Cobalamin Inactivation Decreases Purine and Methionine Synthesis in Cultured Lymphoblasts

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Abstract

The megaloblastic anemia of cobalamin deficiency appears secondary to decreased methionine synthetase activity. Decreased activity of this enzyme should cause 5-methyltetrahydrofolate to accumulate intracellularly, and consequently, decrease purine and DNA synthesis; this is the basis of the "methylfolate trap" hypothesis of cobalamin deficiency. However, only some of the clinical and biochemical manifestations of cobalamin deficiency can be explained by the methylfolate trap. We investigated cobalamin deficiency by treating cultured human lymphoblasts with N_2O since N_2O inhibits methionine synthetase activity by inactivating cobalamin. We found that 4 h of N_2O exposure reduced rates of methionine synthesis by 89%. Rates of purine synthesis were not significantly reduced by N_2O when folate and methionine were present at 100 μM in the medium; however, at the physiologic methionine concentration of 10 μM, N_2O decreased rates of purine synthesis by 33 and 57% in the presence of 100 μM folate and in the absence of folate, respectively. The dependency of rates of purine synthesis on methionine availability would be expected in cells with restricted methionine synthetic capacity because (*a*) methionine is the immediate precursor of *S*-adenosylmethionine, a potent inhibitor of 5-methyltetrahydrofolate synthesis; (*b*) methionine serves as a source of formate for purine synthesis; and (*c*) rates of purine synthesis are dependent on the intracellular availability of essential amino acids. We conclude that cobalamin inactivation decreases purine synthesis by both methylfolate trapping and reduction of intracellular methionine synthesis.

Introduction

In animals only two enzymes are known to require cobalamin (vitamin B_{12}) as a coenzyme, methionine synthetase (N^5-methyltetrahydrofolate-homocysteine methyltransferase, EC 2.1.1.13) and methylmalonyl coenzyme A mutase (EC 5.4.99.2) (1). Cobalamin deficiency leads to megaloblastic anemia, which appears secondary to decreased methionine synthetase activity, and to subacute combined degeneration of the spinal cord, which may be secondary to decreased methylmalonyl CoA mutase activity (2, 3). A decrease in methionine synthetase activity would be expected to cause 5-methyltetrahydrofolate to accumulate at the expense of other forms of folate because (*a*) 5-methyltetrahydrofolate appears to be the major storage form of intracellular folates (4), and (*b*) the conversion of 5,10-methylene tetrahydrofolate to 5-methyltetrahydrofolate by 5,10-methylene tetrahydrofolate reductase (EC 1.1.1.68), although fully reversible in vitro, may be irreversible in vivo (Fig. 1) (5). These data form the basis of the "methylfolate trap" hypothesis of cobalamin deficiency (6, 7). Trapping folates as 5-methyltetrahydrofolate should lead to decreased purine, thymidylate, and *S*-adenosylmethionine synthesis (Fig. 1); and thus, to decreased DNA synthesis and methylation; and ultimately, to megaloblastosis. Support for the methylfolate trap hypothesis is that the megaloblastosis of cobalamin deficiency responds to large doses of folate (8). However, physiologic doses of folate capable of producing a maximal response in folate deficiency produce only a partial or no response in cobalamin deficiency (9–10). Moreover, animal studies only partially support methylfolate trapping in cobalamin deficiency (11, 12).

Nitrous oxide (N_2O) induces megaloblastic changes in bone marrow cells within 6 h when used as an anesthetic and has caused fatal megaloblastic anemia when used in the treatment of tetanus (13, 14). In rats, N_2O rapidly inhibits methionine synthetase activity by oxidizing the active cobalamin I to the inactive cobalamin III (15). N_2O may be used, therefore, to create a model of cobalamin deficiency (15, 16).

To determine the sequelae of cobalamin inactivation, we measured the following parameters in N_2O-treated cultured human lymphoblasts: (*a*) rates of methionine and serine synthesis; (*b*) rates of purine synthesis at variable folate concentration; (*c*) rates of purine synthesis at variable methionine concentration; and (*d*) rates of purine synthesis in the presence of homocysteine. We found that N_2O decreased rates of purine synthesis and this decrease was attributable to both methylfolate trapping and decreased methionine synthesis.

