THE INACTIVATION OF COBALAMIN BY NITROUS OXIDE:
application in experimental chemotherapy of leukemia

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HET INACTIVEREN VAN COBALAMINE DOOR LACHGAS:
toepassing in experimentele chemotherapie van leukemie

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Ter nagedachtenis aan mijn moeder
Aan mijn vader
Aan Christi

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INTRODUCTION

1.1. The significance of cobalamin in normal and malignant hematopoiesis.

1.1.1. Cobalamin, folic acid and megaloblastic anemia.

Cobalamin, also widely known as vitamin B12, and folic acid are two constituents of the diet which are essential to maintain the continuous production of blood cells in the bone marrow. Blood cells fulfil a wide range of vital functions, but most types have only a limited life span, and therefore the lifelong process of their production, called hematopoiesis, is of great importance to prevent disease.

The discovery in this century of cobalamin and folic acid as dietary factors involved in hematopoiesis has been a major medical breakthrough, because this has made possible the treatment and prevention of serious diseases caused by a lack of these vitamins. Most important in this respect has been the successful treatment of pernicious anemia. In this disease, which was already recognized by Addison in 1849 and by Ehrlich in 1880, hematopoiesis is severely disturbed, resulting in a serious and usually fatal anemia with abnormally large cells in the bone marrow, which were called megaloblasts. In 1926, Minot and Murphy discovered a successful therapy for this disorder, consisting of a diet rich in liver (1). This approach fitted well in the concept of specific nutritional deficiencies as causes of disease, which was just emerging at that time. The pathogenesis of pernicious anemia appeared to be more complex, however, when it was shown by Castle in 1929 (2), that not only this dietary (extrinsic) factor was necessary to prevent anemia, but also a factor present in gastric juice, which he called intrinsic factor. This factor appeared to facilitate specifically the absorption of the dietary factor. Subsequently, the extrinsic factor was purified from the crude liver extracts and crystallized in 1948, by Rickes et al. (3), who named this substance vitamin B12, and by Lester Smith (4). Finally, its complex structure as a porphyrin-like molecule containing cobalt, hence its name cobalamin, was elucidated by Hodgkin in 1956 (5). For their contributions, Minot and Murphy (1934) and Hodgkin (1964) were awarded Nobel Prizes. Pernicious anemia, which is the consequence of the selective malabsorption of cobalamin, due to a lack of Castle's gastric intrinsic factor, had become a treatable disease. The cause of the underlying gastric disease, autoimmune atrophic gastritis, is still not clear, nor can it be cured, but its life-threatening hematologic complication is now completely prevented by

the parenteral administration of cobalamin.

Some confusion resulted in the 1930s from the recognition, by Wills (6), of a second dietary factor which was effective in the treatment of megaloblastic anemia, in particular of cases occurring as a complication of pregnancy and tropical sprue. This factor was present in yeast (6) and appeared to be distinct from the liver factor (7). It turned out to be identical to an ubiquitous growth factor that was named folic acid in view of its isolation from plant leaves (8). The structure of this compound was described in 1946 (9). The existence of folate deficiency as another vitamin deficiency syndrome, with similar hematological consequences but separate from pernicious anemia, was clearly established around 1950 (10).

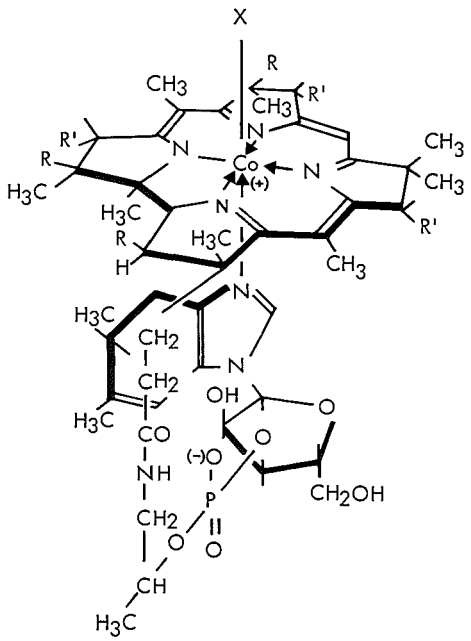
A deficiency of either cobalamin or folic acid clinically results in the same, rather typical, aberration in the blood and bone marrow. The intense cellular proliferation, which is characteristic of hematopoiesis, is severely impaired in this condition and maturation of blood cells is much slower, resulting in reduced numbers of abnormally large cells. These clinical observations on the effects of deficiency states immediately suggest that the two vitamins are functionally closely related, and ever since their characterization, the interrelationships of cobalamin and folic acid have been the subject of intensive research. Apart from the clinical relevance of this problem, this interest is also explained by its obvious importance to the understanding of metabolic processes involved in cellular proliferation. The issue is still not completely settled to date, but the expansion of knowledge on this subject is apparent from the many reviews that have been published from 1951 until recently (10-20). The subject of this introductory chapter will be a discussion of our present knowledge of the function of cobalamin and folic acid in normal and malignant hematopoiesis.

1.1.2. Biochemistry of cobalamin and folic acid.

With regard to their chemical structure, cobalamin and folic acid are entirely unrelated. Several forms exist of both vitamins, related to different coenzyme functions. Thus, the terms "cobalamin" and "folic acid" in fact both refer to a group of related compounds.

Cobalamin is the parent compound of a specific subgroup of the corrinoids (21). Corrinoids (from "core" of vitamin B12 structure) are characterized by the corrin nucleus, consisting of four reduced pyrrole rings. Three of the links between these pyrrole rings contain a single carbon, and one link is direct. Corrin derivatives which contain a cobalt atom in the centre of the macrocyclic tetrapyrrole ring, carry a series of trivial names beginning with cob-. The corrinoids have a number of side chains added to the corrin structure as well. In the case of cobalamin, there is a specific pattern of side chains, and of ligands bound to the central cobalt atom, as shown in Figure 1.1. This central cobalt atom has six coordinating bonds,

four of which are occupied by nitrogens of the corrin nucleus. Of the other positions, one is always occupied by the 3-N-atom of a heterocyclic base, 5,6-dimethylbenzimidazole, an addition that is specific for cobalamin. The sixth position can be occupied by various ligands, as shown in 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' = $CH_2CH_2CONH_2$

Figure 1.1. The structure of cobalamin, with the various ligands that can occupy the sixth coordinate bond of the cobalt ion in the naturally occurring forms of this vitamin. The cobalt-containing corrin-nucleus is synthesized by bacteria only and, like the heme-nucleus, derived from the pathway of porphyrin synthesis.

Four forms of cobalamin have been identified in man: cyano-, hydroxo- (or aquo- at lower pH), adenosyl- and methylcobalamin. Only adenosyl- and methylcobalamin are known to have coenzyme functions in human cells (22).

Adenosylcobalamin is the coenzyme of methylmalonylcoenzyme A mutase, a mitochondrial enzyme converting methylmalonylCoA into succinylCoA (23). This reaction involves a unique carbon skeleton rearrangement, of which the exact mechanism is still controversial (24-26). Methylmalonic acid is formed by the degradation of odd-chain fatty acids and some amino acids. A deficiency of cobalamin leads to increased levels of methylmalonic acid in plasma and urine (27). Folic acid is not involved in this pathway.

Methylcobalamin is essential in the enzyme methionine synthetase, or 5-methyltetrahydrofolate homocysteine methyltransferase (28-30). As is evident from the latter name, this enzyme is also involved in folate metabolism. Not surprisingly, therefore, explanations of the related functions of cobalamin and folic acid in hematopoiesis focus on this enzyme. The enzyme catalyzes the transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine, which results in the formation of tetrahydrofolate and methionine (Figure 1.2). Interestingly, no role for cobalamin has been established in any process more directly related to cellular proliferation, like the synthesis of nucleic acids.

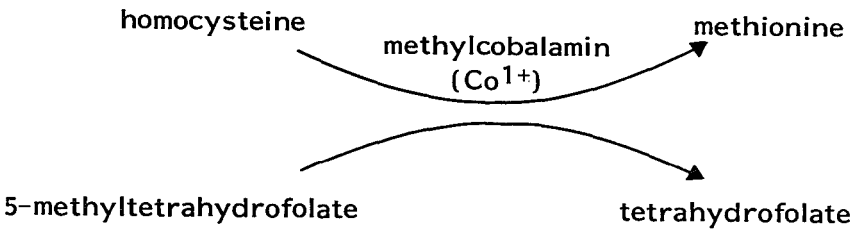


Figure 1.2. The reaction catalyzed by the cobalamin-dependent enzyme methionine synthetase (E.C. 2.1.1.13, recommended name: tetrahydropteroylglutamate methyltransferase).

Regarding the chemistry of cobalamins, the oxidation state of the cobalt atom is also relevant to coenzyme functions. In cobalamin, cobalt can occur in its three usual oxidation states, which are designated cob(I)alamin, cob(II)alamin and cob(III)alamin, to indicate the positive charge of the cobalt-ion. The properties of cobalamin with regard to the binding of organic ligands are strictly dependent on the oxidation state (31). The cobalt-ion, most stable in its trivalent state in naturally occurring cobalamins, has to be reduced to cob(I)alamin prior to the formation of methylcobalamin or adenosylcobalamin. Particularly in the case of methylcobalamin, this oxidation state is essential in the mechanism of the catalyzed reaction, which is based on the acceptance and transfer of the methyl-ligand by cob(I)alamin (32,33). The effect of nitrous oxide on cobalamin, which will be the subject of a separate paragraph (1.3), is explained by oxidation of the cobalt-ion, leading to the complete inactivation of the enzyme.

Other cobalamins, and other cobalamin-dependent enzymes have been found in nature, notably in bacteria. Cobalamins are synthesized by microorganisms only, while plant cells do not contain cobalamin. A second human adenosylcobalamin-dependent enzyme, which isomerizes leucine, has

been described (34), but its significance is still uncertain.

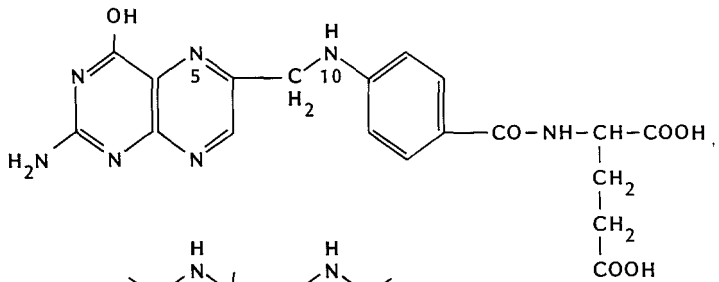
Folic acid, in a limited sense, is pteroylglutamic acid, a compound consisting of a pteridine ring, connected by a single carbon atom to para-aminobenzoic acid, which is joined by a peptide linkage to glutamic acid (35). This structure is shown in Figure 1.3. Folic acid, and folate, are also used as general terms for any member of the group of derivatives, also shown in Figure 1.3. Biologically active folates are reduced, compared to pteroylglutamic acid, and are therefore designated tetrahydrofolate (THF). In addition, these reduced folates can contain a single carbon unit at one of several levels of oxidation by covalent binding to nitrogen atoms 5 or 10, or both. This property is essentially the basis of all folate coenzyme functions, which consist of the uptake and transfer of single carbon units. Some of the folate-dependent enzymes are presented in Figure 1.3. These enzymes catalyze essential steps in the synthesis of thymidylate, with 5,10-methylene-THF as coenzyme, and purines, with 5,10-methenyl-THF and 10-formyl-THF. From their involvement in these pathways, it is immediately apparent that reduced folates are important in DNA synthesis and cell proliferation.

Another important folate-dependent enzyme is methionine synthetase, in which, as mentioned before, methylcobalamin is also necessary. This enzyme catalyzes not only the synthesis of the amino acid methionine, but also the conversion of 5-methyl-THF into THF. This step is essential in the metabolism of folates, because 5-methyl-THF is the reduced folate which is present in plasma and is taken up by cells (36,37). The conversion catalyzed by methionine synthetase represents the only way in which reduced folates, after uptake in the cell, become available for any of the other important folate-dependent reactions. This reaction is the single known point of interaction between folates and cobalamin. Considering the evident role of folates in nucleotide synthesis and in cellular proliferation, and the absence of any direct role of cobalamin in these processes, it is tempting to postulate that the function of cobalamin in the enzyme methionine synthetase should explain its importance in hematopoiesis. In addition, the striking similarity of the consequences of deficiencies of either cobalamin or folic acid, is easily explained if the hematological function of cobalamin concerns an essential step in folate metabolism. Based on these principles, more than two decades ago the "methylfolate trap hypothesis" was formulated to explain the metabolic consequences of cobalamin deficiency, with regard to folate metabolism and hematopoiesis. This hypothesis, and some alternative theories, will be discussed in greater detail in the next paragraphs.

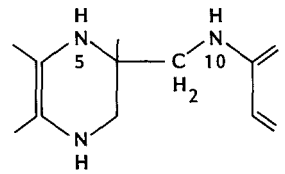
More recently, the important metabolic functions were recognized of a particular structural property of folate coenzymes: additional glutamic acid residues can be coupled to the single glutamate which is part of the folate molecule, resulting in folylpolyglutamates (38,39). These glutamates are linked to each other through peptide bonds between their 1-amino and 3-

STRUCTURE OF FOLATES

Pteroylglutamic acid (folic acid)



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 –	

FOLATE DEPENDENT ENZYMES

Coenzyme	Conversion	Enzyme
5-methyl-THF	homocysteine → methionine	methionine synthetase
5,10-methylene-THF	dUMP → dTMP	thymidylate synthetase
5,10-methenyl-THF	glycinamide ribotide (GAR) → formyl-GAR	GAR formyltransferase
10-formyl-THF	aminoimidazole carboxamide ribotide (AICAR) → formyl-AICAR	AICAR formyltransferase
5,10-methylene-THF	glycine → serine	serine transhydroxymethylase

Figure 1.3. Structure of the folate coenzymes, all of which are reduced derivatives of pteroylglutamic acid (folic acid). The most important folate-dependent enzymatic processes are shown in the table. Not included are the folate interconverting enzymes, which convert one folate derivative into another, like dihydrofolate reductase.

carboxyl groups, in a reaction catalyzed by the enzyme folylpolyglutamate synthetase. Intracellular folates predominantly are polyglutamate derivatives, whereas in plasma only 5-methyl-THF-monoglutamate is present (monoglutamate indicates folate without additional glutamates). The number of glutamates attached to intracellular folates is variable, but generally amounts 3 to 6. This addition of glutamates changes the properties of folate molecules considerably. One important consequence is the prevention of their transport across cellular membranes. Polyglutamation is therefore essential to retain folates in the cell after uptake. This was convincingly shown in studies of mutant CHO cells, which were extremely folate-deficient and consequently auxotrophic for purines and pyrimidines, and in which the only difference from wild type cells was a lack of folylpolyglutamate synthetase (40,41). Coenzyme functions of folates are also modified by polyglutamation of folates. With most folate-dependent enzymes, a distinct preference is observed for a specific number of glutamates, added to their folate coenzymes. The affinity of these enzymes for folylmonoglutamates generally is much lower (39). Certain folates can also function preferentially in one of several possible reactions, depending on their polyglutamate chain length (42,43). This suggests that the level of polyglutamation has a function in the regulation of one-carbon metabolism (44). It appears that cobalamin also has a role in folate polyglutamation. This relates to the observation that 5-methyl-THF is very poorly polyglutamated by folylpolyglutamate synthetase, as is evident from several metabolic studies (45-47), as well as from studies of the partially purified enzyme (41,48-50). Therefore, after its uptake in the cell, 5-methyl-THF has to be demethylated by cobalamin-dependent methionine synthetase before it can be retained and function efficiently as a coenzyme. This further emphasizes the importance of the point of interaction between the two factors which are essential in the prevention of the megaloblastic disorder of hematopoiesis.

1.1.3. The methylfolate trap hypothesis.

Around 1960 it was recognized that the synthesis of methionine from homocysteine is cobalamin-dependent, and also involves the demethylation of the folate coenzyme 5-methyl-THF. This reaction therefore established a metabolic point of interaction between cobalamin and folic acid. The relevance of this reaction to cellular proliferation is not immediately apparent, however. Independently, several investigators subsequently formulated a hypothesis to explain the effect of cobalamin deficiency on hematopoiesis, based on a disturbance of the reaction, which is catalyzed by methionine synthetase (51,52). This hypothesis was called the "methylfolate trap hypothesis", and, at the time it was proposed, there was only limited evidence to support this explanation. After 25 years of research on cobalamin-folate interrelationships the hypothesis still stands and, although some important additions appeared necessary, the explanation in fact is not

hypothetical anymore. Nevertheless, a few alternative theories are still found in the literature. The methylfolate trap hypothesis will now be considered in detail systematically. The relevant metabolic pathways are presented in Figure 1.4.

The methylfolate trap hypothesis states that cobalamin deficiency causes reduced activity of methionine synthetase, which leads to a disturbance of folate metabolism because 5-methyl-THF cannot be utilized by the cell under these circumstances, and will become "trapped". It was expected that eventually the levels of all other folates would be reduced, impairing the important folate-dependent synthesis of nucleotides, comparable to the effects of folate deficiency.

From this description some advantages of the methylfolate trap hypothesis are already apparent. It tries to explain the hematologic effects of cobalamin deficiency on the basis of the only cobalamin-dependent process which is known to be involved in folate metabolism. This obviates the need for postulated additional cobalamin-dependent processes, which, up till now, have not been discovered. The hypothesis also simply explains the identical megaloblastic hematopoiesis of cobalamin and folate deficiency.

The hypothesis can be evaluated on the basis of its four major implications, all of which have been investigated thoroughly:

[1] the activity of methionine synthetase becomes reduced in cobalamin deficiency.

[2] 5-methyl-THF cannot be utilized in any quantitatively significant process other than after demethylation by methionine synthetase. This implies that the reaction which reduces 5-methylene-THF to 5-methyl-THF, catalyzed by 5,10-methylene-THF reductase, is irreversible under intracellular conditions.

[3] all reduced folates can correct the impairment of cellular proliferation in cobalamin deficiency, with the sole exception of 5-methyl-THF, which should be ineffective in this respect.

[4] there is an accumulation of 5-methyl-THF, relative to other reduced folates, in cobalamin deficiency.

Experimental evidence concerning these four points will now be considered.

[1] Reduced activity of methionine synthetase in cobalamin-deficient cells has been observed invariably. This was first shown in vitamin B12-deficient rats, in which the activity of hepatic methionine synthetase was markedly reduced without effect on some other enzymes in folate metabolism (53). Subsequently, methionine synthetase activity in bone marrow from pernicious anemia patients was shown to be extremely low, and to be correctable in vitro and in vivo by vitamin B12 addition (54). Several other studies confirmed these findings (55-57). In addition, the activity of methionine synthetase in various cultured cell lines was clearly dependent on the supply

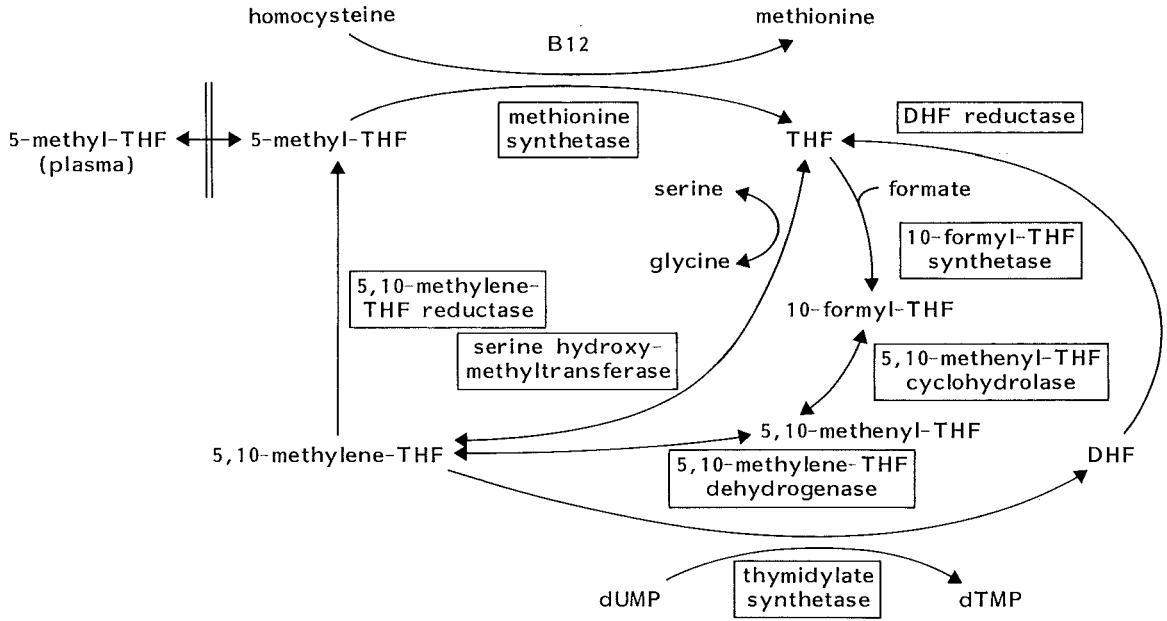


Figure 1.4. Outline of the intracellular interconversion of reduced folates, after the uptake of 5-methyl-THF from plasma. The cobalamin-dependent methionine synthetase reaction provides the only way to enter the cellular pool of active folate coenzymes. In addition, 5-methyl-THF appears not to be polyglutamated by folylpolyglutamate synthetase, and therefore is not retained in the cell if its metabolism is blocked. The three consecutive steps leading from THF and formate to 5,10-methylene-THF are catalyzed by a single "trifunctional" enzyme.

of cobalamin (58-60). The relationship between megaloblastic hematopoiesis and reduced activity of methionine synthetase was further substantiated by observations on inborn errors of cobalamin metabolism. Until recently four types were recognized, designated Cbl A, B, C and D mutants (61), and in all of these there is methylmalonic aciduria, caused by a deficiency of the adenosylcobalamin-dependent mutase. In two of these types, Cbl C and D, there is also homocystinuria, and a reduced activity of methionine synthetase. Only in these cases megaloblastic anemia is found. Recently, the existence of an additional mutant, deficient in methionine synthetase only, has been firmly established (62-64). This disorder is characterized by homocystinuria and megaloblastic anemia without methylmalonic aciduria. In conclusion, cobalamin deficiency leading to megaloblastic hematopoiesis is correlated with reduced activity of methionine synthetase in bone marrow cells.

[2] Except for the cobalamin-dependent conversion catalyzed by methionine synthetase, there is only one other reaction in which 5-methyl-THF is involved. This concerns the reaction catalyzed by 5,10-methylene-THF reductase (indicated in Figure 1.4). However, it is not probable that in this reaction 5-methyl-THF is utilized as a substrate, because the reduction of 5,10-methylene-THF appears to be irreversible. This was first found in studies of the bacterial enzyme (65), and was confirmed by kinetic studies of the mammalian enzyme (66,67). Some conflicting results were reported with regard to a possible role of 5-methyl-THF and 5,10-methylene-THF reductase in the methylation of biogenic amines in brain (68-70). When this pathway would indeed exist, it is probably quantitatively insignificant. The irreversible nature of the methylene-THF-reductase reaction under physiological conditions was clearly demonstrated in cultured L1210 cells, in which the labelled methyl-group of 5-methyl-THF could not be recovered from thymidylate (71). This indicates that demethylation by methionine synthetase, and not oxidation by methylene-THF reductase, is the way by which 5-methyl-THF enters the cellular pool of reduced folates.

[3] It is well established clinically that the hematological effect of cobalamin deficiency can be corrected by folic acid (72,73). In this respect, the demonstration of a differential effect of 5-methyl-THF compared to other reduced folates is of crucial importance to the methylfolate trap hypothesis, which postulates a selective block in the utilisation of 5-methyl-THF to be the fundamental defect in cobalamin deficiency. The effects of different folate derivatives cannot easily be studied in vivo, because reduced folates are readily interconverted upon administration. There is one observation which clearly demonstrates the ineffectiveness of parenteral 5-methyl-THF without cobalamin in a patient with megaloblastic anemia (74). To study the metabolic disturbance in cobalamin deficiency, and its correction by folate analogues, a specific in vitro test is usually applied. This metabolic test is the deoxyuridine suppression test (75,76), which can detect a

disturbance of the folate-dependent synthesis of deoxythymidylate. Essentially, it measures the suppression of the incorporation of labelled thymidine into DNA by the addition of deoxyuridine. The degree of this suppression will depend on the conversion of deoxyuridylate to thymidylate. Alternatively, the incorporation of labelled deoxyuridine in DNA can be measured, which also directly depends on this folate-dependent reaction (76,77). The test is described in more detail in the following chapter. Results of this test are abnormal in bone marrow cells and lymphocytes of cobalamin-deficient patients (56,57,76-84). Important information is obtained by comparing the effects of various additives on the test values. In cobalamin deficiency the addition of cobalamin invariably corrects the test values, although in most cases only partially, compared to normal controls (57,76-84). Interestingly, of the different cobalamin coenzymes, methylcobalamin is always far more effective than adenosylcobalamin (57,79,81). In all studies, a complete correction of the test results was possible with the addition of folic acid or 5-formyl-THF, whereas in all cases 5-methyl-THF appeared to be ineffective (56,57,76,77,81,82,84).

These studies on cobalamin-deficient cells should be compared to those on cells from folate-deficient subjects, in which cobalamin compounds have no effect at all on test results (76,77,80-84), but 5-methyl-THF is as effective as all other reduced folates in stimulating the disturbed thymidylate synthesis (76,77,81,82,84). The results of the deoxyuridine suppression test in bone marrow cells thus clearly support the methylfolate trap hypothesis by demonstrating a specific defect in the utilisation of 5-methyl-THF in cobalamin deficiency. Furthermore, the possibility to completely correct the effect of cobalamin deficiency in this test with the addition of folates is in agreement with the proposed indirect role of cobalamin in nucleotide synthesis. Studies on the growth of L5178Y and L1210 mouse leukemia cells in culture have confirmed the results of metabolic tests. When these cells had been made severely cobalamin-deficient, folic acid and 5-formyl-THF could restore normal growth, whereas 5-methyl-THF was totally ineffective, except with the simultaneous addition of cobalamin (85,86). Finally, several studies in which cobalamin was inactivated by nitrous oxide have also clearly demonstrated that 5-methyl-THF cannot be utilized without the methionine synthetase reaction. These studies will be dealt with in section 1.3.

[4] The final implication of the methylfolate trap hypothesis concerns the accumulation of 5-methyl-THF in cobalamin deficiency as the ultimate cause of the disturbance of folate-dependent reactions. Over the past twenty years this point has raised serious problems, which eventually appear to have been solved satisfactorily. These problems were both technical, with regard to the difficulties in tissue determinations of folate coenzymes, and conceptual, relating to the unexpected central role of folylpolyglutamates in folate metabolism.

The elevation of serum levels of 5-methyl-THF in many patients with

cobalamin deficiency was an early observation (52), which directly contributed to the development of the methylfolate trap hypothesis. This finding has been confirmed in other studies (87,88), although in patients with chronic disease or additional dietary folate deficiency, folate levels may be low (87). The high serum folate levels, which fall to normal following the supply of cobalamin, are easily explained as a consequence of methylfolate trapping. On the other hand, however, it was found that tissue levels of folate were reduced in cobalamin deficiency. This was already observed in 1954, when folate levels in "whole blood" were measured (89), and has been confirmed, not only in erythrocytes (88), but also in leukocytes (90) and liver cells (53,91). This finding appears not to be compatible with the original methylfolate trap hypothesis, which implies an accumulation of 5-methyl-THF. More recently, this cellular folate depletion has also been explained as direct consequence of the failure to convert 5-methyl-THF in cobalamin deficiency, because this will lead to severely impaired synthesis of folylpolyglutamates (92). The recognition of the important role of folylpolyglutamate synthesis has been rather slow. At first, the observations of increased serum levels of folate, with reduced tissue levels, have led to the hypothesis that the cellular uptake of folate was a cobalamin-dependent process (93-95). Others recognized that decreased polyglutamation, resulting in decreased cellular retention of folates, could also explain these effects (53,92). This in turn has led to the suggestion that the polyglutamation of folates was directly cobalamin-dependent (96). It is important to notice that these presumed additional cobalamin-dependent processes have not been characterized, and in fact are not necessary. The decreased polyglutamation, and the resulting decreased cellular uptake, can be explained as a consequence of the failure to convert 5-methyl-THF. This folate derivative is not used in the synthesis of polyglutamates, as is evident from several studies (45-50) that were discussed in the former section. Other studies have made it unlikely that the membrane transport of folates is cobalamin-dependent by itself (92,97), and the enzyme activity of folylpolyglutamate synthetase was not affected by the absence of any form of cobalamin (41). Thus, with an expansion which includes the role of folylpolyglutamates in the cellular retention of folates, the methylfolate trap hypothesis can explain the reduction of total intracellular folate in cobalamin deficiency.

The observed absolute reduction of tissue folates does not, however, exclude a relative increase of 5-methyl-THF, which would provide additional support for the methylfolate trap hypothesis. Several difficulties have hampered the study of folate coenzyme distribution in cobalamin deficiency. Reliable extraction and assay of the various intracellular folates, some of which are rather labile, has been difficult for a long time, so much the more in this case, because of: a) the low cellular folate content in cobalamin deficiency; b) the abundance of 5-methyl-THF, even in cobalamin-replete cells. Methylated folates amount from about 83% in normal fibroblasts (98), to

39% in liver cells (99), and even 100% in erythrocytes (100). These high percentages limit the detection of a relative accumulation of 5-methyl-THF with regard to other folates. Nevertheless, in several studies a relative increase in methylated folates has been found in cobalamin deficiency, to a variable extent and duration. The determination of methylfolates in most studies has been simplified by the application of microbiological assays, which can differentiate between total folates (detected by Lactobacillus casei) and non-methylated folates (detected by Pediococcus cerevisiae or Streptococcus faecalis, which cannot use 5-methyl-THF). A relative increase of 5-methyl-THF was demonstrated in the livers of cobalamin-deficient rats (51,101) and sheep (91,102), as well as in L1210 leukemia cells (103) and Euglena gracilis (104), cultured without cobalamin. In some of these studies (101-103) it was also shown that this relative increase was caused by a decrease of polyglutamate derivatives of other reduced folates. A decrease of folylpolyglutamates appeared to be the primary event in the development of intracellular folate deficiency resulting from cobalamin deficiency. This was also shown in erythrocytes of pernicious anemia patients, but not of patients with primary folate deficiency (100,105). With regard to the confirmation of the (relative) trapping of 5-methyl-THF, studies employing the nitrous oxide model of cobalamin deficiency have also been useful. In general, a rapid initial rise of 5-methyl-THF has been found (106-108), followed by severe folate loss due to decreased polyglutamation (47,109). Thus, the postulate that cobalamin deficiency increases the proportion of tissue folates in the methylated form is also supported by a large number of experiments.

It can be concluded that the main implications of the methylfolate trap hypothesis have been confirmed experimentally. An important addition to the original hypothesis has been the recognition of the impairment of folylpolyglutamation, which explains the occurrence of intracellular folate depletion in cobalamin deficiency. For this reason, it is probably not entirely correct to refer to methylfolate "trapping" as the ultimate cause of the metabolic disturbance. Perhaps, the explanation of the effects of cobalamin deficiency should be called simply methylfolate "blockade". This more appropriately emphasizes the mechanism responsible for the hematological effects of cobalamin deficiency: a secondary intracellular deficiency of metabolically active folates, caused by a failure to convert the methyl-form after its uptake from the plasma. In current literature, some alternative views can still be found, which will be discussed in section 1.1.5.

1.1.4. Cobalamin deficiency and methionine metabolism.

Methionine is also involved in the metabolic consequences of cobalamin deficiency. The role of cobalamin in methionine metabolism is evident from its function in the enzyme methionine synthetase. In the methylfolate trap hypothesis the function of this enzyme in folate metabolism is

emphasized, but the methylation of homocysteine to methionine is also of great importance. In fact, as a result of the dual function of this cobalamin-dependent enzyme, methionine and folate metabolism are closely related. The folate-dependent synthesis of methionine is an essential step in the "methylation cycle", in which S-adenosylmethionine (SAM) serves as a methyldonor in a wide variety of metabolic processes. On the other hand, methionine has profound influence on folate metabolism as well, because the metabolism of one carbon-units is carefully regulated, as discussed in a paper by Krebs et al. (110). In this regulation, the intracellular concentration of SAM determines the activity of the enzyme methylene-THF-reductase, by a strong inhibitory effect (66,111). This enzyme catalyzes the conversion of reduced folates to 5-methyl-THF, which is the methyl donor in the synthesis of methionine. A low concentration of methionine, directly leading to reduced concentrations of SAM, therefore causes a shift of folates towards 5-methyl-THF at the expense of other reduced folates. High levels of methionine and SAM will, on the other hand, reduce 5-methyl-THF, favouring all other folate-dependent processes. Conceivably, in cobalamin deficiency, when 5-methyl-THF cannot be utilized anymore, this regulatory mechanism can further complicate folate metabolism. If methionine and SAM are low, as is to be expected in cobalamin deficiency (112), the increased activity of methylene-THF-reductase will further increase the pool of 5-methyl-THF. A large number of studies, in particular by the group of Stokstad, has demonstrated that methionine deficiency indeed increases the metabolic disturbance of folate metabolism in cobalamin deficient cells, and that supplementation of methionine reduces the consequences of cobalamin deficiency. These studies were performed in rats (53,101,113-120) or sheep (102), and generally liver cells of these animals were used. In all of these studies methionine corrected one or more aspects of disturbed folate metabolism in cobalamin-deficient animals, such as the increased proportion of 5-methyl-THF, the intracellular depletion of folates and the impairment of folate-dependent processes, most often histidine degradation. Animals, which were methionine deficient in addition to their cobalamin deficiency, were more severely affected with regard to these aspects of folate metabolism. These experimental results thus are in agreement with the regulatory effect of methionine as proposed by Kutzbach et al. (66) and Krebs et al. (110), and support the methylfolate trap hypothesis. It should be noted however, that although the existence of these effects in liver cells is beyond doubt, these results may not be directly applicable to hematopoietic cells. Different results were obtained when methionine was added to bone marrow cells of cobalamin deficient patients. In the deoxyuridine suppression test methionine further impaired the *de novo* synthesis of thymidylate (121,122). In one study it was shown that folate metabolism of bone marrow and liver cells reacted differently on methionine addition. In the marrow cells, methionine increased the proportion of 5-methyl-THF, disturbing folate-

dependent nucleotide synthesis (123). The mechanism of this was proposed to be the increased inhibition of methionine synthetase by excess methionine. This has indeed been observed in earlier studies on this enzyme (30,53,59). A recent study has shown that the effects of methionine in bone marrow cells are in fact dose-dependent (124). A low dose of methionine was able to cause a nearly complete correction of the disturbed deoxyuridine suppression test in cobalamin deficient bone marrow. However, higher concentrations of methionine, as used in the earlier studies, resulted in further disturbance of the test. With folate-deficient bone marrow cells there was only minimal improvement by low doses of methionine, while high doses again produced severe disturbance. Thus, the effects of methionine in cobalamin deficiency can be rather variable. The enhancement of folate coenzyme functions by the low doses of methionine can be explained not only by the shift towards non-methylated reduced folates, caused by the inhibition of 5,10-methylene-THF reductase, but possibly also by a catalytic action of SAM on the enzyme methionine synthetase. SAM is an essential cofactor in the enzyme complex (30,33,125), and low levels of SAM could limit the enzyme activity. A summary of these effects is presented in Figure 1.5.

