exposure to nitrous oxide (7-12 h) on hematopoiesis was investigated, using *in vitro* assays to determine the number of myeloid and erythroid progenitors, present in the bone marrow. Bone marrow samples from 14 patients (pts) before and after nitrous oxide anesthesia were obtained. Four pts, operated on under total intravenous anesthesia, served as controls. Macrocytic changes of the bone marrow were noticed in only two pts exposed to more than 24 h nitrous oxide over a 3 day period. Disturbance of the deoxyuridine suppression test value of the bone marrow cells at the end of anesthesia occurred in all pts undergoing nitrous oxide exposure. Both myeloid and erythroid progenitors, cultured *in vitro*, increased in 6 pts (160%) and decreased in 6 other pts (40%). Increase occurred in pts with a normal holo- transcobalamin II and folate level. Decrease of the hematopoietic progenitors was related to nitrous oxide exposure for more than 24 h (2 pts) or low plasma holo-transcobalamin II and/or low plasma folic acid (4 pts). Still, the number of progenitors present in the bone marrow after nitrous oxide anesthesia never decreased below the lower limit of normal values. It can be concluded that although nitrous oxide induces changes in the proliferation kinetics of the hematopoietic compartment within the studied exposure periods, the toxic effect on bone marrow functioning is limited.

6.2. Introduction.

Early clinical observations in tetanus patients have shown that prolonged exposure to nitrous oxide could result in megaloblastic bone marrow depression (1). Later investigators correlated this clinical effect of nitrous oxide exposure to the chemical reactions which can occur between cobalt(I) complexes, like cobalamin, and nitrous oxide (2,3). Since then further research, both in man and test animal, has been aimed at elucidation of the effects of prolonged exposure to nitrous oxide on cobalamin dependent metabolism (for review see: 4,5). It was found that nitrous oxide specifically inactivates the methylcobalamin coenzyme of methionine synthase. This enzyme plays a crucial role in the cellular retention of reduced folates by conversion of homocysteine and methyltetrahydrofolate, the major extracellular folate, to methionine and tetrahydrofolate which subsequently can be polyglutamated and converted to other active folate coenzyme forms. Prolonged exposure to nitrous oxide therefore causes cellular folate depletion and disturbance of folate-dependent methylation reactions such as the *de novo* synthesis of thymidylate (dTMP) and purines. Obviously the rapidly dividing cells of the hematopoietic compartment are very dependent on the intact function of the methylcobalamin coenzyme.

Several studies have revealed a relation between the duration of exposure to nitrous oxide, inactivating human methionine synthase with a half time of ±46

minutes (6), and the changes that occur in the bone marrow. The deoxyuridine suppressiontest (dU test) value is a reliable assay for measuring disturbance of the folate-dependent dTMP synthesis. In fit patients, increase of the dU test requires 5-6 h of nitrous oxide exposure (7). Megaloblastic changes of the bone marrow only occur after 12-24 h of nitrous oxide exposure (2,8,9). Data on the events which occur between the first sign of biochemical changes in the bone marrow and the onset of morphological changes are not available.

In this study, we determined the number of myeloid and erythroid progenitors cells, present in the bone marrow of patients exposed to nitrous oxide. During *in vitro* culturing of bone marrow cells in semi- solid culture medium, proliferation of these myeloid and erythroid progenitors produces recognizable groups of descending cells (called "colonies" and "bursts" respectively) which enables their quantitation in marrow aspirates. Both progenitors (called Colony Forming Unit- Granulocyte/Macrophage (CFU-GM) and Burst Forming Unit-Erythroid (BFU-E) respectively) represent less than 1% of the bone marrow cells and are involved in self renewal and proliferation of the hematopoietic compartment. This immediate importance to hematopoiesis, together with their ability to produce colonies during *in vitro* culture, has resulted in the application of clonogenic assays for the estimation of bone marrow functioning.

In the present study, correlations between these data, bone marrow morphology, dU test values and the cobalamin/folic acid status were established.

6.3. Materials and methods.

Patients. Eighteen patients undergoing prolonged anesthesia for plastic surgery (10 pts), tumor resection (6 pts) or trauma treatment (2 pts) were studied. None of them were critically ill. Nitrous oxide was used in 14 cases, total intravenous anesthesia without nitrous oxide in the other 4 patients. Patients'informed consent was obtained. All subjects were hematologically normal as assessed by preoperative values for hemoglobin, hematocrit, mean corpuscular volume and leucocyte count. Patients previously treated with cytostatics or radiation were excluded.

Anesthesia.

-Nitrous oxide exposed group (14 pts): induction: thiopentone (3-6 mg/kg), maintenance: nitrous oxide (70%), oxygen (30 %), enflurane (0,5-1%) and fentanyl, relaxation: pancuronium (0,1 mg/kg).

-Control group (4 pts): induction: propofol (2-3 mg/kg), maintenance: oxygen enriched air, propofol (10-6 mg/kg/h) and fentanyl, relaxation: pancuronium (0,1 mg/kg).

Except for nitrous oxide, none of the applicated drugs is known to interfere with the activity of methionine synthase. Physiological parameters were always kept within normal limits. Transfusions of erythrocyte concentrates were given to all patients when necessary.

Procedures. Venous blood (10 ml) was collected preoperatively in an EDTA containing tube for determination of plasma cobalamin, holo-transcobalamin II and folate. Bone marrow was sampled from the anterior iliac crests immediatly after induction and at the end of anesthesia. It was collected in EDTA containing tubes. This material was used for bone marrow smears, the dU test, and clonogenic assays.

Plasma folic acid and cobalamin were determined simultaneously with a radioassay (Becton-Dickinson, Orangeburg, New York, USA). Plasma holo- transcobalamin II was determined according to Van Kapel et al. (10). Bone marrow smears were made directly after aspiration and stained with May Grunwald-Giemsa.

DU suppressiontest. Cells were isolated from the bone marrow sample by Ficoll-Paque centrifugation. A portion of the obtained cells was subjected to the dU test as described by Metz et al. (11). Briefly, cells preincubated with 0,1 mM deoxyuridine for 1 h are incubated with [H^3] thymidine for an extra hour. If the folate dependent dTMP synthesis is normal, the preexposure to deoxyuridine will suppress [H^3] thymidine incorporation to less than 10% of the control incubations without deoxyuridine. However, in case of disturbed folate metabolism, this ratio increases rapidly.

Clonogenic assays. Bone marrow cells (10^5) were plated in triplicate in 1 ml Alpha medium containing 0,9% methylcellulose, 20% fetal calf serum, 1% bovine serum albumin, 3.10^{-5} M lecithin, 3.10^{-6} M transferin and 2.10^{-5} M mercaptoethanol. Myeloid growth was supported by addition of 10% Giant Cell Tumor-Conditioned Medium. Erythroid growth was supported by 10% Phytohaemagglutine Leukocyte-Conditioned Medium and 2 units r-erythropoietin per ml. Colony Forming Unit-Granulocyte/Macrophage (CFU-GM) and Burst Forming Unit-Erythroid (BFU-E) were counted respectively on day 10 and day 14 of incubation at 37 C, 5% CO_2.

Data analysis. Non-parametric statistical analysis of the obtained data was performed on the ratio's of post-and preoperative values of the deoxyuridine suppression test, and the ratio's of post-and preoperative numbers of CFU-GM and BFU-E present in 10^5 bone marrow cells. Differences between patients operated on under nitrous oxide anesthesia and patients operated on under TIVA were tested with the Mann-Whitney U test. The same test was used to evaluate the difference between normal patients and patients with a low plasma cobalamin and/or low folate. Correlation between nitrous oxide exposure time and the post and preoperative ratio's was calculated with the Spearman rank correlation test. All p values are given as one-tailed.

6.4. Results.

Preoperative blood parameters. The preoperative hematological values of all patients (see M&M) were within the normal range. Table 6.1 reveals that this study included 7 patients with a low plasma folate (<7 nM) of which 5 were operated on under nitrous oxide and 2 under TIVA. Two patients had a low serum cobalamin (<120 pM) and 3 low holotranscobalamin II (<20 pM). All others had normal plasma levels and will be referred to as "normal patients". One patient had a low serum cobalamin but a normal holo- transcobalamin II and was therefore also considered "normal".

Effect of anesthesia on marrow morphology and dU test values. Preoperative dU tests and bone marrow morphology were normal in all patients. Continuous anesthesia varied from 390 to 720 minutes. After anesthesia, macrocytic changes were noticed only in bone marrow aspirates from 2 patients who had undergone multiple nitrous oxide exposures. The dU test increased to abnormal values (>10%) in all patients who underwent nitrous oxide anesthesia ($p<0.001$, table 6.1) but no correlation was seen between the exposure time and the observed increases. However, the

Table 6.1:

Age (in years), sex, duration of anesthesia (in minutes), preoperative plasma parameters and preoperative (T_1) and postoperative (T_2) bone marrow parameters of patients involved in this study.

PATIENTS OPERATED ON UNDER NITROUS OXIDE ANESTHESIA WITH NORMAL PLASMA COBALAMIN AND/OR FOLATE LEVELS.