Methods

The B lymphoblast line MGL8B2 was established in the Genetics Unit of the Massachusetts General Hospital, Boston, MA, by Epstein-Barr virus transformation of peripheral blood lymphocytes from a normal donor. The cells were routinely cultured as previously described (17).

Preparation of methionine-deficient or folate-deficient medium. The methionine-deficient medium was prepared as previously described (17) and supplemented to 1.5 μM cobalamin. The folate-deficient medium was prepared similarly, replacing the modified Eagle's medium vitamin solution with 11 μM myoinositol, 8 μM nicotinamide, 4 μM pantothenic acid, 5 μM pyridoxine, 0.25 μM riboflavin, and 3 μM thiamine. Both media were serum-free and contained 5 g/liter of extensively dialyzed fatty acid-free bovine serum albumin (no. A7030, Sigma Chemical Co., St. Louis, MO).

Exposure of cells to N_2O. Cells were recovered by centrifugation from the growth medium and resuspended at a density of 1.5×10^6/ml in the experimental medium. The cell suspension was transferred to 16 × 100-mm glass tubes which were flushed with either 95% air/

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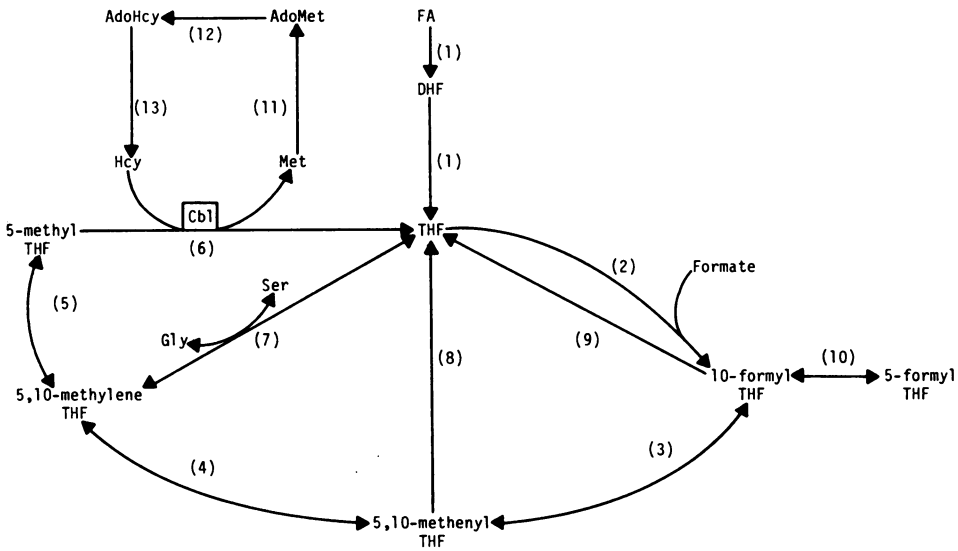

Figure 1. Folate and cobalamin reactions involved in methionine and purine synthesis. Enzymes: (1) dihydrofolate reductase; (2) 10-formyl tetrahydrofolate (THF) synthetase; (3) 5,10-methenyl THF cyclohydrolase; (4) 5,10-methylene THF dehydrogenase; (5) 5,10-methylene THF reductase; (6) methionine synthetase; (7) serine transhydroxymethylase; (8) glycineamide transformylase; (9) aminoimidazole carboxamide transformylase; (10) formyl THF isomerase; (11) methionine adenosyltransferase; (12) methyltransferase reactions; and (13) S-adenosylhomocysteine hydrolase. FA, folic acid (pteroylglutamic acid); DHF, dihydrofolate; Gly, glycine; Ser, serine; Cbl, cobalamin; Met, methionine; AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; and Hcy, homocysteine. Enzymes 2, 3, and 4 compose the trifunctional enzyme C_1-tetrahydrofolate synthetase. Reactions (8) and (9) contribute the carbon atoms to position eight and two of the purine ring, respectively.

5% CO_2 or 75% N_2O/20% O_2/5% CO_2, tightly capped and incubated at 37°C in a shaking water bath. In preliminary experiments we found that cell viability, as measured by trypan blue exclusion, exceeded 90% after 18 h under both the control and N_2O-treated conditions.