The interaction of methionine with folate metabolism is apparently complex and intricately regulated. Unfortunately, there are no reports of either a beneficial or a detrimental effect of methionine administration in pernicious anemia patients. It has been reported, however, that serious protein malnutrition (kwashiorkor), in which methionine deficiency is a prominent feature, may aggravate or even precipitate severe megaloblastic anemia (126,127). Scott and Weir have underlined the importance of disturbed methionine metabolism for the mechanism of methylfolate trapping in cobalamin deficiency (128,129). Interestingly, these authors also presented evidence that decreased synthesis of methionine is related to the occurrence of neurological disease (subacute combined degeneration of the spinal cord) in longstanding cobalamin deficiency (130). In this investigation, they made use of the nitrous oxide model for cobalamin deficiency, which will be dealt with in section 1.3. In several other studies employing the nitrous oxide effect, methionine or SAM corrected various aspects of disturbed folate metabolism (131-134).

Finally, studies on inherited metabolic disorders also have provided insight in the way in which methionine is involved in folate metabolism. A recent comparison of the effects of deficiencies of either methylene-THF-reductase or methionine synthetase is most interesting in this respect (135). In methylene-THF-reductase deficiency, there is no trapping of 5-methylfolate, no impairment of folate coenzyme functions related to cell division, and consequently, no megaloblastic anemia. This may be comparable to the SAM-induced inhibition of this same enzyme, which in cobalamin deficiency restores folate metabolism by preventing the formation of

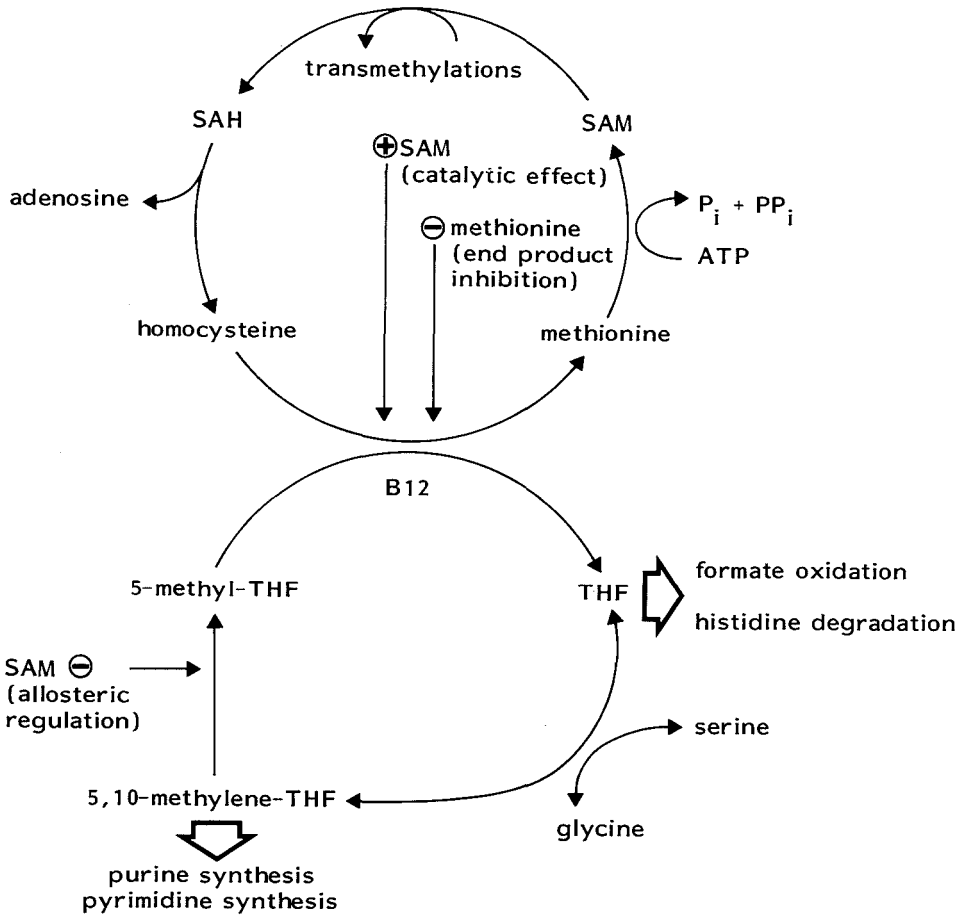


Figure 1.5. The regulatory effects of methionine and S-adenosylmethionine (SAM) on folate metabolism. It should be realized that these three mechanisms are not necessarily active at the same time. Probably physiologically most important is the inhibition of 5,10-methylene-THF reductase by SAM. In cobalamin deficiency, this effect reduces the accumulation and subsequent loss of 5-methyl-THF, which explains the ameliorating influence of methionine observed in most experiments.

methylfolate. In methylene-THF-reductase deficiency, intracellular and plasma levels of 5-methyl-THF are low, but total intracellular folate, mainly as THF, is normal. The lack of 5-methyl-THF as a cofactor in methionine synthesis explains the homocystinuria and low methionine levels found in this disease (136,137), which gives rise to neurological symptoms only. When the activity of methionine synthetase is reduced, there is also homocystinuria, low methionine and neurological disease, but in contrast, 5-methyl-THF is high, and the total folate level is reduced. In this disease, folic acid coenzyme functions are severely disturbed, and there is megaloblastic anemia, in accordance with the methylfolate trap hypothesis.

It can be concluded that in the pathways of one carbon-metabolism methionine plays a important role, and for this reason, the involvement of methionine in folate metabolism is also essential to the understanding of the role of cobalamin in the cell.

1.1.5. Alternative theories on the role of cobalamin in hematopoiesis.

Experimental results of a large number of studies have been in agreement with the role of cobalamin in hematopoiesis as proposed in the methylfolate trap hypothesis. In some instances, this hypothesis, as formulated nearly 25 years ago, appeared to be not entirely sufficient to explain new experimental observations. Most prominently, this was the case with the role of folylpolyglutamates in folate homeostasis. It has been possible to include this aspect of folate metabolism in the role of cobalamin proposed by the methylfolate trap hypothesis, thereby accounting for the cellular depletion of folates in cobalamin deficiency. The way in which methionine is involved in folate metabolism and cobalamin deficiency, which appeared to be rather complicated, is as well in agreement with the methylfolate trap hypothesis. Finally, results obtained with the nitrous oxide model for cobalamin deficiency were in general also as predicted by this hypothesis. However, several other explanations of the effects of cobalamin deficiency have appeared in the literature. Most of these theories had to be discarded, when they failed to fit new experimental data. This has been the case with the theories that cobalamin is directly involved in the entry of folates in the cell, or is a cofactor in polyglutamation of folates. Some alternatives to the methylfolate trap hypothesis can still be found in recent literature, and will be described briefly now.

1: *A cobalamin function in thymidylate synthetase* has been proposed by Haurani (138-140). He assumed a direct role of cobalamin in nucleotide synthesis, and this would be the induction or activation of the enzyme thymidylate synthetase. This theory is based on the observation that thymidylate synthetase activity is low in PHA-stimulated lymphocytes obtained from cobalamin-deficient persons. However, this finding has never been confirmed. Indications of an involvement of cobalamin in the enzymatic activity of thymidylate synthetase are lacking in the vast literature on this subject. On the contrary, an increased activity of thymidylate synthetase in bone marrow cells from cobalamin-deficient subjects (141,142), and in marrow cells of nitrous oxide-treated rats (143) has been reported. This increase is easily explained as a regulatory effect, since the enzyme activity will be impaired by the lack of its folate coenzyme. Haurani et al. have reported that nitrous oxide exposure in their experiments also reduced thymidylate synthetase activity (144). It may be that either PHA-stimulated lymphocytes are not a useful model to study hematopoiesis, or that the results depend on the enzyme assay system employed by these workers. Their recent observation that methotrexate induces an increase in

thymidylate synthetase activity is also in contrast with other data (145). This theory is not easily compatible with the large number of observations on an involvement of folate metabolism in cobalamin deficiency.

2: *The "formate starvation hypothesis"* was put forward by Chanarin and coworkers (146,147). They agree that the prime defect in cobalamin deficiency is the reduced activity of methionine synthetase, but do not accept that the conversion of 5-methyl-THF to THF, catalyzed by this enzyme, is of functional significance. Instead, it is proposed that the impairment of methionine supply, another consequence of inactive methionine synthetase, is the ultimate cause of the effect of cobalamin deficiency on folate metabolism. This is explained by suggesting a rather complicated pathway, in which methionine is eventually the source of formate, which subsequently is essential in the synthesis of formyl-THF. Finally, formyl-THF is supposed to be the optimal substrate for the synthesis of folylpolyglutamates. This would explain the fall in intracellular folate levels and the impairment of folate coenzyme functions, induced by cobalamin deficiency. The hypothesis is based on the observation that in vitro addition of formyl-THF more efficiently corrects the disturbance by nitrous oxide of the deoxyuridine suppression test in rat bone marrow cells, if compared to the addition of THF (148). As THF was largely ineffective in this test system, it was concluded that the conversion of 5-methyl-THF to THF could have no essential function. They therefore reject the methylfolate trap hypothesis. In addition to their observations on the nitrous oxide model in rats, they also have found THF to be less effective than formyl-THF when using bone marrow cells of cobalamin deficient patients (149,150). These findings, however, have not been confirmed. On the contrary, in some recent studies, THF was quite effective in correcting the metabolic effect of cobalamin deficiency, although somewhat less than formyl-THF (57,84). Furthermore, several authors suggested that the observed differential effect of formyl-THF may reflect its higher stability, and the increased capacity of cells to transport this compound (19,84). In addition, THF may also be more rapidly converted into 5-methyl-THF, and thus be lost in cobalamin deficiency. It is noteworthy that in the studies of Chanarin's group, like in all other studies discussed before, 5-methyl-THF was totally ineffective in deoxyuridine suppression tests of cobalamin deficient cells, leaving this essential implication of the methylfolate trap hypothesis unaffected. Chanarin and coworkers have produced a large number of studies in recent years, of which they interpreted the results as confirming the essential role of "formate starvation" in cobalamin deficiency. In most cases, they have used nitrous oxide exposure as a model for cobalamin deficiency. They found that methionine and SAM, presumed to be precursors of formate, corrected impaired folylpolyglutamate synthesis (109,133). This is also explained by other theories, as described in section 1.1.4. They also reported that formyl-THF was a substrate in folylpolyglutamate synthesis, while THF and 5-methyl-THF

were not (151). This pattern, however, has not been found in other studies, even after purification of the polyglutamate synthetase enzyme (50). Usually, THF appeared to be the preferred substrate in folylpolyglutamate synthesis (46). According to the methylfolate trap hypothesis, any reduced folate, except 5-methyl-THF, may be a substrate in polyglutamate synthesis, and the results of Chanarin's group do not contradict this assumption.

Furthermore, the formate starvation hypothesis implicates a fairly complicated connection between methionine and the presumed production of formate. Cobalamin deficiency would involve reduced levels of several

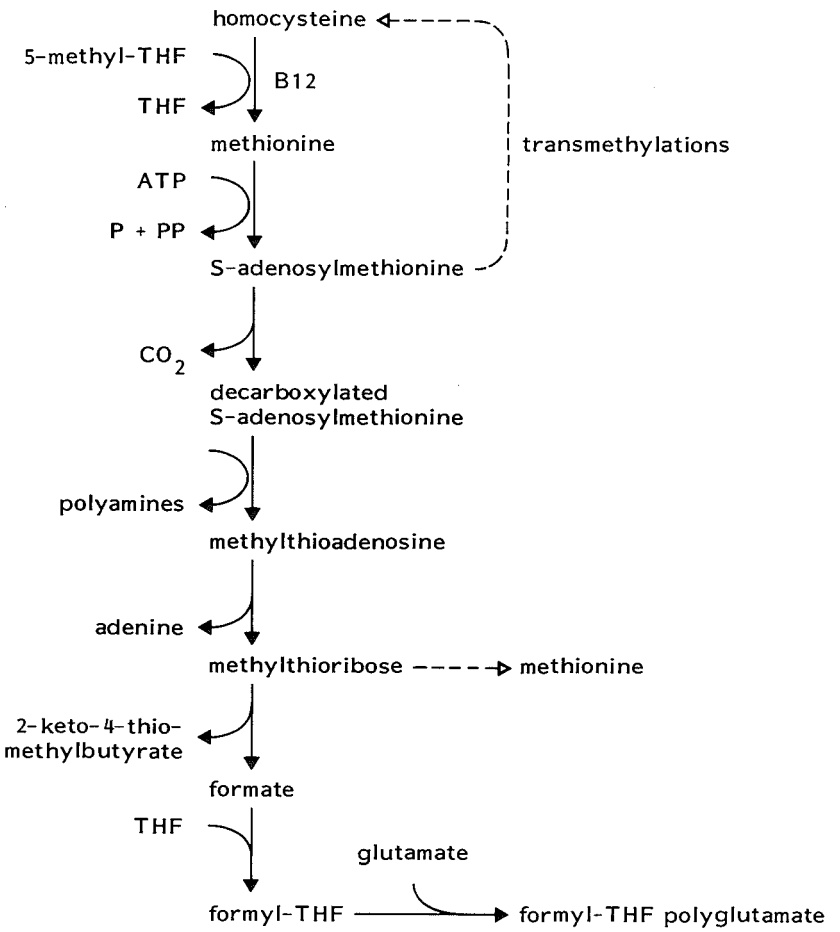


Figure 1.6. The pathway from methionine to formate, which according to Chanarin et al. is essential to prevent megaloblastic anemia. It is assumed that the reduced activity of methionine synthetase in cobalamin deficiency ultimately will result in decreased levels of formyl-THF, supposed to be the preferred substrate for folylpolyglutamate synthesis (from ref. 20 and 133).

important intermediates, according to the pathway described in their papers (20,133), and shown in Figure 1.6. Among these are methionine and SAM, and also decarboxylated SAM, a precursor of polyamines. Inhibition of the synthesis of SAM by cycloleucine, or of its decarboxylation by mitoguanozone (MGBG), is not known to cause megaloblastic anemia, however. Another difficulty with the hypothesis of Chanarin and coworkers is the failure of direct administration of sodium formate to correct folate metabolism upon nitrous oxide treatment (133). For these reasons, the hypothesis that the hematologic consequences of cobalamin deficiency are caused by formate starvation, does not seem to be a likely alternative to the more direct involvement of folate in cobalamin deficiency, as suggested in the methylfolate trap hypothesis.

An additional alternative hypothesis seems to be the recent suggestion of Sourial et al. (152), that the primary lesion in megaloblastic anemia is an inability to synthesize SAM. SAM would be required for DNA synthesis and hematopoiesis. It appears, however, that this suggestion only differs from the conventional view of methylfolate trapping by a more pronounced emphasis on the role of SAM in folate metabolism. In their report, based on the use of nitrous oxide-induced inactivation of cobalamin, Sourial et al. do not suggest other ways in which SAM could be required for DNA synthesis than an effect on the enzyme methionine synthetase.

Finally, it should be mentioned that controversy still exists on the mechanism of the neurological lesions in cobalamin deficiency. At present, an impairment of SAM-mediated methylation reactions in the nervous system, as a consequence of reduced activity of methionine synthetase, seems to be the most probable explanation. An earlier hypothesis assumes reduced activity of the second cobalamin-dependent enzyme in man, methylmalonyl-CoA mutase, to be the cause. This explanation does not fit with the well-established occurrence of neurological lesions with long term nitrous oxide exposure, which only inactivates methionine synthetase (132,153,154). In addition, a congenital deficiency of the mutase enzyme does not cause neurological impairment. A detailed discussion of the active research in this area is beyond the scope of this chapter.

1.1.6. The role of cobalamin in malignant hematopoiesis.

The importance of cobalamin in normal hematopoiesis is clearly demonstrated by the clinical effects of cobalamin deficiency. It is the subject of this paragraph to describe some observations relating to the role of cobalamin in malignant hematopoiesis, or: the growth of leukemia. This obviously is fundamental to the potential therapeutic application of cobalamin antagonists, which will be described in the following sections.

The cobalamin-dependent enzyme methionine synthetase, which has an essential function in folate metabolism, is also present in malignant

leukocytes (155-158). It appears that, in general, the activity of this enzyme in malignant cells is somewhat higher than in normal cells. Lymphoid leukemia cells have a higher activity than myeloid leukemia cells, while activity in the stationary phase of chronic myeloid leukemia (CML) is very low, in contrast to the accelerated phase. Observations on lymphoid cell lines (155) and (normal) CHO cells (60) have also indicated that the enzyme activity is highest during rapid growth of the cells. The presence of this enzyme in leukemic cells suggests that cobalamin is also essential to the growth of these malignant cells, and that methionine synthetase consequently is a potential target in cancer chemotherapy (159). Mouse leukemia cells effectively take up cobalamin, bound to its transport protein transcobalamin II (TC II) (160), and in cultures the presence of TC II-bound cobalamin is essential to the utilization of 5-methyl-THF and growth of these cells (85,86).

Two cases have been described which demonstrate the importance of cobalamin for the growth of human leukemia (161,162). In these patients, the administration of cobalamin accelerated the growth of leukemia. In the first case (161), a patient with coexisting pernicious anemia and CML was "treated" by withholding vitamin B12 as long as possible, because supplementation appeared to cause stimulation of leukemic growth. The other case (162) also demonstrated an acceleration of leukemic blast cell proliferation on cobalamin administration. The controlling effect of cobalamin deprivation in this case was apparent from bone marrow cultures with and without the addition of cobalamin. In another report of concomitant pernicious anemia and leukemia (163), the authors speculated that vitamin B12 therapy had resulted in progression of the disease. On the other hand, one case has been reported in which maturation of leukemic blasts apparently resulted from cobalamin supplementation (164).

Although the evidence is still limited at present, a role of cobalamin in malignant hematopoiesis seems probable, which is not surprising in view of the established role of folate metabolism in tumor growth. To some extent, the situation with regard to the application of cobalamin antagonists seems comparable to the introduction of folate antagonists nearly 40 years ago. Farber first observed the importance of folic acid to the growth of tumors, which was evident from an accelerated tumor growth in some cases (165,166), before he realized the therapeutic value of antagonists (167). The remainder of this introductory chapter will be devoted to a discussion of the present knowledge on cobalamin antagonists.

Cobalamin and folic acid are intimately involved in the metabolism of malignant cells, and consequently their antagonists are of importance to the treatment of malignant disease. Interestingly, in malignant cells alterations of methionine metabolism and transmethylation reactions have been observed in vitro, a phenomenon which is called "methionine dependence", and which may be related to the process of carcinogenesis (168). Although

cobalamin and folic acid both are essential in the biosynthesis of methionine, it is as yet unclear whether a disturbance in the metabolism of these vitamins is also implicated in the development of malignant disease, as has been suggested (166).

1.2. Cobalamin antagonists: an overview.

The interest in antagonists of cobalamin started early after the elucidation of its chemical structure in 1956. One of the pioneers in this field has been Lester Smith, who has investigated several structural analogues of the vitamin (166-171). His motivation was twofold (171): firstly, antimetabolites of cobalamin might provide information on the mode of action of this vitamin, and secondly, they might be used in the treatment of leukemia and possibly other malignant diseases. The same reasons still explain the current revived interest in cobalamin antagonists, which began in 1978 with the discovery of the inactivation of cobalamin by nitrous oxide.

Several different substances have been found to interfere with cobalamin functions, but usually their biological activity and possible application have not been investigated in detail. Their characterization often was limited to a single aspect of cobalamin metabolism. In fact, only the recent interest in nitrous oxide has resulted in extensive metabolic studies of a cobalamin antagonist. This does not necessarily imply, however, that nitrous oxide is more effective than any other antagonist. Nitrous oxide is probably only unique because of its easy administration by inhalation, its rapid action and, in particular, because of its widespread clinical use in anesthesia, which has necessitated the study of this side-effect. It is important to realize that the observations on the effects of nitrous oxide may also apply to other antagonists that are already known, or to new ones that could be developed. The properties of different types of cobalamin antagonists will be discussed now.

1: *Structural analogues of cobalamin* represent the most obvious approach in the search for cobalamin antagonists. The interest in these compounds has been inspired by the therapeutic results obtained with folate analogues. Considering the complicated structure of the cobalamin molecule, the number of possible structural analogues is virtually unlimited. A large number of cobalamin analogues has been prepared, often by chemical modification of the vitamin, or by isolation from bacterial cultures. The systematic screening of the biological properties of these compounds has been very difficult, especially in the time when the metabolic functions of cobalamin were still ill-defined. Therefore, the rather extensive literature on cobalamin antimetabolites published around 1960 is nowadays of limited value. Some important results of these and more recent studies will be summarized.

Chemical modification of cobalamin often started with mild acid hydrolysis, which converts the propionamide side chains to carboxylic acids. Three isomeric monocarboxylic acids may be formed, which subsequently may be converted into substituted amides by reactions with several different amines or aniline (aminobenzene). These products, called B12-amides and B12-anilide, were tested on biological activity mainly in bacterial systems. Several of these compounds, including the unsubstituted monocarboxylic acids, had antagonistic effects with regard to cyanocobalamin (169-175). Results were different dependent on the type of microorganism used in the test, however. Inhibition in one system did not exclude vitamin-like stimulation in another. Further investigations showed that B12-anilide produced megaloblast-like cells when added to human bone marrow cultures (173), and that monocarboxylic acid derivatives caused granulocytopenia in rats (174), but not in primates (175). In the latter study, methylmalonic acid excretion and serum folates were increased by the treatment with these substances. B12-anilide failed to disturb the deoxyuridine suppression test (76). Treatment of malignant disease with these antagonists has not been encouraging (171) and results have not been published in detail. In one case of human chronic myeloid leukemia Herbert and Hoogstraten found a striking remission with either B12-anilide (166) or B12-ethylamide (176).

In another class of structural analogues the aminopropanol moiety in the large side chain attached to the D-ring of cobalamin is replaced by other alkanolamines. These substances were produced by partial synthesis and some of them strongly inhibited bacterial growth, which could be corrected by the addition of methionine (177). The 2-amino-2-methylpropanol derivative (called S102) was further tested with equivocal results (178).

In addition, analogues have been produced with modifications in the nucleotide part of the cobalamin structure. In these compounds the 5,6-dimethylbenzimidazole heterocyclic base, which is specific for cobalamin, is substituted or replaced by another base (178). These analogues in general were not antagonists but active coenzymes in several bacterial cobalamin-dependent reactions.

Antagonists of cobalamin occurring in bacterial cultures have been studied (179), also mainly by testing their ability to inhibit the growth of cobalamin-requiring microorganisms. These antagonists are not necessarily structural analogues, although most of them were related to at least a part of the cobalamin molecule. However, not all were fully characterized. In cultures of Bacillus cereus a substance inhibiting methionine synthetase was found, and from cultures of Corynebacteria compounds with a high affinity towards cobalamin-binding proteins were isolated. No further details on these and other naturally occuring antagonists have been reported.

A wide variety of metal-substituted cobalamins has been investigated, like the rhodibalamins, which contain rhodium (180). These were found to

be weak antagonists. Manganibalamin, cupribalamin and zincobalamin have also been described, but appear to have no significant biological effects.

Treatment of cobalamin with mild alkali at high temperature can result in an oxidative cyclization of the *c*-acetamide side chain at the B-ring, yielding a *c*-lactam. Recently, the administration of hydroxocobalamin(*c*-lactam) to rats during 14 days was reported to cause inhibition of methionine synthetase, comparable to nitrous oxide (181).

Finally, structural analogues have been made by replacing the ligand at the sixth coordinating bond of the central cobalt atom. Fluoro- and chloroalkyl-groups, replacing methyl in methylcobalamin, yielded cobalamin analogues inhibiting cobalamin-dependent methane formation by bacteria (182), and the synthesis of cobalamin coenzymes in human lymphocytes (183). A variety of nucleosides was used to prepare analogues of adenosylcobalamin (184). Nearly all of these inhibited bacterial adenosylcobalamin-dependent ribonucleotide reductase, and, moreover, all analogues effectively inhibited transcobalamin II-mediated transport of cobalamin into L1210 mouse leukemia cells. Methylcobalamin analogues were also expected to inhibit methionine synthetase (159), but this has not been substantiated.

2: *Complexes of cobalamins with heavy metals* are readily formed in vitro, with platinum (185) and palladium (186), and, in the case of platinum, lead to transfer of the methylgroup of methylcobalamin to the metal (187). The complexes of cobalamin with these heavy metals attained biological significance when it was speculated that the serious peripheral neuropathy occurring with cisplatin chemotherapy might be caused by inactivation of cobalamin. This neuropathy is clinically similar to the neurological effects of cobalamin deficiency, but in a recent study cisplatin treatment did not cause alterations in serum cobalamin, or in the excretion of methylmalonic acid or homocystine (188). A palladium-methylcobalamin complex was shown to inhibit the synthesis of cobalamin coenzymes in lymphocytes (183), and to reduce the growth of a transplanted tumor in mice (189).

3: *Nitrous oxide* (N_2O) is an effective antagonist of the coenzyme function of methylcobalamin, causing rapid and specific inactivation of methionine synthetase (190-193). At present, it is the only cobalamin antagonist which has been investigated in considerable detail in metabolic and clinical studies. The effect of nitrous oxide on the methylcobalamin coenzyme appears to be due to oxidation of the central cobalt ion, which disrupts the catalytic activity of the enzyme. This immediate action on the enzyme is essentially different from the effects of structural analogues, which will impair the synthesis of new enzyme by competition with the normal coenzyme. Although nitrous oxide indeed rapidly inactivates methionine synthetase, the resulting disturbance of folate metabolism develops gradually. Nitrous oxide is a gas, and this will contribute to its effective tissue penetration. An important aspect of this cobalamin antagonist is its widespread use

in anesthesia and for industrial purposes, and its abuse, as a "recreational drug". The consequences of prolonged exposure, which are megaloblastic bone marrow depression and myeloneuropathy, are therefore of clinical importance. The effects of nitrous oxide are considered in more detail in the next paragraph.

4: *Alkylnitrites* were recently reported to affect both methylcobalamin and 5-methyl-THF in vitro (194). It was suggested that these interactions were due to the oxidizing properties of the nitrites comparable to the effect of nitrous oxide. As yet, it is uncertain whether these effects are biologically relevant. Abuse of volatile alkylnitrites is not known to cause symptoms of cobalamin or folate deficiency.

5: *Other approaches* in the development of cobalamin antagonists have been described. A variety of nucleotides was tested for antagonism towards the cobalamin-dependent growth of Euglena gracilis (195), and some indeed appeared to have such an effect. However, there was only little evidence of specificity for cobalamin. Inhibition of the enzyme methionine synthetase, the main objective of the application of cobalamin antagonists as inhibitors of cellular proliferation, may also be pursued by other means, like analogues of the substrate 5-methyl-THF. Effective inhibitors of this kind are not known, however. It has been argued that the cobalamin coenzyme represents the most vulnerable and specific facet of this enzyme (159), as illustrated by the great care to absorb and conserve this substance in mammalians.

Interestingly, another inhibitor has been described with clear clinical relevance. Ethanol appears to impair the activity of methionine synthetase (196), resulting in the accumulation of 5-methyl-THF (197). This may contribute to the megaloblastic anemia frequently observed with alcoholism, but should not be considered to be specific enough to merit further study of the alcohol treatment of myeloproliferative disorders.

1.3. The effect of nitrous oxide on cobalamin.

Nitrous oxide, or dinitrogen monoxide (N_2O) is a colorless gas without appreciable odor or taste, which was prepared and recognized as a distinct gas by Joseph Priestley in 1772. The gas was extensively studied for the first time by Humphry Davy between 1798 and 1800 (198-200). Davy discovered and vividly described the particular mental effects produced by breathing this gas, but also noted that it could relieve pain, concluding that nitrous oxide "may probably be used with advantage during surgical operations" (198). Although breathing the "laughing gas" became a popular entertainment, it was not until 1844 before it was applied as an anesthetic by Colton and Wells, initially with only limited success. Nitrous oxide is not a

very potent anesthetic agent, and if administered alone, high concentrations are necessary to produce unconsciousness, with the danger of causing hypoxia. It appeared to be of great value, however, as an adjuvant to other anesthetics. In modern anesthesia, 70% nitrous oxide is commonly used to reduce the concentration of more potent anesthetic agents. The distinct analgesic properties are an additional advantage, and are the reason of its use in dentistry and obstetrics, which is widespread especially in the United States. During the long period of its application in anesthesia, nitrous oxide became known as a safe agent, without important side effects. Recovery from its effects is rapid, and there is no detectable biotransformation.

In the 1950s nitrous oxide was applied in the treatment of tetanus, to relieve patients from the emotional stress caused by the application of curare and artificial respiration. Exposure to nitrous oxide therefore lasted several days. It was observed that in this period severe bone marrow depression developed, which could even be fatal (201-203). Lassen, the first to describe this effect, found on examination of the bone marrow of these patients that "erythropoiesis was strikingly megaloblastic and the changes in granulocytopoiesis were so pronounced and typical that, unless one knew the clinical history, one would not hesitate to diagnose pernicious anaemia" (201). This serious megaloblastic bone marrow depression was observed specifically in patients who had received nitrous oxide. Thus, it appeared that exposure to nitrous oxide for periods longer than in ordinary surgery, could be very toxic. Nothing was known in these days about the possible mechanism of this toxicity.

Remarkably, not much later, in two studies this unexplained side-effect was deliberately applied in patients with leukemia. The first report, also from Lassen and coworkers (204), described two patients with chronic myeloid leukemia (CML) in which the leukocyte counts decreased rapidly to about normal values on nitrous oxide exposure. This effect was rather quickly reversible, however, when treatment was discontinued. In addition, Lassen noticed a dramatic fall of serum vitamin B12 during the treatment. In one case, the level was reduced from 4900 pg/ml, an increased value as found often in CML, to 1280 pg/ml in about 14 days, followed by a rapid increase after exposure had been stopped. The second publication, by Eastwood et al. (205), described nitrous oxide administration to two children with acute myeloid leukemia, resulting in a similar rapid fall of initially high leukocyte counts. In one patient, continued intermittent application of nitrous oxide could maintain the leukocyte counts at normal levels and relieve severe joint pain for more than five weeks.

This cytotoxic effect of nitrous oxide appeared reproducible in vitro, in cultured embryonal mouse cells (206). Subsequently, several studies in rodents (207-211) have demonstrated suppression of hematopoiesis, resulting in a marked leukopenia. Typical megaloblastic anemia was not observed, but it should be noted that dietary manipulation of laboratory

animals also has failed to produce this, probably strictly human, phenomenon. Nitrous oxide was also tested in animal tumor systems. The growth of a transplanted fibrosarcoma in mice was reduced by nitrous oxide (212), and the same was found with Ehrlich ascites tumor (213). As an incidental finding, it was reported that nitrous oxide reduced the growth of lymphoma in mice (214). In another study no effect was found on a mouse mammary carcinoma (215,216). Another manifestation of cytotoxicity on chronic exposure to nitrous oxide was the inhibition of spermatogenesis in rats (217).

In the meantime, inorganic chemists investigated the reactions of a variety of transition-metal complexes with nitrous oxide. Nitrous oxide appeared to be much more reactive than was previously assumed. In 1968, Banks et al. (218) described the rapid reaction of some cobalt(I)-complexes, including cob(I)alamin, with nitrous oxide, which yielded cob(II)alamin and nitrogen. This reaction occurred under very mild conditions, i.e. room temperature, atmospheric pressure and about neutral pH. Apparently these chemists were not aware of the potential biological consequences of this reaction. Their publication escaped the attention of medical investigators, so this important observation with regard to the toxic effects of nitrous oxide remained unrecognized for a long time. Referring to the observations by Banks et al., Schrauzer and Stadlbauer subsequently described the oxidative inactivation in vitro by nitrous oxide of the bacterial cob(I)alamin-dependent enzyme ethanolamine ammonia-lyase (219). Other chemists further characterized the reaction of cob(I)alamin and nitrous oxide in 1977 (220). These observations were still not connected to the fact that nitrous oxide exposure could disturb hematopoiesis in vivo, in a way closely similar to cobalamin deficiency.

In 1978, Amess et al. reported the frequent occurrence of megaloblastic hematopoiesis in patients who had been treated postoperatively with nitrous oxide for about one day (190). This complication apparently was already a problem for some time (221). Searching for an explanation, these authors not only recovered the early reports on nitrous oxide in the treatment of tetanus, but also the chemical observations on nitrous oxide and cobalamin. With their recognition of the relation between these findings, this example of poor interdisciplinary communication had finally ended. Amess and coworkers used the deoxyuridine suppression test to demonstrate the similarity between nitrous oxide exposure and cobalamin deficiency, and suggested that the cobalamin-dependent enzyme methionine synthetase was inactivated by nitrous oxide. This report was shortly afterwards followed by a confirmation by Deacon et al. (191), and became the start of extensive studies on the metabolic effects of nitrous oxide, which will now be summarized. With respect to the theory on the function of cobalamin in hematopoiesis, the research on nitrous oxide has produced important results, which have already been mentioned in the discussion of the methylfolate trap hypothesis

(1.1.3).

First, it should be noted that in contrast to the large number of medical and biological studies that recently have appeared after the recognition of the nitrous oxide-cobalamin interaction, no further observations have been reported on the chemical basis of this effect. Perhaps, this is attributable to the cleft still existing between workers on the biological aspects of cobalamin, and those interested in its chemistry. Not much has been added to the earlier reports (218,220), which demonstrated that nitrous oxide, under conditions that are present intracellularly, oxidizes cob(I)alamin to cob(II)alamin. In view of the knowledge on the enzyme methionine synthetase, as reviewed by Taylor (33), it is to be expected that the oxidation of cob(I)alamin results in a loss of its coenzyme function and, hence, in the inactivation of the enzyme. In the mechanism of its reaction, the monovalent cobalt-ion appears to be indispensable (222,223). However, more detailed information on this effect would be useful to its biomedical application, and to the development of other cobalamin antagonists.

To discuss the studies on the nitrous oxide effect on cobalamin in a logical order, they are to be divided in the different aspects of cobalamin-dependent metabolism.

1: *Effects on methionine synthetase* are unequivocal, as all studies in which this fundamental aspect of cobalamin antagonism was investigated demonstrated the inactivation of this enzyme by nitrous oxide. Deacon et al. (191,192) first demonstrated its reduced activity in liver cells of rats exposed to nitrous oxide; subsequently this was confirmed in cells from mouse liver (224), rat liver (97,134,225-230), brain (226) and testis (231), rat embryonic tissue (229,230), sheep liver, heart and brain (232), fruit bat liver (233), human liver (234), in cultured human lymphocytes (235), and also in human bone marrow cells (236). Several conclusions can be drawn from these studies:

- the inhibition of methionine synthetase by nitrous oxide is nearly maximal within a few hours; in most studies 4 to 6 hours exposure was used to study the metabolic effects of nitrous oxide. Nevertheless, in some studies it was shown that prolonged exposure may lead to a further decrease in activity of the enzyme (225,233).
- the inhibition is virtually complete, as in most studies a residual activity of 10% or less of control values was found.
- no recovery of enzyme activity takes place during exposure to nitrous oxide, as is evident from studies with exposure of up to 33 days (225) or even 14 weeks (233).
- cessation of exposure is followed by a slow recovery of methionine synthetase activity, which does not reach control values until 3 to 4 days after exposure in most studies. In general, nitrous oxide was used in a concentration of 50%, mixed with 50% oxygen. Because this oxygen

concentration is higher than atmospheric, it could be argued that methionine synthetase is inactivated by the increased oxygen pressure. This was ruled out by a study (237) demonstrating that even prolonged exposure to hyperbaric oxygen did not affect methionine synthetase activity.