Age	Sex	Anesthesia duration	Cobalamin (pM)	TC II (pM)	Folate (nM)	DU test T_1	DU test T_2	CFU-GM T_1	CFU-GM T_2	BFU-E T_1	BFU-E T_2
56	M	390	225	30	7.9	10	15	66	78	72	83
74	F	480	204	53	10.0	5	27	75	115	20	35
20	M	530	182	23	37.3	10	53	62	82	66	77
49	F	600	322	86	7.7	11	29	ND	ND	ND	ND
39	M	615	88	21	8.3	3	32	87	163	80	148
40	M	630	309	51	8.4	8	21	ND	ND	ND	ND
65	F	720	272	67	12.0	ND	ND	35	82	48	88
53	M	1440*	330	65	18.1	11	22	250	107	258	100
18	M	1680*	287	49	16.3	10	24	45	30	108	83

PATIENTS OPERATED ON UNDER NITROUS OXIDE ANESTHESIA WITH LOW PLASMA COBALAMIN AND/OR FOLATE LEVELS.

Age	Sex	Anesthesia duration	Cobalamin (pM)	TC II (pM)	Folate (nM)	DU test T_1	DU test T_2	CFU-GM T_1	CFU-GM T_2	BFU-E T_1	BFU-E T_2
65	M	400	165	32	6.4	6	37	37	26	ND	ND
18	M	420	167	34	4.4	8	54	278	168	197	142
27	F	425	109	8	4.8	9	34	44	25	27	17
54	M	460	185	43	4.7	10	39	75	90	42	42
65	M	580	70	17	6.8	9	61	169	63	64	33

PATIENTS OPERATED ON UNDER TOTAL INTRAVENOUS ANESTHESIA.

Age	Sex	Anesthesia duration	Cobalamin (pM)	TC II (pM)	Folate (nM)	DU test T_1	DU test T_2	CFU-GM T_1	CFU-GM T_2	BFU-E T_1	BFU-E T_2
70	M	520	186	16	4.4	8	7	57	54	40	46
17	M	540	138	14	15.7	9	9	180	188	91	86
51	M	560	184	23	14.2	5	4	221	216	128	134
18	M	600	296	51	6.5	10	9	90	93	93	89

Deoxyuridine suppression test (DU test) values are expressed as the ratio x 100 % between [^{3}H] thymidine incorporation in bone marrow cells with and without deoxyuridine preincubation (see Materials and Methods). Myeloid (CFU-GM) and erythroid (BFU-E) progenitors present in bone marrow are given as the number per 10^5 marrow cells. *: Total exposure to nitrous oxide over 3 days. (Normal range cobalamin: 120-800 pM, transcobalamin II: 20-220 pM, folate: 7-30 nM)

postoperative dU test values from patients with a low folate and/or cobalamin level differed significantly from normal patients ($p<0.05$).

Effect of anesthesia on CFU-GM and BFU-E (Table 6.1). In patients with total intravenous anesthesia no changes in the number of assayable hematopoietic progenitors occurred. However in nitrous oxide treated subjects two patterns seemed to evolve. In patients with normal folate/cobalamin levels, nitrous oxide exposure caused accumulation of both CFU-GM and BFU-E. The correlation between the

increase of CFU-GM in bone marrow of normal subjects was statistically significant ($n=5$, $r_s=0.90$, $p<0.01$). On the other hand, in patients with low plasma cobalamin and/or folate the exposure to nitrous oxide decreased the number of both progenitors present in bone marrow. The difference between normal patients and patients with a low plasma cobalamin and /or folate was significant ($p<0.05$). The 2 patients who received several nitrous oxide exposures over a short period also showed a decrease in CFU-GM and BFU-E. However, in none of the patients the number of both types of progenitors present in bone marrow decreased below the lower limit for normal values (in our laboratory: 25 colonies per 10^5 cells).

6.5. Discussion.

So far, nitrous oxide is the only drug known to inactivate the function of the cobalamin coenzym in methionine synthase. This eventually causes disturbance of the folate-dependent dTMP and purine synthesis and slowing down or arrest of cells shortly after starting DNA synthesis. The final result of this process is an asynchronous pattern between nuclear and cytoplasmic maturation, which is characteristic for megaloblastic bone marrow.

Most investigations on hematological toxicity of nitrous oxide have focused on exposure periods of 12-24 h. However, in seriously ill patients already short term exposure to nitrous oxide may lead to macrocytic/megaloblastic changes in the bone marrow (12). The purpose of the present study was to investigate the effect of 7-12 h nitrous oxide exposure on hematopoiesis of patients who were not critically ill as this situation occurs rather frequently. The application of clonogenic assays for quantification of myeloid and erythroid progenitors (CFU-GM and BFU-E) permitted the early detection of changes in the proliferation capacity of the hematopoietic compartment.

The presented data show that the folate-dependent dTMP synthesis, as measured by the dU test, is indeed disturbed after exposure to nitrous oxide. The observed increase in CFU-GM and BFU-E by nitrous oxide exposure in patients with normal preoperative plasma folate/cobalamin levels is difficult to explain. However, a similar phenomenom has been described in rats (13) and human bone marrow cells during *in vitro* pertubation of the folate metabolism (14). Possibly the nitrous oxide-induced arrest of proliferating cells in S phase (15) results in accumulation/synchronisation of hematopoietic progenitor cells. Nitrous oxide-exposed patients with a low preoperative plasma folate/cobalamin level had the highest postoperative dU test values and in contrast a decrease in CFU-GM and BFU-E. Low plasma cobalamin and low plasma folate are not synonymous to a cobalamin or folate deficiency (16). However, studies in patients with subnormal plasma cobalamin or folate values have revealed elevated plasma homocysteine concentrations which suggests reduced methionine synthase activity in this particular group of subjects (17,18). Previous studies have also demonstrated that patients with deranged cobalamin and /or folate metabolism are more susceptible to the toxic effects of nitrous oxide (9,19,20). An accelerated onset of toxicity on hematopoietic progenitors, therefore seems obvious. The decrease in progenitors, found in the two patients who underwent multiple nitrous oxide exposures, is probably caused by surpassing the critical period during which these cells

can survive the nitrous oxide-induced disturbance of folate metabolism.

Still the postoperative values of CFU-GM and BFU-E present in the bone marrow after nitrous oxide exposure are within the normal range of our laboratory which indicates the sufficient functioning of hematopoiesis. Therefore the presented data suggest that although changes in the proliferation kinetics occur, the effect of continuous nitrous oxide exposure (up to 12 h) on bone marrow functioning in non-critically ill patients is rather limited. However, the observed accumulation of progenitors by nitrous oxide may contribute to an increased sensitivity of the hematopoietic compartment to S phase-specific cytostatics after withdrawal of anesthesia (7,13). Especially in compromized patients (e.g. low plasma folate/cobalamin) this phenomenom could rapidly result in unexpected myelosuppresion, even after short exposures to nitrous oxide (21,22).

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Chapter 7

Cobalamin inactivation during nitrous oxide anesthesia. Effects on homocysteine and folate in plasma and urine.

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7.1. Summary

Exposure to the anesthetic gas nitrous oxide impairs the activity of methionine synthase by oxidation of its cobalamin coenzyme. This enzyme catalyses the conversion of homocysteine and methyltetrahydrofolate to methionine and tetrahydrofolate respectively. Earlier studies have shown that hematological and/or neurological damage, resembling cobalamin deficiency, may occur after prolonged or repeated inhalation of nitrous oxide. In the present study, effects of nitrous oxide anesthesia on homocysteine and folate metabolism have been investigated.

Plasma levels of cobalamin, folate, homocysteine and methionine were determined in 40 patients before and after operation under nitrous oxide anesthesia (range of exposure time: 70-720 minutes). Twelve patients anesthetized with total intravenous anesthesia served as controls (range of exposure time: 115-600 minutes). Post-operative plasma levels of folate and homocysteine were found to be increased ($p<0,001$) up to 220 and 310%, respectively in nitrous oxide exposed patients while plasma levels of methionine were decreased ($p<0.025$). Response already occurred after 75 minutes of nitrous oxide exposure. The percentage increase of plasma folate and homocysteine correlated significantly with exposure time ($p<0,025$ and $p<0,0001$ respectively). After withdrawal of nitrous oxide, plasma levels of folate and homocysteine usually continued to rise for several hours.

Return of plasma homocysteine to the preoperative level took more than 1 week ($p<0.01$) in 8 patients after anesthesia with nitrous oxide (range of exposure time:215-370 minutes).

Urinary excretion of folate and homocysteine increased during and after nitrous oxide exposure ($p<0,01$ and $p<0.002$ respectively), and correlated with exposure time ($p<0,01$ and $p<0.005$ respectively).

It can be concluded from this study that nitrous oxide-induced cobalamin inactivation can be monitored by determining plasma homocysteine. Moreover the presented data suggest that effects of nitrous oxide anesthesia on homocysteine and folate metabolism develop more rapidly than presently assumed and that return to normal requires several days.

7.2. Introduction

The widely used anesthetic gas nitrous oxide is known to oxidize the coenzyme methylcobalamin, causing irreversible inactivation of methionine synthase (1,2). This enzyme is responsible for the methyltransfer from 5-methyltetrahydrofolate to homocysteine (figure 7.1). Exposure to nitrous oxide may therefore disturb the intracellular folate metabolism and reduce the folate-dependent thymidylate and purine synthesis, necessary for DNA replication. In addition, the reduction of methionine synthase activity may result in a decrease of plasma methionine concentrations (3).