Measurement of methionine and serine synthesis. Rates of methionine and serine synthesis were measured as previously described (18, 19). Briefly, 20 μCi of [^{14}C]formate (42 mCi/mmol, ICN Pharmaceuticals, Irvine, CA) were added to 2 ml of cell suspension; after 2 h the tubes were cooled on ice, centrifuged at 500 *g* for 5 min, and the medium discarded. The cell pellet was extracted in 5% TCA for 30 min, heated at 80°C for 30 min, and then, cooled on ice for 30 min. The resulting protein precipitate, free of nucleic acids, was washed three times in ice-cold 10% TCA and resuspended in ultrapure 6 N HCl. After 20 h of hydrolysis at 110°C, the HCl was evaporated under vacuum at 70°C and the hydrolysate was resuspended in H_2O. Methionine and its oxidation productions, methionine sulfoxide and methionine sulfone, were separated from serine and cysteine and its oxidation products, cystine and cysteic acid, in a phenol/ethanol/H_2O/NH_4OH (65:20:20:2) one-dimensional solvent system on cellulose thin-layer chromatography sheets. Nonradioactive marker amino acids were visualized under ultraviolet light after the plates were dipped in a 0.01% fluorescamine acetone solution. The appropriate spots were cut out of the chromatograph and counted at ~95% efficiency. Since serine condenses with homocysteine to yield cystathionine, which is converted to cysteine, radioactivity in cysteine originates from the serine and is thus included as part of serine. The assay measures newly synthesized methionine or serine incorporated into cellular protein; the data are expressed as picomoles of [^{14}C]formate incorporated into methionine or serine per hour per 10^6 cells. The assay was linear with time to at least 4 h and with cell density from 0.5 to 2 × 10^6 cells/ml.

Measurement of rates of de novo purine synthesis. Rates of *de novo* purine synthesis were measured as previously described (17). Briefly, 10 μCi of [^{14}C]formate were added to 1 ml of cell suspension for 1 h. The tubes were cooled on ice, and cells and media were extracted separately in 0.4 N perchloric acid. After heating at 100°C for 70 min to hydrolyze the ribose-purine bond, the tubes were centrifuged and the supernatants were applied to Dowex-50 cation exchange columns. The columns were washed twice with 10 ml of 0.1 N HCl and the purines were eluted in 5 ml of 6 N HCl of which 1 ml was used to measure radioactivity at ~90% efficiency. Total *de novo* purine synthesis was calculated from the sum of radioactive purines in the cells and in the medium. Since the radioactive formate may not be incorporated equally into position 2 and 8 of the purine ring, the data are expressed as cpm per hour per 10^6 cells instead of as picomoles per hour per 10^6 cells. The assay was linear with time to 2 h and with cell density from 0.5 to 2 × 10^6 cells/ml.

Results

Methionine and serine synthesis. When cells were exposed to N_2O, rates of methionine synthesis decreased rapidly by 52% after 30 min and 89% after 4 h (Fig. 2 *A*). Rates of serine synthesis decreased after N_2O exposure by 18% at 30 min and 49% at 4 h (Fig. 2 *B*). The rapid and profound decrease in methionine synthesis after N_2O exposure is compatible with the previously demonstrated rate of N_2O inactivation of methionine synthetase (16). The more gradual decrease in serine synthesis after N_2O exposure may also be secondary to methionine synthetase inhibition, if such inhibition leads to trapping of intracellular folates as 5-methyltetrahydrofolate (Fig. 1).