The absence of an effect of nitrous oxide on the other cobalamin-dependent enzyme, methylmalonylCoA mutase, is demonstrated by the lack of increased excretion of methylmalonic acid in urine (191). In one study, the activity of this enzyme was directly determined (225), and was found to be decreased after a rather extreme exposure to nitrous oxide of 16 days. This effect may be ascribed to a general intracellular depletion of cobalamin that occurs on chronic exposure to nitrous oxide, as oxidized cobalamin is rapidly lost from the cell, probably after the formation of cobalamin analogues (238). A recent study found no significant change in methylmalonyl-CoA mutase activity after multiple short exposures of 2 hours per day (232). The absence of a direct effect of nitrous oxide on the mutase enzyme may be related to its different mechanism, in which the oxidation state of the adenosylcobalamin coenzyme is not as essential as it is in the methylcobalamin coenzyme of methionine synthetase. A study in which the levels of individual cobalamin coenzymes were determined (239) confirmed that, at least initially, only the intracellular level of methylcobalamin became decreased on exposure to nitrous oxide.

2: *Effects on cobalamin levels in tissue and plasma* consist of a gradual decrease during exposure to nitrous oxide. This has been demonstrated in fruit bats (154), and in rats in two studies (225,238), in which in addition evidence was found that after oxidation by nitrous oxide analogues of cobalamin were formed, which were rapidly excreted. In another study a selective decrease of methylcobalamin was found in lymphocytes after nitrous oxide exposure (239), but in general not much work has been done on this subject.

3: *Effects on folate metabolism* have been studied extensively, mainly in rats, and in general appeared to confirm the methylfolate trap hypothesis.

The intracellular folate levels are reduced by exposure to nitrous oxide (108,131,240). There is a decrease in net uptake of 5-methyl-THF in liver cells (97), and, consequently, an increase in plasma concentrations of folate (241,242). This in turn leads to an increased urinary loss of folates (243). Although the total intracellular folate content is reduced, there is a relative increase in the amount of 5-methyl-THF (107,108,240,244), which is in support of methylfolate trapping. As discussed, the impaired cellular retention of folates is explainable by a failure of folylpolyglutamate synthesis. This has also been demonstrated in nitrous oxide-treated rats (47,109,133, 240,245).

In a well-documented study (240), Lumb et al. recently showed that in

rat liver the ratio of 5-methyl-THF to non-methyl-THF was increased from 0.76 to 3.60 (or, expressed in percentages, 5-methyl-THF increased from 43% to 78%), after 10 days of exposure to nitrous oxide. Furthermore, they showed that the methylgroup of 5-methyl-THF was not separated from the THF-moiety in the first 3 days of exposure, while in non-exposed control rats this occurred very rapidly. After 3 days, there was some demethylation of 5-methyl-THF, although extremely slow as compared to the normal rate. They also found evidence that with longer duration there was some folylpolyglutamate synthesis with 5-methyl-THF as a substrate. The mechanism of this potential adaptation is not clear, and its significance is uncertain, as folate levels continued to decrease until 10 days. In control rats, 5-methyl-THF was not used to synthesize polyglutamates.

Recovery of tissue folates after a period of nitrous oxide exposure has also been studied by Lumb et al. (246). It appeared that rat liver folate levels returned to control values in about 5 days after exposure, provided that the diet contained adequate amounts of folate. This demonstrates that there is indeed a loss of folate from the body, rather than a simple redistribution of folates.

It is to be expected that this disturbance of folate metabolism has considerable effects on the various folate-dependent biochemical processes. Several of these have been studied during nitrous oxide-induced inactivation of cobalamin:

- **the oxidation of formate** is an important process in toxicology, being responsible for the elimination of formic acid, which accumulates in methanol poisoning. This reaction is dependent on the availability of THF as a cofactor for formyl-THF synthetase. The subsequent action of formyl-THF dehydrogenase oxidizes formate to CO_2, releasing THF again. Rats are normally not susceptible to methanol poisoning. Only in primates the rate of formate oxidation is too slow to prevent metabolic acidosis in this situation. Eells et al. have shown that with exposure to nitrous oxide rats will also develop metabolic acidosis on administration of methanol (244), and that in monkeys the toxicity of methanol is considerably enhanced by nitrous oxide (247). It was further demonstrated that the decreased oxidation of formate correlated with the reduction of intracellular THF levels (132,227). The metabolism of other substances which lead to the formation of formic acid may also be affected (248). In primates, the capacity of this folate-dependent pathway is apparently limited, but it is not known whether this also relates to their unique susceptibility to develop megaloblastic anemia.
- **the degradation of histidine** is another folate-dependent catabolic pathway. A part of the imidazole ring of histidine is transferred to THF, the resulting 5-formimino-THF is converted into 5,10-methenyl-THF, and eventually via formyl-THF, to CO_2 and THF again. The excretion of

formiminoglutamic acid (FiGlu) is a sensitive marker of impairment of this pathway. Accordingly, in nitrous oxide-treated rats (134) and sheep (232) the excretion of FiGlu in urine was markedly increased, while the rate of histidine oxidation was substantially reduced (132).

- **the synthesis of nucleotides:** impairment of the folate-dependent steps in nucleotide synthesis directly affects cellular proliferation. In view of the special importance of this effect, it will be discussed separately.

4: *Effects on methionine metabolism* are as would be expected with the inhibition of methionine synthesis: methionine levels are reduced, both in plasma (232,242,249,250) and cells (226). In addition, the intracellular concentration of S-adenosylmethionine (SAM) is lowered by nitrous oxide treatment (132,226,251). There is some recovery of intracellular methionine in rat liver after 6 days of exposure (226), possibly due to the induction of another pathway converting homocysteine into methionine, through the reaction catalyzed by betaine-homocysteine methyltransferase. Nevertheless, SAM levels continue to fall with exposure of up to 11 days.

The functional significance of methionine and SAM levels with regard to folate metabolism, which has already been discussed, was also established in experiments with nitrous oxide. The addition of methionine or SAM restored histidine degradation (132,134), formate oxidation (132,247), folate retention (131), folylpolyglutamate synthesis (133) and purine synthesis (252). Methionine can also correct the nitrous oxide effect on the deoxyuridine suppression test (152), but at higher concentrations it causes further disturbance (236). This discrepancy was recently discussed by the authors of these articles (253), and has also been dealt with in 1.1.4. (Figure 1.5). It has also been discussed that a disturbance of SAM-mediated methylation reactions is implicated in the neurological effects of chronic nitrous oxide exposure (130,153,154). Interestingly, the conversion of methionine to SAM, catalyzed by methionine adenosyltransferase, appeared to be more active during nitrous oxide exposure (251).

It would be expected that decreased activity of methionine synthetase not only leads to a low level of methionine, but also to increased homocystine excretion. This was not the case in patients after nitrous oxide anesthesia (254), but it was in sheep (232), and in rats homocysteine in serum was found to be increased (181).

Although it has been found that protein and immunoglobulin synthesis by lymphocytes is decreased in cobalamin deficiency (255), there is no evidence that protein synthesis is affected by the reduced levels of methionine on nitrous oxide exposure. However, this apparently has not yet been investigated in detail.

Another metabolic process dependent on methionine and SAM supply is the synthesis of the polyamines spermidine and spermine. Although these compounds are specifically related to cell proliferation, no clear evidence of

their involvement in the effects of nitrous oxide or cobalamin deficiency has been established. In chapter 7, this will be discussed in more detail.

5: *Effects on the synthesis of nucleotides*, which are fundamental to the influence of nitrous oxide on cellular proliferation, are a consequence of the effects on folate metabolism described above. Folate coenzymes are involved in purine synthesis and in the conversion of deoxyuridylate to thymidylate. Purine synthesis appears to be impaired by exposure to nitrous oxide in rats (252,256,257) and in human lymphoid cells (235). The significance of these changes in purine metabolism has not been firmly established at present, while much more is known on the impairment of thymidylate synthesis by nitrous oxide exposure. The folate-dependent methylation of deoxyuridylate is the last step in the *de novo* synthesis of thymidine, which is essential in DNA synthesis only. Usually, the deoxyuridine (dU) suppression test is applied in the evaluation of this pathway. This test is clearly disturbed in bone marrow cells of man and animals after nitrous oxide exposure (148,152,190,228,236,242,258-260). The dU suppression test can also be used to study the effect of various additives on the synthesis of thymidylate, as has been done in cells from cobalamin or folate deficient patients. In these experiments, nitrous oxide-treated cells appeared to be fully comparable to cobalamin-deficient cells. The results, therefore, are as discussed before (in 1.1.3): incomplete correction by cobalamin addition, and complete correction by reduced folates, but not by 5-methyl-THF (242,258). As described in 1.1.5, in studies of Chanarin's group on rats, THF did not correct the test results (148), but in one, more recent study of this group on human marrow cells (242), only 5-methyl-THF failed to produce correction. The results obtained with the nitrous oxide model are therefore, in this respect as well, in support of the methylfolate trap hypothesis. In a recent study in fruit bats, the dU suppression test was not disturbed by nitrous oxide, although methionine synthetase activity was reduced (233). Although fruit bats are extremely sensitive to the neurological effects of cobalamin deficiency, they may be rather resistant towards the effects on folate metabolism.

6: *The effects on cellular proliferation* are a logical consequence of the impairment of nucleotide synthesis. The bone marrow depression in patients after prolonged nitrous oxide anesthesia obviously is the result of reduced cellular proliferation. This effect was analyzed in detail by Skacel et al. (242), who emphasized the morphological and biochemical similarities with classical cobalamin deficiency. The growth of several hematopoietic cell lines was shown to be inhibited by nitrous oxide exposure, with disturbance of the dU suppression test (261). The nature of the inhibition of proliferation was analyzed in a study employing DNA flow cytometry (262). It was found that in marrow cells of patients treated with nitrous oxide for 1 day, there was a significant increase of cells in the early S-phase of the cell cycle, and a decrease in the late S, G_2 and M phases. This is in

agreement with the presumed impairment of DNA synthesis as the mechanism of the effects on cellular proliferation.

7: *Clinical effects* of the nitrous oxide-cobalamin interaction are of considerable interest, in view of the widespread application of nitrous oxide in anesthesia, and the potentially serious consequences of its toxicity. The manifestations of this interaction, as mentioned above, can lead to unwanted side-effects of the use of nitrous oxide. Fortunately, the duration of ordinary anesthesia is too short to give rise to serious hematological toxicity. It appears, however, that in some situations the interaction of nitrous oxide and cobalamin may lead to clinical manifestations. It has been reported that in seriously ill patients, who needed intensive care after surgery under nitrous oxide anesthesia, there was a more severe disturbance, and a slower recovery, of the dU suppression test compared to other patients, after operations of equal duration (263). Furthermore, the occurrence of a megaloblastic bone marrow change in some of these patients correlated with a very high mortality. It has also been argued that repeated exposure to nitrous oxide in a short period may more rapidly induce megaloblastic hematopoiesis (264). Another problem may be the existence of unrecognized cobalamin or folate deficiency in some patients, because it has been shown in rats that the deleterious effect of cobalamin deficiency is enhanced by nitrous oxide exposure (265). It was recently reported that this can result in the precipitation of neurologic disease in patients (266). The interaction of nitrous oxide with folate antagonists is also a potential problem (267), as this may lead to a synergistic effect on folate metabolism (236). There is one report of unexpectedly severe toxicity of adjuvant chemotherapy including methotrexate, which had been started immediately after surgery for breast cancer (268). The authors attributed this effect to a potentiation of the effects of methotrexate by nitrous oxide.

A judgement on the acceptability of these risks is obviously extremely difficult, considering the advantages of nitrous oxide, and the toxic effects of alternative anesthetics. It should be noted that it is possible to avoid the hematological toxicity simply by the administration of folinic acid (257,269,270). Measurement of serum cobalamin and folic acid, and awareness of the existence of this interaction, are necessary to be able to recognize the probably rare patient who should be protected against the consequences of cobalamin inactivation.

1.4. Scope of this thesis.

The subject of this thesis is the investigation of the role of cobalamin in leukemic cell proliferation. This has been studied by the application of the nitrous oxide-induced inactivation of the cobalamin coenzyme function in methionine synthetase. In most of the studies, the well-characterized

myeloid leukemia of BN rats (BNML) has been employed as a model for human leukemia. In some studies leukemic bone marrow cells from patients have been used. The intention of the studies was to provide answers to the following questions:

- does inactivation of the cobalamin coenzyme function by nitrous oxide result in reduced growth of leukemia in vivo? *(chapter 3)*;
- are there accompanying metabolic effects, on the deoxyuridine suppression test, and on plasma cobalamin and folate levels? *(chapter 3)*;
- is it possible to apply the inactivation of cobalamin in combination chemotherapy with agents active in related pathways, to achieve synergistic effects? This can be expected with combinations involving:
  1. inhibitors of the synthesis of S-adenosylmethionine (SAM), in view of the effects of SAM on folate metabolism, discussed in 1.1.4. *(chapter 4)*;
  2. folate antagonists, because cobalamin inactivation has been shown to disturb folate metabolism and to cause folate depletion *(chapter 5)*;
  3. inhibitors of thymidylate synthetase, because cobalamin inactivation will impair folate-dependent thymidylate synthesis *(chapter 6)*;
  4. inhibitors of the decarboxylation of SAM, which cause impairment of polyamine synthesis; their action could be enhanced by a reduction of SAM synthesis as occurs with cobalamin inactivation *(chapter 7)*; Appropriate metabolic studies are essential in the evaluation of all these combinations, along with the effects on leukemic growth.
- can the effects of cobalamin inactivation by nitrous oxide, alone or in combination with other agents, be reproduced in human leukemic cells, as an essential step towards the application of this approach in clinical chemotherapy *(chapter 8)*?

The results of these studies may lead to a tentative conclusion with regard to the perspectives of cobalamin inactivation in antileukemic chemotherapy. Prior to the presentation of these studies in chapters 3 to 8, some essential experimental methods are described in chapter 2. This involves the BN rat leukemia model, the exposure of rats to nitrous oxide, and the deoxyuridine suppression test.

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Chapter two
GENERAL
EXPERIMENTAL PROCEDURES

2.1. The Brown Norway Myeloid Leukemia.

In most studies presented in this thesis, the Brown Norway Myeloid Leukemia (BNML) has been used as a model for human myeloid leukemia. This leukemia, which is transplantable in BN rats, is well characterized and its properties appear to be very suitable for experimental chemotherapeutic studies. Typical advantages of the BN leukemia are: its slow growth rate, leading to the gradual suppression of normal hematopoiesis by bone marrow infiltration, and to the development of hepatosplenomegaly, quite similar to the pattern observed in human acute myeloid leukemia; its response to chemotherapy, which is very well comparable to human AML; the exact reproducibility of its growth chararacteristics, which facilitates the application of this model.

Origin of the BNML. This rat leukemia model has been developed at the Radiobiological Institute TNO, Rijswijk, The Netherlands, by Hagenbeek and Van Bekkum. The leukemia was induced in a female BN rat in 1971, by injections of 9,10-dimethyl-1,2-benzanthracene, 100 days earlier. It was introduced in 1977 (1-3). Although the leukemia can be maintained by continuous in vivo passages, this procedure has the great risk of changing growth properties. Therefore, it is preferable to minimize in vivo transfer by using cells from a large cryopreserved stock. The studies presented in this thesis were all carried out with cells from the same stock, which was built up after a single passage in vivo of an initial sample that was kindly provided by dr A. Hagenbeek.

Characteristics of the BNML. The BN rat leukemia has morphologically and cytochemically been classified as an acute promyelocytic leukemia. The cells are large, 10-18 μm in diameter, and of irregular shape. Their large oval nucleus has a coarse chromatin pattern with a nucleolus. With May-Grunwald-Giemsa staining, cytoplasmic granules are not observed as clearly as in human promyelocytes, and Auer rods are not present, but the cytochemistry is almost identical. Peroxidase and Sudan black are strongly positive. The survival time of leukemic rats is strictly dependent on the number of the injected leukemic cells. With 10^7 cells, often used as a standard dose, rats invariably die between 20 and 24 days. A tenfold increase, or decrease, of this dose shortens, or prolongs, survival with 3 days. This

rather precise exponential dose-survival relationship can be applied in chemotherapeutic studies to calculate the proportional cell kill in various treatment groups. The growth pattern of this leukemia has been described in considerable detail (2-6). It has been shown that various organs are infiltrated by the leukemic cells, notably bone marrow, liver and spleen, but also lungs and kidneys. The increase in spleen weight is particularly impressive: in terminally leukemic rats this organ can be enlarged tenfold to 4-5 g. Normal hematopoiesis gradually disappears (7,8). The cause of death in leukemic rats usually is bleeding, due to severe thrombocytopenia, but infection may also occur. In our studies, splenic rupture was a rare, but occasionally observed cause of premature death in rats older than about 16 weeks.

Evaluation of treatment results. The effects of chemotherapy on this rat leukemia can be evaluated by comparing several of the characteristics of this disease between treatment groups. In Table 2.1, some of the techniques used for that purpose are listed. Most simple is the study of survival times. A disadvantage of this method is that it precludes metabolic or hematologic studies on the rats. Moreover, it generally requires large numbers of rats to demonstrate a difference in tumor reduction of less than tenfold, which still can be relevant in experimental chemotherapy. Furthermore, the extent of supportive care provided to the leukemic animals has a great influence on the results. Advanced supportive care, like blood transfusions, can make the model more realistic, and may also lead to more pronounced effects of treatment (9), but this approach is experimentally cumbersome. With most other methods, the duration of the disease is limited to a fixed period, usually chosen as long as possible, that is: until the untreated controls would die of the disease. This approach has the additional advantage of avoiding the terminal suffering of rats in all groups.

Table 2.1

EVALUATION OF TREATMENT IN THE BN RAT LEUKEMIA

1. Survival of rats (with or without hematological supportive care)
2. Bio-assay of leukemic clonogenic cells in bone marrow (LCFU-s)
3. Leukemic infiltration of organs: liver, spleen
4. Hematological determinations in blood and bone marrow
5. Depression of normal hematopoiesis in vivo (CFU-s) or in vitro (CFU-GM, CFU-E, BFU-E)

One of these methods is the quantification of leukemic clonogenic cells in a bio-assay (LCFU-s). Bone marrow contents of leukemic rats, either or not treated, are injected into normal BN rats. On the surface of the spleen

of these rats, leukemic colonies will appear after about 19 days, and can be counted. This is only possible within a narrow range of the injected number of cells. With more than 5 x 10^4 cells, individual colonies become confluent (9). The assay also has a rather large standard deviation. Although the technique has a specific advantage in measuring only those leukemic cells that are able to resume proliferation, its narrow range, and the large number of animals required, severely limit its application.

The leukemic infiltration of organs, in particular of liver and spleen can be determined simply, because the weight of these organs is a direct measure of their infiltration. Spleen weight can increase about tenfold, and liver weight about threefold, in untreated BNML rats. This excessive splenomegaly of leukemic rats is the reason that spleen weight is a sensitive parameter of leukemic growth, applicable in a wide range of tumor load. The determination of organ weights can be combined with studies of survival, by autopsies of rats dying spontaneously, but this appeared to be unreliable in some of our studies. It is likely that in terminally ill rats the infiltration of liver and spleen does not increase further, and may even decrease substantially, as is the case with their total body weight. Therefore, organ weights should be determined before rats tend to die spontaneously.

Hematological parameters are essential in the evaluation of leukemia. The total peripheral leukocyte count is a simple and sensitive technique, showing a gradual increase in the course of this leukemia. The BNML does not give rise to excessively high leukocyte counts, except sometimes in terminally ill rats. In normal rats, the leukocyte count is 3-4 x 10^9/l, and in leukemic rats this may rise to about 30 x 10^9/l. The differential counts demonstrate that the increase of leukocytes indeed is due to the appearance of leukemic cells (promyelocytes) in the blood. It appears that there is also an increase in the absolute number of normal lymphocytes in the leukemic rats. Differential counts of bone marrow cells provide additional information only in the case of nearly complete remissions. Other hematological determinations have a limited significance in the evaluation of treatment: erythrocyte and hemoglobin values fall slowly during the disease (except in the case of bleeding), and thrombocytes are nearly always low. Finally, by measuring directly the residual normal hematopoiesis in vivo or in vitro, the destructive influence of leukemic growth can be demonstrated. Counting the hematopoietic colonies on the spleen of irradiated mice (CFU-s) after the injection of a bone marrow sample is a well-established technique. In bone marrow cultures, colonies of several hematopoietic precursors can be counted (CFU-GM: granulocytes/macrophages, CFU-E and BFU-E: colony and burst forming units, consisting of erythroid precursors). In general, depression of normal hematopoiesis will be less severe with effective treatment, although the therapy is usually also toxic to the bone marrow.

In the studies presented in this thesis, leukemic growth has been evaluated by the determination of spleen and liver weights, by total and

differential leukocyte counts, and occasionally by survival studies, without supportive care. In addition, it was found that the plasma level of cobalamin gradually increased in the course of the leukemia. This may be comparable to the frequent occurrence of increased cobalamin levels in human acute promyelocytic leukemia (10). As will be discussed in the following chapters, this finding has made possible the use of plasma or serum cobalamin levels as an additional parameter of leukemic growth.

Relevance of this leukemia as a model for human AML. It has already been described that several characteristics of the BNML model are well in agreement with human AML. This relates to the cytology, which is very similar to the human AML subtype M3, the growth pattern and symptoms of the disease, notably the gradual suppression of normal hematopoiesis, and finally, its similar sensitivity to chemotherapy. This latter point has been substantiated by a large number of studies since the introduction of this leukemia (9,10-15), in which the response to chemotherapy of this rat leukemia appeared to be largely comparable to human AML. It has also been shown that its sensitivity is very different from human ALL (9). Thus, the BNML model may indeed be considered a relevant model with regard to experimental chemotherapy of AML.

Finally, some further technical details concerning the use of this model will be described.

Animals. Rats of the Brown Norway inbred strain were used, obtained from REPGO-TNO in Rijswijk, or CPB-TNO, Zeist, The Netherlands, who deliver the same strain of rats. Only male rats were used, at the age of 12 to 16 weeks (body weight 200-275 g). In all studies, the rats received standard food pellets and water ad libitum.

Transfer of leukemia. Leukemia may be transferred to rats, either by using cells from the frozen stock, or by preparing a cell suspension directly from the spleen of a leukemic rat (Figure 2.1). Rats injected with thawed cells were not used in experiments, however, because of rather large variations in growth kinetics, probably due to varying damage by freezing and thawing. The spleen of these rats is used exclusively to transfer leukemia to the experimental series. This serial transplantation was subsequently limited to one further generation only, to avoid a gradual change in growth properties. Consequently, after two experimental series, spleen cells were again obtained from a rat freshly inoculated with cells of the frozen stock. A monocellular suspension of leukemic spleen cells was prepared by cutting the organ into small pieces and gently pressing these fragments through a fine (50 μm) nylon gauze, while rinsing with Hanks' balanced salt solution (HBSS). The cells were washed with HBSS once before use. Cells frozen in 10% dimethylsulfoxide and 20% fetal calf serum were kept in liquid nitrogen. Before use, ampoules were rapidly thawed in a water bath of 37 ^{o}C, slowly diluted stepwise in about 30 min, and washed in HBSS.

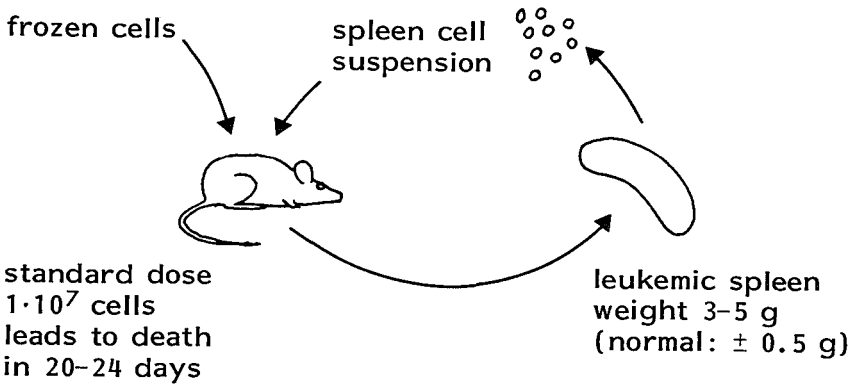

Figure 2.1. Transfer of leukemic cells in the BN myeloid leukemia is possible either by using cells from a large frozen stock, or by direct transplantation of spleen cells from fully leukemic animals. The latter procedure is used in the experimental series, but is limited to only two serial transplantations, to prevent a gradual change in growth properties.

Viability of the cells, checked by eosin uptake, had to be less than 50%. Leukemic cell suspensions were counted electronically and 10^7 cells were injected intravenously in rats under ether anesthesia.

2.2. Exposure of rats to nitrous oxide.

The investigation of the effects of nitrous oxide-induced inactivation of cobalamin usually requires exposure to this gas of more than about one day. As discussed in the former chapter, biochemical evidence of this effect can be found already after a few hours of exposure, but it is important to realize that the severe disturbance of folate metabolism, with subsequent depletion of cellular folates develops only gradually. If it is the objective of a study to examine the effects of this disturbance on the growth of a malignant tumor, it is likely that even longer periods of exposure will be necessary, to find a measurable degree of growth inhibition. This is indeed the case in the studies presented in this thesis, and for that reason an exposure system has been developed that allows such prolonged exposure of leukemic rats to nitrous oxide, for periods of one week or slightly longer.

Chronic exposure of rats to an atmosphere containing about 50% nitrous oxide is surprisingly simple, as this has no appreciable effects on the consciousness of the animals. This means that exposure, after an adaptation period to the system of a few days, does not affect the nutritional condition of the rats. In the present studies, body weight of the rats did not change

significantly by exposure of more than one week. Parbrook (15) has investigated the effects of nitrous oxide exposure on food consumption and weight of several experimental animals, and demonstrated that rats were the least sensitive of the species tested. He also described an effective exposure system, including a cleaning circuit in which the atmosphere of the cage is recirculated. This circuit provides for humidity and temperature control by cooling, carbon dioxide absorption by soda lime and absorption of contaminating volatile compounds by activated charcoal. A similar recirculating system was described by Rupreht and Dzoljic (17). The system used in the studies of this thesis is based on the same principles, as is shown in Figure 2.2. The main advantage of a recirculating system is that a stable atmosphere can be maintained without an excessive flow of fresh gases. The

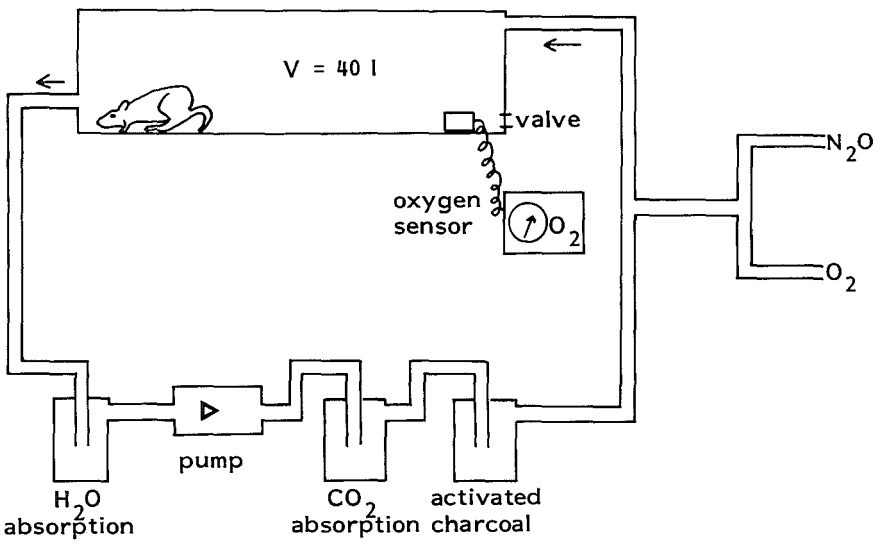

Figure 2.2. Exposure system, designed to allow prolonged exposure of leukemic rats to a mixture of nitrous oxide (N_2O) and oxygen (O_2).

flow is regulated by rotameters, which are calibrated with an oxygen analyzer only, because no effective method is available for the continous monitoring of nitrous oxide. As in most studies, an atmosphere of 50% nitrous oxide with 50% oxygen was chosen. This allows the use of a relatively simple two cylinder system, and minimizes the risk of hypoxia. It also means that the partial pressure of oxygen is increased, compared to air. It has been shown, however, that no effect is observed even from hyperbaric oxygen on the cobalamin-dependent methionine synthetase (18). Moreover, oxygen transport in the circulation is hardly influenced by this increase in partial pressure, because hemoglobin is already saturated at normal atmospheric pressure. Rats can be maintained in this system without any interruption as long as in normal cages in air, that is for 3 or 4 days if necessary.

The continuous flow of fresh gases, in the volume of about 40 l, can be as low as 500 ml/min for the mixture of the two gases, except for the first few hours, when 1000-1500 ml/min was blown in. Rats not exposed to nitrous oxide, in the same experiment, were kept in ambient air, but otherwise treated identically as much as possible.

2.3. The deoxyuridine suppression test.

The deoxyuridine (dU) suppression test is designed to demonstrate a failure of the de novo synthesis of thymidylate, as occurs in cobalamin or folate deficiencies. The principles of this test are essentially simple, and were first described by Killmann in 1964 (19). The cells to be investigated, usually bone marrow cells, are first incubated with dU, and after a few hours labelled thymidine is added.

The incorporation of [^{3}H]-thymidine into DNA in these cells is then compared with the incorporation in the same cells, which were not incubated with dU. In normal cells, it appears that dU considerably suppresses this incorporation of [^{3}H]-thymidine. This is explained by the conversion of dU, after its phosphorylation to deoxyuridylate (dUMP), to thymidylate by the enzyme thymidylate synthetase. An increase in thymidine nucleotides will then dilute the labelled exogenous thymidine, and suppress its metabolism through the salvage pathway. The activity of thymidylate synthetase is, as described previously, dependent on its folate coenzyme, 5,10-methylene-THF. In the case of cobalamin or folate deficiency the activity of this enzyme will be impaired, and consequently, there will be much less suppression of [^{3}H]-thymidine incorporation by dU. In Figure 2.3, the pathways relevant to this test are shown.

Metz et al. (20) started to use this test in metabolic studies of cobalamin and folate deficient cells, and ever since this test has played an essential role in this field. Although the test is mainly used in research of cobalamin and folate metabolism, it has also been applied clinically as a diagnostic aid in the evaluation of megaloblastic anemia (21). In a recent report, it was suggested that the dU suppression test could uncover subtle cobalamin deficiency, prior to the development of anemia (22). A particular advantage of the test is the possibility to study the effects of different additions to the incubations, which can clarify the nature of a metabolic disturbance. It has been described (1.1.3) that in cobalamin deficiency, 5-methyl-THF cannot correct the abnormal test results, in contrast to folate deficiency. On the other hand, cobalamin addition (partially) corrects the test in cobalamin deficiency, and is completely ineffective in folate deficiency. These and similar observations have been essential in the study of cobalamin and folate

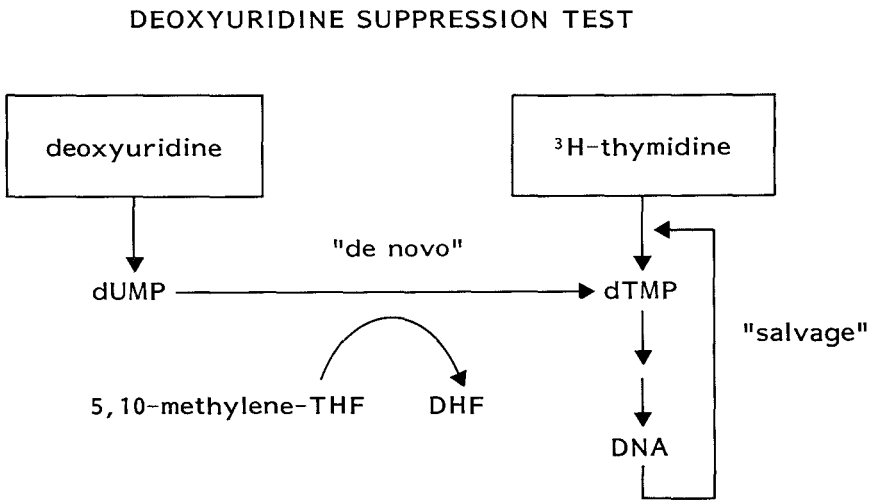

Figure 2.3. The deoxyuridine suppression test measures the effect of deoxyuridine addition on the incorporation of labelled [^{3}H]-thymidine in DNA. There will be a significant suppression of this incorporation if dUMP is converted into dTMP by the folate-dependent enzyme thymidylate synthetase. There is much less suppression if folate metabolism is disturbed, as is the case in deficiency of either cobalamin or folate.

deficiencies, and also in the study of cobalamin antagonists, since the introduction of nitrous oxide. It is noteworthy, that these are not the only causes of a disturbed dU suppression test. Several cytostatic drugs, notably 5-fluorouracil, which directly inhibits thymidylate synthetase, and methotrexate, will cause abnormal test values (21). Therefore, the dU suppression test has also been applied in studies of combinations involving these drugs (23). Although the usefulness of this test is clearly beyond doubt, some of its principles have been challenged in recent years. It has been shown that there are other factors that influence [^{3}H]-thymidine incorporation in this test, apart from the rate of conversion of dUMP to dTMP (24,25). These include the inhibition of thymidine uptake and thymidine kinase in the salvage pathway of thymidine synthesis by dUMP, a certain degree of inhibition of [^{3}H]-thymidine incorporation by other deoxynucleosides than dU, the inhibition of incorporation of other labelled nucleotides besides [^{3}H]-thymidine by dU, and the stimulation of the catabolism of dUMP by some drugs, notably methotrexate. These mechanisms may be important with respect to the specificity of the dU suppression test, which is probably less than presumed, but do not interfere with the validity of earlier observations in cobalamin and folate deficiencies. Of all factors influencing this test, it appears that the efficacy of the de novo synthesis of thymidine is the most

important.

Considerable confusion exists on the technical details of the dU suppression test. As described by Wickramasinghe (21), differences are found in the literature concerning all aspects of the test: concentrations of cells, dU, [^{3}H]-thymidine; incubation times; temperature during the incubations; methods to extract DNA. These variations do not lead to important differences in results, but they may explain that the test results generally are not quantitatively comparable between studies. A more basic point is the need for appropriate controls, if the influence of additions is investigated (26), which has not always been recognized. A different approach to the test has been described, in which [^{3}H]-dU is used instead of labelled thymidine (20,27). A disadvantage of this method is that the effect of correcting additives is necessary to demonstrate a disturbance of the test (27,28). "Thymidine suppression" does not discriminate sufficiently between normal and megaloblastic cells.

The dU suppression test as applied in the studies of this thesis was carried out rather closely according to the original technique described by Metz et al.(20). Some modifications will be summarized now, and can also be found in the various chapters concerning more specific points. The dU suppression test was applied to fresh suspensions of leukemic spleen cells, which were washed once in Hanks' balanced salt solution. Incubations were performed in triplicate, with about 5 x 10^6 cells in each tube. dU (Sigma) was generally used in a concentration of 0.1 mmol/l, and 0.3 μCi of [^{3}H]-thymidine (Amersham, specific activity of 25 Ci/mmol) was used per test. Incubations, at 37 ^{o}C, lasted for 4 hours (2 hours pre-incubation with or without dU, and 2 hours with [^{3}H]-thymidine). DNA was extracted conventionally with cold 1% trichloroacetic acid.