Hematopoiesis in man depends strongly on the function of the methylcobalamin coenzyme and inactivation by prolonged exposure to nitrous oxide can cause megaloblastic changes of the bone marrow (1,3) similar to cobalamin deficiency. Moreover, neurological impairment which is well known in cobalamin deficiency, may

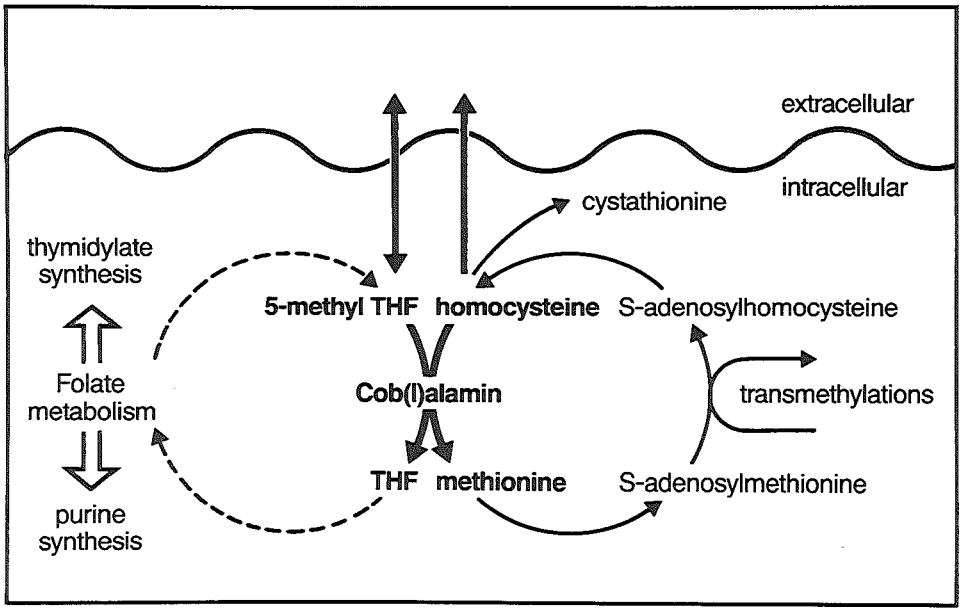


Figure 7.1: Outline of the methylcobalamin-dependent conversion of 5-methyltetrahydrofolate (5-methylTHF) and homocysteine to tetrahydrofolate (THF) and methionine.

occur after prolonged, intermittent, exposure to nitrous oxide (4). In critically ill patients (5) and folate and/or cobalamin deficient subjects (6) these effects may be provoked by nitrous oxide within few hours. However, in the majority of surgical patients receiving nitrous oxide anesthesia, the oxidation of methylcobalamin is regarded to be unharmful (7).

Studies on hematological toxicity of nitrous oxide have mainly focused on bone marrow morphology and quantitation of the folate-dependent thymidylate synthesis in hematopoietic cells by means of the deoxyuridine suppression test (1,3,8).

Recently, elevated serum levels of homocysteine have been reported in cobalamin and/or folate deficient subjects (9) and cancer patients treated with high-dose methotrexate (10). The concentration of homocysteine in extracellular media like plasma and urine may therefore reflect perturbations of cobalamin-dependent folate metabolism.

In the present study, we determined plasma levels of homocysteine, methionine, folate and cobalamin and renal excretion of homocysteine and folate in patients subjected to nitrous oxide anesthesia.

Our results reveal an increase in plasma and urinary homocysteine and folate concentrations by nitrous oxide exposure within several hours, indicating that cobalamin inactivation occurs rapidly. This study shows that, among the studied parameters, plasma and urinary homocysteine are the most responsive to nitrous oxide-induced cobalamin inactivation.

7.3. Methods

Patients. Fifty four patients requiring plastic, otolaryngological or neurological surgery who underwent anesthesia with nitrous oxide (n=42) or total intravenous anesthesia (TIVA, n=12) were included in this study. Informed consent was always obtained. Their ages varied from 18-81 years (Table 7.1). The duration of nitrous oxide anesthesia varied from 70 to 720 minutes (mean: 318±26), and for patients anesthetized with TIVA from 115 to 600 minutes (mean: 332±49).

Two traumatized patients admitted for reconstructive surgery undergoing several operations during a three day period, were exposed to nitrous oxide for totally 24 hours and 28 hours, respectively. Sixteen patients received 1-8 erythrocyte concentrates (200 ml per unit) and two of them also received 1-2 units fresh frozen plasma (200 ml per unit) to compensate for blood loss during surgery (table 7.1).

Anesthesia. The group of patients exposed to nitrous oxide received anesthesia including nitrous oxide (70%), oxygen (30 %), enflurane (0,5-1%) and fentanyl. The control group received anesthesia including oxygen enriched air, propofol (10-6 mg/kg/h) and fentanyl.

Protocol. Venous blood from all patients was collected at the start and end of anesthesia, and the following parameters were routinely determined in plasma: total cobalamin, holotranscobalamin II, folate, methionine and total homocysteine.

Voided urine was collected for 24 hours after the start of anesthesia from 19 patients exposed to nitrous oxide and 10 patients anesthetized with TIVA, for determination of folate and homocysteine content.

Blood samples from 6 patients exposed to nitrous oxide (patient 6, 7, 14, 19, 25 and 38 in table 7.1) and 4 patients anesthetized with TIVA (patient 49, 50, 51 and 54 in table 7.1), were also collected hourly during anesthesia and repeatedly afterwards during 24 hours, for the above mentioned determinations. In addition free homocysteine was determined. Of the same patients, fractionated urine samples collected during 24 hours for determination of urinary folate and homocysteine excretion.

From 8 patients (patient 10, 15, 17, 18, 20, 21, 26 and 27 in table 7.1) having being anesthetized between 215-370 minutes with nitrous oxide anesthesia (mean: 290±19) the long term effect of nitrous oxide was studied in blood samples collected 1, 2 and 7 days after the day of surgery. Six patients (patient 46-51 of table 7.1) anesthetized for 195 to 520 minutes with TIVA (mean: 313±44) were used as controls.

Sample collection and processing. Ten ml of venous blood was collected into cooled, EDTA containing tubes and immediatedly placed on ice. Plasma was prepared by centrifugation within 5 minutes. For determination of free, acid soluble homocysteine, a portion (500 ul) of the sample was immediatly deproteinized with perchloric acid as descibed (11). The samples of deproteinized and whole plasma were stored at -20 C until analysis.

Analytical methods. Total homocysteine in plasma was determined by a fully automated assay recently developed in our laboratory (12). Free, acid soluble homocysteine in plasma as well as urinary homocysteine were assayed by a radioenzymic method (11). Protein-bound homocysteine is total homocysteine minus free homocysteine.

Table 7.1:

Patient characteristics and preoperative plasma values of studied parameters.

A Nr	Age (yrs)	Sex	Anesthesia (minutes)	Transf.	Cobal. (pM)	TC II (pM)	FA (nM)	HCY (μM)	MET (μM)
1	41	M	70	—	157	50	10.7	14.2	25.5
2	26	F	75	—	147	24	5.9	16.8	21.9
3	70	M	80	—	309	90	5.6	13.8	27.3
4	50	F	130	—	719	298	21.0	7.2	14.4
5	49	F	135	—	196	34	10.6	19.0	20.9
6	32	M	135	—	254	49	5.0	12.0	19.3
7	21	F	175	—	283	113	8.6	6.8	15.0
8	26	F	175	—	184	51	3.1	16.3	24.1
9	36	M	185	—	167	29	6.0	13.5	19.1
10	24	F	215	—	435	155	6.4	7.8	20.9
11	50	M	220	—	199	58	3.3	23.8	22.5
12	50	M	220	—	612	249	16.0	11.2	19.4
13	32	M	225	—	226	46	7.8	4.6	19.7
14	74	F	225	—	224	40	4.2	14.2	23.3
15	63	M	230	—	273	44	5.7	8.8	14.3
16	33	F	230	1 EC	179	27	5.2	14.7	18.5
17	40	M	255	—	220	48	5.8	9.6	27.3
18	45	F	280	—	212	43	6.3	9.8	39.5
19	29	M	300	—	287	45	5.0	8.1	18.5
20	59	M	300	—	180	50	10.6	12.1	24.5
21	72	M	305	—	121	24	8.4	12.7	19.0
22	26	F	310	—	143	89	2.9	14.2	26.0
23	41	F	310	—	233	67	4.8	12.9	27.9
24	52	F	315	—	172	36	14.5	9.3	35.7
25	47	F	320	3 EC	515	82	2.8	8.4	24.9
26	45	M	360	—	382	71	3.8	9.2	27.7
27	46	M	370	—	315	102	16.4	11.4	25.8
28	56	M	390	1 EC	325	30	7.8	11.2	17.6
29	65	F	400	—	165	32	6.4	10.8	17.9
30	18	M	420	—	167	34	4.1	14.6	31.1
31	27	F	425	—	109	8	4.8	10.6	6.2
32	54	M	460	2 EC	185	43	4.7	8.3	18.8
33	74	F	480	1 EC	204	53	10.0	6.7	13.6
34	52	M	520	3 EC	163	56	19.7	9.0	12.7
35	71	M	555	—	174	34	8.0	10.3	16.3
36	65	M	580	4 EC	70	17	6.8	7.2	7.2
37	49	F	600	1 EC	322	86	7.7	15.9	26.7
38	39	M	615	8 EC,2 FFP	88	21	8.3	10.9	19.9
39	27	M	660	—	279	110	3.1	33.5	30.0
40	65	F	720	6 EC,1 FFP	272	67	12.2	7.1	11.1
41	53	M	1440*	4 EC	350	90	18.1	13.6	18.6
42	18	M	1680*	4 EC	287	49	16.3	15.0	25.7
B Nr	**Age (yrs)**	**Sex**	**Anesthesia (minutes)**	**Transf.**	**Cobal. (pM)**	**TC II (pM)**	**FA (nM)**	**HCY (μM)**	**MET (μM)**
43	32	F	115	-	189	83	6.0	15.0	20.8
44	54	M	125	-	475	163	24.2	11.6	21.6
45	30	M	165	-	389	225	5.4	18.9	24.4
46	40	M	195	-	167	23	23.8	13.2	14.7
47	81	M	240	-	196	31	6.3	15.1	11.4
48	24	F	245	-	213	41	17.8	8.1	13.4
49	40	F	310	-	272	61	7.0	7.5	10.5
50	35	M	370	-	171	16	5.3	9.6	19.0
51	70	M	520	4 EC	86	16	4.7	19.6	18.4
52	18	M	540	2 EC	138	14	15.7	11.7	14.0
53	51	M	560	1 EC	184	23	14.2	16.4	21.4
54	18	M	600	4 EC	317	51	7.0	12.3	9.6