Purine synthesis at variable folate concentration. If methionine synthetase inhibition causes 5-methyltetrahydrofolate to accumulate at the expense of free tetrahydrofolate, then rates of purine synthesis should decrease in N_2O-treated cells (Fig. 1). In untreated control cells, rates of purine synthesis were independent of the folate concentration in the medium: only the data at 100 μM folate are shown (Fig. 3, solid circles) but similar data were obtained at 1 or 10 μM folate or in the absence of folate, indicating that for the duration of the experiments the intracellular folate stores were sufficient under control conditions. However, in N_2O-treated cells, rates of purine synthesis decreased with time and the rate of decrease was greater in the absence of folate. At 1, 10, 100, or 1,000 μM folate, 4 h of N_2O exposure did not affect rates of purine synthesis appreciably, but after 14 h of exposure purine synthetic rates decreased by 25% ($P < 0.05$, *t* test); only the data at 100 μM folate are shown (Fig. 3, solid triangles). Similar data were obtained in the presence of 100 or 1,000 μM 5-formyltetrahydrofolate. In the absence of folate, N_2O decreased rates of purine synthesis more rapidly—by 20% at 4 h ($P < 0.05$, *t*

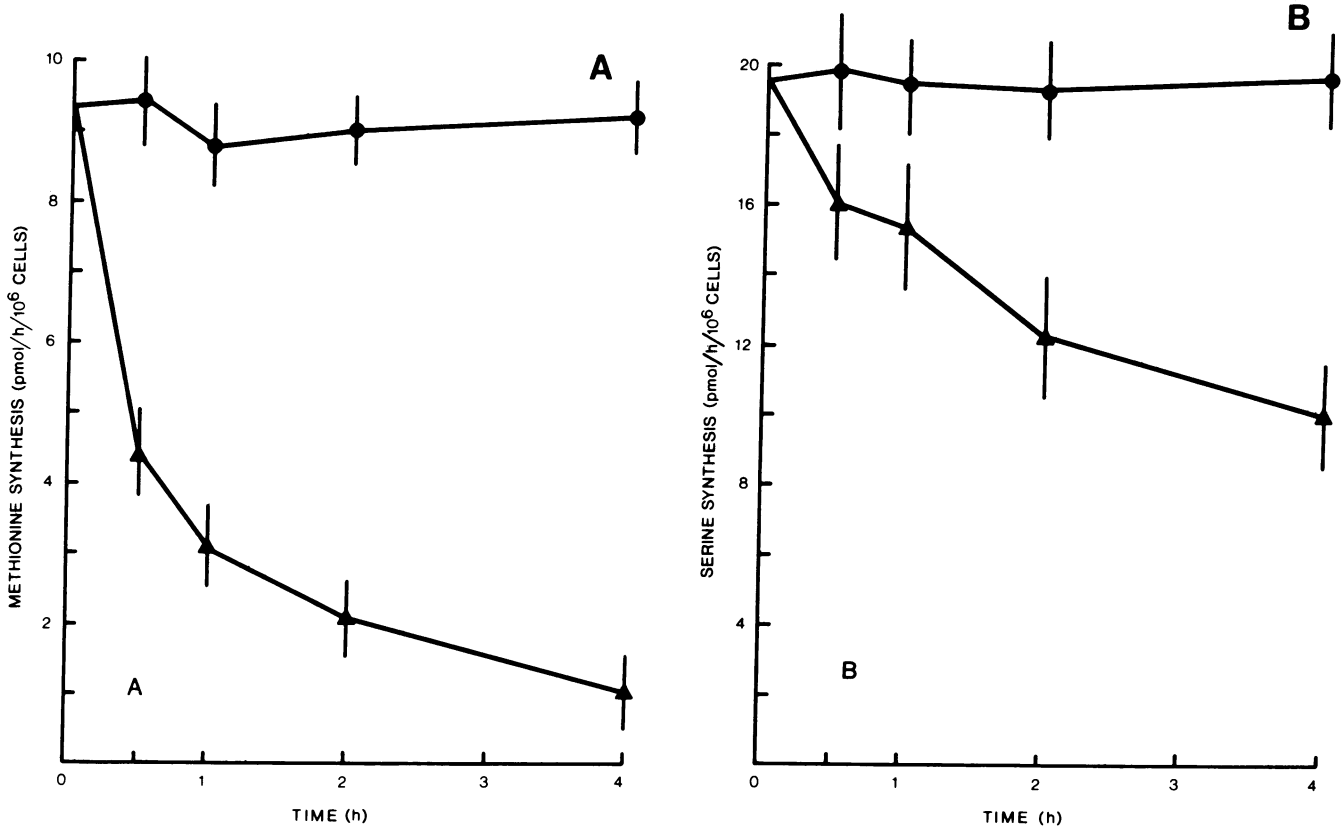

Figure 2. Rates of methionine (*A*) and serine (*B*) synthesis in cells incubated in the absence (— ● —) or presence of N_2O (— ▲ —). Cells were equilibrated in tubes for 10 h in 95% air/5% CO_2; at zero time, half of the tubes were flushed with 75% N_2O/20% O_2/5% CO_2. Rates of methionine and serine synthesis were measured by following [^{14}C]formate incorporation into methionine and serine residues of cellular protein as described fully in Methods. Each point is the mean±SD of at least three independent experiments performed in duplicate.