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Chapter three
NITROUS OXIDE AND GROWTH OF RAT LEUKEMIA

This chapter has been published in Leukemia Research, 8:441-8 (1984), titled "Nitrous oxide reduces growth of experimental rat leukemia", by A.C.M. Kroes, J. Lindemans, A. Hagenbeek and J. Abels.

Abstract. The ability of nitrous oxide to inhibit the in vivo growth of hematological neoplasms was investigated in a rat model for acute myeloid leukemia (BNML). Nitrous oxide, administered in a concentration of 67% with 33% oxygen, resulted in a reduction of spleen and liver weights of approx. 30%, as compared with leukemic rats kept in ambient air. Peripheral white cell counts were also considerable lower in the treated rats. Plasma levels of vitamin B12 were found to be elevated in untreated leukemia, but fell to about normal levels after nitrous oxide exposure. On the contrary, folic acid levels were low in untreated leukemic rats, and significantly higher in animals exposed to nitrous oxide. The observed effects of nitrous oxide appeared to be dose dependent. The deoxyuridine suppression test performed with leukemic cells became abnormal with nitrous oxide inhalation, in accordance with the effect on normal bone marrow. These results indicate that the interference of nitrous oxide with vitamin B12-related metabolism, which leads to impairment of *de novo* thymidine synthesis, has the potency to reduce leukemic proliferation in vivo.

3.1. Introduction.

The anesthetic gas nitrous oxide (N_2O) has the side effect of suppressing hematopoiesis. This was first described in 1956, when patients with tetanus who were treated with nitrous oxide anesthesia for several days developed pancytopenia with megaloblastic changes in the bone marrow (1). Subsequently it was found that in rats, after prolonged exposure to nitrous oxide, there was a marked but reversible leukopenia (2-5), while other hematological effects were much less apparent. Preliminary trials utilizing this effect in the treatment of human chronic (6) and acute (7) leukemia have resulted in regression of leukemia upon prolonged exposure to nitrous oxide, in all four instances tried.

In spite of these remarkable results, further reports on the antileukemic effect of nitrous oxide did not appear.

In 1978, it was discovered that nitrous oxide oxidizes the cobalt-moiety of vitamin B12 (8), leading to inactivation of the methylcobalamin-requiring enzyme methionine synthetase or 5-methylhydrofolate homocysteine methyltransferase (9,10). The resulting impairment of folate-dependent do novo synthesis of thymidine will ultimately affect proliferation of fast dividing cells, like hematopoietic precursors. In recent years, the effects of nitrous oxide on vitamin B12-related metabolism have been studied extensively. These data were recently reviewed (11). It appears that nitrous oxide effectively can be used as an anti-cobalamin, inducing a state of functional vitamin B12-deficiency. The present study was designed to investigate the possibility of reducing leukemic proliferation by nitrous oxide exposure. A rat leukemia model was used: the Brown Norway myelocytic leukemia

(BNML). This transplantable leukemia has been described in detail (12-14). Its most important characteristics are the slow net growth rate, the gradual suppression of normal hematopoiesis and the similarity with human acute myeloid leukemia in response to chemotherapy, which make it a suitable model for experimental treatment of leukemia (15).

3.2. Materials and methods.

Animals. Male rats of the Brown Norway inbred strain were used, at the age of 12-16 weeks (body weight 200-250 g). Food and water were supplied *ad libitum* during the experiments.

Brown Norway myelocytic leukemia (BNML). Cryopreserved leukemic cells were provided by the Radiobiological Institute TNO, Rijswijk, the Netherlands, where this transplantable rat leukemia model was developed. Origin, classification and proliferation kinetics were described before (12-14).

For leukemia transfer in experimental series, spleen cells of fully leukemic animals were used. 10^7 cells were suspended in 1 ml of Hanks' balanced salt solution and injected i.v.. This standard dose leads to death in 20-24 days. During this period massive infiltration of spleen liver and bone marrow takes place; spleen and liver weights therefore can be used as indicators of tumor load. To avoid a gradual change in growth properties, serial transplantations were limited to only two passages in order, after which spleen cells were used from animals of a separate series freshly inoculated with cells from a cryopreserved stock.

Exposure to nitrous oxide. Rats were exposed in a flow chamber to a mixture of 67% nitrous oxide and 33% oxygen at a rate of at least 500 ml/min. Exposure started 7 or 8 days after inoculation with leukemia cells, and lasted until termination of the experiments at 17-19 days after inoculation. For each group treated with N_2O, consisting of 4-6 rats, a matched control group, inoculated with the same cell suspension, was maintained in ambient air during the same period. In one experiment a separate group of four leukemic rats was exposed intermittently to nitrous oxide, with change from air to nitrous oxide/oxygen atmosphere and reverse every 2 days. In one nitrous oxide-treated group, normal (non-leukemic) rats were included.

Methods of evaluation. Termination of the experiments at 17-19 days after inoculation of rats with leukemia is just before their expected death. Rats of both exposed and control groups were killed by exsanguination under deep ether anesthesia. Liver and spleen weights were recorded. White blood cells were counted electronically and routine differential blood cell counts were made. In some experiments, cellular contents of bone marrow were determined by flushing femurs with a constant volume of Hanks' solution and electronic counting of the resulting suspension. Plasma vitamin B12 and

folic acid levels were determined simultaneously, essentially as described by Gutcho and Mansbach (16). Methyltetrahydrofolate was used as a folate standard. In one additional experiment, the orbital venous plexus was punctured every 8 days to obtain 0.5 ml of blood, for regular assays of vitamin B12 and folic acid. This was done in 14 rats, who at 8 days after inoculation were divided in a treatment and a control group, each of seven rats. This experiment was excluded from the studies of organ weights. Normal values for organ weights, as shown in the figures, were derived from earlier experiments with large series of the same strain of rats (14).

Deoxyuridine suppression test. This test was done in some experiments to evaluate the metabolic inactivation of vitamin B12. It measures the reduction of [^{3}H]-thymidine uptake (salvage pathway) by the addition of deoxyuridine, thus stimulating *de novo* synthesis of thymidine. Decreased reduction is seen as a result of folate or vitamin B12 deficiency (17), as in this experiment, as a result of the inactivation of methylcobalamin (18). The test was carried out according to Metz (19). Leukemic spleen cells were used (ca. 5 x 10^6 per test). Incubation with deoxyuridine (Sigma Chemical Co., St.Louis, Missouri, USA) in various concentrations for 1 h, was followed by addition of [^{3}H]-thymidine (ca. 3 μCi per test, specific activity 25 Ci/mmol, from Amersham International, UK), and incubation for another 3 h, after which the incorporation of labelled thymidine in DNA was determined. This incorporation is expressed as a percentage of the maximum incorporation, obtained by omitting deoxyuridine.

3.3. Results.

The results of four separate experiments in each of which 10-12 leukemic rats were divided in control and treatment groups are shown in Figures 3.1 and 3.2. Figure 3.1a demonstrates a consistent reduction in spleen weights of the nitrous oxide-treated groups, as compared to the control groups breathing air. This reduction amounted to 29.4 $\pm$ 8.0% (mean $\pm$ S.D.), after subtraction of a normal average spleen weight of 0.6 g. Included in this as well as in other figures is one experiment in which a group of leukemic rats (n=4) was exposed intermittently to nitrous oxide; treatment periods of 2 days alternated with equal periods in air. In most cases, the results are intermediate between untreated and fully treated groups. Figure 3.1b shows a reduction in liver weight, averaging 32.6 $\pm$ 4.1%, as calculated after subtraction of a normal liver weight of 8 g. Figure 3.1c indicates a considerable decrease in peripheral white cell counts after nitrous oxide exposure. The differences in spleen and liver weights, and white cell counts, were statistically significant in each experiment, using Wilcoxon's non-parametric rank-serum test; p-values were less than 0.05 or 0.01, except

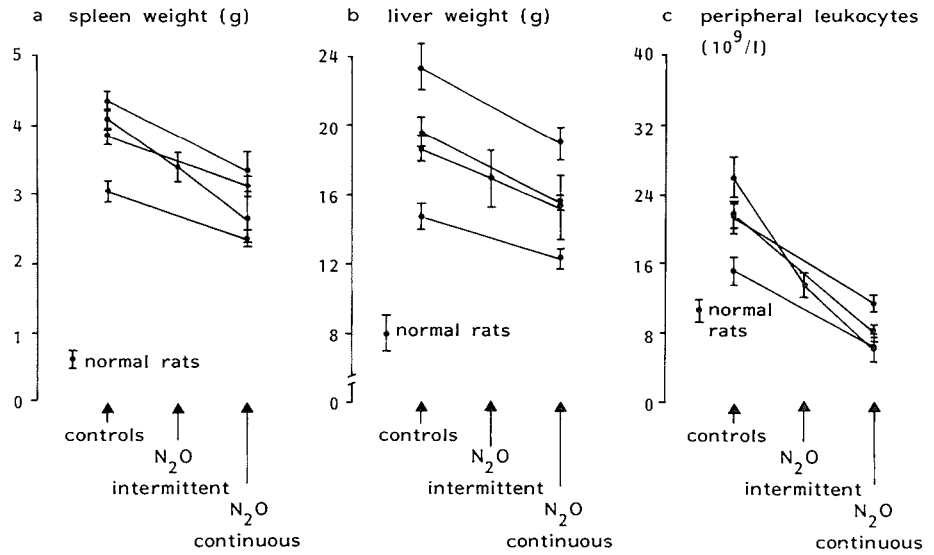

Figure 3.1. Effect of nitrous oxide (N_2O) on (a) spleen weight, (b) liver weight, (c) peripheral white cell count. Controls are untreated leukemic rats. Lines connect control and treatment groups of the same experiment. Standard errors of the mean are indicated. Values in comparable normal (non-leukemic) rats are shown.

for the differences in the experiment with intermittent exposure.

Results of a differential count of white cells can be found in Table 3.1, which demonstrates there is also a relative decrease in leukemic cells after nitrous oxide exposure.

In one experiment, normal (non-leukemic) rats exposed to nitrous oxide during the same period showed no hematological abnormalities.

Plasma levels of vitamin B12 were found to be raised in untreated leukemia and about normal after nitrous oxide exposure (Figure 3.2a). Folic acid levels, on the contrary, were decreased in untreated leukemic rats, but again about normal, or slightly raised, in treated animals (Figure 3.2b). In order to gain more insight in the development of the changes in vitamin B12 and folic acid levels, a separate experiment was carried out with venous puncture at days 0, 8 and 16 after inoculation (Figures 3.3a,b). Nitrous oxide apparently reversed the gradual changes observed in the untreated leukemic animals.

In some experiments the, purely leukemic, bone marrow cells were collected from femurs of treated and control groups, and counted. Values obtained are (mean $\pm$ S.D.) $3.73 \pm 0.42 \times 10^7$ cells/femur for the nitrous oxide-treated animals, and $3.14 \pm 0.32 \times 10^7$ cells/femur for the controls.

The Wilcoxon rank-sum test of the values in these groups did not yield a significant difference: $p = 0.05$.

Table 3.1

DIFFERENTIATED BLOOD CELL COUNTS, EXPRESSED AS PERCENTAGES OF THE TOTAL NUMBER OF NUCLEATED CELLS, COUNTING 200 CELLS

	Promyelocytes (leukemic cells)	Lymphocytes	Neutrophils	Eosinophils	Monocytes
Leukemic rats, untreated controls (n = 6)	55.8 ± 3.7	40.3 ± 3.4	3.8 ± 1.2	0	0
Leukemic rats, N_2O-treated (n = 5)	41.6 ± 7.0	56.0 ± 7.1	2.3 ± 0.4	0	0
Normal rats (n = 4)	0	91.0 ± 1.8	5.8 ± 2.0	0.5	2.8 ± 0.3

Values represent means of each group, with indication of the standard error of the mean

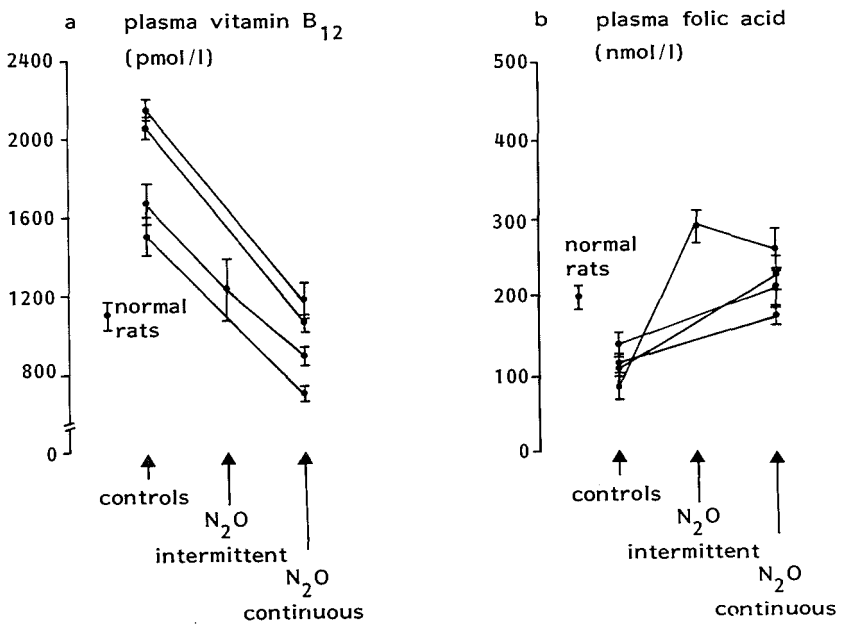

Figure 3.2. Effect of nitrous oxide (N_2O) on plasma levels of (a) vitamin B12 and (b) folic acid. Controls are untreated leukemic rats. Lines connect control and treatment groups of the same experiment. Standard errors of the mean are indicated. Values in comparable normal (non-leukemic) rats are shown.

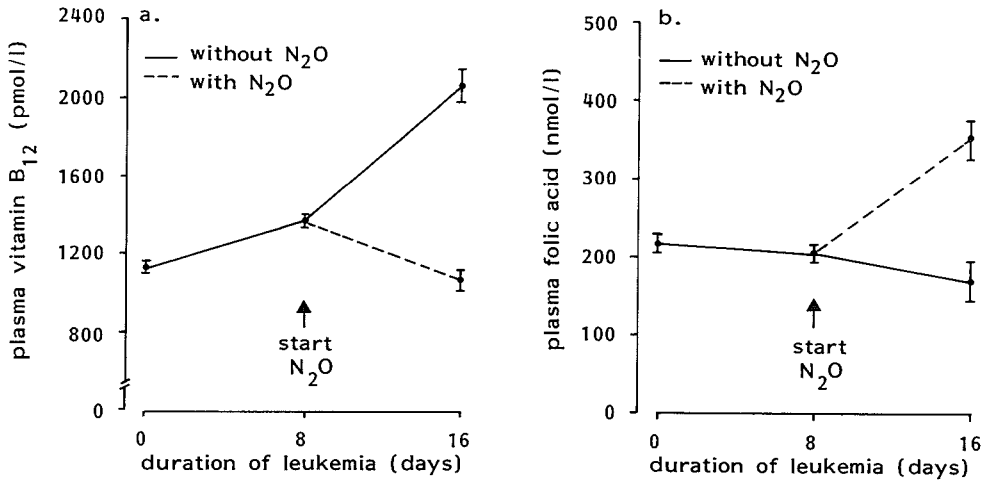

Figure 3.3. Effect of nitrous oxide (N_2O) on plasma levels of (a) vitamin B12 and (b) folic acid during progression of leukemia. The rats were inoculated with leukemia at day 0. Standard errors of the mean are indicated.

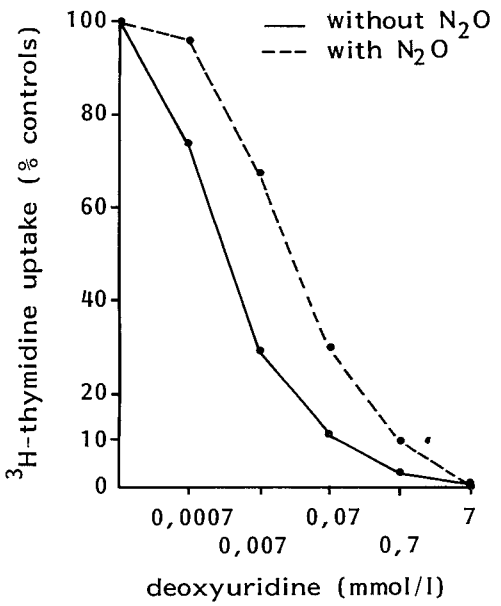

Figure 3.4. Effect of nitrous oxide (N_2O) on deoxyuridine-induced suppression of [^{3}H]-thymidine uptake by leukemic spleen cells. Values are expressed as a percentage of the maximum incorporation of [^{3}H]-thymidine, obtained by omitting deoxyuridine. Results of a nitrous oxide-treated rat (interrupted line) are compared with an untreated control (continuous line), at various concentrations of deoxyuridine.

A deoxyuridine suppression test was performed in some rats, using leukemic spleen cells. Various concentrations of deoxyuridine were used. A representative result is shown in Figure 3.4, which confirms the inactivation of methylcobalamin in the rats treated with nitrous oxide.

Finally, total body weight was determined for all treated and untreated rats at the termination of each experiment. Body weight of untreated rats was 241.7 ± 24.5 g (mean ± S.D.), and of treated rats 232.7 ± 34.6 g, demonstrating that long-term exposure to nitrous oxide did not cause a significant weight loss.

3.4. Discussion.

In recent years information has become available about the metabolic changes underlying the hematological effects of nitrous oxide. The possible antileukemic application of this anesthetic therefore deserves renewed attention. Recent in vitro studies (20) have demonstrated a growth inhibition by nitrous oxide in several cell lines derived from hematological neoplasms. The results of the present study indicate that nitrous oxide also has distinct inhibitory properties against leukemic proliferation in vivo. The relative decrease in spleen and liver weights in leukemic animals after nitrous oxide exposure is evidence of a reduced tumor load.

The specific effect of nitrous oxide on vitamin B12 function was verified by means of the deoxyuridine suppression test, which showed, as expected, abnormal values after treatment. The established mechanism of methylcobalamin inactivation, resulting in impaired *de novo* synthesis of thymidine, therefore remains a probable explanation of the observed effects, rather than less specific and weaker inhibition of mitosis that is known to occur with a variety of anesthetics (21). Halothane, for example, did not influence experimental leukemia (22), whereas nitrous oxide, in another study, differed from halothane in having an inhibitory effect on lymphoma cell proliferation (23).

Exposing animals to nitrous oxide can cause some weight loss due to decreased appetite (24). In rats, at the concentrations of nitrous oxide that were used in this study, no influence on consciousness is evident, and their nutritional condition is not affected. No significant reduction in body weight was observed.

The effects of nitrous oxide appeared to be dose-dependent, as indicated by the experiment with intermittent exposure, in accordance with earlier findings (5). This means that, at least from this experiment, there is no evidence for adaptation to the effects in the treatment period.

A recent report, suggesting that nitrous oxide causes impaired movement of leukocytes out of extravascular bone marrow stores into circulation

(25), led us to investigate femur cellularity in treated and control groups. The small increase in femur cell content, found in the treated group, cannot have contributed substantially to the reduction in spleen and liver weights by means of a shift to the bone marrow.

The effects on plasma folate levels are as expected: it has been observed in several studies that nitrous oxide-induced inactivation of vitamin B12 leads to a decreased cellular uptake of folates, which causes a fall in tissue folates (26), and, consequently, a rise in plasma and urinary folates (27-29). The observed low folate levels in untreated leukemic rats may be related to the increased demands caused by intensive cellular proliferation. The radical changes in plasma vitamin B12 however, are not easy to explain. A rise in vitamin B12 levels was observed in untreated leukemia. A similar effect is well known in human chronic myeloid leukemia (30,31), and also occurs in human acute promyelocytic leukemia (31). The BNML rat model is also considered to be a promyelocytic leukemia (12). In human cases, the rise in vitamin B12 levels is explained by release of excessive R-type vitamin B12-binding protein (transcobalamin I), which, however, is not found in rat plasma (32). The reversal of this rise by nitrous oxide seems too drastic to be explained just as an aspect of normalization. Inactivation of vitamin B12 by nitrous oxide is not known to cause deficiency of this vitamin by affecting plasma levels (8). In human CML, Lassen observed in 1959 a rather similar decrease of vitamin B12 levels during nitrous oxide exposure (6). The cause of these effects, however, awaits further investigations.

The result of this study demonstrates that nitrous oxide has a growth reducing effect towards the BNML rat leukemia, which has generally proved to be a good model for the chemotherapeutic response in human AML (13,15). However, humans are more susceptible to deficiency and inactivation of vitamin B12 than most animals (11,33). The phenomenon of megaloblastic anemia, e.g. occurs only in man (11,34), and therefore human leukemia might show a different, perhaps greater, sensitivity to nitrous oxide. It is important to realize that the therapeutic abilities of nitrous oxide, albeit probably moderate when used alone, may be exploited more fully when combined to other agents. This is supported by the present knowledge of the way nitrous oxide influences vitamin B12 and folate metabolism. A synergistic effect in vitro with agents active in related pathways, like methotrexate, has already been reported (35). Further analysis of the cytostatic effect of nitrous oxide, alone or in combination, and its relevance to the therapy of human leukemia, seems justified. It is worth noting that the well-known pain-relieving and sedative effects of this easily administered gas might be of additional benefit when used in the treatment of cancer.

Chapter four
NITROUS OXIDE AND CYCLOLEUCINE IN RAT LEUKEMIA

This chapter has been published in the British Journal of Cancer, 50:793-800 (1984), titled "Synergistic growth inhibiting effect of nitrous oxide and cycloleucine in experimental rat leukemia", by A.C.M. Kroes, J. Lindemans and J. Abels.

Abstract. Nitrous oxide (N_2O) inactivates the vitamin B12-dependent enzyme methionine synthetase with subsequent impairment of folate metabolism and a reduction of cellular proliferation. Indications exist that this effect is antagonized by S-adenosylmethionine (SAM), and it was investigated whether combination with an inhibitor of SAM synthesis, cycloleucine, would result in increased inhibition of growth in rat leukemia model (BNML). Leukemic growth was compared in untreated rats, in rats treated with either nitrous oxide/oxygen (1:1) or cycloleucine (50 mg/kg i.p.), and in rats receiving both agents. Combined treatment resulted in the strongest reduction of leukemic infiltration in spleen and liver, and this reduction often was more than the added effects of single treatments. Peripheral leukocyte counts were also lowest after combined treatment. The deoxyuridine suppression test, measuring folate-dependent *de novo* synthesis of thymidine was more severely disturbed with combined treatment. Levels of vitamin B12 in plasma were reduced in rats receiving N_2O, but an increase in plasma folate occurred in all treated rats. These results indicate that a reduction of SAM synthesis by cycloleucine can increase the disturbance of folate metabolism that is caused by nitrous oxide, with a potentiation of the effects of leukemic growth.

4.1. Introduction.

The well known anesthetic gas nitrous oxide (N_2O) is able to suppress hematopoiesis, giving rise to megaloblastic anemia in man (1) and marked leukopenia in rats (2-4). This effect could be explained by a specific oxidative action of nitrous oxide on the cobalt-moiety of vitamin B12 (5), which causes a nearly complete inactivation of the methylcobalamin-requiring enzyme methionine synthetase or 5-methyltetrahydrofolate homocysteine methyltransferase (E.C. 2.1.1.13). This results in a decreased availability of tetrahydrofolate coenzymes, which impairs folate dependent *de novo* synthesis of thymidine and ultimately affects cellular proliferation. Attempts to utilize this hematological side effect of nitrous oxide in the treatment of human leukemia were already reported before its biochemical mechanism was revealed (6,7). These preliminary trials showed some promising results with a rapid, though reversible, regression of leukemia on nitrous oxide exposure. Recent investigations have confirmed inhibition of tumor growth in vitro, in several human cell lines (8), and in vivo, in leukemic rats (9).

The use of nitrous oxide in metabolic studies made clear that the effects of this agent, through a reduction of methionine supply, also involve a decreased synthesis of S-adenosylmethionine (SAM) (10,11). In addition, it appeared that SAM antagonized the disturbance of folate metabolism which is caused by nitrous oxide (12,13). These observations suggest that a further reduction of SAM might enhance the inhibiting effects of nitrous

oxide on cellular proliferation.

We now report the effect of combining nitrous oxide with cycloleucine (NSC-1026, 1-aminocyclopentane carboxylic acid). Cycloleucine is a potent inhibitor of the enzyme methionine adenosyltransferase (E.C. 2.5.1.6.) (14) which converts methionine into SAM. The combined action of nitrous oxide and cycloleucine can cause a sequential blockade of SAM synthesis, possibly resulting in increased inhibition of tumor growth. Interestingly, cycloleucine by itself also has cytostatic properties (15,16). However, in recent clinical trials this drug, in high doses, was unsuccessful mainly because of severe neurological and hematological toxicity (17,18).

This study describes the effects of nitrous oxide exposure, combined with the administration of cycloleucine, on proliferation of an experimental rat leukemia: the Brown Norway Myeloid Leukemia (BNML). This transplantable leukemia has been described in detail (19) and is considered to be a suitable model for experimental chemotherapy (20). To evaluate the metabolic effects of vitamin B12-inactivation by nitrous oxide, the deoxyuridine suppression test is used, and plasma levels are determined of vitamin B12 and folic acid.

4.2. Materials and methods.

Animals. Male rats of the Brown Norway inbred strain were used, at the age of 14-18 weeks (body weight 200-290 g). Food and water were supplied *ad libitum* during the experiments.

Brown Norway myelocytic leukemia (BNML). Cryopreserved leukemic cells were kindly provided by dr A. Hagenbeek from the Radiobiological Institute TNO, Rijswijk, The Netherlands, where this transplantable rat leukemia model was developed. Origin, classification and proliferation kinetics were described before (17). For leukemia transfer in experimental series, spleen cells of fully leukemic animals were used. 10^7 cells, suspended in 1 ml of Hanks balanced salt solution were injected i.v.. This standard dose leads to death in 20-24 days. During this period massive leukemic infiltration of spleen, liver and bone marrow takes place. Spleen and liver weights, therefore, are reliable indicators of tumor load and, along with hematological determinations, can be used effectively to assess the effects of chemotherapy, correlating well with studies of survival time (19). To avoid a gradual change in growth properties, serial transplantations were limited to only two passages in order, after which spleen cells were used from animals of a separate series, freshly inoculated with cells from a cryopreserved stock.

Treatment with nitrous oxide and cycloleucine. Two sets of identical experiments were carried out separately (Experiments 1 and 2). In each 4 groups of 4 rats were inoculated with leukemic cells at day 0. Mean body weight in

each group was nearly identical. Treatment of these leukemic rats started at day 7. To detect any synergistic action, one group received no treatment (controls), while the other 3 groups were treated with nitrous oxide, cycloleucine, or both. Rats were exposed to nitrous oxide in a 36 l flow chamber, in which a mixture of 50% nitrous oxide and 50% oxygen was blown at a rate of about 1000 ml/min. Oxygen concentration was monitored with an oxygen analyzer (Teledyne Analytical Instruments). Exposure was interrupted only for short cleaning periods. Rats not exposed to N_2O were kept in air, but otherwise treated identically. Cycloleucine was administered as a single i.p. injection at day 7, of 50 mg/kg cycloleucine (Sigma Chemical Co., St.Louis, USA), dissolved in water containing 0.15 mol/l NaCl. Rats not receiving cycloleucine were injected with 0.15 mol/l NaCl solution i.p..

Evaluation of treatment. To allow a simultaneous investigation of several parameters of leukemic growth, including metabolic tests, both experiments were terminated after a fixed period of 19 days of leukemia, which is just before the rats would die spontaneously. Rats were killed by exsanguination under either anesthesia, after recording their body weights. Liver and spleen were weighed. Leukocytes and thrombocytes were counted electronically. Hemoglobin concentration was measured by the hemoglobin cyanide spectrophotometrical assay. Plasma vitamin B12 and folic acid were determined simultaneously, essentially as described (21). Methyltetrahydrofolate was used as a folate standard. Normal values for organ weights, hematological parameters, plasma folate and vitamin B12 concentrations were derived from at least 12 comparable non-leukemic Brown Norway rats.

Deoxyuridine suppression test. This test is used to evaluate the metabolic inactivation of vitamin B12. [^{3}H]-Thymidine incorporation in DNA is measured with and without added deoxyuridine. Deoxyuridine is able to suppress incorporation of [^{3}H]-thymidine very significantly if it can be converted to thymidine by folate-dependent methylation. This suppression is decreased in bone marrow cells as a result of folate or vitamin B12 deficiency, and as a result of the inactivation of methylcobalamin by nitrous oxide (22).

In this test, leukemic spleen cells were used (ca. 5 x 10^6 per test) from rats of the various groups in Experiment 2, as described above. In addition, leukemic rats were included who were similarly treated for 1 day only (day 16-17), after which they were sacrificed. In some cases, cycloleucine was added to the leukemic cell suspensions used in the test to compare in vitro and in vivo effects of this drug. The test was carried out (23) with some modification as described before (9). Deoxyuridine (Sigma Chemical Co., St.Louis, USA), was used in a concentration of 0.1 mmol/l. Incorporation of [^{3}H]-thymidine (ca.0.3 μCi per test, specific activity 25 Ci/mmol, from

Amersham International, UK), is expressed as a percentage of the maximum incorporation in each case, obtained by omitting deoxyuridine.

4.3. Results.

In each of the two experiments, four groups of leukemic rats were compared: one untreated (controls), one treated with nitrous oxide, one treated with cycloleucine, and one group treated with both agents. Data on leukemic growth in these groups can be found in Tables 4.1 and 4.2. All rats survived until termination of the experiments, except for one untreated rat in Experiment 2, dying spontaneously a few hours before. Organ weights of this rat have been included in the results.

The treated rats had reduced spleen and liver weights (Table 4.1) and reduced leukocyte counts (Table 4.2), compared to the untreated controls. With Wilcoxon's non-parametric rank sum test, applied to the values of individual rats in both experiments, these differences are statistically significant ($p<0.01$). Combined treatment resulted in the strongest inhibition of leukemic growth, with an increase in organ weights of less than half of control values, as shown in Figure 4.1. The differences between groups treated with a single agent, and groups with combined treatment are also statistically significant using Wilcoxon's test ($p<0.01$).

No significant difference was observed between the two groups treated with nitrous oxide or cycloleucine alone. In fact, after preliminary experiments, the lowest dose of cycloleucine was selected which produced inhibition of growth about comparable to nitrous oxide exposure alone. This would facilitate recognition of a potentiating effect after combined treatment and minimize possible side effects of cycloleucine. With regard to the hematological values shown in Table 4.2, platelet counts and hemoglobin values do not suggest adverse effects of treatment on normal hematopoiesis. Although all animals remained thrombopenic, hemoglobin values showed a small increase with treatment and were highest after combined treatment. These differences were, however, not statistically significant.

Treatment with N_2O, as in these experiments, is well tolerated by rats, without a noticeable influence on consciousness. A limited loss of body weight is observed, which is not aggravated by cycloleucine (Table 4.1).

Plasma concentrations of vitamin B12 and folic acid as determined in the various groups of both experiments, are shown in Figure 4.2. In untreated leukemic rats a strong increase in plasma levels of vitamin B12 is found, whereas decreases are observed after treatment. A pronounced effect of nitrous oxide is evident, in particular when compared with the effect of cycloleucine. Combined treatment resulted in the lowest levels of vitamin B12. Folic acid levels are low in untreated rats, with treatment

Table 4.1

EFFECTS OF TREATMENT ON LEUKEMIC INFILTRATION IN SPLEEN AND LIVER, AND ON BODY WEIGHT

Treatment	Spleen weight[a]		Liver weight[b]		Body weight
	Experiment 1 g ± s.e.	Experiment 2 g ± s.e.	Experiment 1 g ± s.e.	Experiment 2 g ± s.e.	Experiment 1 + 2 % change
None (controls)	4.52 ± 0.18	4.49 ± 0.15	21.38 ± 0.60	20.54 ± 1.58	− 1.0
Cycloleucine	3.77 ± 0.13	4.04 ± 0.13	16.79 ± 0.54	16.95 ± 0.65	+ 0.2
Nitrous oxide	3.57 ± 0.14	3.34 ± 0.23	18.81 ± 0.30	15.50 ± 0.93	− 5.9
Cycloleucine and nitrous oxide	2.32 ± 0.08	2.42 ± 0.05	13.04 ± 0.54	13.48 ± 0.51	− 4.1

All treatment groups in both experiments consisted of 4 rats.
s.e. = standard error of the mean in each group.
[a]normal spleen weight in comparable non-leukemic Brown-Norway rats: 0.45 ± 0.07 g.
[b]normal liver weight in comparable non-leukemic Brown-Norway rats: 8.25 ± 0.99 g.

Table 4.2

EFFECTS OF TREATMENT ON HEMATOLOGICAL VALUES

Treatment	Leukocytes[a]		Thrombocytes[b]	Hemoglobin
	Experiment 1 $10^9\ l^{-1}$ ± s.e.	Experiment 2 $10^9\ l^{-1}$ ± s.e.	Experiment 1 ± 2 $10^9\ l^{-1}$ ± s.e.	Experiment 1 + 2 mmol l^{-1} ± s.e.
None (controls)	32.7 ± 2.3	19.2 ± 1.2	81 ± 13	8.0 ± 0.2
Cycloleucine	30.3 ± 3.9	11.8 ± 0.6	94 ± 13	8.7 ± 0.1
Nitrous oxide	10.2 ± 0.7	8.9 ± 1.4	60 ± 5	8.2 ± 0.3
Cycloleucine and nitrous oxide	5.3 ± 0.3	5.2 ± 0.6	61 ± 8	9.2 ± 0.1

All treatment groups in both experiments consisted of 4 rats.
s.e. = standard error of the mean in each group.
[a]normal value of leukocyte count in Brown Norway rats: 3.7 ± 0.3. $10^9\ l^{-1}$.
[b]normal value of thrombocyte count in Brown Norway rats: 790 ± 22. $10^9\ l^{-1}$.

resulting in higher levels. In this case, no differences are observed between the various treatments.