A: Patients anesthetized with nitrous oxide, B: Patients anesthetized with total intravenous anesthesia. Normal ranges for plasma total cobalamin: 120 - 800 pM., holotranscobalamin II: 20 - 220 pM., homocysteine: 5 - 15 uM., folate: 7 - 25 nM., methionine: 10 - 55 uM. *: total exposure time over a 3 day period. (EC: erythrocyte concentrate, FFP: fresh frozen plasma)

Methionine in plasma was determined in deproteinized plasma, by a method involving derivation of the amino acids with orthophtaldialdehyde, followed by reversed-phase liquid chromatography and fluorescence detection (13).

[^{57}]Co cobalamin and [^{125}]I PGA were obtained from Becton Dickinson, Orangeburg, New York, USA. Total cobalamin and cobalamin analogues in plasma were determined with a radioassay using purified intrinsic factor and salivary R-binder (14). Folate was also determined with a radioassay using milk-binder. Holotranscobalamin II was determinded according to van Kapel et al.(15).

Statistical evaluation. Only patients undergoing one period of anesthesia were included in the statistical analysis. Differences between pre- and postoperative plasma values were statistically evaluated with the Wilcoxon signed rank test for paired observations. Urinary excretion of folate and homocysteine of the nitrous oxide and TIVA groups were tested with the Mann-Whitney U test. Percentage changes of plasma homocysteine, folate and methionine, urinary folate and urinary homocysteine excretion were correlated with anesthesia duration using the Spearman rank correlation coefficent.

To evaluate the long term effects of anesthesia, values at the start of anesthesia were tested against the values at the end and also 1, 2 and 7 days afterwards using the Wilcoxon signed rank test for paired observations. All p values are given as two-tailed.

7.4. Results

Preoperative plasma values. Initial values for total cobalamin, holotranscobalamin II, folate, homocysteine and methionine are summarized in table 7.1. Three patients had a low plasma cobalamin, together with low holo-transcobalamin II. Two of these patients also had a subnormal methionine level. Folate concentrations were frequently below the normal range. Homocysteine above the normal range was observed in 10 patients. The plasma folate concentrations correlated inversely with the homocysteine level ($n=52$, $r_s=-0.30$, $p<0{,}025$).

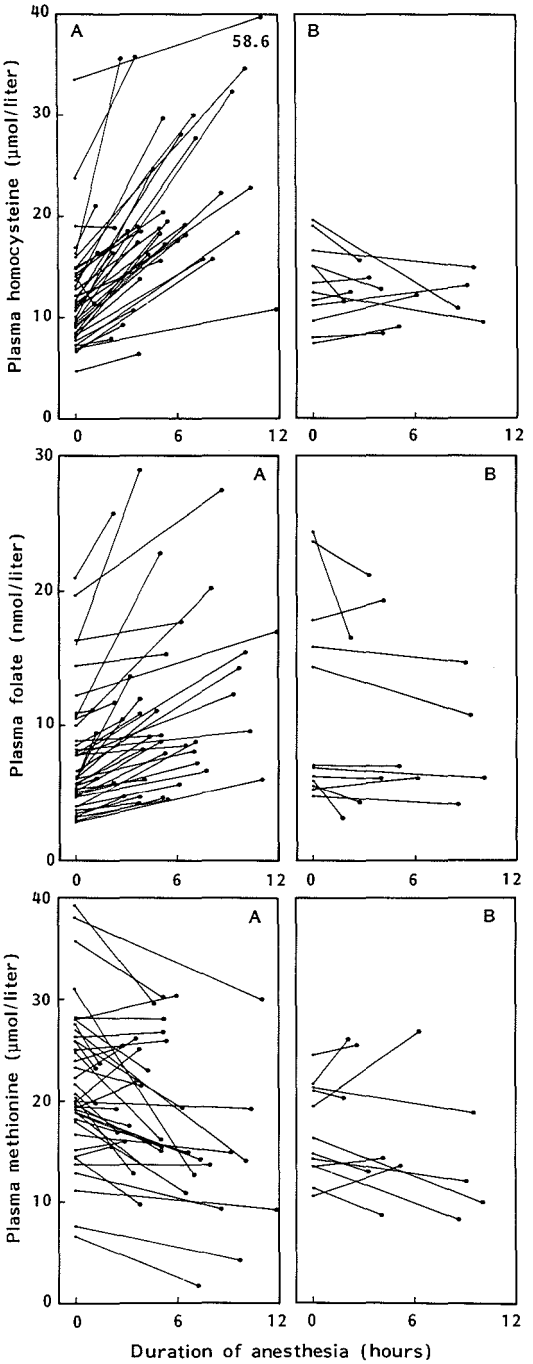
Effect of anesthesia on postoperative plasma parameters. In both groups of patients, total cobalamin and holotranscobalamin II had not significantly changed at the end of anesthesia. Increase in plasma concentration of cobalamin analogues was not observed.

In patients anesthetized with TIVA, plasma folate decreased moderately ($n=12$, $p<0{,}05$) without significant changes in plasma homocysteine or methionine.

During nitrous oxide exposure, postoperative levels of plasma homocysteine and folate significantly increased (figure 7.2 top and middle) ($n=40$, $p<0.001$).

In only 5 out of 42 patients anesthetized with nitrous oxide the postoperative folate levels exceeded the normal range. In 35 of the 42 patients operated on under nitrous oxide the elevated homocysteine level surpassed the upper limit of the normal range. The percentage change in plasma homocysteine and folate correlated significantly

Figure 7.2 (next page): Pre- and postoperative levels of plasma homocysteine, folate and methionine. A: Patients anesthetized with nitrous oxide (n=40), B: Patients anesthetized with total intravenous anesthesia (n=12). Normal range for plasma homocysteine: 5-15 μM., normal range for plasma folate: 7-25 nM., normal range for plasma methionine: 10-55 μM.


A
58.6
B
Plasma homocysteine (µmol/liter)
40
30
20
10
0
0
6
12
A
B
Plasma folate (nmol/liter)
30
20
10
0
A
B
Plasma methionine (µmol/liter)
Duration of anesthesia (hours)

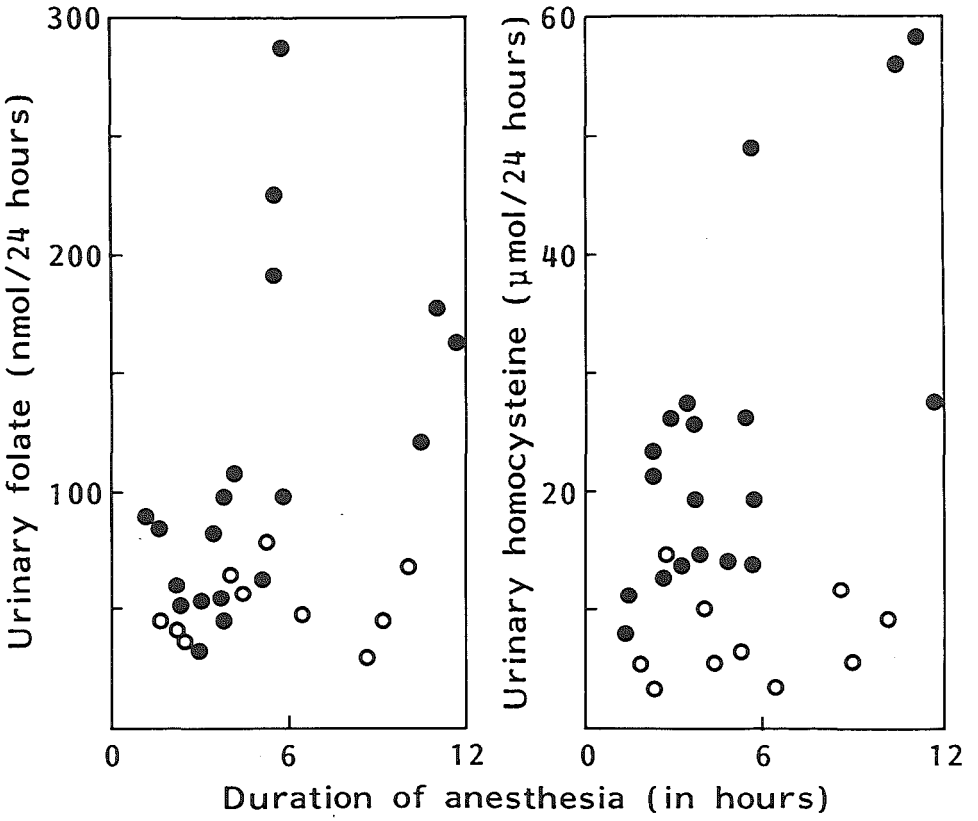


Figure 7.3: Urinary excretion of folate and homocysteine during the first 24 hours after start of anesthesia.(•: Patients anesthetized with nitrous oxide (n=19), o: Patients anesthetized with total intravenous anesthesia (n=10).) Normal urinary folate excretion: < 75 nmol/24 hrs. Normal urinary homocysteine excretion: 3.5-9.5 μmol/24 hrs.