test) (Fig. 3, solid squares). These data are further evidence that methionine synthetase inhibition decreases the availability of free tetrahydrofolate.

Purine synthesis at variable methionine concentration. To determine if the decrease in purine synthesis during methionine synthetase inhibition was secondary to methionine deprivation of the cell (17), rates of purine synthesis were measured in N_2O-treated cells over a range of methionine concentrations that included the normal plasma concentration of 6–30 μM (20–22). In untreated control cells, rates of purine synthesis were dependent on the methionine concentration but were independent of the folate concentration; only the data at 100 μM folate are shown (Fig. 4, solid circles). The marked decrease in rates of purine synthesis at low methionine concentrations also occurs at low concentrations of any of the other essential amino acids; this is secondary to decreased phosphoribosylpyrophosphate production by the pentose phosphate pathway (17, 23, 24). When cells were treated with N_2O, rates of purine synthesis decreased more in the absence of folate than in the presence of folate: only the data in the absence of folate and at 100 μM folate are shown (Fig. 4, solid squares and solid triangles, respectively) with the data at 1 and 10 μM folate similar to those at 100 μM folate. N_2O reduced rates of purine synthesis most efficiently at the physiologic methionine concentrations of 10 and 30 μM; thus, rates of purine synthesis at 30 and 10 μM methionine were decreased in the presence of 100 μM folate by 22 and 33%, respectively, and in the absence of folate by 37 and 57%, respectively.

Purine synthesis in the presence of homocysteine. Since N_2O inhibits methionine synthesis from homocysteine and rates of purine synthesis are reduced at low methionine concentrations, purine synthesis should decrease even more dramatically in N_2O-treated cells incubated in medium in which homocysteine replaces methionine. After 30 min and 4 h of N_2O exposure, rates of purine synthesis decreased by 56 and 75%, respectively, when cells were incubated in methionine-free medium containing 200 μM homocysteine thiolactone, 100 μM folic acid, and 1.5 μM cobalamin (Fig. 5). These experiments were performed only at a folate concentration of 100 μM because cells cultured in the presence of homocysteine require relatively high concentrations of folate to synthesize purines and grow normally (data not shown) (25).

Discussion

Previous studies in rats have shown that N_2O rapidly inhibits liver methionine synthetase activity by inactivating cobalamin (16). Since enzyme activity measured in cell extracts under nonphysiologic conditions does not always correlate with physiologic activity of the enzyme (26), we decided to measure rates of methionine synthesis in N_2O-treated cultured human lymphoblasts. We found that N_2O rapidly decreased methionine synthesis from homocysteine.