Results of deoxyuridine suppression test are presented in Figure 4.3. In this test, higher values are caused by a reduced suppressive effect of

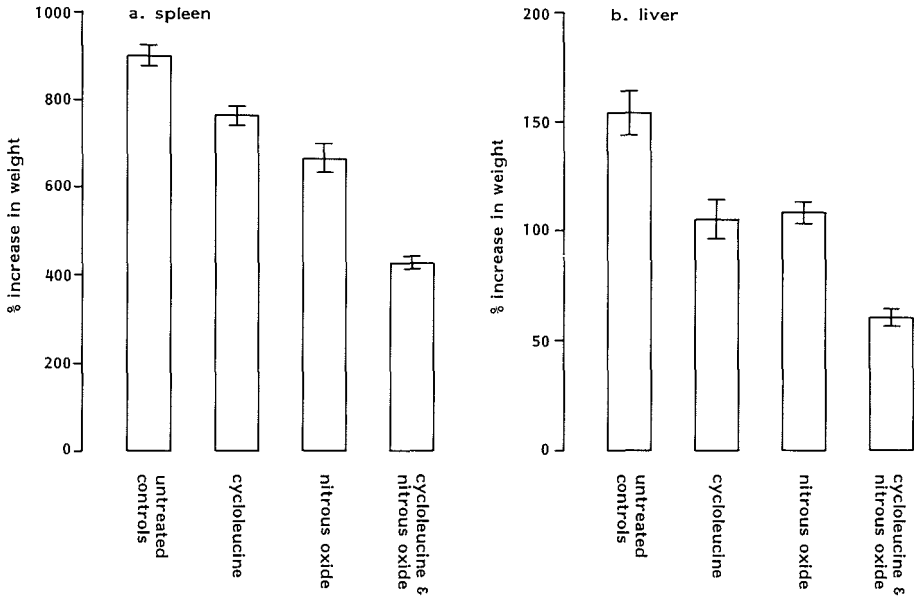

Figure 4.1. The percentages of increase in weight are indicated of (a) spleen and (b) liver in leukemic rats, relative to normal weights of spleen: 0.45 g and liver: 8.25 g in comparable non-leukemic Brown Norway rats. Data of Experiments 1 and 2 are combined. Each group consists of 8 rats. Standard errors of the mean are indicated.

deoxyuridine on the incorporation of [^{3}H]-thymidine, which is indicative of impaired *de novo* synthesis of thymidine. Figure 4.3a shows suppression values, obtained with leukemic spleen cells from rats of the four groups in Experiment 2. Although the differences are small, they are suggestive of a limited increase in suppression values after N_2O or cycloleucine as single treatments, with a more severe disturbance after combined treatment. These values, however, were obtained 12 days after the initiation of treatment in Experiment 2. In this period, the level of cycloleucine may be appreciably reduced, although this agent in normal rats has a plasma half life of about 22 days (24). Therefore, in a separate experiment leukemic spleen cells were used of rats 1 day after the administration of cycloleucine (Figure 4.3b). This resulted in a more pronounced effect after combined treatment. About the same values were observed with in vitro addition of cycloleucine to leukemic cell suspensions of untreated rats and rats treated with N_2O. At a concentration of 1 mmol/l, effects on the deoxyuridine suppression test are comparable with in vivo administration of 50 mg/kg cycloleucine.

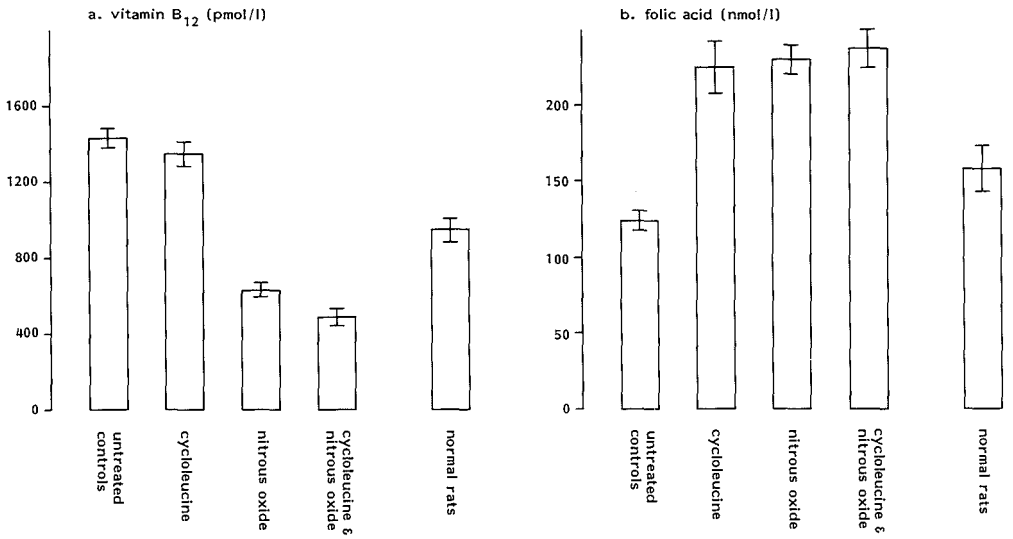

Figure 4.2. Plasma levels of (a) vitamin B12 and (b) folic acid in leukemic rats of Experiments 1 and 2. Values in normal (non-leukemic) rats are also shown. Standard errors of the mean are indicated.

4.4. Discussion.

Exposure to N_2O is known to cause a selective and virtually complete inhibition of the methylcobalamin-dependent enzyme methionine synthetase (25). This enzyme is essential both for the generation of tetrahydrofolate (THF) and the synthesis of methionine (see Figure 4.4). The disturbance of folate metabolism, and in particular of folate-dependent *de novo* synthesis of thymidine, is considered to be primarily responsible for the impairment of cellular proliferation caused by nitrous oxide. However, it appears that methionine metabolism is also involved in this effect. From metabolic studies in rats it has become evident that the disturbed folate metabolism on N_2O exposure can be completely restored by the administration of methionine (12). An explanation for this effect is the established ability of methionine after its conversion to S-adenosylmethionine (SAM), to inhibit the enzyme 5,10-methylene-THF reductase (26). This will prevent the detrimental accumulation of the substrate of methionine synthetase, 5-methylTHF, which is caused by nitrous oxide (Figure 4.4). An alternative explanation is to assume direct activation of methionine synthetase by SAM (27). Thus, it appears that SAM antagonizes the effect of nitrous oxide on folate metabolism. This conclusion is supported by other reports (13,28), while it is interesting to note that methionine also protects against specific neurological disorders caused by nitrous oxide in some species (29,30).

In this study, an effect is demonstrated which can be considered a direct

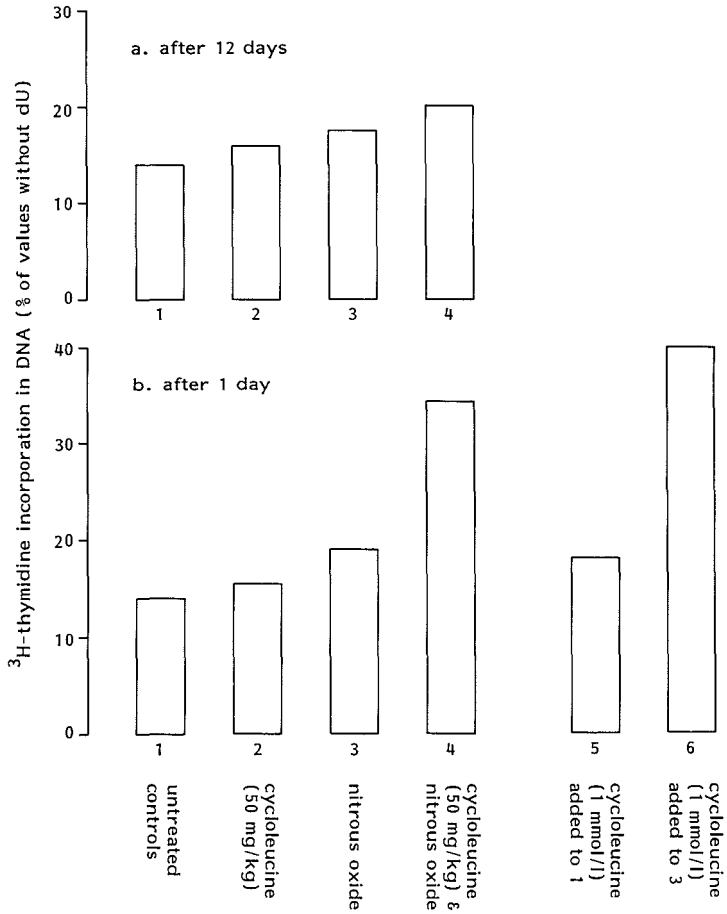

Figure 4.3. Deoxyuridine suppression values in leukemic cells: (a) from four groups of two rats each, 12 days after the administration of cycloleucine and initiation of nitrous oxide exposure; (b) from four rats, after treatment for 1 day, with cycloleucine administered in vivo (columns 2 and 4), and cycloleucine added in vitro to leukemic cell suspensions (columns 5 and 6). Each value is the mean of triple incubations, with a maximal difference between estimations of 10%. dU: deoxyuridine.

consequence of the same interaction. The effects of nitrous oxide alone on leukemic growth in vivo appear to be limited to the extent which is achieved in this study and in previous work (9). In the present study, however, it is shown that the inhibitory effects on leukemia can be increased by an additional reduction of SAM synthesis, which is induced by cycloleucine. A combination of nitrous oxide exposure with the administration of cycloleucine resulted in the strongest reduction of leukemic proliferation. With regard to spleen weight, the amount of this reduction seemed to be even more than the added effects of both agents when given separately. This is also reflected in the deoxyuridine suppression test, which measures the

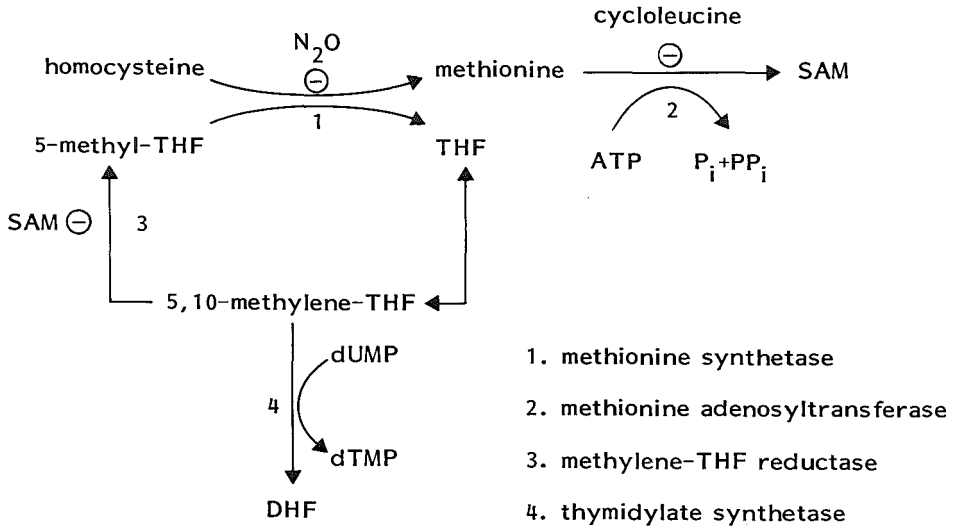

Figure 4.4. Relations between the reactions discussed in the text, with indication of enzymes and inhibitors. N_2O: nitrous oxide, THF: tetrahydrofolate, SAM: S-adenosylmethionine.

capacity of *de novo* synthesis of thymidine. In this test, the effect of nitrous oxide is enhanced by cycloleucine: this applied both to the in vivo combination of these two agents and the in vitro addition of cycloleucine to leukemic cells of rats treated with nitrous oxide.

The most likely explanation of this synergistic action therefore is further impairment of folate metabolism. The cytostatic properties of cycloleucine generally are attributed to its inhibition of SAM-dependent methylation reactions (31). Although it was recently shown that N_2O also decreases tissue levels of SAM (10,11), it remains uncertain to what extent this effect directly contributes to the inhibition of cellular proliferation. In this respect, the impairment of thymidine synthesis is an established mechanism. It should not be excluded that N_2O and cycloleucine mutually can potentiate distinctive cytostatic effects.

Peripheral leukocyte counts clearly are lowest with combined treatment. It appears that nitrous oxide more effectively reduces peripheral leukocytes, when compared with cycloleucine as a single agent.

Nitrous oxide and cycloleucine also appear quite different with regard to their effects on plasma levels of vitamin B12. We have previously shown that a continuous rise of plasma vitamin B12 is a feature of this leukemia in rats (9). The reduction of leukemic growth, as caused by cycloleucine, is not very effective in decreasing vitamin B12 levels. Nitrous oxide treatment, however, with the same inhibition of leukemic growth, caused a

striking fall in plasma vitamin B12 to subnormal levels. This is evidence of a specific effect of this agent, which oxidizes the methylcobalamin coenzyme. Reduced vitamin B12 levels in plasma with prolonged N_2O exposure were already reported in fruit bats (30), and, remarkably, in the first case of human leukemia treated with N_2O (6). Kondo et al. (32) showed that with N_2O exposure analogues of cobalamin are formed which are rapidly excreted, resulting in a depletion of vitamin B12.

Folic acid levels were low in untreated leukemic rats, which is probably related to the increased demands of rapid cellular proliferation (33). In all treated groups folate levels were increased, and with regard to N_2O treatment this effect has been observed before (34). This is explained by the blockade in folate metabolism with a reduction of cellular uptake of folates and a consequent accumulation in plasma. Cycloleucine causes the same increase in folate levels, however, and this supports the presumed role of SAM in the regulation of folate metabolism, as discussed before. Finally, it should be noted that with a low dose of cycloleucine as used in this study, no adverse effects were apparent when comparing treated and untreated rats.

It can be concluded that it is possible to achieve increased inhibition of leukemic proliferation when N_2O is combined with a low dose of cycloleucine, an inhibitor of SAM synthesis. This effect supports a role of SAM in modulating the effects of N_2O, and should stimulate further research to establish cobalamin-dependent metabolism as an additional target in cancer chemotherapy. On the other hand, these results suggest that cycloleucine can be used more effectively, than in the toxicity limited dosages used before, when it is combined with N_2O, an agent which has the additional advantage of notable analgesic properties.

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Chapter five
NITROUS OXIDE AND METHOTREXATE IN RAT LEUKEMIA

This chapter has been published in Cancer Chemotherapy and Pharmacology, 17:114-20 (1986), titled "Enhanced therapeutic effect of methotrexate in experimental rat leukemia after inactivation of cobalamin (vitamin B12) by nitrous oxide", by A.C.M. Kroes, J. Lindemans, M. Schoester and J. Abels.

Abstract. Exposure to nitrous oxide interferes selectively with the coenzyme function of vitamin B12 and causes inactivation of methionine synthetase, with subsequent impairment of folate metabolism and reduction of cellular proliferation. In a rat leukemia model (BNML) we investigated the combined administration of nitrous oxide, inactivating vitamin B12, and methotrexate (MTX), a folate antagonist inhibiting the enzyme dihydrofolate reductase. Through different mechanisms, both agents decrease the availability of tetrahydrofolate, and subsequently of other reduced folates, with increased impairment of folate-dependent synthesis of thymidylate. Effects on leukemic growth and on hematological values in rats demonstrated enhancement of the therapeutic effect of MTX by exposure to nitrous oxide. With several treatment schedules, the results of combined treatment were seen to be better than additive when compared with the effects of single agents. In particular, pretreatment of leukemic rats with nitrous oxide for 3 days before administration of MTX, concomitant exposure to nitrous oxide even resulted in toxic effects. These findings were in accordance with the results of some metabolic studies performed in leukemic rats. De novo synthesis of thymidylate in leukemic cells, when studied by means of the deoxyuridine suppression test, showed the most severe disturbance with combined treatment consisting in MTX (0.5 mg/kg) and nitrous oxide pretreatment for 3 days. Intracellular levels of folate and dTTP were lowest with 2 and 3 days' pretreatment before MTX, respectively. It is concluded that this interaction of nitrous oxide and MTX can result in enhanced metabolic and therapeutic effects of low doses of MTX. Inactivation of vitamin B12 appears to be a potentially useful addition in cancer chemotherapy.

5.1. Introduction.

The similarity of hematological disturbances caused by deficiency of either folic acid or cobalamin (vitamin B12) is well established. Both vitamins are involved in pathways essential in the synthesis of nucleotides, and consequently of DNA. The important function of folic acid in cellular proliferation is also reflected in the striking cytostatic activity of folate antagonists of which methotrexate (MTX) is the best known example. This antimetabolite is widely used in cancer chemotherapy (1). In contrast, until recently, the role of vitamin B12 in neoplastic growth has remained unclear, because no effective method was available to interfere with its coenzyme function. In 1978, however, it was recognized that selective inactivation of vitamin B12 could be achieved with exposure to the anesthetic gas nitrous oxide, or N_2O (2,3). Megaloblastic hematopoiesis after prolonged exposure to nitrous oxide had ben observed much earlier (4), and chemical interaction of nitrous oxide with complexes of cobalt was also known for some time (5). It appeared that a specific oxidative action of nitrous oxide on the cobalt

moiety of vitamin B12 caused a nearly complete inactivation of the methylcobalamin-requiring enzyme methionine synthetase, or 5-methyltetrahydrofolate homocysteine methyltransferase (E.C.2.1.1.13). Nitrous oxide effectively established a state of functional deficiency of vitamin B12, with severely disturbed folate metabolism (6) and toxic effects on hematopoiesis (7). Methionine synthetase is essential in folate metabolism, because it provides the only pathway by which 5-methyltetrahydrofolate, the major extracellular folate, can be converted into tetrahydrofolate (THF). THF and other, subsequently formed, reduced folates can be converted into folylpolyglutamates, which are then retained in the cell and are important coenzymes in one-carbon transfer reactions. De novo synthesis of thymidylate requires such folate-dependent methylation. MTX also interferes with folate metabolism by limiting the generation of THF, but through a different mechanism. Its inhibition of dihydrofolate (DHF) reductase prevents the reconversion of DHF to THF.

It has been shown that the effect of nitrous oxide on vitamin B12 can be utilized to reduce growth of leukemia in vitro (8) and in vivo, in rats (9). This effect is associated with a disturbance of folate-dependent *de novo* synthesis of thymidylate. Therefore, it appears that nitrous oxide and MTX, through inhibition of different pathways, ultimately may have similar effects on folate metabolism. These observations suggest that the inactivation of vitamin B12 can modify, and possibly enhance, the efficacy of MTX. In vitro studies on human bone marrow have demonstrated a synergistic effect of MTX and nitrous oxide with regard to impairment of nucleotide synthesis (10). The purpose of the present study is to investigate the influence of nitrous oxide on the effects of MTX in vivo, using a rat leukemia model: the Brown Norway myeloid leukemia (BNML). This transplantable acute promyelocytic leukemia has been described in detail elsewhere (11) and is considered to be a suitable model for chemotherapeutic studies (12). In addition to experiments intended to assess effects on leukemic growth, a number of metabolic studies were performed in leukemic rats, to investigate some effects of treatment on folate metabolism. These studies included deoxyuridine suppression tests, and determinations of intracellular folate and deoxythymidine triphosphate (dTTP) levels.

5.2. Materials and methods.

Animals. Male rats of the Brown Norway inbred strain were used at the age of 12-16 weeks (body weight 200-275 g). Food and water were supplied ad libitum during the experiments.

Brown Norway myeloid leukemia (BNML). Cryopreserved leukemic cells were kindly provided by dr A. Hagenbeek from the Radiobiological Institute (TNO), Rijswijk, the Netherlands, where this transplantable rat

leukemia model was developed. Origin, classification and proliferation kinetics were described elsewhere (11). For leukemia transfer in experimental series, spleen cells of fully leukemic animals were used. A standard dose of 10^7 cells suspended in Hanks' balanced salt solution was injected i.v., which leads to progressive leukemic infiltration of bone marrow, spleen and liver, with death after 20-24 days. Spleen and liver weights, steadily increasing in the course of leukemia, are reliable indicators of tumor load and, along with hematological determinations, can be used effectively to assess effects of chemotherapy (11). To avoid a gradual change in growth properties, serial transplantations were limited to only two passages, after which spleen cells were used from rats freshly inoculated with cells from a cryopreserved stock.

Treatment with nitrous oxide and MTX. Leukemic rats were treated according to different schedules in groups of at least four. 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/min. Oxygen concentration was monitored with an oxygen analyzer (Teledyne Analytical Instruments). Carbon dioxide, water, and contaminating volatile compounds were eliminated in a cleaning circuit, essentially as described by Rupreht and Dzoljic (13). Rats not exposed to nitrous oxide were kept in air, but otherwise treated identically. Sodium methotrexate (Ledertrexate SP, from Lederle) was injected i.p. Rats not receiving MTX received injections of 0.15 M NaCl i.p. instead.

Evaluation of leukemic growth. Experiments intended to assess effects on leukemic growth were all evaluated by the same procedure. To allow a simultaneous investigation of several aspects of leukemia these experiments were terminated after a fixed period of 18 days (in some instances: 19 days) of leukemia, just before death from leukemia was to be expected. Rats were killed by exsanguination, after recording of body weights. Liver and spleen were carefully removed and weighed. Leukocytes were counted electronically, and in some experiments differential blood cell counts were done. Plasma levels of vitamin B12 were measured in a competitive radioisotope binding assay using purified intrinsic factor (14). Normal values for organ weights, leukocyte counts and plasma vitamin B12 were derived from at least 12 comparable non-leukemic Brown Norway rats.

Metabolic studies. In separate experiments, rats with advanced leukemia were treated for short periods, after which leukemic cells were used in metabolic studies. Three similar experiments were carried out separately. In each, eight leukemic rats inoculated at day 0 were divided in four pairs. These pairs were treated with nitrous oxide for 3, 2, 1 or 0 days. Immediately afterwards one rat in each pair received MTX, 0.5 mg/kg i.p., the other rat receiving saline only. At 18 h after administration of MTX, on day 15 of leukemia, leukemic cells were obtained from the spleens of all rats, washed, and resuspended in Hanks' balanced salt solution. These cell

suspensions were counted electronically and used in deoxyuridine suppression tests and determinations of intracellular folate and dTTP.

Deoxyuridine suppression test. This test demonstrates impaired *de novo* synthesis of thymidylate. [^{3}H]-Thymidine incorporation into DNA is measured with and without addition of deoxyuridine. Deoxyuridine will suppress incorporation of [^{3}H]-thymidine in DNA if it can be converted to thymidylate through folate-dependent methylation. This suppression is reduced by vitamin B12 or folate deficiency (15), inactivation of vitamin B12 by nitrous oxide (16), and treatment with other agents interfering with *de novo* synthesis of thymidylate (17).

Leukemic spleen cells (approx. 5 x 10^6 per test) were used from rats of various groups, as described above. The test was carried out essentially according to Metz (18), with some modifications as described elsewhere (9). Deoxyuridine (Sigma, St.Louis, USA) was used in a concentration of 0.1 mmol/l. All incubations were performed in triplicate. Incorporation of [^{3}H]-thymidine (0.3 μCi per test, specific activity 25 Ci/mmol, from Amersham, UK) is expressed as a percentage of the maximal incorporation, measured in each case in incubations without addition of deoxyuridine.

Intracellular folate. In suspensions of leukemic spleen cells, intracellular folate content was determined. After centrifugation a pellet of approx. 10^8 cells was resuspended in a total volume of 1 ml 10% (= 1.3 mol/l) mercaptoethanol, heated in a water bath of 100 °C for 5 min, and cooled. Hog kidney polyglutamate hydrolase, prepared as described by McMartin et al. (19), was added and allowed to incubate at room temperature for 2 h. The samples were frozen at -20 °C until assayed. After thawing, the extracts were centrifuged (1500 g for 10 min at 4 °C), and aliquots of the supernatants were used in a folate radioisotope dilution assay, essentially as described by Dunn and Foster (20), with [^{125}I]-folic acid (Becton Dickinson, Orangeburg, N.Y., USA), as a tracer, and β-lactoglobulin (Sigma, St.Louis, USA) as a folate binder. 5-Methyl-THF was used as a standard, and results are expressed as picomoles of folate per 10^6 cells.

Intracellular dTTP. In leukemic cells obtained from rats as described above, dTTP was determined using the DNA polymerase assay system originally developed by Solter and Handschumacher (21), with the modifications and corrections published by Hunting and Henderson (22). A different extraction method was used, however. After being washed once in Hanks' balanced salt solution, cell suspensions were centrifuged and the supernatant was removed. To the pellet of about 10^8 cells, 1.2 μg cyano[^{57}Co]cobalamin, or 10^5 dpm, (Amersham, UK) was added, as an internal standard for cells quantities in the assay. The pellet was then extracted with 5 ml ice-cold 60% methanol and stored at -20 °C until

assayed. After centrifugation the supernatant was dried using a rotary evaporator at 25 ^{o}C dissolved in 50 mM potassium, cacodylate and subsequently used in the DNA polymerase catalyzed assay. DNA polymerase I from E.coli, dATP, dTTP, and poly(d(A,T)) were all obtained from Boehringer Mannheim (FRG) and [^{3}H]-dATP and [^{3}H]-dTTP were from Amersham (UK). Procedures, and calculated corrections for dilution of specific activities by the endogenous nucleotides were carried out as described elsewhere (22). Concentrations of dTTP are expressed as picomoles per 10^{6} cells, based upon counts of the internal standard and cell concentration of the initial suspension.

5.3. Results.

Effects of growth of leukemia in rats. In Table 5.1, results of six experiments with several different treatment schedules are summarized. In all these experiments, one group of leukemic rats treated with MTX only is compared with one or more groups of rats treated with a combination of MTX and exposure to nitrous oxide. Most experiments included a group receiving nitrous oxide continuously throughout the treatment period (day 7 to day 18 or 19 of leukemia). In other groups, nitrous oxide treatment was limited to either 3 days before administration of MTX (pretreatment) or 3 days after administration of MTX (post-treatment). This is illustrated in Figure 5.1, which shows the treatment schedules as applied in experiment 3 of Table 5.1. In addition, all experiments included rats receiving no treatment or nitrous oxide only (days 7-19), without MTX. Cumulative results in these rats are also shown in Table 5.1. From the data in Table 5.1, it follows that low doses of MTX alone do not have substantial effects on leukemic growth. In all experiments the addition of nitrous oxide enhanced the therapeutic effects of MTX. The differences between rats treated with MTX only and rats treated with both MTX and nitrous oxide (continuously) are statistically significant according to Wilcoxon's non-parametric rank sum test. For all three parameters of leukemia, results did not overlap, yielding p-values of 0.05 or less, in any of the experiments 1-4. In addition, the results of combined treatment appeared to be better than additive in these experiments, with the possible exception of experiment 1, in which the lowest dose of MTX was administered. In other cases, the reduction of leukemic growth obtained with combined treatment is often greater than the added effects of the two single agents, which is indicative of a synergistic interaction. From experiments 3 and 5 it can be derived that by far the major part of the added effect is contributed by the period of exposure to nitrous oxide before administration of MTX (pretreatment schedules). In experiment 6 the highest dose of MTX was used (4 mg/kg). In this

Table 5.1

NITROUS OXIDE AND METHOTREXATE

Effects on growth of BNML in rats

Treatment	Spleen (g ± s.e.m.)	Liver (g ± s.e.m.)	Leukocytes (10^9/l ± s.e.m.)
None (controls)	3.90 ± 0.09	17.39 ± 0.45	24.8 ± 2.1
N_2O, continuous	2.89 ± 0.09	14.33 ± 0.44	11.1 ± 1.0
MTX 1 × 0.5 mg/kg	3.64 ± 0.10	15.60 ± 0.69	18.1 ± 1.2
with N_2O, continuous	2.34 ± 0.10	12.50 ± 0.55	7.7 ± 0.7
MTX 2 × 0.5 mg/kg	3.38 ± 0.18	16.04 ± 0.80	20.2 ± 1.4
with N_2O, 2 × 3d. (after)	3.32	17.05	20.0
with N_2O, 2 × 3d. (before)	2.37 ± 0.11	13.08 ± 0.44	8.1 ± 0.6
with N_2O, continuous	1.97 ± 0.17	11.68 ± 0.81	7.0 ± 1.1
MTX 1 × 2 mg/kg	3.44 ± 0.14	14.00 ± 0.46	20.8 ± 2.9
with N_2O, 1 × 3d. (before)	2.58 ± 0.24	12.59 ± 0.94	7.7 ± 1.0
with N_2O, continuous	1.95 ± 0.08	11.47 ± 0.17	6.1 ± 0.6
MTX 1 × 4 mg/kg	2.85 ± 0.32	13.30 ± 0.74	14.9 ± 1.1
with N_2O, 1 × 3d. (before)	1.47 ± 0.43	10.12 ± 0.49	4.2 ± 1.4
with N_2O, continuous	all rats died (without evidence of leukemia)		

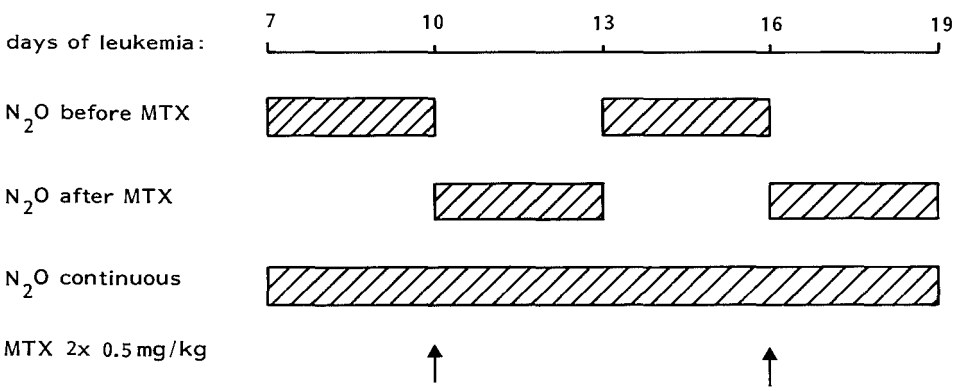

Figure 5.1. Treatment of leukemic rats with nitrous oxide (N_2O) and methotrexate (MTX), as given in experiment 3 of Table 5.1. Periods of exposure to nitrous oxide are shaded, and arrows indicate time of administration of MTX. Rats receiving MTX or nitrous oxide only, and untreated rats, were also included.

Table 5.2

NITROUS OXIDE AND METHOTREXATE

Effects of treatment on differential blood cell counts (percentages ± s.e.m.)

Treatment	No. of rats	Promyelocytes (leukemic cells)	Lymphocytes	Neutrophils
None (untreated controls)	8	17.4 ± 1.9	74.4 ± 2.0	8.3 ± 1.6
N_2O, continuous (day 7-18/19)	6	13.5 ± 3.4	83.2 ± 2.7	3.3 ± 0.8
MTX, 2x 0.5 mg/kg (day 10 and 14)	4	13.5 ± 2.2	74.5 ± 1.8	12.0 ± 1.0
+ N_2O, continuous (day 7-18)	4	0.8 ± 0.2	98.3 ± 0.6	1.0 ± 0.4
MTX, 1x2 mg/kg (day 10)	3	30.6 ± 1.5	65.0 ± 1.5	4.3 ± 1.2
+ N_2O, 1x3 d. before MTX (day 7-10)	3	8.0 ± 6.0	90.3 ± 6.2	1.7 ± 0.3
+ N_2O, continuous (day 7-19)	4	3.3 ± 0.9	95.5 ± 1.0	1.3 ± 0.2
MTX, 1x4 mg/kg (day 10)	4	13.3 ± 3.9	81.5 ± 5.2	5.3 ± 1.6
+ N_2O, 1x3 d. before MTX (day 7-10)	4	2.0 ± 0.7	97.5 ± 0.9	0.5 ± 0.3
Normal BN-rats (non-leukemic)	5	0	91.4 ± 1.4	6.0 ± 1.5

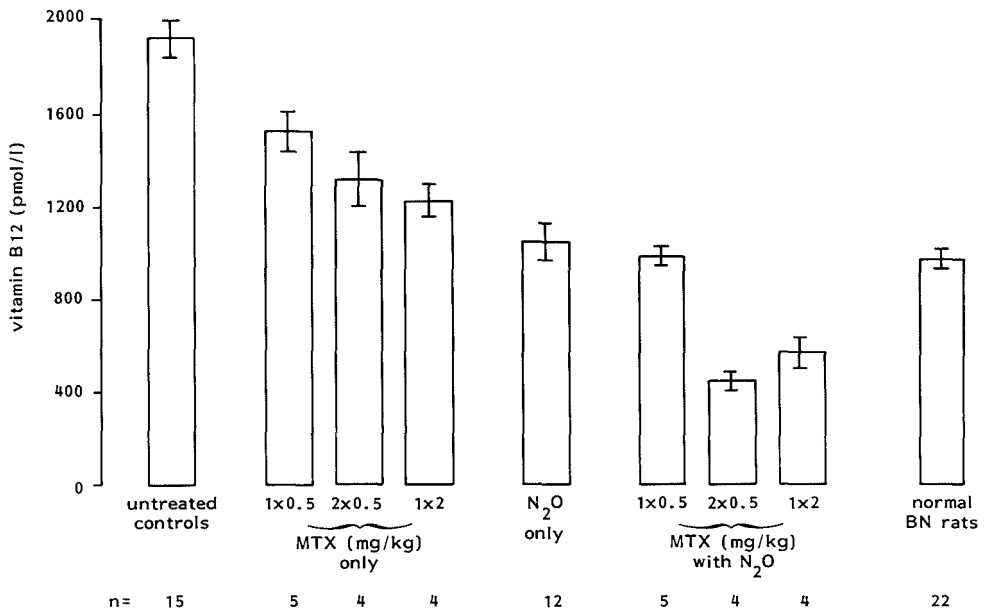

Figure 5.2. Plasma levels of vitamin B12 in leukemic rats treated in several experiments with different doses of MTX. Groups with combined treatment received nitrous oxide continuously (days 7-18/19 of leukemia). Values in normal (non-leukemic) BN rats are also shown. Bars indicate the standard error of the mean.

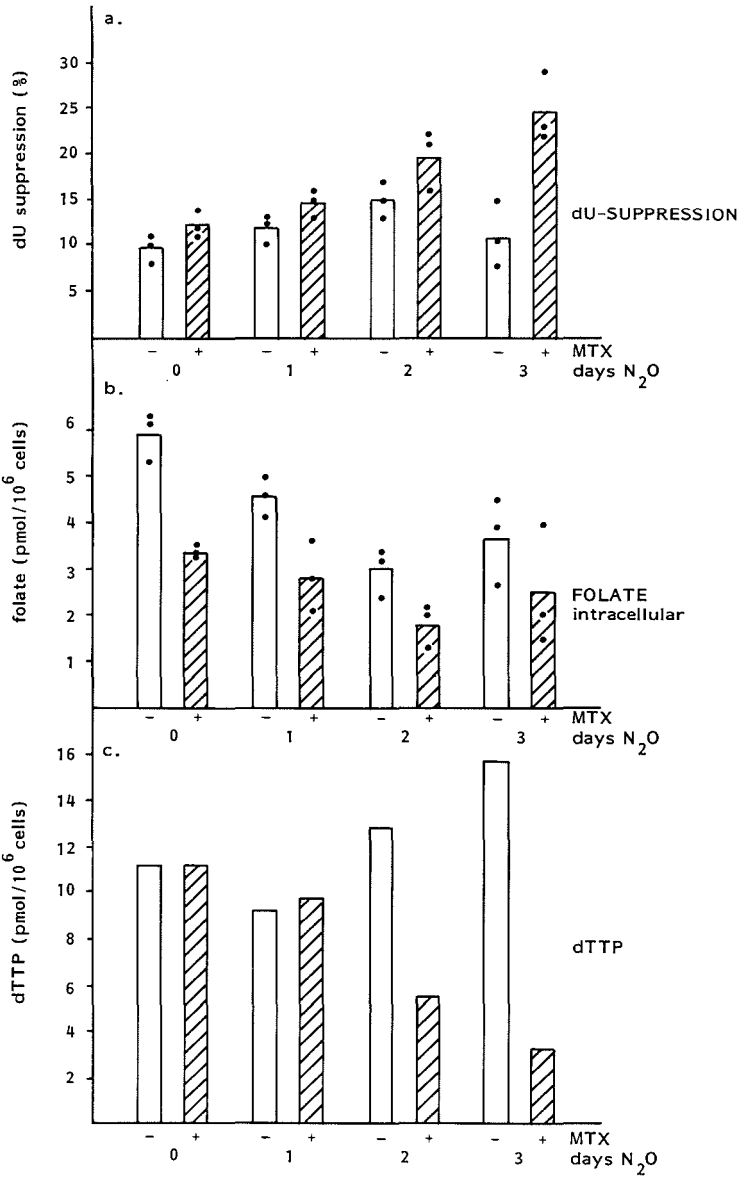

Figure 5.3. Results of metabolic experiments with leukemic spleen cells after in vivo treatment of rats with 0-3 days' exposure to nitrous oxide, followed or not by administration of MTX (0.5 mg/kg): (a) deoxyuridine suppression values, expressed as percentages of maximal incorporation of [^{3}H]-thymidine, in incubations without deoxyuridine; (b) intracellular levels of folate (pmol/10^6 cells); (c) intracellular levels of dTTP (pmol/10^6 cells). Shaded columns indicate MTX treatment. The experiments were performed 18 h after administration of MTX and/or exposure to nitrous oxide.

experiment, rats treated with both MTX and nitrous oxide (continuously) died prematurely at 14-16 days after inoculation, without any evidence of leukemia. Spleen and liver weights at autopsy were subnormal, and body weight was seriously reduced (77% of weight before treatment). This was considered to be a toxic effect of treatment. In other experiments, however, no toxicity was observed and nitrous oxide treatment was well tolerated by rats without evident effects on consciousness. Even with combined treatment, loss of body weight was always less than 10%.