with nitrous oxide exposure time (n=40, r_s=0.73, p<0.0001 and n=40, r_s=0.36, p<0.025 respectively). Moreover, preoperative folate values inversely correlated with the absolute increase in plasma homocysteine concentrations (n=40, r_s=-0.34, p<0.025).

Interestingly, patient 41 and 42, who had been exposed to nitrous oxide for more than 24 hours within three days, showed a remarkable strong increase in plasma homocysteine (from 13.6 to 134.5 μM and from 15.0 to 67.4 μM respectively). Plasma folate only increased twofold (from 18.1 to 39.6 nM and from 16.3 to 27.7 nM). After the last nitrous oxide anesthesia, bone marrow smears from both patients revealed macrocytic changes. As reported by others (3), we found that during nitorus oxide exposure plasma methionine decreased (n=40, p<0.025) (figure 7.2, bottom). The percentage change in plasma methionine inversely correlated with the duration of nitrous oxide anesthesia (n=40, r_s=- 0.29, p<0.025).

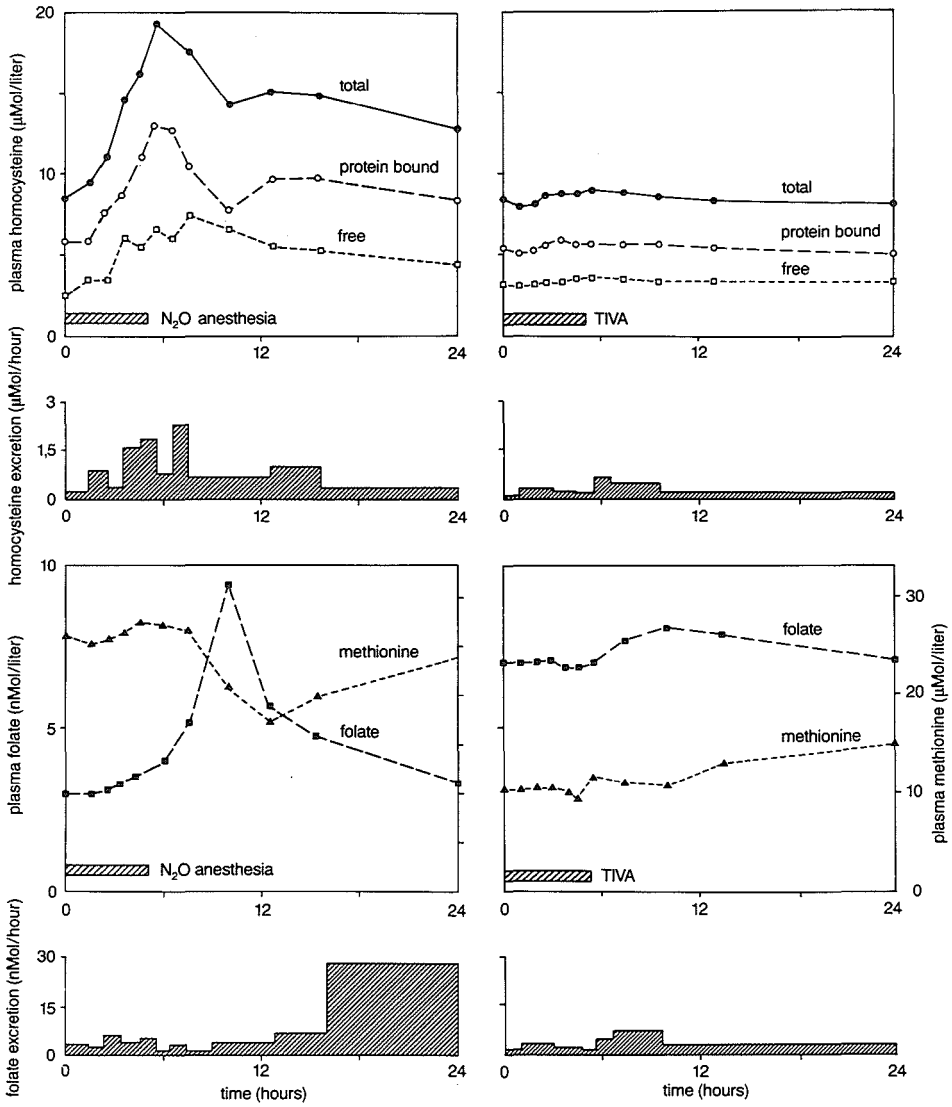


Figure 7.4: Time course of changes in studied plasma and urinary parameters in a patient (nr 25) anesthetized with nitrous oxide (left) and a patient (nr 49) anesthetized with total intravenous anesthesia (right).

Effect of anesthesia on urinary excretion of folate and homocysteine. During the first 24 hours after the start of anesthesia, the urinary folate (figure 7.3A) and homocysteine excretions (figure 7.3B) of patients anesthetized with nitrous oxide were significantly higher than those of patients anesthetized with TIVA ($p<0.002$ and $p<0.01$

Table 7.2:

Plasma folate and homocysteine levels 1, 2 and 7 days after anesthesia with nitrous oxide or total intravenous anesthesia.

	PLASMA FOLATE (nM)		PLASMA HOMOCYSTEINE (μM)	
Group:	N_2O (n = 8)	TIVA (n = 6)	N_2O (n = 8)	TIVA (n = 6)
Start of anesthesia:	7.9 ± 1.3	10.8 ± 3.0	10.2 ± 0.6	13.1 ± 1.8
End of anesthesia:	11.7 ± 1.9**	9.0 ± 2.3	18.0 ± 1.9**	11.1 ± 0.8
1 day after anesthesia:	10.5 ± 1.2**	9.2 ± 2.6	18.4 ± 2.1**	8.9 ± 1.1*
2 days after anesthesia:	8.0 ± 1.1	10.3 ± 3.0	15.6 ± 1.4**	12.0 ± 1.9
1 week after anesthesia:	6.4 ± 1.3	N.D.	15.1 ± 1.6**	N.D.

(*: $p < 0.05$, **: $p < 0.01$)

respectively). The increased homocysteine and folate excretion in the patients correlated with the duration of nitrous oxide anesthesia ($n=19$, $r_s=0.62$, $p<0.005$ and $n=19$, $r_s=0.60$, $p<0.01$ respectively). Postoperative plasma homocysteine, correlated with urinary homocysteine ($n=19$, $r_s=0.43$, $p<0.05$) whereas no correlation was found between postoperative plasma folate and urinary folate excretion.

Time course of induced changes. From six patients anesthetized with nitrous oxide for various time periods and 4 patients anesthetized with TIVA, blood samples were collected at regular intervals for 24 hours in order to study changes in plasma folate, homocysteine and methionine as a function of time. Figure 7.4 shows the typical results of patients anesthetized with nitrous oxide (left) and TIVA (right) respectively. In patients anesthetized with TIVA only minor fluctuations in the studied parameters occured and no significant pattern evolved.

In patients operated on under nitrous oxide anesthesia, plasma methionine usually declined after a lag phase of 3-6 hours. Plasma folate and homocysteine progressively increased during nitrous oxide anesthesia. In most patients, maximal plasma folate and homocysteine were reached at the end or a few hours after termination of anesthesia. Then, plasma folate and homocysteine gradually declined. Increase and decrease of total homocysteine did not change the ratio between free and protein-bound homocysteine (figure 7.4).

From the same group of 6 patients operated on under nitrous oxide anesthesia and 4 patients operated on under TIVA, fractionated urine was collected during 24 hours for determination of folate and homocysteine excretion. Elevated homocysteine and

folate excretion occured several hours after the start of nitrous oxide anesthesia (figure 7.4, left), causing up to 20-fold increase of urinary concentrations of both compounds. In patients anesthetized with TIVA only diurnal variations in urinary folate and homocysteine excretion occured (figure 7.4, right).