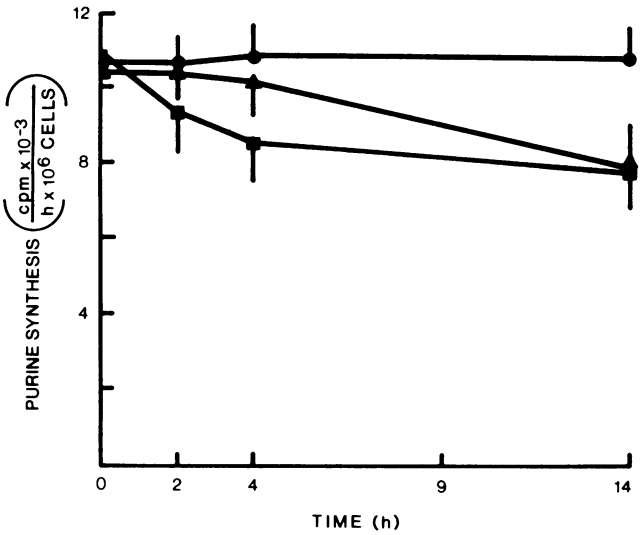

Figure 3. Rates of purine synthesis in cells incubated in the absence of N_2O (— ● —) or in the presence of N_2O at either 100 μM folate (— ▲ —) or in the absence of folate (— ■ —). Cells were incubated in either 95% air/5% CO_2 or 75% N_2O/20% O_2/5% CO_2; at the indicated times, rates of purine synthesis were measured by following [^{14}C]formate incorporation for 1 h into all intracellular and extracellular purines as described fully in Methods. Each point is the mean±SD of at least three independent experiments performed in duplicate.

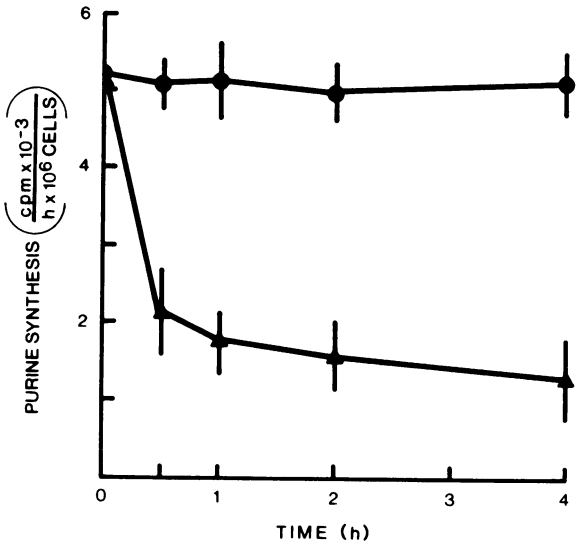

Figure 5. Rates of purine synthesis in cells incubated in the absence (— ● —) or presence of N_2O (— ▲ —). Cells were incubated in either 95% air/5% CO_2 or 75% N_2O/20% O_2/5% CO_2 in medium lacking methionine but containing 200 μM homocysteine thiolactone; after 4 h, rates of purine synthesis were measured as described in the legend to Fig. 3. Each point is the mean±SD of at least three independent experiments performed in duplicate.

Since 5-methyltetrahydrofolate appears to be the major storage form of intracellular folates and the reaction of 5,10-methylenetetrahydrofolate reductase seems to be essentially irreversible in vivo, cobalamin deficiency may lead to 5-methyltetrahydrofolate accumulation and a relative lack of other folate forms. We found that two pathways which require tetrahydrofolate derivatives, serine and purine *de novo* synthesis, were decreased in N_2O-treated cells, thus providing further evidence that supports the methylfolate trap hypothesis. However, the considerably less pronounced decrease in rates of serine and purine synthesis compared with methionine synthesis suggest that the trap is incomplete. Furthermore, if 5-methyltetrahydrofolate trapping were the only sequelae of cobalamin inactivation, other folate forms should return rates of purine synthesis towards control values in N_2O-treated cells. We found that neither folic acid nor 5-formyltetrahydrofolate in concentrations as high as 1 mM fully reversed the N_2O effect on purine synthesis. Thus, our data suggest that cobalamin inactivation decreases purine synthesis by an additional mechanism which is independent of 5-methyltetrahydrofolate trapping.