In some experiments differential blood cell counts were made, which demonstrated that the observed reductions in peripheral leukocyte counts were also accompanied by a striking relative decrease of leukemic cells (promyelocytes), as shown in Table 5.2.

Plasma levels of vitamin B12, as determined in these experiments, are presented in Figure 5.2. Compared with normal BN rats, vitamin B12 levels in untreated leukemic controls, are very high. With treatment these levels are reduced, as is shown for three doses of MTX. Exposure to nitrous oxide alone has marked effects on the vitamin B12 level in plasma, but much lower and even subnormal levels are found after combined treatment.

Metabolic effects. In separate experiments, pairs of leukemic rats were treated with nitrous oxide for 0, 1, 2 or 3 days. One rat of each pair subsequently received MTX (0.5 mg/kg), and 18 h afterwards leukemic cells of all rats were used for deoxyuridine suppression tests and determinations of intracellular folate and dTTP. This experimental procedure was repeated twice, and results of all rats used in this study are presented together (Figure 5.3).

Deoxyuridine suppression tests (Figure 5.3a) show increased disturbance with longer duration of nitrous oxide exposure before the administration of MTX. Higher values in this test indicate a decreased ability of deoxyuridine to suppress the uptake of [^{3}H]-thymidine, demonstrating impaired *de novo* synthesis of thymidylate. MTX without nitrous oxide treatment is clearly less effective: mean values are 12% without nitrous oxide and 25% with 3 days of pretreatment. Figure 5.3b shows the results of intracellular folate determinations. Both nitrous oxide and MTX decreased folate levels, and the lowest levels are found with combined treatment after 2 days of nitrous oxide exposure. With 3 days of nitrous oxide exposure it appears that folate contents recover to some extent. Figure 5.3c, presenting levels of intracellular dTTP, is based upon the last metabolic experiment only, involving eight rats, in contrast to the other results. From this experiment it can be concluded that dTTP levels in leukemic cells are lowest with combined treatment consisting in MTX and 2 or 3 days' pretreatment with nitrous oxide. Nitrous oxide treatment alone appears to cause increased levels of dTTP, but it should be emphasized that these and

other values were obtained 18 h after exposure and some recovery may have occurred.

5.4. Discussion.

Nitrous oxide interferes specifically with the coenzyme function of vitamin B12 and thereby inactivates methionine synthetase (23). This severely affects folate metabolism, because methionine synthetase is required in the conversion of 5-methyl-THF, the predominant extracellular folate, into THF. This conversion is essential for folate coenzyme functions, and also for the cellular retention of folates. In contrast to other reduced folates, 5-methyl-THF is not a substrate for synthesis of folylpolyglutamates, as is evident from metabolic studies (24,25) and from properties of the purified enzyme (26). The synthesis of folylpolyglutamates is decreased by nitrous oxide (25,27,28), which explains the serious cellular depletion of folates occurring on exposure (29). The decreased availability of reduced folates impairs folate-dependent synthesis of thymidylate, and subsequently reduced DNA synthesis and cellular proliferation. The inhibition of leukemic growth by nitrous oxide in vitro has been demonstrated (8), and in a previous study we described in vivo antileukemic effect of nitrous oxide in rats (9). We also showed that these effects were enhanced in combined treatment with cycloleucine, which inhibits the conversion of methionine into S-adenosylmethionine and indirectly interferes with folate metabolism (30). A number of studies have investigated the combination of nitrous oxide, as a vitamin B12-inactivating agent, and MTX, as a typical folate antagonist. Kano et al. (10) have demonstrated synergistic effects with regard to inhibition of thymidylate synthesis in normal human bone marrow. Black and Tephly (31) compared metabolic effects of both agents in rat liver cells. The inhibition of methionine synthetase by nitrous oxide considerably decreased the availability of THF while the inhibition of DHF reductase by MTX was much less effective. This difference probably can be explained by the low activity of thymidylate synthetase in liver cells. Dudman et al. (32) found increased sensitivity of leukemic cell lines to MTX with nitrous oxide-induced inhibition of methionine synthetase, which was further exploited by the use of 5-methyl-THF instead of 5-formyl-THF as a rescue agent.

The present study shows effects of combined therapy with nitrous oxide and MTX on in vivo growth and metabolism of rat leukemia. The exposure of rats to nitrous oxide enhanced inhibition of leukemic growth by low doses of MTX. A period of exposure before the administration of MTX (pretreatment) appeared essential for this effect. The effects on leukemic infiltration in spleen and liver correlated well with hematologic results (total

and differential leukocyte counts) and with determinations of vitamin B12 in plasma. An interesting feature of this leukemia is a continuous rise of vitamin B12 levels in the course of leukemic growth (9), which is also observed in human acute promyelocytic leukemia (33). Plasma vitamin B12 can be used as a kind of tumor marker, and treatment leads to reduced levels. The particularly striking decrease caused by nitrous oxide, however, is explained by its specific effect on cobalamin. Analogues of cobalamin are formed after oxidation and are excreted rapidly (34). In addition to effects on leukemic growth, some metabolic aspects of this interaction were studied in leukemic cells after in vivo treatment of rats. On the basis of the results discussed before, pretreatment with nitrous oxide was administered for periods up to 3 days. Deoxyuridine suppression test demonstrated increased disturbance of *de novo* synthesis of thymidylate with longer duration of nitrous oxide pretreatment before MTX. Treatment with a single agent was clearly less effective. Simultaneous determination of intracellular folates, to confirm the presumed cellular folate depletion, indeed showed a decrease in folate content, particularly with combined treatment. Some comments on these folate measurements are warranted, however. In the radioisotope competitive binding assay the β-lactoglobulin folate binder is used, and probably not all relevant folate derivatives show the same affinity for this binder. Our own observations (not included in this study) indicated that 5-methyl-THF has slightly lower affinity than THF. As these folates are intracellularly predominant (19,31), the observed decrease in total folate content could also be explained, at least partially, by a shift of folates from THF toward 5-methyl-THF. This, however, is functionally about equivalent, because the conversion of 5-methyl-THF is blocked on nitrous oxide exposure, as discussed before. Moreover, the results of this folate radioassay in the measurement of tissue folates were recently found to be comparable to microbiological assays (35). Changes in cellular folate on nitrous oxide exposure in our study are similar to earlier observations (26,28), including an indication of recovery at more than 2 days of exposure, ad adaptive mechanism which is not yet understood. Finally, decreased dTTP levels show that the impairment of folate-dependent synthesis of thymidylate has noticeable effects on this direct precursor of DNA. Considered together, the findings in metabolic experiments are in accordance with the results of studies directed at inhibition of growth, showing maximum effects after about 3 days of pretreatment with nitrous oxide before MTX.

Several potential mechanisms could explain the results of this interaction. First, a reduction of intracellular folates by nitrous oxide pretreatment can obviously induce greater susceptibility to folate antagonists (36). A second mechanism is closely related and concerns the reduced synthesis of folylpolyglutamates on nitrous oxide exposure. Polyglutamation of MTX, leading to increased activity and cellular retention (37), occurs in competition with normal folate substrates (36,38). In cells pretreated with nitrous

oxide a larger proportion of MTX may be converted into polyglutamate forms. A third potential mechanism to explain the results relates to the observed marked increase in activity of thymidylate synthetase, after nitrous oxide exposure of up to 3 days (39). It is well established that thymidylate synthetase has a central role in mediating the cytotoxic effects of MTX (40,41), because this enzyme actually causes THF depletion by its conversion of reduced folates into DHF. Increased activity of this enzyme, as a possible adaptation to nitrous oxide treatment, thus may result in enhanced effects of MTX. All these mechanisms can explain the observed importance of pretreatment with nitrous oxide before MTX.

The results presented in this study demonstrate that in vivo metabolic manipulation of leukemic cells with nitrous oxide can enhance metabolic and therapeutic effect of MTX. The clinical relevance of this interaction is illustrated by the recent observation of increased toxicity of adjuvant chemotherapy involving MTX started directly post-operatively (42), which the authors attributed to inactivation of vitamin B12 by nitrous oxide during anesthesia. Our findings lend support to this suggestion. In experimental chemotherapy, the inactivation of vitamin B12 represents a new method to enhance activity of MTX. The relative contributions of increased polyglutamation of MTX, and increased activity of thymidylate synthetase, should be subjects of further research. It also remains to be demonstrated that the effects described are applicable to human leukemia, but it is known that man is more susceptible to vitamin B12 deficiency than any animal (6). Finally, these results indicate the significance of vitamin B12 in leukemic proliferation and the value of vitamin B12-related metabolism as an additional target in cancer chemotherapy.

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Chapter six
NITROUS OXIDE AND 5-FLUOROURACIL IN RAT LEUKEMIA

This chapter has been published in Anticancer Research, 6:737-43 (1986), titled "Effects of 5-fluorouracil treatment of rat leukemia with concomitant inactivation of cobalamin", by A.C.M. Kroes, A.A.M. Ermens, J. Lindemans and J. Abels.

Abstract. The cytostatic activity of 5-fluorouracil (5-FU) can be modified by the addition of reduced folates, as well as antifolates. This is indicative of the complex involvement of folate metabolism in the effects of 5-FU. In the BN rat leukemia model, 5-FU treatment was combined with the inactivation of cobalamin (vitamin B12) by nitrous oxide (N_2O). Exposure to nitrous oxide causes severe disturbance of folate metabolism through the inhibition of the cobalamin-dependent enzyme methionine synthetase, and leads to loss of folates from the cell. With regard to the effects on growth of leukemia, the addition of nitrous oxide did not antagonize 5-FU. On the contrary, therapeutic effects were enhanced by combined treatment, as was evident from a further reduction of leukemic infiltration in spleen and liver, from a decrease or even disappearance of leukemic cells in the peripheral blood, and from extended survival of rats. These findings were in accordance with metabolic studies in isolated leukemic cells of treated rats, in which combined treatment caused further impairment of thymidylate and DNA synthesis. Pretreatment with nitrous oxide, for a period of 3 days, was more effective than treatment after the administration of 5-FU. Folate levels, in plasma and intracellular, were reduced after combined treatment. It is concluded that in this leukemia, unlike observations in some models of solid tumors, the activity of 5-FU is enhanced with a depletion of folates. This effect is probably comparable to the combination of methotrexate pretreatment with 5-FU, and might be important to applications of 5-FU in combination chemotherapy of hematological neoplasms.

6.1. Introduction.

The chemotherapeutic activity of 5-fluorouracil (5-FU) is closely related to folic acid metabolism. Thymidylate synthetase, the target enzyme of 5-FU with regard to inhibition of DNA synthesis, is dependent on folate coenzyme function. Recently, in several ways it has been tried to combine the inhibition of thymidylate synthetase by 5-FU with treatment interfering with folate metabolism. 5-FU has been combined with antifolates, like methotrexate, which, through a depletion of folate coenzymes, also cause impairment of thymidylate synthesis (1). On the other hand, it has also been tried to increase the effects of 5-FU by the concomitant administration of reduced folates. This approach is based on biochemical evidence that the inhibitory complex of thymidylate synthetase and 5-FdUMP, an anabolite of 5-FU, is more stable in the presence of increased levels of folate coenzyme (2,3). Both methods are still under investigation in clinical trials, but so far varying results have been obtained (4-7). Clearly, the relationship between 5-FU and folate metabolism is rather complex, and more information will be useful for the optimal design of these chemotherapeutic combinations.

In this study, we present results of experiments in which 5-FU therapy

is combined with inactivation of cobalamin (vitamin B12), in a rat model for acute myeloid leukemia (BNML, Brown Norway Myeloid Leukemia). Inactivation of the cobalamin coenzyme function in the enzyme methyltetrahydrofolate homocysteine methyltransferase, or methionine synthetase, is possible by exposure to nitrous oxide, or N_2O, a specific side effect of this anesthetic gas which was recognized only recently (8-10). Methionine synthetase is essential in folate metabolism, and therefore exposure to nitrous oxide represents yet another method to interfere with folate coenzyme functions. The interaction of nitrous oxide with 5-FU could, theoretically, lead to increased therapeutic effects, as well as to antagonism. We have previously described the reduction of leukemic growth by nitrous oxide in the BNML model (11), and have also reported on the therapeutic effects of 5-FU in this rat leukemia (12). The combination of both agents in the present study is directed at effects on leukemic growth and survival time of rats, and at some metabolic changes caused by this treatment, including the plasma and intracellular levels of folates, and tests of thymidylate and DNA synthesis in leukemic cells.

6.2. Materials and methods.

Animals. Male rats of the Brown Norway inbred strain were used, at the age of 12-16 weeks (body weight 200-275 g). Food and water were supplied *ad libitum* during the experiments.

Brown Norway Myeloid Leukemia (BNML). Properties and use of this transplantable rat leukemia have been described before (11,13). It is considered to be a suitable model for experimental chemotherapy (14). In this study, rats were injected with 10^7 leukemic cells i.v., which after progressive leukemic infiltration of bone marrow, liver and spleen leads to death after 20-24 days. Spleen and liver weights, steadily increasing in the course of leukemia, are reliable indicators of leukemic growth and, along with hematological determinations, can be used effectively to assess effects of chemotherapy (13).

Treatment with nitrous oxide and 5-FU. Exposure of leukemic rats to nitrous oxide was carried out in a 40 l flow chamber in which a mixture of 50% nitrous oxide and 50% oxygen was blown at a rate of 500 ml/min. Oxygen concentration was monitored with an oxygen analyzer (Teledyne Analytical Instruments). Carbon dioxide, water and contaminating volatile compounds were eliminated in a cleaning circuit (15). Rats not exposed to nitrous oxide were kept in air, but otherwise treated identically. 5-FU (Fluorouracil Roche, from Hoffmann-La Roche) was administered i.p., at a dose of 15 or 25 mg/kg, according to different treatment schedules. Rats not receiving 5-FU were injected with 0.15 M NaCl i.p..

Evaluation of therapeutic effects. In most experiments, rats from all treatment groups were compared after a fixed period of leukemia of 19 days, which is just before untreated rats would die spontaneously. Rats were then killed by exsanguination, after recording their body weights. Liver and spleen were carefully removed and weighed. Leukocytes were counted electronically and differential blood cell counts were done. Normal values for organ weights and leukocyte counts were derived from 16 comparable non-leukemic Brown Norway rats. As an alternative method, in one experiment the extension of survival time in the treatment groups was compared.

Plasma vitamin B12 and folic acid. Plasma vitamin B12 and folic acid were determined simultaneously using a radioisotope dilution assay, essentially as described by Gutcho and Mansbach (16).

Studies using leukemic spleen cells. In separate experiments, leukemic rats were examined shortly after treatment, and leukemic spleen cells were obtained to perform additional metabolic studies. After washing and resuspension in Hanks' balanced salt solution, these cell suspensions were counted and used in an assay of intracellular folate levels and in deoxyuridine suppression tests.

Intracellular folate. A pellet of ca. 10^8 cells was resuspended in a total volume of 1 ml 10% (=1.3 M) mercaptoethanol, heated for 5 min. at 100 °C, and cooled. After incubation with hog kidney polyglutamate hydrolase (17) at room temperature for 2 hours, the samples were frozen at -20 °C until assayed. After thawing and centrifugation (1500 g for 10 min. at 4 °C) aliquots of the supernatant were used in a folate radioisotope dilution assay (18) with [^{125}I]-folic acid (Becton Dickinson, Orangeburg NY, USA) as a tracer, and β-lactoglobulin (Sigma, St.Louis, USA) as a folate binder. 5-methyltetrahydrofolate was used as a standard and results are expressed as pmol of folate per 10^6 cells.

Deoxyuridine suppression test. This test is used to demonstrate impaired *de novo* synthesis of thymidylate. It measures the incorporation of [^{3}H]-thymidine into DNA without and with the addition of deoxyuridine. Deoxyuridine will suppress [^{3}H]-thymidine incorporation if it can be converted to thymidylate by the enzyme thymidylate synthetase. This suppression will be reduced under all circumstances with impaired activity of this enzyme (19), and the test therefore can be used to evaluate effects of chemotherapeutic agents on this metabolic pathway (20). The test was carried out essentially as described by Metz (21), with some modification as described (11). All incubations were carried out in triplicate, in each 5 x 10^6 cells were used with and without the addition of 0.1 mM deoxyuridine (Sigma, St.Louis, USA). Incorporation of [^{3}H]-thymidine (0.3 μCi, specific activity 25 Ci/mmol, from Amersham, UK) in incubations with deoxyuridine is expressed as a percentage of the incorporation in the incubations without

deoxyuridine. In one experiment, however, incorporation after treatment was too low to detect significant differences with and without deoxyuridine, and in this case only [^{3}H]-thymidine incorporation in dpm/10^6 cells is given, as determined in tests without deoxyuridine.

6.3. Results.

Inhibition of leukemic growth. Leukemic rats were treated with 5-FU, and the effect of concomitant exposure to nitrous oxide was investigated. After a fixed period of leukemia (19 days) several groups were compared with regard to leukemic infiltration in spleen and liver, and leukemic cells in the peripheral blood. Results of these experiments are presented together in At a dose of 3x15 mg/kg 5-FU (administered at days 7, 12 and 17 of leukemia) the addition of nitrous oxide causes a further reduction of leukemic growth, which is evident from all parameters that were studied. In addition, as indicated in Table 6.1, most of these differences are statistically significant if Wilcoxon's non-parametric rank sum test is applied to values of individual rats. The same result is obtained with a combination of 3x25 mg/kg 5-FU and nitrous oxide. In this experiment the values of rats after combined treatment are not significantly increased compared to normal (non-leukemic) rats, whereas results after treatment with 5-FU alone are still significantly different from normal values ($p < 0.05$).

In one experiment with 1x25 mg/kg 5-FU, administered at day 14 of leukemia, the effects were studied of timing of exposure to nitrous oxide relative to the administration of 5-FU. It appears that results obtained with 4 days of nitrous oxide before 5-FU are better than with exposure only after 5-FU. In fact, this period of exposure does not add much to the effects of 5-FU alone, as is evident from the rats treated with nitrous oxide for both periods. In Figure 6.1 results are presented of determinations of plasma levels of vitamin B12 and folic acid in these experiments. It is evident that with treatment there is a dose-dependent normalization of the increased levels of vitamin B12 that are found in untreated controls. In all groups, however, the addition of nitrous oxide to 5-FU causes a further fall of vitamin B12 to subnormal levels. The determination of folic acid levels in plasma leads to more varying results, but it can be derived from Figure 6.1b that with the more effective doses of 5-FU the addition of nitrous oxide causes a decrease of folic acid levels to subnormal values.

Survival of leukemic rats. Leukemic rats were treated with 25 mg/kg 5-FU every 5 days, either or not preceded by a period of exposure to nitrous oxide of 3 days. This schedule was chosen as a consequence of the results in the studies presented in Table 6.1, in which pretreatment with nitrous oxide before 5-FU was found to be most effective. Treatment was

Table 6.1

EFFECTS OF TREATMENT WITH 5-FU AND NITROUS OXIDE (N_2O) ON GROWTH OF LEUKEMIA

Treatment	No. of rats	Spleen weight (g ± s.e.m.)	Liver weight (g ± s.e.m.)	Peripheral leukocytes (10^9/l ± s.e.m.)	% Leukemic cells (± s.e.m.)
None (untreated controls)	9	4.10 ± 0.10	19.14 ± 0.67	23.6 ± 2.3	29 ± 4
N_2O only (12 days)	6	2.87 ± 0.14	13.57 ± 0.83	13.5 ± 1.4	23 ± 4
5-FU 3 × 15 mg/kg	10	2.91 ± 0.20	12.52 ± 0.69	15.6 ± 1.6	16 ± 3
5-FU 3 × 15 mg/kg + N_2O (12 days)	9	1.87 ± 0.74**	11.15 ± 0.81	7.0 ± 1.6*	8 ± 4*
5-FU 1 × 25 mg/kg	3	0.85 ± 0.03	8.19 ± 0.67	5.3 ± 1.0	0
5-FU 1 × 25 mg/kg + N_2O (4 d. after 5-FU)	3	0.83 ± 0.06	8.20 ± 0.25	7.2 ± 0.9	0
5-FU 1 × 25 mg/kg + N_2O (4 d. before 5-FU)	4	0.46 ± 0.04	8.49 ± 0.26	3.3 ± 0.6	0
5-FU 1 × 25 mg/kg + N_2O (8 days)	5	0.43 ± 0.01	7.28 ± 0.18	2.7 ± 0.3	0
5-FU 3 × 25 mg/kg	9	0.90 ± 0.19	9.02 ± 0.92	5.7 ± 0.8	1 ± 1
5-FU 3 × 25 mg/kg + N_2O (12 days)	9	0.49 ± 0.07**	7.48 ± 0.16**	2.7 ± 0.6**	0
Normal BN rats	16	0.45 ± 0.02	8.25 ± 0.24	3.9 ± 0.4	0

* Significantly different from results without N_2O with $p < 0.025$

** Significantly different from results without N_2O with $p < 0.01$

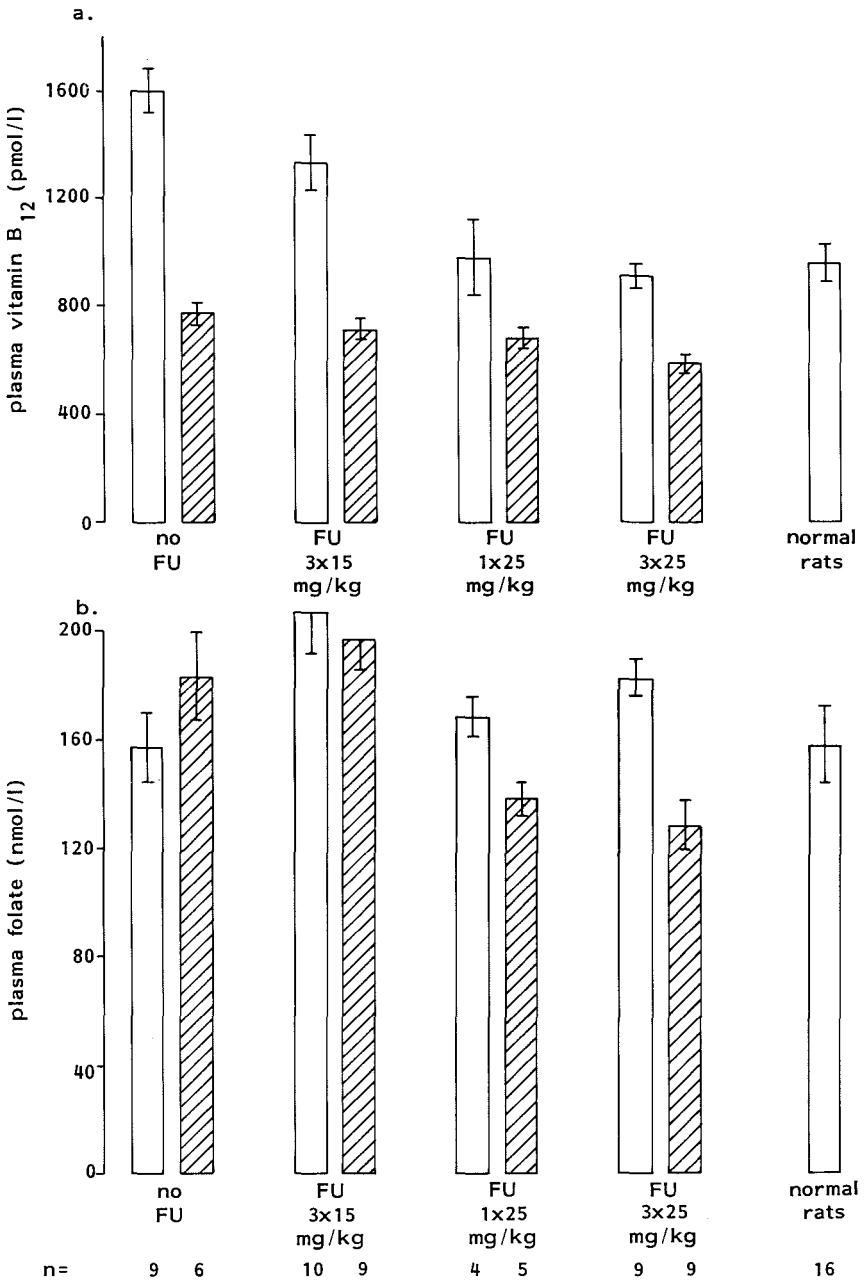

Figure 6.1. Plasma levels of (a) vitamin B12 and (b) folic acid, in leukemic rats after various treatment schedules. Shaded columns indicate results with exposure to nitrous oxide during the treatment period. Values in normal (non-leukemic) BN rats are shown in the last columns. Standard error of the mean is indicated in each column.

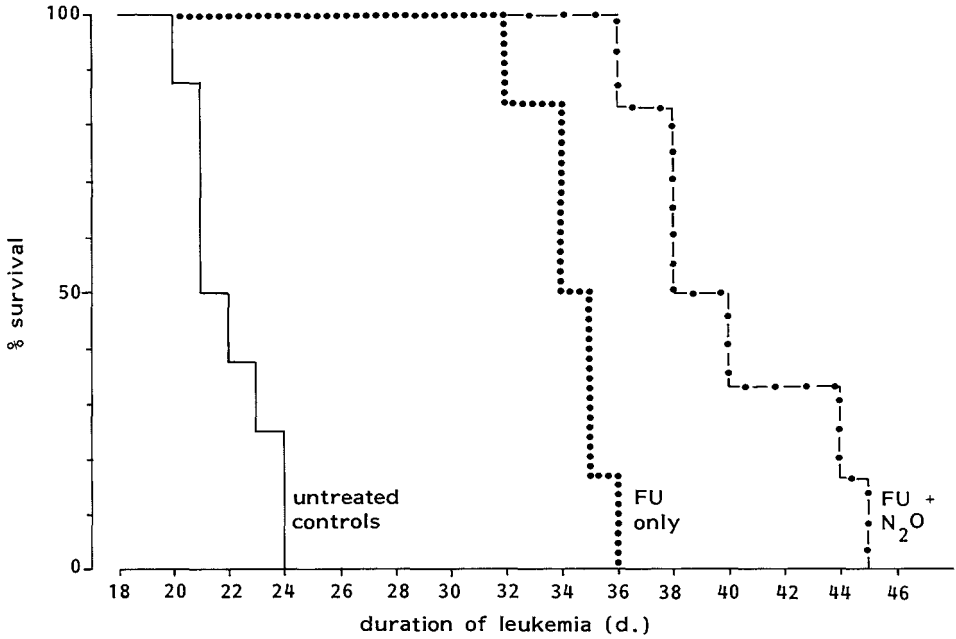

Figure 6.2. Survival of leukemic rats without treatment (controls), with 5-fluorouracil (FU) only, and with the combination of 5-fluorouracil and nitrous oxide (FU + N_2O). 5-FU (25 mg/kg i.p.) was administered every 5 days. With combined therapy, rats were exposed to nitrous oxide 3 days before each administration of 5-FU.

continued until death of the rats. Results of this experiment are presented in Figure 6.2. It appears that with addition of nitrous oxide survival of leukemic rats is extended to 40.2 $\pm$ 1.5 days (mean $\pm$ s.e.m.) compared to 22.0 $\pm$ 0.5 days for untreated rats, and 34.3 $\pm$ 0.5 days for rats treated with 5-FU alone. The results of combined treatment are significantly different from treatment with 5-FU only, with $p < 0.005$ (Wilcoxon's rank sum test). Survival after combined treatment is increased by 18.2 days compared to untreated rats (183%). In this leukemia model, 3 days of increased survival are equivalent to a 10-fold reduction in tumor load (13), which in this case would mean a reduction by a factor 10^6. All rats were examined after death and without exception, leukemia was considered to be the cause of death, in view of increased spleen and liver weights. No evidence for toxicity was found.

Deoxyuridine suppression tests. Treatment of leukemic rats with a single dose of 25 mg/kg 5-FU, was combined with 3 days of exposure to nitrous oxide, either before or after 5-FU, or both. Rats treated with 5-FU or nitrous oxide only, and untreated rats were also included. Leukemic spleen cells of all rats were used in deoxyuridine suppression tests, 3 days after administration of 5-FU, at day 16 of leukemia. Results of these tests are presented in Table 6.2. It appears that there is an enhanced effect of

Table 6.2

DEOXYURIDINE SUPPRESSION TESTS IN LEUKEMIC CELLS AFTER TREATMENT OF RATS WITH 5-FU AND NITROUS OXIDE (N_2O)

5-FU: 1 × 25 mg/kg i.p. at 3 days before examination

Treatment	No. of rats	% Suppression
None	4	12.6 (± 1.7)
5-FU only	4	12.8 (± 0.6)
N_2O only (3 days)	4	15.5 (± 2.1)
5-FU + N_2O (3 d., after 5-FU)	2	14.5 (13,16)
5-FU + N_2O (3 d., before 5-FU)	2	24.0 (22,26)
5-FU + N_2O (6 days)	2	35.5 (33,38)

Mean values of each group are given, with indication of s.e.m. in groups of 4 rats, and of individual results in groups of 2 rats. The result of each rat is the mean of triple incubations.

combined treatment, indicated by a higher percentage of suppression, with nitrous oxide pretreatment and with continuous treatment for 6 days, but not with nitrous oxide exposure after 5-FU. 5-FU alone, after 3 days, does not cause a disturbance of this test.

DNA synthesis in leukemic cells. In a similar experiment, rats were examined 1 day after administration of 25 mg/kg 5-FU. In this case, only pretreatment with nitrous oxide for 3 days is possible. In Table 6.3, results are included of tumor growth in various groups, examined at day 16 of leukemia. As in other experiments, combined treatment is most effective in reducing leukemic growth. In addition, in this table results are given of [^{3}H]-thymidine incorporation in isolated leukemic cells of these rats. DNA synthesis in both groups treated with 5-FU was impaired to such an extent that it was not possible to perform a deoxyuridine suppression test. A small additional effect of nitrous oxide exposure is still detectable, however.

Intracellular folate. In leukemic cell suspensions of rats treated in vivo with 25 mg/kg 5-FU, 1 or 3 days before examination and with or without 3 days of exposure to nitrous oxide prior to the the administration of 5-FU, the intracellular levels of folate were determined. Results can be found in Figure 6.3. For comparison, values in untreated rats and rats treated with 3 days of nitrous oxide only, are also given. With 5-FU treatment there is some decrease of folate content, more apparent at the first day after administration, but with the addition of nitrous oxide folate loss becomes much more severe.

Table 6.3

EFFECTS OF TREATMENT WITH 5-FU AND NITROUS OXIDE (N_2O) ON GROWTH OF LEUKEMIA AND ON DNA SYNTHESIS IN LEUKEMIC CELLS

5-FU: 1 × 25 mg/kg i.p. at day 1 before examination

Examination at day 16 of leukemia

Treatment	No. of rats	Spleen weight (g ± s.e.m.)	Peripheral leukocytes (10^9/l ± s.e.m.)	^{3}H-thymidine incorporation (dpm/10^6 cells ± s.e.m.)
None	3	2.65 ± 0.18	10.8 ± 0.7	3522 ± 942
N_2O only (3 days)	3	1.83 ± 0.06	8.8 ± 1.6	2135 ± 536
5-FU only	3	1.01 ± 0.06	5.1 ± 0.1	243 ± 71
5-FU + N_2O (3 d., before 5-FU)	3	0.68 ± 0.04	4.2 ± 0.2	173 ± 17

Value of ^{3}H-thymidine incorporation in leukemic cells of each rat is the mean of triple incubations.
For normal values of spleen weight and leukocyte count in BN rats, see Table 6.1.

6.4. Discussion.

Nitrous oxide disturbs folate metabolism because of its specific interference with the coenzyme function of cobalamin, which causes inactivation of the enzyme methionine synthetase (22). This prevents the conversion of 5-methyltetrahydrofolate into tetrahydrofolate, which is essential for folate coenzyme functions. The effects of nitrous oxide develop rapidly, in a few hours after exposure is started, and remain remarkably constant even with prolonged exposure of one week or more (23).

In this report exposure to nitrous oxide has been used to study the effects of impaired availability of folate coenzyme forms on the activity of 5-FU towards the the BN rat leukemia. It appeared that the therapeutic effects of 5-FU were increased with the addition of nitrous oxide, with regard to the reduction of leukemic infiltration in spleen and liver, as well as with regard to leukocyte counts. In addition, compared to treatment with 5-FU alone, there was a significantly increased survival of leukemic rats after combined treatment. In none of the treatment schedules there was any indication of antagonism, and no evidence of increased toxicity was found.

Plasma levels of vitamin B12 in this study are interesting for two reasons. First, in this leukemia vitamin B12 steadily increases with progression of disease (11), an effect which is also often observed in human promyelocytic leukemia (24). Treatment of leukemia will reduce vitamin B12 to about normal levels. Separately, however, the specific effect of nitrous oxide on vitamin B12 will cause an additional fall of plasma levels (11,25). These effects explain both the dose-dependent reduction of plasma vitamin

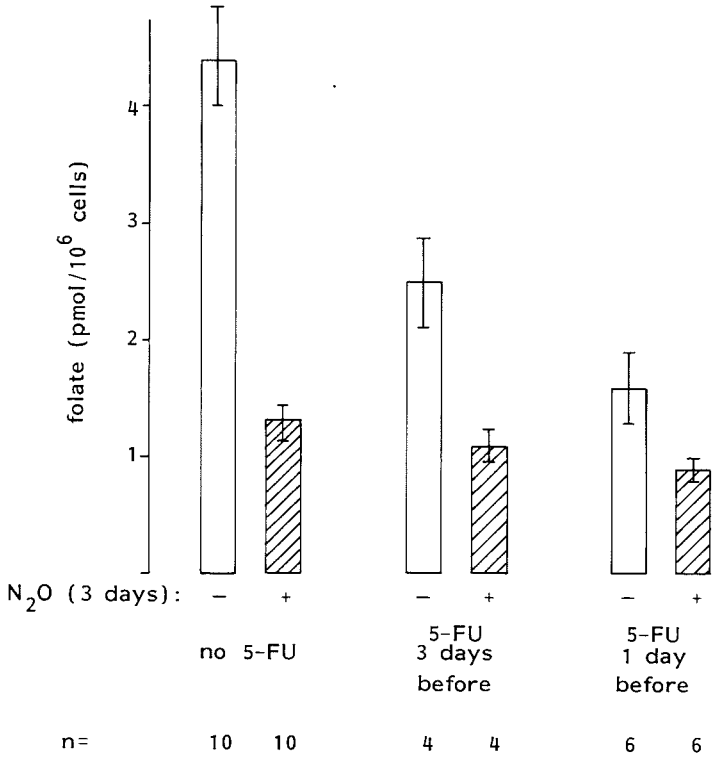

Figure 6.3. Intracellular folate levels in isolated leukemic cells after in vivo treatment of rats. 5-FU (25 mg/kg) was administered at 1 day and 3 days before examination, respectively, either or not preceded by 3 days of exposure to nitrous oxide (shaded columns). Standard error of the mean is indicated in each column.