Long term effects. From 8 patients operated on under nitrous oxide anesthesia and from 6 patients operated on under TIVA, plasma homocysteine and plasma folate were determined 1 day, 2 days and 1 week after anesthesia (table 7.2). No specific patterns developed in the group of patients anesthetized with TIVA. In patients anesthetized with nitrous oxide anesthesia, elevated plasma folate levels usually returned to preoperative values within 2 days. However, in all patients anesthetized with nitrous oxide plasma homocysteine was still increased 2 days and one week after anesthesia ($n=8$, $p< 0.01$).

7.5. Discussion

Nitrous oxide is the only compound known to inactivate the methylcobalamin coenzyme of methionine synthase which is an important enzyme of the folate metabolism. Possible side effects of this interaction are clinically relevant, in view of the widespread application of nitrous oxide in anesthesia.

Earlier studies have shown that in fit surgical patients, disturbance of the folate-dependent thymidylate synthesis occurs after 5-6 hours of nitrous oxide anesthesia (16). Megaloblastic bone marrow changes occur in fit patients after exposure to nitrous oxide for 12-24 hours. The chance that anesthesia including nitrous oxide becomes hematotoxic is generally considered to be small because duration of nitrous oxide exposure is usually too short to interfere seriously with the cobalamin-dependent folate metabolism. However, the rapid rise of plasma homocysteine and folate during nitrous oxide anesthesia found in this study indicates that perturbation of folate and homocysteine metabolism develops with little delay. The megaloblastic bone marrow changes seen in critically ill patients after less than 2 hours exposure nitrous oxide (5) and the highly increased bone marrow toxicity of methotrexate when administered after nitrous oxide anesthesia may well be attributed to this phenomenom (17,18).

The observed differences in the individual response to nitrous oxide induced cobalamin inactivation concurs with the observation of Nunn et al. who showed that the rate of methionine synthase inhibition during nitrous oxide anesthesia is variable (19). In addition, this phenomenom may be related to the differences in the existing cobalamin and folate status because in our study low preoperative folate correlated positively with the increase of plasma homocysteine during nitrous oxide anesthesia. Apparently, low preoperative plasma folate reflects a relative lack of intracellular folates which aggrevates the effects of nitrous oxide on the cobalamin-dependent methylation of homocysteine.

The plasma homocysteine levels after nitrous oxide exposure frequently increased above normal. In several patients the plasma homocysteine levels were comparable with those in cobalamin deficiency (9). These findings further support the suggestion that plasma homocysteine may be a useful parameter to measure disturbances of the cobalamin-dependent folate metabolism (9). Concentration of homocysteine in urine

may also be a good indicator as the enhanced renal excretion of this amino acid strongly correlates with duration of nitrous oxide anesthesia. The demonstrated increase in urinary folate agrees with similar observations in rats (20) but is difficult to explain as folate plasma values rarely became abnormal. Possibly, the reuptake of urinary folates by renal tubular cells is also affected by the inhibition of methionine synthase causing elevated excretion.

In most tissues, the cobalamin-dependent methylation of homocysteine to methionine is a quantitatively important pathway of homocysteine disposal (21). Our finding that plasma homocysteine is still elevated one week after nitrous oxide anesthesia implies that methionine synthase activity recovers very slowly. Slow recovery of enzyme activity may explain why the increase of plasma homocysteine was most pronounced in our 2 patients receiving multiple nitrous oxide exposures. Notably, subjects who have repeatedly been exposed to nitrous oxide within a short period, become particularly vulnerable to developing megaloblastosis (22,23).

In summary, this study demonstrates that the effect of cobalamin inactivation by nitrous oxide can be monitored by the rapid increase in plasma homocysteine. In addition, it shows that nitrous oxide anesthesia perturbs cobalamin-dependent folate and homocysteine metabolism at a faster rate than has been hitherto re cognized and that recovery of methionine synthase activity probably requires several days, even after short exposure.

7.6. References.

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Chapter 8

General discussion

Cobalamin, or vitamin B12, is a coenzyme of methionine synthase. This enzyme concomitantly converts homocysteine and 5-methyltetrahydrofolate to methionine and tetrahydrofolate. The latter favors the polyglutamation of intracellular folates to less exchangeable forms and can be converted to other folate coenzymes. Several steps in the *de novo* synthesis of thymidylate and purines, necessary for DNA-replication and RNA-synthesis respectively, require folate coenzymes. In man, the proliferation of the hematopoietic compartment is very dependent on the intact function of the cobalamin-dependent folate metabolism as is clear from the megaloblastic bone marrow depression which may develop during cobalamin or folate deficiency.

The anesthetic gas nitrous oxide is capable to oxidize the monovalent cobalt atom of the cobalamin coenzyme of methionine synthase. This inactivation of cobalamin causes the complete inhibition of methionine synthase activity. It has been shown that prolonged exposure to nitrous oxide can therefore produce megaloblastic changes in the bone marrow, similar to cobalamin deficiency.

The widespread clinical use of nitrous oxide has evoked general interest in possibly toxic side effect of this anesthetic gas. Since the discovery of *in vivo* cobalamin inactivation by nitrous oxide in 1978, extensive investigations on the hematological and metabolic consequences of this phenomenom have been performed. However, most clinical studies have focused on nitrous oxide exposure periods of 12-24 hours (1,2) and metabolic studies usually have been performed in test animals.

In the present thesis the possible hematological and metabolic effects of shorter periods of nitrous oxide exposure were investigated in patients anesthetized with nitrous oxide (chapter 6,7). The determination of bone marrow progenitors and plasma homocysteine in patients anesthetized with nitrous oxide allowed a more accurate evaluation of the effects of cobalamin inactivation. It can be concluded that nitrous oxide exposure up to 12 hrs changes the proliferation kinetics of the hematopoietic progenitors but does not disturb normal bone marrow function. However, the sustained elevation of plasma homocysteine one week after anesthesia indicates that nitrous oxide may result in long term inhibition of the cobalamin-dependent remethylation of homocysteine to methionine.

The importance of the various folate coenzymes for the DNA synthesis of proliferating cells makes them an attractive target in cancer chemotherapy. This is illustrated by the current application of several structural analogues of reduced folates (e.g. methotrexate) in the treatment of cancer. Studies on the possible application of cobalamin inactivation in the treatment of malignant diseases has always been hampered by the absence of biologically active cobalamin antagonists. The discovery that nitrous oxide effectively disturbs the coenzyme function of cobalamin in methionine synthase provides a tool to study cobalamin inactivation as a potential target in cancer chemotherapy.

Kroes et al. (3) have shown that exposure to nitrous oxide may reduce the proliferation of leukemic cells in a rat model for myeloid leukemia. Moreover they demonstrated that the antileukemic effect of nitrous oxide may be further enhanced by combinations with drugs (4-7) which interfere with other steps of the one carbon metabolism. Especially the antiproliferative effect of nitrous oxide in combination with 5-fluorouracil, an inhibitor of the folate-dependent enzyme thymidylate synthase or with methotrexate, which inhibits the enzyme dihydrofolate reductase, was striking.

This potentiation of methotrexate by nitrous oxide in a rat leukemia model (5) initiated further research on this particular drug interaction. Studies in normal rats (chapter 3) reveal that the cytotoxicity of the nitrous oxide-methotrexate combination is restricted to dividing cells. Biochemical investigations in fresh human leukemic cells (chapter 4,5) show that the antiproliferative effect of nitrous oxide-methotrexate interaction is probably based on the disturbance of the cellular folate metabolism (chapter 5) impairing the folate- dependent thymidylate synthesis (chapter 4). The observed accumulation of hematopoietic progenitors in nitrous oxide exposed rats (chapter 3) concurs with a similar accumulation of both myeloid and erythroid progenitors in patients exposed to nitrous oxide (chapter 6). This finding suggests synchronisation of progenitor cells and may also contribute to observed myelotoxicity of methotrexate in nitrous oxide- exposed patients (8). Altogether the results of these studies contribute to a better understanding of the unexpected toxic side effects which may be seen in patients treated with methotrexate shortly after or during anesthesia including nitrous oxide (8). In addition, the presented data support earlier suggestions (5,9) on the possible application of combined nitrous oxide-methotrexate exposure in the chemotherapeutic treatment of malignancies.

The application of cobalamin inactivation by nitrous oxide in the treatment of hematological malignancies in humans has only been studied casually (10,11,12). Exposure of leukemic rats of the Brown Norway Myeloid Leukemia model to nitrous oxide shows that leukemic cells are more sensitive to nitrous oxide than normal hematopoietic cells (chapter 2). This finding suggests that in humans, leukemic proliferation may also be more vulnerable towards the effects of nitrous oxide-induced cobalamin inactivation.

The present thesis provides *in vitro* evidence that the cobalamin- dependent folate metabolism of most types of human leukemia is sensitive to nitrous oxide; besides disturbance of the folate dependent thymidylate synthesis (chapter 4), a significant redistribution of the various intracellular folate coenzymes has been demonstrated (chapter 5). In addition it is shown that nitrous oxide exposure up to 12 hours hardly interferes with normal hematopoiesis (chapter 6).