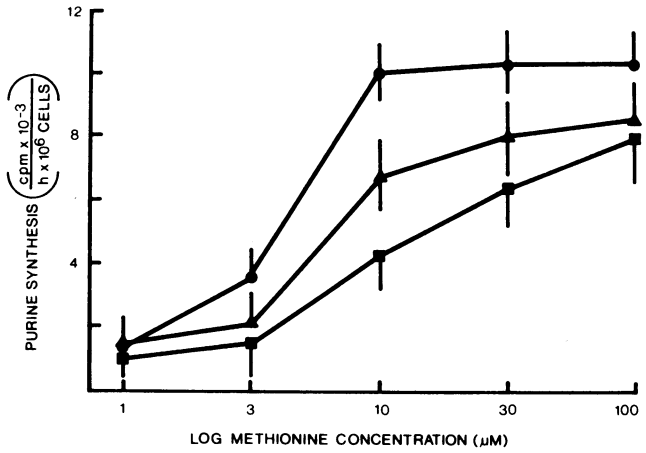

Figure 4. Rates of purine synthesis in cells incubated in the absence of N_2O (— ● —) or in the presence of N_2O at either 100 μM folate (— ▲ —) or in the absence of folate (— ■ —). Cells were incubated in either 95% air/5% CO_2 or 75% N_2O/20% O_2/5% CO_2 at the indicated methionine concentrations; after 4 h, rates of purine synthesis were measured as described in the legend to Fig. 3. Each point is the mean±SD of at least three independent experiments performed in duplicate.

This additional mechanism appears to be reduced methionine synthesis. At physiologic methionine concentrations, i.e., 6–30 μM, rates of purine synthesis were more profoundly inhibited by N_2O than at the standard tissue culture medium concentration of 100 μM. Moreover, rates of purine synthesis were markedly inhibited by N_2O when homocysteine replaced methionine in the culture medium. Thus, when the cell is relatively limited for methionine, inhibition of methionine synthetase has a more pronounced effect than when ample methionine is present. This suggests that a significant proportion of cellular methionine is normally converted to homocysteine, via *S*-adenosylmethionine and *S*-adenosylhomocysteine (Fig. 1), and regenerated by methionine synthetase. Similar conclusions were reached recently by German et al. (27), who found in cultured lymphoblasts that ~20% of intracellular methionine traverses this cycle at any time; and by Finkelstein and Martin (28), who found in an in vitro system which simulates the in vivo conditions of rat liver, that methionine synthetase activity accounted for 27% of the homocysteine consumed. This relatively high rate of homocysteine production from methionine does not account for the greater rate of purine synthesis

at high methionine concentrations in N_2O-treated cells, because, even in the presence of 200 μM homocysteine thiolactone, rates of purine synthesis were markedly reduced by N_2O.

Limiting the cell's ability to synthesize methionine, and thus decreasing intracellular methionine availability, could decrease purine synthesis by at least three different mechanisms. First, decreased methionine synthesis and availability may lead to decreased *S*-adenosylmethionine synthesis (Fig. 1). *S*-adenosylmethionine is a potent inhibitor of 5,10-methylenetetrahydrofolate reductase (29); a decrease in the intracellular *S*-adenosylmethionine concentration could increase 5,10-methylenetetrahydrofolate conversion to 5-methyltetrahydrofolate, thus exacerbating the methylfolate trap (Fig. 1) (7). Moreover, *S*-adenosylmethionine is the main intracellular methyl donor and a decrease in its concentration could have profound effects on many enzymatic reactions (30). Second, Perry and Chanarin (15, 31–33) suggested that methionine may be a major provider of cellular formate and thereby may serve as a precursor of the purine ring. They showed that folate polyglutamate synthesis with tetrahydrofolate as the substrate is markedly impaired in the N_2O-treated rat but is normal when formyltetrahydrofolate is the substrate. The ability to use tetrahydrofolate can be restored when either methionine, *S*-adenosylmethionine, or 5'-methylthioadenosine are infused (15). All three of these compounds are potential sources of 1-phospho-5-methylthioribose, which has been shown recently to be metabolized to formate and 2-keto-4-methylthiobutyrate (34). In addition, the methyl group of methionine may be oxidized intracellularly to formate (35). And third, decreased methionine availability decreases the synthesis of phosphoribosylpyrophosphate, which is the first and a key regulatory substrate of *de novo* purine synthesis (24). We have shown that when cultured human lymphoblasts are starved for an essential amino acid for brief periods of time, rates of *de novo* purine synthesis decrease by as much as 90% (17). This rapid decrease in purine nucleotide synthesis is mostly secondary to decreased phosphoribosylpyrophosphate synthesis by the nonoxidative pentose phosphate pathway (23, 24, 36).

There is clinical evidence for reduced methionine synthesis and a state of partial methionine deprivation in cobalamin deficiency since (a) cobalamin deficiency may be associated with homocystinuria (37–39); and (b) methionine administration to cobalamin-deficient patients decreases the excretion of formiminoglutamic acid, a marker of cobalamin deficiency (40).

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