B12 with treatment, and the occurrence of the lowest, subnormal, levels in rats with combined therapy.

The disturbance of folic acid metabolism by nitrous oxide is evident from the striking decrease of intracellular folates in leukemic cells after 3 days of exposure. Folate depletion with nitrous oxide exposure has often been observed (26), and is explained by a decreased synthesis of folate polyglutamates (27), as these forms are essential for cellular retention. Interestingly, 5-FU by itself also caused a, less pronounced, fall in intracellular folates, compared to untreated rats. This is evidence of a blockade in folate metabolism, as will indeed occur with inhibition of thymidylate synthetase, but the precise mechanism of this effect remains unclear. As would be expected, intracellular folate is lowest with combined treatment, one day after the administration of 5-FU.

The intracellular depletion of folates will ultimately also lead to reduced plasma levels of folate, as is demonstrated by the subnormal levels found after combined treatment with nitrous oxide and higher doses of 5-FU. Temporarily, however, loss of folates from the tissues can give rise to

increased concentrations of folate in plasma, as has been observed before with nitrous oxide exposure (11,28). This can explain the observations in this study of increased folate levels in rats after nitrous oxide alone and after low doses of 5-FU.

With regard to the mechanism of the interaction described in this study, an important observation is the significance of pretreatment with nitrous oxide. With nitrous oxide exposure after 5-FU, there was no enhancement of the effects of 5-FU on leukemic growth, and even some indication of a reduced effect in the deoxyuridine suppression test. A similar schedule-dependency of combination therapy is found with methotrexate and 5-FU (29,30). In that combination, methotrexate pretreatment is essential to achieve synergistic effects. If 5-FU precedes methotrexate, antagonism will result (1). Several mechanisms have been proposed to explain these effects (31). Probably, the inhibition of purine synthesis by methotrexate is important, because this can lead to increased formation of 5-FU-nucleotides, interfering with both thymidylate (DNA) and RNA synthesis (32). This effect could also occur with nitrous oxide treatment, which can impair purine synthesis as well (33,34). Other mechanisms may be involved however. Unlike methotrexate, nitrous oxide treatment for up to 3 days increases the activity of thymidylate synthetase (35), probably as an adaptive mechanism. Subsequent inhibition of this enzyme by 5-FU could render cells more vulnerable to the effects of nitrous oxide. This would also explain the necessity of pretreatment to achieve increased effects. Finally, it has been reported (2) that the addition of vitamin B12 and 5-methyltetrahydrofolate decreased the stability of the binding of 5-FdUMP to thymidylate synthetase. Prolonged exposure to nitrous oxide will decrease both these factors, and could therefore stabilize the inhibitory complex.

An important result of this study is the observation that some degree of folate depletion does not necessarily antagonize the effects of 5-FU. This conclusion obviously only applies to the leukemia model used in this study, but, interestingly, in another experimental leukemia it has been found that the addition of reduced folates to 5-FU did not increase therapeutic effects (36). Furthermore, no increased cytoxicity resulted from folate addition to cultured leukemic cells treated with methotrexate and 5-FU (37). In that study, it was also concluded that the interactions of 5-FU and folates were more complex and variable than was previously understood. The synergistic effect of folates and 5-FU as observed in xenografts of colon carcinomas might well be tissue specific to some degree. As discussed in another study (12), 5-FU is not used clinically in the treatment of leukemia. Recently however, sequential methotrexate/5-FU therapy has been applied to human leukemia with favourable results (38). This report further adds to the potential importance of 5-FU, alone or in combination with other agents interfering with folate-dependent nucleotide synthesis, in the treatment of leukemia.

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Chapter seven
NITROUS OXIDE AND INHIBITORS OF POLYAMINE SYNTHESIS IN RAT LEUKEMIA

This chapter has been submitted for publication, titled "The reduction of intracellular polyamines by sequential inhibition of the synthesis of decarboxylated S-adenosylmethionine: effects on rat leukemia", by A.C.M. Kroes, A.A.M. Ermens, J. Lindemans, M. Schoester, and J. Abels.

Abstract. Decarboxylated S-adenosylmethionine (SAM) is an aminopropyl-donor in the synthesis of the polyamines spermidine and spermine. The decarboxylation of SAM is inhibited by the toxic cytostatic drug MGBG. To achieve more specific and less toxic effects of MGBG, this drug was combined with cycloleucine, which inhibits SAM synthesis, and with nitrous oxide, which inhibits methionine synthetase. This treatment thus aimed at sequential inhibition of the synthesis of decorboxylated SAM, and was studied in a rat leukemia model (BNML). Combined treatment further decreased the level of spermine, but not of spermidine, in leukemic cells, compared to the effects of MGBG alone. The therapeutic effects of this combination were additive or less than additive, however. MGBG was not very effective in reducing leukemic growth and severely toxic, although less with combined treatment. Another inhibitor of SAM decarboxylase, berenil, was also used, and although this drug was about equally active in inhibition of leukemic growth, alterations in intracellular polyamines were not observed. The combination of nitrous oxide and cycloleucine, which effectively reduced leukemic growth at non-toxic dosages, selectively inhibited spermine synthesis, and therefore may be applied to interfere with polyamine metabolism. The relevance of this polyamine deprivation to the treatment of leukemia could not be demonstrated.

7.1. Introduction.

The polyamines spermidine and spermine have essential functions in cellular proliferation (1,2), and the biosynthesis of these compounds is therefore considered a target in cancer chemotherapy (3). In the pathway of polyamine synthesis putrescine is converted into spermidine by the addition of an aminopropyl group, derived from decarboxylated S-adenosylmethionine (SAM), and the subsequent synthesis of spermine requires a second aminopropyl donation. From this pathway, it is evident that decarboxylated SAM is an essential intermediate, and interference with the enzymatic decarboxylation of SAM is an obvious approach to the inhibition of polyamine synthesis (4). Methylglyoxalbis(guanylhydrazone) or MGBG, also known as methyl-GAG and mitoguazone, is a cytostatic agent which is known to inhibit the decarboxylation of SAM (5,6). As the impairment of cellular proliferation caused by this drug can be prevented by the addition of spermidine (7,8), the inhibition of polyamine synthesis is indeed considered to be its mechanism of action. The application of MGBG in the treatment of cancer is limited by its severe toxicity, which probably relates to other metabolic effects of this drug, notably its ability to cause severe mitochondrial damage (9). This action appeared to be independent of the effects on polyamines (10).

In this study, it is attempted to enhance the specificity of the inhibition

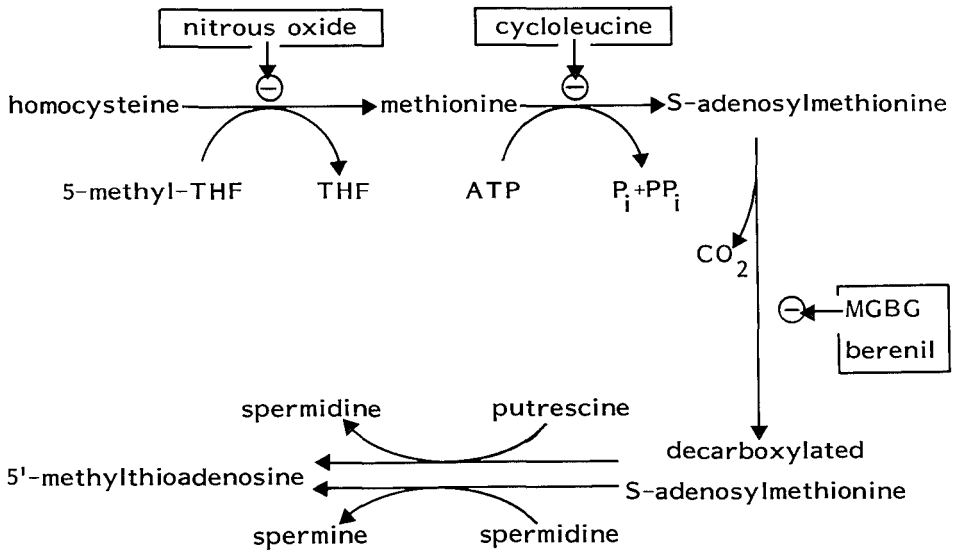

Figure 7.1. Three sequential steps in the synthesis of decarboxylated S-adenosylmethionine, with indication of the inhibitors applied in this study. The role of decarboxylated S-adenosylmethionine in the synthesis of the polyamines spermidine and spermine is shown.

of decarboxylated SAM synthesis by MGBG through concomitant interference with two preceding steps in this pathway. As outlined in Figure 7.1, these steps involve the synthesis of methionine and SAM, catalyzed by methionine synthetase and methionine adenosyltransferase, respectively. Methionine synthetase can be inhibited by exposure to nitrous oxide (N_2O), which inactivates its cobalamin coenzyme (11), and the inhibition of methionine adenosyltransferase is accomplished by the amino acid analogue cycloleucine, or 1-aminocyclopentane carboxylic acid (12). The combined use of nitrous oxide, cycloleucine and MGBG thus represents a sequential inhibition of the synthesis of decarboxylated SAM, which may enhance the selectivity of the effects of MGBG on this pathway. The modulation of polyamine synthesis by interference with the role of SAM was extensively reviewed recently (3).

Nitrous oxide inhibits cellular proliferation in vitro (13) and in vivo (14), and is known to decrease the intracellular levels of methionine and SAM (15,16). Cycloleucine has cytostatic properties as well (17), and in a previous study it was shown that the combination of these two agents resulted in a synergistic inhibition of leukemic growth (18).

We now report on the effects of nitrous oxide and cycloleucine, combined with MGBG in an experimental rat leukemia, the Brown Norway Myeloid Leukemia (BNML). MGBG has been considered a promising

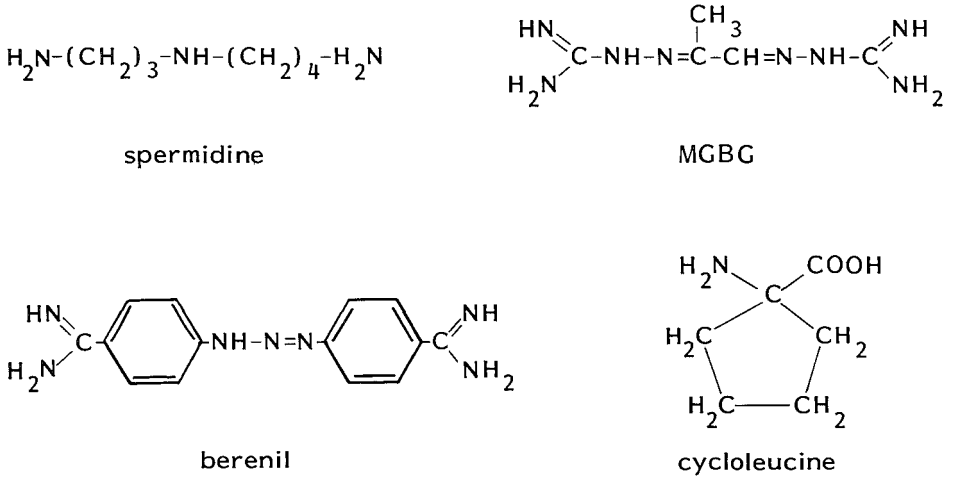

Figure 7.2. Structures of spermidine, MGBG (mitoguazone, methyl-GAG), berenil and cycloleucine.

agent in the treatment of leukemia (6,19-21). Along with treatment results of various combinations, polyamine levels were determined in isolated leukemic cells, after in vivo treatment of rats. In addition to MGBG, the antiparasitic drug berenil was used in some experiments, since it was recently reported that this drug is an inhibitor of SAM decarboxylase as well (22). The structural formulas of these drugs and of spermidine are shown in Figure 7.2.

7.2. Materials and methods.

Animals. Male rats of the Brown Norway inbred strain were used, at the age of 12-16 weeks (body weight 200-275 g). Food and water were supplied *ad libitum* during the experiments.

Brown Norway Myeloid Leukemia (BNML). Properties and use of this transplantable rat leukemia have been described before (14,23). It is considered to be a suitable model for experimental chemotherapy (24). In this study, rats were injected with 10^7 leukemic cells i.v., which after progressive leukemic infiltration of bone marrow, liver and spleen leads to death in 20-24 days. Spleen and liver weights, steadily increasing in the course of leukemia, are reliable indicators of leukemic growth and, along with hematological determinations, can be used effectively to assess effects of chemotherapy (14).

Treatment of rats. MGBG was administered at doses of 20 to 80 mg/kg by i.p. injection of MGBG dihydrochloride (Aldrich, Milwaukee, USA) dissolved in water containing 0.15 mol/l NaCl. Treatment started at day 8 of

leukemia, and was repeated every 3 days until day 17. Exposure of rats to nitrous oxide was carried out in a 40 l flow chamber in which a mixture of 50% nitrous oxide and 50% oxygen was blown at a rate of 500 ml/min. Oxygen concentration was monitored with an oxygen analyzer (Teledyne Analytical Instruments). Carbon dioxide, water and contaminating volatile compounds were absorbed in a cleaning circuit (25). Exposure started at day 7 of leukemia and was continued until the end of the experiments at day 18. Rats not exposed to nitrous oxide were kept in air, but otherwise treated identically. Cycloleucine was administered at day 7 of leukemia, as a single i.p. injection of 50 mg/kg cycloleucine (Sigma, St.Louis, USA), dissolved in water containing 0.15 mol/l NaCl. Berenil or diminazene aceturate (Sigma, St.Louis, USA) was administered at doses of 12.5 to 75 mg/kg, dissolved in water containing 0.15 mol/l NaCl, by the same schedule as described for MGBG.

Evaluation of therapeutic effects. In all experiments, rats from the various treatment groups were compared after a fixed period of 18 days, which is just before untreated rats would die spontaneously. Rats were killed by exsanguination, after recording their body weight. Liver and spleen were carefully removed and weighed. Leukocytes were counted electronically. Normal values for organ weights and leukocyte counts were derived from 16 comparable non-leukemic Brown Norway rats.

Intracellular polyamines. Leukemic spleen cells were washed, resuspended in Hanks' solution and counted. Intracellular polyamines were separated and quantified as their dansylated derivatives by HPLC as described (26). Modifications were the use of a Hypersil ODS (4.6 mm ID x 150 mm) column (Chrompack, Middelburg, The Netherlands), and a methanol gradient of 75 to 100% in 14 min. Retention times were: internal standard 1,6-diaminohexane 9.5 min, putrescine 8.2 min, spermidine 11.8 min and spermine 13.2 min. Interference of polyamine peaks with MGBG or Berenil was excluded. Polyamine values are the mean of duplicate determinations, and are expressed in nmol/10^6 cells.

DNA and protein content of leukemic cells. In one experiment, DNA and protein content of leukemic cells were determined in addition to the assay of polyamines. A DNA assay based on the colorimetric diphenylamine method was used (27), and protein was measured by the Folin-Ciocalteu method (28).

7.3. Results.

Effects of treatment on leukemia. The results are presented in Table 7.1, with indication of the spleen and liver weights, and the leukocytes counts in each treatment group, as determined after 18 days of leukemia. In addition

the number of rats dying prematurely, that is before 18 days, is indicated. As premature death was rare in untreated rats, this result

Table 7.1

THERAPEUTIC EFFECTS IN LEUKEMIC RATS OF MGBG, NITROUS OXIDE, CYCLOLEUCINE AND BERENIL.

Treatment [a]	Initial number of rats	Premature or toxic deaths	Spleen weight (g ± s.d.)	Liver weight (g ± s.d.)	Peripheral leukocytes (10^9/l ± s.d.)
None (controls)	18	2	4.23 ± 0.48	17.81 ± 2.24	19.6 ± 6.4
Nitrous oxide	10	0	2.88 ± 0.53	14.44 ± 1.99	9.0 ± 5.7
Nitrous oxide + cycloleucine	12	0	2.22 ± 0.39	12.13 ± 1.78	6.7 ± 3.7
MGBG 20	3	0	4.00 ± 0.50	15.92 ± 1.38	21.0 ± 1.7
+ nitrous oxide + cycloleucine	3	0	1.97 ± 0.46	11.92 ± 1.52	5.7 ± 1.8
MGBG 40	15	2	3.46 ± 0.32	15.37 ± 1.55	16.6 ± 6.7
+ cycloleucine	5	0	3.25 ± 0.23	13.36 ± 1.32	11.3 ± 1.7
+ nitrous oxide	4	0	2.59 ± 0.60	12.67 ± 1.32	4.1 ± 1.7
+ nitrous oxide + cycloleucine	7	0	2.13 ± 0.35	11.91 ± 1.07	6.7 ± 2.4
MGBG 50	5	3	2.35	15.71	32.5
+ nitrous oxide + cycloleucine	5	0	2.73 ± 0.32	15.06 ± 1.79	10.2 ± 2.1
MGBG 60	6	5	1.69	10.57	15.2
+ nitrous oxide + cycloleucine	6	6	–	–	–
MGBG 80	3	3	–	–	–
Berenil 12.5	4	1	3.46 ± 0.50	15.24 ± 1.74	10.6 ± 5.0
+ nitrous oxide + cycloleucine	4	0	2.42 ± 0.26	11.74 ± 0.51	3.2 ± 0.6
Berenil 25	9	4	3.18 ± 0.27	15.72 ± 2.93	14.2 ± 3.9
+ nitrous oxide + cycloleucine	5	0	1.97 ± 0.11	11.15 ± 0.49	2.3 ± 0.2
Berenil 50	4	4	–	–	–
+ nitrous oxide + cycloleucine	4	1	1.94 ± 0.42	11.70 ± 0.86	2.3 ± 0.8
Berenil 75	3	3	–	–	–
Berenil 12.5 + MGBG 20	4	0	2.96 ± 0.38	13.21 ± 2.18	21.1 ± 10.0
+ nitrous oxide + cycloleucine	4	0	1.82 ± 0.09	11.85 ± 0.89	2.8 ± 1.6
Normal (non-leukemic) rats	16	–	0.45 ± 0.07	8.25 ± 0.99	3.9 ± 1.3

[a] Dose of MGBG and Berenil is indicated in mg/kg (q 3d.)

represents an estimation of the toxicity of treatment. From the data in Table 7.1, it appears that MGBG is severely toxic at doses of 50 mg/kg and higher. Rats receiving 80 mg/kg died 1 or 2 days after the first injection. Most experiments were done with 40 mg/kg MGBG (q.3 d.), since this dose

was only moderately toxic, with still measurable therapeutic effects. The addition of nitrous oxide and/or cycloleucine to this dose of MGBG more effectively inhibited leukemic growth. The results are about additive, if compared with the effects of nitrous oxide and cycloleucine alone. Toxicity with combined treatment was less in rats receiving 50 mg/kg MGBG, but was not different at 60 mg/kg.

Results of treatment with berenil, also summarized in Table 7.1, are not much different from the results obtained with MGBG. Combined treatment with nitrous oxide/cycloleucine and berenil, 25 or 50 mg/kg, was most effective, with apparently very little toxicity. Berenil, administered alone, was increasingly toxic at doses from 12.5 mg/kg.

The simultaneous administration of low doses of MGBG and berenil, combined with nitrous oxide and cycloleucine, resulted in pronounced inhibition of leukemia, without causing toxicity.

Effects on intracellular polyamine levels. Putrescine could be determined reliably in leukemic cells after MGBG treatment only. In all other cases, putrescine was less than 0.08 nmol/10^6 cells. MGBG at a dose of 40 mg/kg resulted in a putrescine level of 0.52 ± 0.08 nmol/10^6 cells ($n=7$). There was no further elevation with the addition of nitrous oxide, cycloleucine or berenil. Spermidine was highest in cells obtained from untreated rats (mean level: 0.74 ± 0.10 nmol/10^6 cells, $n=16$), and reduced by treatment with 40 mg/kg MGBG to 0.56 ± 0.08 nmol/10^6 cells ($n=11$), but was not influenced by nitrous oxide/cycloleucine treatment, with a mean level of 0.74 ± 0.12 nmol/10^6 cells ($n=8$). The most interesting results were obtained with the determination of intracellular spermine levels, which are shown in Figure 7.3. With MGBG treatment, there is a dose-dependent reduction of spermine. Nitrous oxide alone, and nitrous oxide combined with cycloleucine also lead to reduced levels. In addition, if these agents are combined with MGBG there is a further reduction at all doses of MGBG. Surprisingly, berenil alone was not able to cause reduction of spermine or spermidine levels. Neither did this drug have synergistic effects with nitrous oxide and cycloleucine in this respect, and combinations of berenil with these drugs and MGBG result in the same spermine values as found without berenil.

DNA and protein content of leukemic cells. In one experiment, leukemic cells were obtained from four groups of 3 leukemic rats each, which were untreated, or treated with either nitrous oxide/cycloleucine, MGBG 1x60 mg/kg, or a combination of both. The mean DNA content, expressed per cellular protein, was lowest in the controls, 0.126 mg DNA/mg protein, and increased with treatment: 0.151 after nitrous oxide/cycloleucine, 0.167 after MGBG and 0.162 after MGBG with nitrous oxide/cycloleucine.

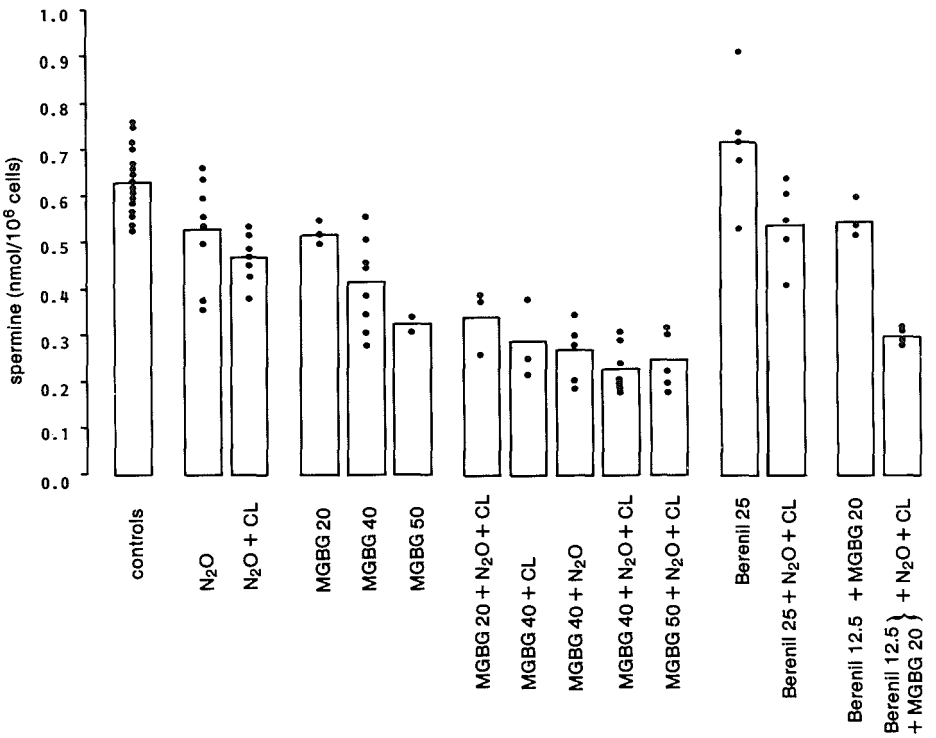

Figure 7.3. Spermine concentrations in leukemic cells after in vivo treatment of rats. Individual results of rats are indicated by dots, and columns indicate the mean of each group. Values are expressed in nmol/10^6 cells. Dose of MGBG and berenil is indicated in mg/kg (q. 3 days). Controls are untreated leukemic rats.

7.4. Discussion.

Cellular polyamine deprivation is a potentially valuable approach in cancer chemotherapy, although it is not easily achieved in vivo. One of the agents most widely applied for this purpose is MGBG, a drug inhibiting SAM decarboxylase but with other biochemical effects as well, which probably cause its severe toxicity. As the particular toxic effect of MGBG on mitochondria appears unrelated to its effect on polyamines (10), an obvious approach in the reduction of toxicity would be a combination with other agents impairing the synthesis of decarboxylated SAM.

In this study, it has been tried to increase the specificity of MGBG by aiming at a sequential inhibition of the biosynthesis of decarboxylated SAM. The addition of nitrous oxide and cycloleucine will reduce SAM synthesis. This could enhance the inhibition of SAM decarboxylase by MGBG, which is known to be competitive with the substrate SAM (5).

The inhibition of leukemic growth by MGBG was unimpressive, compared with results obtained with nitrous oxide and cycloleucine, or with 5-fluorouracil in a previous study, employing the same leukemia model (29). This is remarkable in view of some early clinical studies reporting promising effects in human acute myeloid leukemia (19-21), although not all studies were positive (30). Severe toxicity has limited its use, and was also observed in our experiments. The combination of MGBG with nitrous oxide and cycloleucine further inhibited leukemic growth, but results were about additive, with only slightly reduced toxicity. The results obtained with berenil in leukemic rats were essentially the same as with MGBG. Neither drug was able to improve the results of nitrous oxide and cycloleucine substantially. In the experiment combining low doses of MGBG and berenil, there was an additional effect on leukemia without increased toxicity. Interestingly, the antifungal agent stilbamidine, which is structurally nearly identical to berenil, has been reported to potentiate the effect of MGBG on mouse leukemia in early studies (31).

These observations on the effects on leukemic growth in rats should be interpreted together with the results of the simultaneous determinations of intracellular polyamines in leukemic cells. The alterations of polyamine levels caused by MGBG treatment were as expected: an increase in putrescine, with a decrease of spermidine and spermine. Nitrous oxide, and nitrous oxide with cycloleucine, caused a decrease of spermine levels only, and could further decrease spermine levels if combined with MGBG. This selective effect on spermine may be explained by the fact that the synthesis of this polyamine requires a twofold aminopropyl donation from decarboxylated SAM. With other inhibitors of polyamine synthesis, the reduction of spermine levels often is only limited (32). As spermine is the main polyamine in the nucleus (33), it is expected to be functionally important. Although with combined treatment spermine levels were clearly further reduced, even to 35% of normal values, the accompanying enhancement of therapeutic results was additive at best. Similar absence of growth inhibition by selective spermine depletion has been reported before (34), and in a leukemic cell line only a weak correlation between spermine depletion and growth control was found (35). In spite of its therapeutic effect, which was about equal to MGBG, berenil did not have any measurable influence on polyamine levels.

These results therefore raise some questions concerning the use of polyamine deprivation in cancer chemotherapy. There appears to be no direct correlation between the therapeutic results and the intracellular polyamine levels with a number of metabolic inhibitors. Berenil, although an active inhibitor of SAM decarboxylase in vivo in rats (22), did not have effects on polyamines. Its therapeutic, and toxic, properties thus may as well be the result of some other effect of this drug. This has been suggested before with regard to MGBG (36,37). Furthermore, the rat leukemia

employed in this study, which has been shown to be a reliable model for human acute myeloid leukemia (24), was only minimally sensitive to polyamine deprivation, in contrast to expectations based on clinical observations on the use of MGBG.

An interesting result of this study is the observation of reduced spermine levels with inactivation of the cobalamin coezyme of methionine synthetase by nitrous oxide. This suggests that to some extent the biosynthesis of polyamines is dependent on cobalamin (vitamin B12). A role of cobalamin in polyamine synthesis has not been observed before. In a recent report on the effects of polyamine deprivation in CHO cells (38), it was noticed that these cells became closely resemblant to megaloblastic cells, as observed in cobalamin deficiency. Although it is well established that cobalamin deficiency leads to a disturbance of folate metabolism, it might be that reduced polyamine synthesis contributes to the impairment of cellular proliferation.

The cellular DNA contents after treatment indicated a blockade in the S or G2 phase rather than earlier in the cell cycle. This differs from the results of a recent study, in which depletion of polyamines reduced DNA contents of cultured BHK cells (39). Similar variations between cell types in this respect have been described before, however (40).

It can be concluded that sequential inhibition of the synthesis of decarboxylated SAM further reduces the intracellular level of spermine in the treatment of leukemic rats with MGBG, but that this does not lead to synergistic effects on leukemic growth. The rat leukemia model used in this study was not as sensitive to polyamine depletion as was expected, and both inhibitors of SAM decarboxylase proved very toxic. Nitrous oxide alone, and combined with cycloleucine, effectively can decrease spermine levels, which may be applied in the study of polyamine synthesis, and also supports a potential function of cobalamin in this pathway.

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Chapter eight
THE EFFECT OF NITROUS OXIDE ON HUMAN LEUKEMIA *IN VITRO*

The investigations presented in this chapter were carried out in close co-operation with A.A.M. Ermens. The skillful technical assistance of K. van Lom, M. Schoester and M.J.M. Melsen is gratefully acknowledged.

Abstract Prolonged exposure to the anesthetic gas nitrous oxide (N_2O) decreases cellular proliferation and causes megaloblastic bone marrow depression. This effect is the result of oxidation, by nitrous oxide, of the cobalamin coenzyme of methionine synthetase, an essential enzyme in folate metabolism. This oxidation completely inactivates the enzyme and leads to a severe cellular depletion of folic acid coenzymes, resulting in impaired folate-dependent synthesis of thymidylate (dTMP). As hematopoietic cells are specifically dependent on the function of cobalamin, the effect of nitrous oxide is potentially valuable in the treatment of leukemia. In this study, leukemic cells were used, obtained from bone marrow aspirates and peripheral blood of patients with acute leukemia. Nucleated cells were suspended in culture flasks and exposed to nitrous oxide by gassing the flasks, alone or combined with methotrexate (MTX) dissolved in the culture medium. After a fixed period of 20 hrs, the cultured cells were used in the deoxyuridine (dU) suppression test, which measures the disturbance of folate-dependent dTMP synthesis. In 15 of 16 bone marrow samples, and 6 of 9 peripheral blood samples, exposure to nitrous oxide increased the dU suppression value by about 150%, indicating disturbed *de novo* synthesis of dTMP. Suppression values obtained with MTX alone were further increased by nitrous oxide exposure. In addition, two human hematopoietic cell lines, HL60 and U937, were also tested. A consistent disturbance of the dU suppression test on exposure to nitrous oxide was found with U937 cells only, as HL60 cells appeared to grow largely independent of cobalamin supply. It is concluded that human leukemic cells usually are sensitive towards the effect of cobalamin inactivation in vitro, as evident from a disturbed folate-dependent thymidylate synthesis, which is in accordance with previous in vivo results in a rat leukemia model. Cobalamin-antagonists thus may reduce leukemic proliferation, or enhance the therapeutic effects of related drugs, like MTX, in vivo as well as in vitro.

8.1. Introduction.

Cobalamin, or vitamin B12, is a nutritional factor indispensable for the maintenance of normal hematopoiesis. This is explained by its role as a coenzyme in the enzyme methionine synthetase, or 5-methyltetrahydrofolate homocysteine methyltransferase. This enzyme is essential in the metabolism of reduced folates, and, as several steps in the biosynthesis of nucleotides are dependent on the function of folate coenzymes, 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, have demonstrated the validity of this approach. It has been attempted to find

similar antagonists of the essential function of cobalamin in folate metabolism, but 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 in 1978 (4), which already was known to cause megaloblastic hematopoiesis on prolonged exposure (5). Nitrous oxide inactivates the methylcobalamin coenzyme of methionine synthetase, resulting in a virtually complete inhibition of this enzyme (6). This has been explained by an oxidative effect of nitrous oxide on the cobalt moiety of cobalamin (7). The availability of a genuine cobalamin antagonist has revived the interest in possible applications in chemotherapy. 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 methotrexate therapy (9). Kano et al. (10) have demonstrated that nitrous oxide inhibits nucleotide synthesis and proliferation in a number of human hematopoietic cell lines. In this study, we have investigated the effects of nitrous oxide in vitro on human leukemic bone marrow and peripheral blood cells, of patients with different types of acute leukemia. The assessment of the sensitivity of human leukemia to nitrous oxide should be considered an essential step towards the application of cobalamin inactivation in clinical chemotherapy. The effects on two human leukemic cell lines, HL60 and U937, were studied for comparison. 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.

8.2. 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 5-10 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 classification (11,12), and cases of acute lymphoid leukemia (ALL) were classified according to immunological phenotype. The percentage of leukemic blasts in each sample was determined in a differential count of 500 cells. Marrow cells from one case of a myelodysplastic syndrome, and from four normal aspirates were also studied.

Isolation of nucleated cells from marrow and blood. Nucleated cells from

bone marrow aspirates, diluted with an equal volume of Hanks' balanced salt solution (HBBS), and from peripheral blood samples, were isolated by sedimentation through 2% methylcellulose at an angle of 45° in about 10 min. The cells were washed in HBBS, counted and diluted to a concentration of 10^6/ml with McCoy's medium 5A, modified by replacement of folic acid by 400 nmol/l methyltetrahydrofolate (Sigma, St.Louis, USA), limitation of methionine to 25 μmol/l, and omission of cobalamin. Viability of the cells was checked by trypan blue exclusion.

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, cell viability was checked again by trypan blue exclusion.

Addition of methotrexate. Methotrexate (Ledertrexate SP, from Lederle) was added in a negligible volume to the culture flasks in some experiments, to a final concentration of 50 nmol/l.

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 by nitrous oxide or methotrexate. [^{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 test values are expressed as the percentage ratio of the [^{3}H]-thymidine incorporation with and without deoxyuridine in the same cell suspension. The test was carried out essentially as described by Metz et al. (13), with some modifications. The concentration of deoxyuridine was 0.1 mmol/l. The duration of pre-incubation, either with or without deoxyuridine, as well as of incubation with [^{3}H]-thymidine was 2 hours. 0.3 μCi of [^{3}H]-thymidine with a specific activity of 25 Ci/mmol (Amersham, UK) was used per test, with about 10^6 cells. All results are the mean of triple incubations.

Culture of leukemic cell lines. Two cell lines, HL60 and U937, were also tested. The HL60 cell line originated from a human acute promyelocytic leukemia and has been described extensively (14). U937 is a hematopoietic cell line derived from a patient with true histiocytic lymphoma (15), consisting of monocyte-like cells. A comparative review of the two cell lines was

published recently (16). Samples of these cell lines were kindly provided by dr N. de Both (Dept. of Pathology, Erasmus University Rotterdam). The cells were cultured in suspension, in McCoy's medium with 10% fetal calf serum (Gibco), prepared without the addition of folic acid, cobalamin or methionine, as these constituents have to be carefully controlled in studies on cobalamin and folic acid metabolism. 5-Methyltetrahydrofolate (Sigma) was added to replace folic acid (pteroylglutamic acid). In most experiments, conditions and concentrations were as described by Kano et al., with 200 nmol/l of 5-methyltetrahydrofolate (10). After each series of experiments, the cultures were replaced by fresh cells from the same frozen stock. Experiments 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.

8.3. Results.

Patient characteristics are summarized in Table 8.1. In all of 16 cases, the normal bone marrow was largely replaced by leukemic cells, with a mean of 73.1% (± s.d. 24.1) of leukemic blasts.