Altogether, the acquired knowledge on this subject has reached the point of which the experimental application of nitrous oxide in the chemotherapy of human leukemia seems justified. The existing clinical data on nitrous oxide exposure of leukemia patients (10,11,12) suggest that effective treatment requires long (intermittent) exposure periods. Today, a massive amount of information exists on the toxic effects of nitrous oxide on normal bone marrow function. However, the effect of cobalamin-inactivation on residual normal hematopoiesis in acute leukemia has only been studied in rats (chapter 2). The experimental use in cancer chemotherapy therefore requires a careful approach. This thesis shows that determination of plasma homocysteine may provide a reliable and practical tool to monitor the process of cobalamin inactivation during exposure to nitrous oxide. Moreover, earlier studies (13) have shown that effects of nitrous oxide-induced cobalamin inactivation in man can easily be curtailed by the administration of 5- formyltetrahydrofolate. The availability of this effective antidote in case of nitrous oxide-induced bone marrow depression further favors the treatment of a selected group of leukemia patients with nitrous oxide exposure.

The use of an anesthetic gas for chemotherapeutic purposes may be considered unpractical. Still, the extensive knowledge on the administration of nitrous oxide among anesthesiologists should make its application feasible. In this way, the common interest among hematologists and anesthesiologist for the toxic side effects of nitrous oxide-induced cobalamin inactivation may perhaps evolve in a interdisciplinary method to reduce the proliferation of malignant blood cells.

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SUMMARY

This thesis deals with the biochemical and clinical sequelae of nitrous oxide-induced cobalamin inactivation. Cobalamin or vitamin B_{12} is, as methylcobalamin a necessary coenzyme in methionine synthase. This particular enzyme converts internalized 5-methyltetrahydrofolate to tetrahydrofolate, which can further be metabolized to other, less exchangeable folate forms. In this reaction homocysteine is concomitantly methylated to methionine. Several steps in the biosynthesis of nucleotides which are necessary for DNA replication, require folate coenzymes. The immediate importance of cobalamin-dependent folate metabolism for proliferating cells is evidenced by its principal role in the maintenance of human hematopoiesis. As far as known, the anesthetic gas nitrous oxide is the only agent capable of inactivating the methylcobalamin coenzyme. Nitrous oxide thereby reduces methionine synthase activity and disturbs intracellular folate metabolism. In humans, prolonged exposure to nitrous oxide leads to impaired hematopoiesis as a result of decreased folate-dependent thymidylate synthesis, and hence DNA replication.

In chapter 1 the biochemistry of cobalamin-dependent folate metabolism and the biochemical and clinical effects of nitrous oxide exposure are reviewed.

In chapter 2, studies on the differential effect of nitrous oxide on normal and malignant hematopoiesis are presented. It was shown that significant reduction of leukemic proliferation can be achieved in the Brown Norway Myeloid Leukemia rat model for myeloid leukemia by exposure to 50% nitrous oxide/50% oxygen. This treatment did not further enhance the suppression of normal hematopoiesis which already was present because of the leukemic bone marrow infiltration. In normal rats a similar exposure to nitrous oxide also did not suppress normal hematopoiesis as wittnessed by peripheral blood parameters and hematopoietic progenitor cells present in the bone marrow. The BNML is considered to be a good model for the chemotherapeutic response of human myeloid leukemia. We therefore concluded that the presented data may also implicate a greater sensitivity of human leukemia for nitrous oxide-induced cobalamin inactivation compared with normal hematopoiesis.

Chapter 3 contains the results of an extensive toxicological study on the interaction between nitrous oxide and the folate antagonist methotrexate. It was shown that nitrous oxide exposure of rats for 48 hours decreased the LD_{50} of methotrexate from 60 mg/kg to 10 mg/kg. Major toxicity occurred in the gastrointestinal tract, causing severe diarrhoea, and the bone marrow, causing leucocytopenia and thrombocytopenia. No important damage to liver or kidney occurred. Lethal toxicity could be prevented by the administration of 5-formyl THF. It was concluded from this study that as nitrous oxide strongly potentiates the cytotoxicity of methotrexate, the unintentional use of this anesthetic gas shortly before or during treatment with methotrexate should be avoided. However, the antiproliferative effect of the nitrous oxide-methotrexate combination merits further investigation of its possible application in the treatment of malignancies.

In chapter 4 the effect of nitrous oxide on the folate metabolism of human leukemia cells was studied *in vitro*, employing the deoxyuridine suppression test. Leukemic cells from bone marrow and peripheral blood of 18 patients and from the myelomonocytic cell line U937 were exposed to nitrous oxide for 20 hours before they

were subjected to the deoxyuridine suppression test. In all leukemic samples, nitrous oxide induced at least 200% increase of the deoxyuridine suppression test value, implicating a disturbance of folate-dependent thymidylate synthesis. Incubation of leukemic cells with drugs, which interfere with the one-carbon metabolism (methotrexate, 5-fluorouracil, cycloleucine) also caused decrease of folate-dependent thymidylate synthesis which was further enhanced by concomitant exposure to nitrous oxide. From these data it appeared that many types of human leukemia are sensitive to cobalamin inactivation by nitrous oxide. Moreover it was concluded that the effect of drugs interfering with folate metabolism on the folate- dependent thymidylate synthesis, may be enhanced by nitrous oxide.

Further biochemical investigations on the effects of nitrous oxide and methotrexate on the folate metabolism of leukemic cells are presented in chapter 5. Folate pools of blast cells from 10 cases of acute myeloid leukemia and 5 cases of lymphoid leukemia were radiolabeled by incubation with [^{3}H] 5-formyltetrahydrofolate. Folate pools were subsequently analysed by means of High Performance Liquid Chromatography. It was found that concentrations of 10-formyltetrahydrofolate and dihydrofolate in cells from acute myeloid leukemia and acute lymphoid leukemia are significantly different. Incubation of the radiolabeled leukemic cells with nitrous oxide or methotrexate caused accumulation of respectively 5-methyltetrahydrofolate or dihydrofolate, at the expense of other folate forms. The presence of substantially more dihydrofolate in cells from acute lymphoid leukemia together with a specific decrease of 10-formyltetrahydrofolate during methotrexate treatment probably contributes to the efficacy of methotrexate in the treatment of acute lymphoid leukemia.

Chapter 6 presents a clinical study in which the effects of nitrous oxide anesthesia for 7-12 hours on normal hematopoiesis were investigated. Bone marrow samples from 14 patients operated on under nitrous oxide were obtained at start and end of surgery. Macrocytic changes of the bone marrow cells only occurred in 2 patients exposed to nitrous oxide for more than 24 hours in a three day period. Disturbed deoxyuridine suppression test values were noticed in all patients operated on under nitrous oxide anesthesia but not in the control group of 4 patients operated on under total intravenous anesthesia. Myeloid and erythroid progenitors present in the bone marrow and cultured *in vitro*, increased during nitrous oxide exposure of patients with normal plasma cobalamin and folate levels. However, in patients with a low plasma cobalamin and/or folate level, both type of hematopoietic progenitors decreased in bone marrow during nitrous oxide exposure. Still, the number of progenitor cells present in bone marrow after nitrous oxide exposure never decreased below the lower limit of normal values. It was therfore concluded that although nitrous oxide induced changes in the proliferation kinetics of the hematopoietic progenitor cells, the toxic effect of this anesthetic gas on hematopoiesis is rather limited within the studied exposure periods.

Chapter 7 comprises an extensive study on the effects of nitrous oxide anesthesia on homocysteine and folate levels in plasma and urine of surgical patients. In patients subjected to anesthesia with nitrous oxide or operated on under total intravenous anesthesia, plasma levels of homocysteine, methionine and folate were determined at start and end of anesthesia. It was found that already short term nitrous oxide exposure elevated plasma homocysteine and folate and decreased plasma methionine.

Increased urinary excretion of both folate and homocysteine was also observed. Moreover it was shown that plasma levels of homocysteine and folate continued to rise for several hours after withdrawal of nitrous oxide anesthesia. As the increase in plasma homocysteine correlated highly with the length of nitrous oxide exposure, it was concluded that the rise of plasma homocysteine is a reliable indicator for nitrous oxide-induced cobalamin inactivation. Moreover, the presented data suggested that effects of nitrous oxide on homocysteine and folate metabolism develop more rapidly than presently supposed.

Finally in chapter 8 the results of these investigations are discussed in view of the current knowledge on the role of the cobalamin-dependent folate metabolism in normal and malignant hematopoiesis.

SAMENVATTING

Cobalamine, of vitamine B_{12} speelt een belangrijke rol in de intracellulaire foliumzuurstofwisseling door zijn functie als coenzym van methionine synthase. Dit enzym is van essentieel belang voor het behoud van intracellulaire gereduceerde foliumzuurvormen. Nieuw opgenomen 5-methyltetrahydrofolaat wordt door methionine synthase gedemethyleerd tot tetrahydrofolaat en vervolgens omgezet in andere folaatvormen welke minder snel de cel kunnen verlaten. Verschillende folaten zijn als coenzym betrokken by de biosynthese van nucleotides, welke noodzakelijk zijn voor DNA synthese. De instandhouding van de humane hematopoëse is dan ook afhankelijk van een normale activiteit van het cobalamine coenzym in de foliumzuurstofwisseling. Voor zover bekend is het anestheticum lachgas de enige verbinding die in staat is het cobalamine coenzyme van methionine synthase te inactiveren. Lachgas remt daardoor de aktiviteit van het enzym methionine synthase en kan zodoende de intracellulaire foliumzuurstofwisseling verstoren. Bij de mens leidt langdurige lachgasblootstelling tot een verminderde hematopoëse doordat de foliumzuurafhankelijke thymidylaatsynthese verstoord raakt en daarmee de DNA replicatie geremd.