Table 8.1

CHARACTERISTICS OF PATIENTS

Number of patients	16
male	11
female	5
Mean age	37.7 y.
Diagnosis	
acute myeloid leukemia	9
FAB type M1	1
M2	2
M4	4
M5	2
acute lymphoid leukemia	6
null-ALL	1
c-ALL	2
B-ALL	1
T-ALL	2
myelodysplastic syndrome	1

The results of deoxyuridine suppression tests after nitrous oxide exposure of the leukemic marrow cells, compared to the same cells exposed to air, are shown in Figure 8.1a. In 15 of the 16 cases, the exposure to nitrous oxide

caused a marked increase of the test value, indicating an impaired ability to use deoxyuridine as a substrate in the *de novo* synthesis of thymidine. The suppression values are presented on a logarithmic scale, because it is likely that the ratios of the values obtained in air and in nitrous oxide are more relevant than their absolute differences. There is a wide variation of suppression values after exposure to air, from 5.4 to 45%, with a mean of 17.9, probably related to the heterogeneity of cell types in different forms of leukemia. After nitrous oxide exposure of these same cells, the test values range from 8.4 to 58%, with a mean of 30.0. The only non-responding cells were from a case of T-ALL. Figure 8.1b shows that normal bone marrow cells are also sensitive to the inactivation of cobalamin. Results obtained with blood cells of 9 leukemic patients, who all had a high number of peripheral blasts cells, are presented in Figure 8.1c. The increase in mean suppression values, from 20.4 to 31.1 %, is comparable to the result obtained with the marrow cells. However, as apparent from the figure, the pattern is somewhat less consistent, as of the 9 cell samples 3 were found to be unresponsive to nitrous oxide, all from cases of ALL.

Methotrexate was added, prior to the incubation, to 8 of the leukemic and 2 of the normal marrow cell suspensions. The results are shown in Figure 8.1d. The addition of methotrexate alone to the marrow cells increased the suppression value in all cases, resulting in a mean value of 47.0%, with a range of 19 to 69%. If the cells were, in addition, exposed to nitrous oxide, this resulted in a further increase of the mean suppression value to 55.1%, with a range of 37 to 74%. 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 test, and that in this range the deoxyuridine suppression test is probably not sensitive anymore. In all of the other 5 cases, nitrous oxide enhanced the effect of methotrexate.

Finally, results obtained with the cell lines HL60 and U937, with culture conditions as described by Kano et al. (10), are presented in Tables 8.2 and 8.3. Only U937 cells appeared to be consistently sensitive towards nitrous oxide exposure, as is evident from results in three consecutive experiments in Table 8.2. The test values appear rather variable quantitatively, between experiments, and in the same experiment on successive days, but there remains a clear difference between the cultures in air and nitrous oxide. The cultures exposed to air were always in the normal range of the test. In contrast, cultures of HL60 cells only showed a clearly increased dU suppression value on nitrous oxide exposure after 1 day, but this difference largely disappeared with continuation of the experiment, when all cells became about equally disturbed (Table 8.3). This cell line therefore appears not suitable for this kind of experiments, as illustrated by the experiment shown in Figure 8.2. In this experiment, the growth of HL60 cells was compared

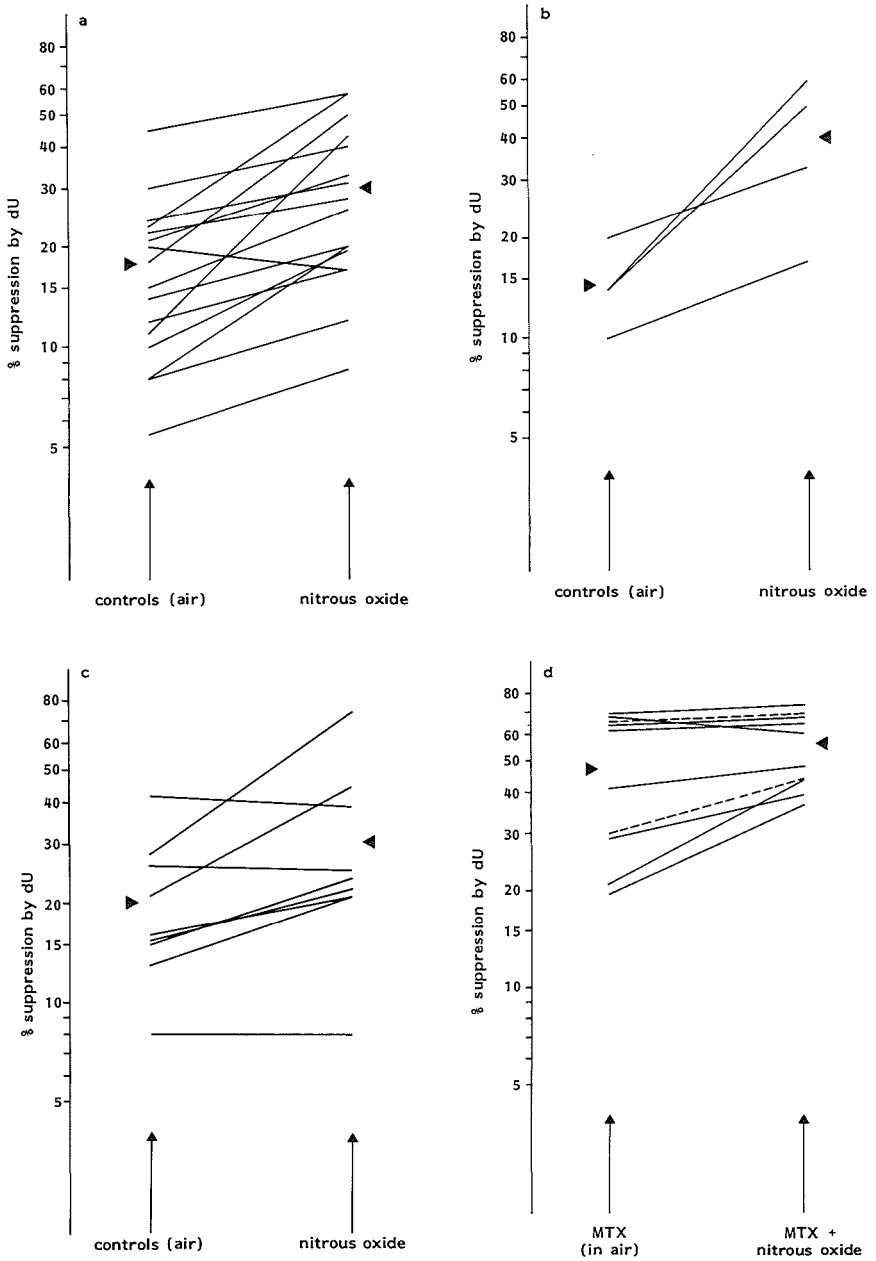

Figure 8.1. Results of in vitro exposure to nitrous oxide on deoxyuridine suppression tests in fresh human marrow or peripheral blood nucleated cells: (a) leukemic marrow cells (b) normal marrow cells (c) peripheral blood cells of leukemia patients (d) marrow cells, either leukemic (continuous line) or normal (interrupted line), treated previously in vitro with methotrexate (MTX). Mean suppression values of groups without and with nitrous oxide exposure are indicated.

Table 8.2

DEOXYURIDINE SUPPRESSION TESTS IN U937 CELLS AFTER EXPOSURE TO NITROUS OXIDE

Days of culture	Percentage of suppression by deoxyuridine					
	Experiment 1		Experiment 2		Experiment 3	
	Air	N_2O	Air	N_2O	Air	N_2O
0	18	18	8	8	15	15
1	3	5	6	34	11	11
2	8	24	2	9	10	14
3	9	91	3	27	6	16

Table 8.3

DEOXYURIDINE SUPPRESSION TESTS IN HL60 CELLS AFTER EXPOSURE TO NITROUS OXIDE

Culture conditions according to Kano et al. (10)

Days of culture	Percentage of suppression by deoxyuridine	
	Air	*Nitrous oxide*
0	8.6	8.3
1	7.1	17.5
3	37.1	39.9
4	39.6	46.6
7	33.2	66.2

in fully supplemented medium, and in medium deprived of methyltetrahydrofolate and/or cobalamin, to study the degree of dependence on this constituents. It appears that after 24 days of culture, there still is a minimal difference in growth between the supplemented and deprived cultures.

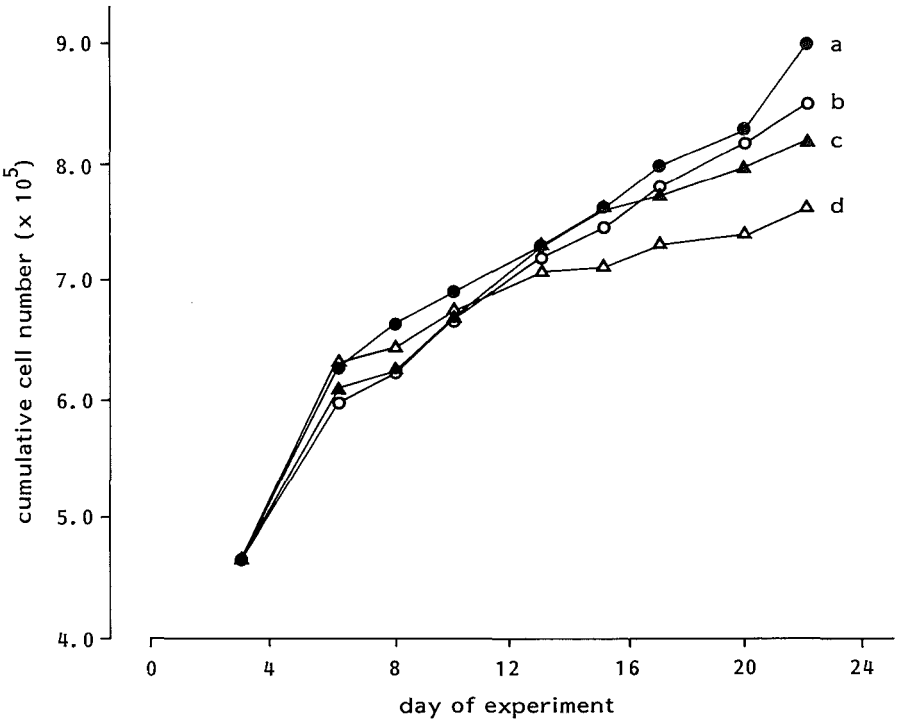

Figure 8.2. Culture of HL60 cells in four different media for 24 days. The cumulative cell number of each culture is shown. All cultures started with 4.5 x 10^9 cells. In all groups McCoy's Medium 5A was used with 10% fetal calf serum, 25 mol/l L-methionine and mmol/l glutamine, with: (a) 400 nmol/l 5-methyl-THF and 40 nmol/l hydroxocobalamin; (b) 200 nmol/l 5-methyl-THF only; (c) 40 nmol/l 5-methyl-THF hydroxocobalamin only; (d) no additions.

8.4. 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. Nitrous oxide, the only available effective antagonist of the metabolic function of cobalamin, has been shown to inhibit rat leukemia in vivo (8), and human leukemic cell lines in vitro (10). There are also two early observations of a distinct antileukemic effect of nitrous oxide exposure in patients (17,18), dating from the time before this agent was known to inactivate cobalamin. Two cases have been described of patients with leukemia, in which the administration of cobalamin was considered to enhance leukemic proliferation (19,20). A disturbance of the deoxyuridine suppression test in normal bone marrow cells after nitrous oxide exposure of about 24 hours is

well established (21), and this effect was also found to be enhanced by methotrexate addition (22).

The results of the present study provide evidence that in nearly all cases of acute leukemia the leukemic cells are sensitive to cobalamin inactivation, as is apparent from a disturbance 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.

The synergistic effect of nitrous oxide and methotrexate, with regard to the disturbance of deoxyuridine suppression in leukemic cells in vitro, is in accordance with the results obtained before in leukemic rats (9) and normal human bone marrow (22).

As nearly all cases of leukemias were responsive to cobalamin inactivation, it is difficult to draw conclusions on the differential sensitivity of particular types. It may be relevant, however, that the single marrow cell suspension, and the three blood cell samples, that were found to be unresponsive, were all cases of ALL.

With regard to the results obtained with cultured leukemic cell lines, it is remarkable that for HL60 cells, the conditions as described by Kano et al. (10) were not suitable to perform reliable experiments. In addition, it appeared that, in contrast to results obtained by these authors, there was no indication of growth inhibition of the cells by nitrous oxide, and neither was complete deprivation of cobalamin and folate sufficient to cause a significant effect on proliferation. Not included in the results are the similar effects of several modifications of the culture conditions. The HL60 cell line therefore probably is not a useful model in the study of cobalamin-dependent metabolism. The U937 cell line also appeared to be sensitive towards minor variations in culture conditions, but after nitrous oxide exposure the deoxyuridine suppression test was more consistently disturbed in these cells.

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, in a way comparable to normal marrow cells. The deoxyuridine suppression test may well be predictive for the clinical sensitivity of acute leukemias to nitrous oxide, as results obtained with nitrous oxide in vitro are in agreement with those obtained treating rat leukemia in vivo. These findings encourage further investigations, eventually directed at the administration of nitrous oxide to selected patients with leukemia.

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Chapter nine
SURVEY AND CONCLUSIONS

Cobalamin, historically known as vitamin B12, is indispensable in normal hematopoiesis. Its metabolic role has been the subject of intensive study in the past thirty years, and is fairly well understood now, as reviewed in the first chapter of this thesis. The inactivation of cobalamin by nitrous oxide has provided a valuable new method to investigate the function of this vitamin in hematopoiesis. At the same time, this particular side-effect of the widely-used anesthetic agent can be used to study the role of cobalamin in the growth of malignant hematopoietic cells, and to evaluate the value of cobalamin-dependent metabolism as a target in tumor chemotherapy. The purpose of the investigations presented in this thesis is to study the application of the nitrous oxide effect on cobalamin in the treatment of leukemia, in a systematic and orienting way. In each of the preceding chapters only a single aspect of the results obtained with nitrous oxide has been discussed, and in this chapter it will be tried to formulate more general conclusions on the perspectives of cobalamin inactivation in the therapy of leukemia.

In most experiments, a realistic rat model for acute myeloid leukemia, the Brown Norway Myeloid Leukemia (BNML) has been used. The results of experimental chemotherapy in this leukemia can be evaluated by a number of methods, which are discussed in Chapter 2. The main subject of the studies described in the previous chapters has been the search for an influence of cobalamin inactivation on leukemic growth, and its correlation with biochemical alterations. Therefore, the method that was usually chosen to evaluate the effects of treatment consisted of the simultaneous analysis of the rats of all treatment groups, after a fixed period of leukemic growth. This method of evaluation correlated well with the established technique of measurement of survival. The latter method, however, does not permit metabolic studies in the same experiment.

Referring to the scope of this thesis, as outlined in section 1.4, the most essential results of the investigations will be reviewed.

In the first studies, a significant retardation of the growth of the leukemia resulted from exposure of the rats to nitrous oxide alone. This was accompanied by metabolic effects, suggesting that the inactivation of cobalamin was indeed responsible for the therapeutic result. The deoxyuridine suppression test was disturbed in leukemic cells of treated rats, plasma cobalamin was decreased, and plasma folate was elevated. The effect on leukemic growth in this model was readily measurable and appeared very

well reproducible, but a complete remission of the leukemia was not observed. In the following studies, it was tried to impair cobalamin-dependent metabolism more effectively by combining nitrous oxide exposure with the administration of selected other agents. Agents with potentially synergistic actions were chosen on the basis of the present knowledge on the metabolic role of cobalamin. The results have been presented of experiments combining nitrous oxide with cycloleucine, methotrexate, 5-fluorouracil and inhibitors of polyamine biosynthesis. With the first three of these agents, it is assumed that a depletion of intracellular folates, as brought about by nitrous oxide, will result in enhanced activity. On the other hand, with polyamine inhibitors, an interaction is expected based on the reduced synthesis of methionine, which is as well a consequence of the inactivation of methionine synthetase.

The synergistic growth inhibiting effect of nitrous oxide and cycloleucine demonstrates the importance of S-adenosylmethionine as a regulatory intermediate in one-carbon metabolism, as cycloleucine is a selective inhibitor of the synthesis of this compound. Cycloleucine was considered to be a promising cytostatic agent in the early sixties, and even in some recent trials, but has been discarded mainly because of severe side-effects. The increased inhibition of leukemic growth in rats apparently does not lead to more pronounced side-effects. The addition of nitrous oxide to cycloleucine, or one of the other inhibitors of S-adenosylmethionine synthesis, appears to be a promising example of metabolic manipulation. Decreased levels of S-adenosylmethionine enhance the disturbance of folate metabolism, but in addition, the effects of cycloleucine could also be enhanced by the inhibition of methionine synthesis by nitrous oxide.

Considering the essential role of cobalamin in folate metabolism, it is not surprising that a significant enhancement of the therapeutic efficacy of methotrexate can be produced by exposure to nitrous oxide. It is important, however, to study the mechanism of this interaction, to be able to apply the most effective treatment schedules in further experiments. It appears that the intracellular folate depletion, produced by exposure to nitrous oxide of up to three days, correlates well with the therapeutic results of the combination. This is in accordance with the finding that exposure to nitrous oxide before methotrexate is necessary to result in increased therapeutic effects. The development of severe folate depletion probably takes one or two days. In this respect, the pretreatment with nitrous oxide can be considered the reverse of folinic acid rescue after methotrexate therapy. Likewise, nitrous oxide can possibly be applied to modulate the cytotoxicity of methotrexate. It has not been investigated so far whether this combination also increases polyglutamation of methotrexate. Polyglutamation of this antifolate, which considerably enhances its therapeutic effects, is known to occur in competition with normal folate derivatives, and could therefore be stimulated by the induced folate depletion.

The results of the nitrous oxide/5-fluorouracil combination appear to be important to the actual problem of the involvement of folate metabolism in the mechanism of 5-fluorouracil cytotoxicity. The increase in therapeutic effects, brought about by nitrous oxide pretreatment before 5-fluorouracil administration, is rather small compared to the interaction of nitrous oxide with methotrexate. However, as it has been suggested that an increase of intracellular folates with 5-fluorouracil therapy is essential to obtain an optimal therapeutic effect, even the small enhancement of its effect by nitrous oxide addition demonstrates that this hypothesis is, at least, not always true. A depletion of cellular folates clearly can result in increased activity of 5-fluorouracil. Furthermore, these experiments have demonstrated an unexpectedly high activity of fluorouracil towards the rat leukemia, emphasizing the need for further evaluation of this drug in the treatment of human leukemia.

Thus, all three combinations that were based on interference with folate metabolism or folate dependent nucleotide synthesis resulted in an enhancement of antitumor activity. The final combination that has been tested is involved in the other part of the reaction catalyzed by the cobalamin-dependent methionine synthetase: the conversion of homocysteine to methionine. In cobalamin-deficient or nitrous oxide treated cells methionine is low, and this in turn results in low S-adenosylmethionine (SAM) levels. SAM is involved in a wide variety of metabolic processes, but only one of these is definitely related to cell proliferation. This is the synthesis of the polyamines spermidine and spermine, requiring decarboxylated S-adenosylmethionine as a donor of aminopropyl groups. The cytostatic drug mitoguazone, or MGBG, inhibits the decarboxylation of SAM, but appeared to be little specific, resulting in severe toxicity in clinical trials. Nitrous oxide can limit the supply of methionine, and thereby impair the synthesis of spermidine and spermine. Cycloleucine, inhibiting the conversion of methionine to SAM, could increase this effect. It appeared that this approach to the inhibition of polyamine synthesis, consisting of nitrous oxide, cycloleucine and mitoguazone, was indeed effective with regard to the reduction of intracellular levels of one of these polyamines, spermine. There appeared to be no synergistic effect on tumor growth, however, as the therapeutic result of the combination was about additive, compared to the results of individual drugs. For this reason, there was neither much reduction in the toxicity of mitoguazone. These experiments illustrate the difficulties frequently encountered when polyamine biosynthesis is used as a target in cancer chemotherapy. Although this study has demonstrated that cobalamin may have a role in the synthesis of spermine, it was not yet possible to increase substantially the therapeutic value of nitrous oxide by aiming at a reduction of polyamine synthesis.

From these experiments in leukemic rats, it can be concluded that the inactivation of cobalamin, as a new approach in chemotherapy, is able to

reduce leukemic growth, and to modify significantly the response of leukemic cells to certain cytostatic drugs, without evidence of increased toxicity. It would be of interest to compare the results obtained with nitrous oxide with those of more established cytostatic drugs. In the studies described in this thesis, the results of methotrexate treatment were not much different from those with nitrous oxide, while 5-fluorouracil appeared, surprisingly, more effective. Differences in evaluation techniques make comparisons with other studies rather difficult. From a number of extensive chemotherapeutic studies in this rat leukemia, the impression would be that the extent of growth inhibition by exposure to nitrous oxide, as evident from spleen and liver weights, is about comparable to the effect of low or moderate doses of cytarabine and adriamycine (Colly LP. Chemotherapy in a transplantable leukaemia in Brown Norway rats. Thesis, Erasmus University Rotterdam. Delft: Meinema, 1980). It has appeared from several studies that results obtained with the BNML model agree well with clinical effects, but still the results of these animal studies may only be cautiously applied to predict the value of nitrous oxide in humans. Man appears to be rather vulnerable with regard to the consequences of cobalamin deficiency.

If nitrous oxide is combined with other agents, its strong enhancement of the effects of the antifolate methotrexate is most conspicuous. Further analysis of this interaction seems warranted, in particular with regard to its mechanism. In addition, the combination of nitrous oxide with other antifolates could be investigated. It is also interesting to study the influence of nitrous oxide on the development of resistance towards methotrexate. Nitrous oxide exposure appears to result in enhanced enzyme activity of thymidylate synthetase. Depression of the activity of this enzyme is one of the ways in which cells may become resistant to methotrexate.

In order to obtain information on the sensitivity of human leukemia to the metabolic effects of nitrous oxide, experiments were carried out with bone marrow cells from patients, and with human leukemic cell lines. In nearly all cases, the deoxyuridine suppression test was clearly disturbed after exposure to nitrous oxide, demonstrating the sensitivity of these human malignant cells. The application of nitrous oxide to selected patients with leukemia, under carefully controlled conditions, logically should be the next step. Nitrous oxide is in fact already a well known and widely used drug, but this radically different application, with a longer duration of administration, and involving other clinical departments, requires a cautious approach. The results of the experiments with human leukemic cells can be considered an essential step in this direction.

The practical application of nitrous oxide in chemotherapy is obviously hampered by its gaseous state. "Breathing" a drug is rather uneasy, compared to oral and parenteral administrations. However, some early trials have demonstrated the feasibility of this way of administration. Nevertheless, it seems worthwhile to search for a solid or liquid alternative for

nitrous oxide, and some candidates in fact exist, like the alkylnitrites. The importance of the studies presented in this thesis also lies in the demonstration of the dependence of tumor growth on the metabolic function of cobalamin, and of the increased vulnerability towards other drugs after inactivation of cobalamin. These findings may as well apply to other cobalamin antagonists.

The application of nitrous oxide in this series of investigations has demonstrated once again that toxicity, from a pharmacological point of view, is only a relative phenomenon. Effects of a drug that are not desired in a certain clinical situation are designated toxic, which does not preclude that the same effect may be useful in another clinical situation. Unwanted, toxic effects of several substances have found an application in medicine. Side-effects turn into desired effects, if drugs are administered to patients with a different disease. Some examples are the antithrombotic effects of acetylsalicylic acid, the use of the vasodilator diazoxide to inhibit insulin secretion, the interference with ADH effects by the antibiotic demeclocycline, the enzyme induction in neonates by the anti-epileptic phenobarbital, or recently, the introduction of the antihypertensive minoxidil as the long-awaited drug promoting hair growth. The nitrous oxide/cobalamin interaction can also serve to illustrate this concept. Nitrous oxide causes severe impairment of blood cell proliferation, which can result in a megaloblastic bone marrow depression. This effect is obviously unwanted in surgical patients receiving nitrous oxide during anesthesia. On the other hand, the selective suppression of the growth of blood cells is of great interest in hematology. Therefore, this interaction has been studied extensively both in anesthesiology and hematology, although for different reasons. In this thesis, the interaction of nitrous oxide and cobalamin has been studied from the hematological point of view. The toxic effect has been applied deliberately, as a new method to influence the growth of malignant blood cells.

Cobalamin, an essential constituent of food for all animals, is intimately involved in the metabolic pathways concerned with the transfer of single carbons. These metabolic steps are in particular important in the synthesis of the various nucleotides. This is the reason that cobalamin, and the functionally closely related vitamin folic acid, appear essential to the rapidly proliferating hematopoietic cells. It also explains that antagonists of both these vitamins may be used to impair the process of cellular proliferation. In contrast to the folate antagonists, probably the most widely used class of chemotherapeutic agents, cobalamin antagonists have not yet contributed to the treatment of malignant growth. The studies presented in this thesis could stimulate the development of this new class of metabolic inhibitors applicable in the fight against cancer.

Summary

The essential role of cobalamin (vitamin B12) in the metabolism of folates is based on its coenzyme function in the enzyme methionine synthetase. Exposure to the anesthetic gas nitrous oxide leads to oxidation of the cobalamin coenzyme, and thereby to a virtually complete inactivation of this enzyme. The consequences of this interaction are fully comparable to a severe deficiency of cobalamin, with a disturbance of folic acid metabolism, resulting in a pronounced impairment of hematopoiesis.

The subject of this thesis is the investigation of the applicability of this specific side-effect of nitrous oxide in chemotherapy of hematologic malignancies. In contrast to the well-known antagonists of folic acid, up till now no effective antagonist of cobalamin is available.

The introductory *Chapter 1* contains a review on the biochemistry of cobalamin and folic acid and their interrelationships, with emphasis on the "methylfolate trap hypothesis". In addition, an overview is presented of cobalamin antagonists in general, and of the the nitrous oxide-cobalamin interaction in particular.

Chapter 2 describes the most essential experimental methods employed in the investigations presented in this thesis: the Brown Norway rat myeloid leukemia, the system used for nitrous oxide exposure and the deoxyuridine suppression test.

In *Chapter 3* experiments are discussed in which leukemic rats were exposed to nitrous oxide. Results included a retardation of leukemic proliferation, as measured by organ infiltration and leukocyte counts, accompanied by a fall of plasma cobalamin, a rise of plasma folate and a disturbance of the deoxyuridine suppression test in leukemic cells.

Chapter 4 discusses the first of a series of investigations in which it was tried to exploit the effects of nitrous oxide by combined treatment with chemotherapeutic agents active in the same or related pathways. In this chapter, experiments involving cycloleucine are described which resulted in a synergistic inhibition of leukemic growth and an additional disturbance of the deoxyuridine suppression test. It seems probable that cycloleucine further disturbs folate metabolism, and the pathways involved in this effect are described.

In *Chapter 5* the results of nitrous oxide exposure combined with methotrexate treatment are discussed. Substantially enhanced therapeutic activity towards the rat leukemia was observed, along with an additional reduction of intracellular folates and of deoxythymidylate, and with a further disturbance of the deoxyuridine suppression test. From these

experiments it appeared that pretreatment with nitrous oxide before methotrexate was essential for an optimal therapeutic interaction.

Chapter 6 contains observations on the combination of nitrous oxide with 5-fluorouracil. 5-Fluorouracil appeared to be an effective treatment of the rat leukemia, which effect still could be enhanced by the addition of nitrous oxide. This was accompanied by intracellular folate depletion, demonstrating that, in this leukemia, the effect of 5-fluorouracil was not dependent on high levels of intracellular folate, as observed with some other tumors.

Chapter 7 deals with the experiments aimed at a reduction of polyamine synthesis, by means of combined treatment consisting of nitrous oxide, cycloleucine and mitoguazone (MGBG). It was found that treatment directed at polyamine deprivation was toxic and only minimally active in the rat leukemia, and that there was at best an additive effect when MGBG was combined with nitrous oxide and cycloleucine. On the other hand, it was possible to cause a further, selective, reduction of the intracellular level of spermine by nitrous oxide and cycloleucine. This could mean that cobalamin has a role in polyamine synthesis.

In *Chapter 8*, in vitro experiments are described in which nitrous oxide was applied to human leukemic cells, from bone marrow and peripheral blood of patients with leukemia. For comparison, cells from human continuous leukemia cell lines (U937 and HL60) were also used. These experiments demonstrated that the results obtained by in vivo treatment of leukemic rats were applicable to the human leukemic cells, with regard to nitrous oxide exposure as well as to the combinations involving methotrexate.

Finally, in *Chapter 9* the results are discussed in coherence with more general conclusions on the perspectives of cobalamin inactivation as a new therapeutic strategy in the chemotherapy of leukemia.

Samenvatting

De belangrijke rol van cobalamine ofwel vitamine B12 in de foliumzuurstofwisseling komt voort uit een functie als coenzym in het enzym methionine synthetase. Blootstelling aan het uit de anesthesie bekende gas lachgas veroorzaakt een oxidatie van het cobalamine coenzym, die leidt tot een vrijwel volledige inactivatie van dit enzym. De gevolgen van deze interactie zijn volledig vergelijkbaar met een ernstige cobalaminedeficientie, met een verstoring van de foliumzuurstofwisseling die resulteert in een sterke remming van de hematopoëse.

Het onderwerp van dit proefschrift is het onderzoek van de toepasbaarheid van deze specifieke bijwerking van lachgas in de chemotherapie van hematologische maligniteiten. In tegenstelling tot de bekende foliumzuurantagonisten zijn er tot nu toe geen werkzame antagonisten van cobalamine beschikbaar.

Het inleidende *Hoofdstuk 1* bevat een overzicht van de biochemie van cobalamine en folaat en met name van hun onderlinge relatie, zoals die wordt beschreven door de methylfolaat-val ("trap") hypothese. Daarnaast wordt samengevat wat bekend is over cobalamine-antagonisten in het algemeen, en over de interactie van lachgas en vitamine B12 in het bijzonder.

Hoofdstuk 2 behandelt de belangrijkste experimentele methoden zoals toegepast in het onderzoek beschreven in dit proefschrift. Dit betreft de myeloide leukemie in Brown Norway ratten (BNML), het systeem waarin deze ratten werden blootgesteld aan lachgas en de deoxyuridine suppressietest.

Hoofdstuk 3 beschrijft de resultaten van de blootstelling van ratten met leukemie aan lachgas. Daarbij werd een vertraging van de groei van de leukemie vastgesteld, gemeten door vermindering van de infiltratie van leukemie in lever en milt en een verminderd aantal leukemiecellen in het bloed. Tegelijk daalde het plasmagehalte van cobalamine, steeg het plasmafolaat en was de deoxyuridine suppressietest in de leukemiecellen verstoord.

Hoofdstuk 4 is gewijd aan de eerste van een serie onderzoeken waarbij werd getracht de resultaten van lachgasblootstelling te versterken door gerichte combinaties met middelen die op dezelfde stofwisselingswegen aangrijpen. In dit hoofdstuk werd daarvoor cycloleucine toegepast, waarmee een synergistische remming van de leukemiegroei werd bereikt en verdere verstoring van de deoxyuridine suppressietest. Een verdere verstoring van het foliumzuurmetabolisme door cycloleucine bleek op te treden, en het

mogelijke mechanisme daarvan wordt beschreven.

Hoofdstuk 5 behandelt de resultaten van lachgasblootstelling gecombineerd met methotrexaatbehandeling. Hierdoor werd de therapeutische activiteit ten opzichte van de ratteleukemie aanzienlijk verhoogd. Het intracellulaire gehalte aan folaat en deoxythymidylaat bleek verder verlaagd dan bij iedere behandeling afzonderlijk, en er was een toegenomen verstoring van de deoxyuridine suppressietest. Uit deze experimenten bleek tevens dat een voorbehandeling met lachgas, voor het toedienen van methotrexaat, een vereiste was voor deze therapeutische interactie.

Hoofdstuk 6 beschrijft de waarnemingen aan de behandeling met de combinatie van lachgas en 5-fluorouracil. 5-Fluorouracil bleek reeds zelf een effectief middel bij de behandeling van de ratteleukemie, doch de resultaten konden nog verbeterd worden door de toevoeging van lachgas. Omdat daarbij een verlaging van het intracellulair foliumzuur werd gevonden lijkt het effect van 5-fluorouracil in dit geval niet afhankelijk te zijn van het folaatgehalte, zoals bij andere tumoren wel is beschreven.

Hoofdstuk 7 bespreekt de resultaten van experimenten gericht op een reductie van de synthese van polyamines, door middel van een gecombineerde behandeling bestaande uit lachgas, cycloleucine en mitoguanozone (MGBG). Het bleek dat behandeling gericht op het verlagen van polyamines toxisch was en slechts weinig actief bij de ratteleukemie. In het gunstigste geval was er een additief effect van MGBG in de combinatie met lachgas en cycloleucine. Wel bleek het mogelijk om een verdere, selectieve, vermindering van spermine te veroorzaken door lachgas en cycloleucine. Dit kan betekenen dat cobalamine een rol speelt in de polyaminesynthese.

Hoofdstuk 8 is gewijd aan in vitro experimenten waarbij lachgas werd toegepast op humane leukemiecellen, verkregen uit beenmerg en perifeer bloed van leukemiepatienten. Ter vergelijking werden ook cellen van continue humane leukemiecellijnen onderzocht (U937 en HL60). Uit deze experimenten bleek dat de resultaten verkregen door in vivo behandeling van ratten met leukemie ook opgaan voor humane leukemiecellen, zowel met betrekking tot de lachgasbehandeling als tot de combinatie met methotrexaat.

Hoofdstuk 9 tenslotte bespreekt de resultaten van de verschillende hoofdstukken in samenhang, waarbij getracht wordt om meer algemene conclusies te trekken over de perspectieven van het inactiveren van cobalamine als een nieuwe behandelingsstrategie in de chemotherapie van leukemie.

"I existed in a world of new connected and newly modified ideas, I theorized, I imagined that I made discoveries. When I was awakened from this semi-delirous trance by Dr Kinglake, who took the bag from my mouth, indignation and pride were the first feelings produced by the sight of persons about me.... I exclaimed to Dr Kinglake: Nothing exists but thoughts! The universe is composed of impressions, ideas, pleasure and pain"

Humphry Davy, describing the effect of breathing nitrous oxide in 1799

In: "Researches, chemical and philosophical, chiefly concerning nitrous oxide, or dephlogisticated nitrous air, and its respiration", a volume of 580 pages, published in 1800.

Nawoord

Het schrijven van dit proefschrift is slechts mogelijk geweest door de steun van zeer velen. Graag wil ik al diegenen bedanken voor de prettige samenwerking in de afgelopen jaren.

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Tenslotte wil ik professor Schalekamp, mijn opleider na mijn indiensttreding op de afdeling Inwendige Geneeskunde I, mw. Tan-Tjiong, mijn chef de clinique in het eerste jaar, andere stafleden en collega-assistenten bedanken voor de manier waarop zij mij ondersteunden bij de niet-eenvoudige overgang naar het werken in de kliniek, juist in de periode waarin dit boekje moest verschijnen.

Curriculum vitae

De schrijver van dit proefschrift werd geboren op 21 mei 1957 te Amsterdam. Het Atheneum B-diploma werd behaald in 1975 aan het Waterlant College te Amsterdam, waarna de studie Geneeskunde werd begonnen aan de Universiteit van Amsterdam. Na het behalen van het kandidaatsexamen in 1978 (cum laude) was hij van 1978 tot 1980 werkzaam als kandidaatsassistent op het Laboratorium voor Biochemie (B.C.P. Janseninstituut). In 1980 werd het doctoraalexamen afgelegd (cum laude). Het basisartsexamen volgde in 1983, en aansluitend werd begonnen met het in dit proefschrift beschreven onderzoek op het Instituut Hematologie van de Erasmus Universiteit Rotterdam onder leiding van prof.dr. J. Abels en dr. J. Lindemans. Sinds 1 september 1986 is hij in opleiding tot internist op de afdeling Inwendige Geneeskunde I van het Academisch Ziekenhuis Rotterdam/Dijkzigt (hoofd: prof.dr. M.A.D.H. Schalekamp).