In dit proefschrift worden studies beschreven welke betrekking hebben op de hematologische, biochemische en klinische facetten van lachgas geinduceerde cobalamine inactivatie. Hoofdstuk 1 omvat een overzicht van de biochemie van de cobalamine afhankelijke foliumzuurstofwisseling. Tevens worden hierin de huidige inzichten in de biochemische en klinische gevolgen van lachgasblootstelling samengevat.

In hoofdstuk 2 worden studies met betrekking tot het differentiele effect van lachgas op de normale en maligne (=leukemische) hematopoëse gepresenteerd. Indien leukemische ratten van het Brown Norway Myeloid Leukemia rattemodel blootgesteld werden aan een gasmengsel van 50% lachgas/50% zuurstof, trad er een duidelijk vertraging van het leukemische proces op. Tevens werd waargenomen dat lachgas blootstelling van de leukemische BN ratten geen extra schade toebracht aan de normale hematopoëse welke onder invloed van de leukemische beenmerginfiltratie reeds sterk verminderd was. In gezonde BN ratten had lachgas evenmin nadelige effecten of de normale hematopoëse, hetgeen onderzocht werd aan de hand van perifere bloedparameters en het aantal aanwezige hematopoëtische voorlopercellen in het beenmerg. Aangezien het BNML ratteleukemie model beschouwd wordt als een goed model voor de chemotherapeutische respons van myeloide leukemie, lijken de beschreven bevindingen te wijzen op een grotere gevoeligheid van humane leukemie voor lachgas geinduceerde cobalamine inactivatie dan voorlopercellen van de normale hematopoëse.

Hoofdstuk 3 bevat de resultaten van een studie naar de toxicologische interactie tussen lachgas en de foliumzuurantagonist methotrexaat. In ratten welke voor 48 uur aan lachgas waren blootgesteld verminderde de LD_{50} waarde voor methotrexaat van 60 mg/kg naar 10 mg/kg. Toxiciteit trad met name op in de darmen, met ernstige diarrhee als gevolg, en in het beenmerg hetgeen tot leukopenie en thrombopenie leidde. Toxiciteit voor lever en nieren was niet aantoonbaar. Lethale effecten van de lachgas-methotrexaat combinatie konden voorkomen worden door de toediening van

5-formyltetrahydrofolaat. Deze studie toonde duidelijk aan dat lachgas in staat is de cytotoxiciteit van methotrexaat te potentiëren. Gekonkludeerd werd dat het onbedoelde gebruik van lachgas in combinatie met methotrexaat voorkomen moet worden. De toepassing van de lachgas-methotrexaat combinatie in de chemotherapeutische behandeling van maligniteiten verdient echter verdere studie.

In hoofdstuk 4 wordt het *in vitro* effect van lachgas op de foliumzuurstofwisseling van humane leukemiecellen bestudeerd met behulp van de deoxyuridine suppressietest. Leukemiecellen uit het beenmerg en/of bloed van 18 patienten en van de myelomonocytaire cellijn U937 werden gedurende 20 uur blootgesteld aan lachgas waarna de deoxyuridine suppressie van de thymidine inbouw werd gemeten. In alle gevallen veroorzaakte de lachgasblootstelling een verhoging van de deoxyuridine suppressietestwaarde van ongeveer 200%, hetgeen een sterke verstoring van de foliumzuurafhankelijke thymidylaatsynthese impliceert. Indien leukemische cellen werden geincubeerd met andere verbindingen die interferen met de foliumzuurstofwisseling (methotrexaat, 5- fluorouracil, cycloleucine) trad er eveneens een verstoring van de foliumzuurafhankelijke thymidylaat synthese op welke versterkt kon worden door de gelijktijdige blootstelling aan lachgas. De gepresenteerde gegevens duiden erop dat bijna alle vormen van humane leukemie gevoelig zijn voor lachgas-geinduceerde cobalamine inactivatie. Tevens kan geconcludeerd worden dat de effecten van enkele verbindingen die eveneens interfereren met de foliumzuurstofwisseling versterkt kunnen worden met lachgas.

Hoofdstuk 5 bevat de resultaten van verdergaande biochemische studies naar de effecten van lachgas en/of methotrexaat op de foliumzuurstofwisseling van humane leukemiecellen. De intracellulaire foliumzuurvormen van leukemiecellen afkomstig van 10 acute myeloide leukemieen en 5 lymfatische leukemieen werden radioactief gemerkt door incubatie met [^{3}H]5-formyltetrahydrofolaat. De verschillende foliumzuurvormen werden vervolgens gekwantificeerd met behulp van een High Performance Liquid Chromatography-scheiding. Cellen van acute myeloide en lymfatische leukemie verschilden duidelijk in de verdeling van foliumzuurcoenzymen. Blootstelling van leukemische cellen aan lachgas of methotrexaat veroorzaakte de intracellulaire ophoping van respectievelijk 5-methyltetrahydrofolaat en dihydrofolaat ten koste van de andere foliumzuurvormen. In cellen van acute lymfatische leukemieën werden aanzienlijke concentraties dihydrofolaat aangetoond. Tevens werd in cellen van deze vorm van leukemie tijdens blootstelling aan methotrexaat een specifieke daling van 10-formyltetrahydrofolaat waargenomen. Deze waarnemingen kunnen mogelijkerwijs de relatief grote effectiviteit van methotrexaat in de behandeling van lymfatische leukemie mede verklaren.

Hoofdstuk 6 omvat de resultaten van een klinische studie waarin het effect van lachgasblootstelling op de normale hematopoëse onderzocht werd. Van 14 patienten welke geopereerd werden onder anesthesie met lachgas (varierend van 7-12 uur) werd aan het begin en aan het einde van de ingreep beenmerg afgenomen. Macrocytaire veranderingen van het beenmerg werd alleen waargenomen in 2 patienten welke meer dan 24 uur lachgasblootstellingen ondergingen in een tijdsbestek van 3 dagen. De deoxyuridine suppressietest was na afloop van de anesthesie alleen verstoord in beenmergcellen van patienten welke aan lachgas blootgesteld waren. In de 4 controle patienten welke geopereerd waren onder totale intraveneuze narcose trad geen

verandering in de deoxyuridine suppressietestwaarde op. In patienten met normale plasma cobalamine en foliumzuur waarden veroorzaakte de lachgasblootstelling een stijging van het aantal erythroide en myeloide voorlopercellen in het beenmerg. In patienten met lage plasma cobalamine en foliumzuur waarden leidde lachgasblootstelling tot een daling van deze hematopoëtische voorlopercellen in het beenmerg. Het aantal voorlopercellen in het beenmerg bleef bij praktisch alle patienten echter binnen het normaalwaarden gebied. De bevindingen van deze studie impliceren een verandering in de proliferatiekinetiek van de hematopoetische voorlopercellen tijdens lachgasblootstelling. Binnen de bestudeerde blootstellingsperiode (7-12 uur) blijkt het hematotoxische effect van lachgas voor niet ernstig zieke patienten gering.

Hoofdstuk 7 beschrijft een onderzoek naar de effecten na lachgas anesthesie op homocysteine en foliumzuurconcentraties in bloed en urine van operatie patienten. Daartoe werd van een aantal patienten die geopereerd werden onder anesthesie met lachgas en van een aantal patienten die geopereerd werden onder totale intraveneuze anesthesie aan het begin en eind van de ingreep bloed afgenomen van de bepaling van homocysteine, foliumzuur en methionine in plasma. Al na kortdurende lachgasblootstelling (75 minuten) bleken homocysteine en foliumzuur gestegen te zijn terwijl plasma methionine daalde. De excretie van homocysteine en foliumzuur via de urine was in de patienten die anesthesie met lachgas ondergingen eveneens gestegen. Afname van plasma homocysteine en foliumzuur na afloop van de lachgas anesthesie verliep meestal zeer langzaam. De toename in plasma homocysteine correleerde zeer sterk met de blootstellingsduur aan lachgas. Plasma homocysteine werd daarom beschouwd als een indicator voor cobalamine inactivatie. Tevens blijkt uit de gepresenteerde gegevens dat de effecten van lachgas op de homocysteine en foliumzuurstofwisseling zich sneller manifesteerden dan tot nu toe werd aangenomen.

In hoofdstuk 8 worden de resultaten van dit proefschrift besproken in samenhang met de huidige kennis omtrent de rol van de cobalamine-afhankelijke foliumzuurstofwisseling in de normale en maligne hematopoëse.

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 30 juli 1959 geboren te Bavel. Hij doorliep de middelbare school in Breda en behaalde in 1977 het eindexamen Atheneum-B aan de Newman scholengemeenschap. In 1978 werd begonnen met de studie biologie aan de Rijks Universiteit Utrecht. Na het behalen van het kandidaatsexamen in 1982 werd de studie voortgezet aan de Landbouwhogeschool te Wageningen. Het ingenieursexamen werd aldaar in 1985 afgelegd. Vanaf april 1985 tot jan 1990 werd het hier beschreven onderzoek verricht op het Instituut Hematologie van de Erasmus Universiteit Rotterdam onder leiding van Prof.dr. J. Abels en dr. J. Lindemans.

NAWOORD

Het hier voor u liggende proefschrift had nooit tot stand gekomen zonder de hulp van een groot aantal personen.

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