0022-3565/92/2613-1096$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 261, No. 3
Printed in U.S.A.

Homocysteine Remethylation During Nitrous Oxide Exposure of Cells Cultured in Media Containing Various Concentrations of Folates[1]

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Accepted for publication February 24, 1992

ABSTRACT

Nitrous oxide irreversibly inactivates cob(I)alamin, which serves as a cofactor of the enzyme methionine synthase catalyzing the remethylation of homocysteine to methionine. In patients exposed to nitrous oxide, increase in plasma homocysteine is a responsive indicator of cob(I)alamin inactivation. In the present work, we measured the inactivation of methionine synthase and the concurrent homocysteine export rate of two murine and four human cell lines during nitrous oxide exposure. When cultured in a standard medium with high content (2.3 μM) of folic acid, the methionine synthase of all cell types was inactivated at an initial rate of 0.05 to 0.14 h^{-1}. The inactivation curves leveled off, and a residual activity of 15 to 45% was observed after 48 h of nitrous oxide exposure. The rate and extent of the nitrous oxide-induced inactivation were markedly reduced when the cells were transferred and cultured (>10 days) in a medium containing low concentration (10 nM) of 5-methyltetrahydrofolate. The methionine synthase inactivation increased in a dose-dependent manner when the 5-methyltetrahydrofolate content of the medium was increased from 3 nM to 2.3 μM. The inactivation of methionine synthase was associated with a marked enhancement of homocysteine export rate of murine fibroblasts and a moderate increase in export from two human glioma cell lines. In contrast, in three leukemic cell lines (murine T-lymphoma R 1.1 cells, human promyelocytic leukemia HL-60 cells and human acute myelogenous leukemia KG-1a cells), the homocysteine export rates were not increased during nitrous oxide exposure. In the responsive murine fibroblasts and the glioma cells, the homocysteine export rate varied inversely to the changes in methionine synthase activity induced by nitrous oxide exposure at different concentrations of folate in the medium. The enhancement of homocysteine export rate of some cell types during nitrous oxide exposure probably reflects inhibition of homocysteine remethylation in intact cells, and highlights the utility of extracellular homocysteine as an indicator of metabolic flux through the methionine synthase pathway. No enhancement of homocysteine export despite inactivation of methionine synthase in three leukemic cell lines questions the functional state of the enzyme in these cells.

Prolonged exposure of humans and some animals to the anesthetic agent nitrous oxide may lead to megaloblastic bone marrow changes, neurological impairment and metabolic effects resembling the state of cobalamin deficiency (reviewed by Nunn, 1987). The side effects of nitrous oxide have been explained by the ability of the agent to inactivate the enzyme methionine synthase (N^5-metyltetrahydrofolate-homocysteine methyltransferase, EC 2.1.1.13) (Chanarin *et al.*, 1985). This enzyme catalyzes the transfer of a methyl group from 5-methyl-THF to Hcy. The products are methionine and tetrahydrofolate, and cobalamin serves as a cofactor. The enzyme operates at the point of convergence of folate metabolism and the transmethylation/transsulfuration pathway, and major functions are Hcy salvage to methionine and provision of reduced folates from 5-methyl-THF (Finkelstein, 1990). Nitrous oxide exerts its effect by oxidation of enzyme-bound cob(I)alamin, and the enzyme is irreversibly inactivated (Chanarin *et al.*, 1985).

The inactivation of methionine synthase has been studied *in vivo* in animals exposed to nitrous oxide and demonstrated in patients subjected to nitrous oxide anesthesia (Nunn, 1987). The rate of inactivation of the enzyme is rapid in rodents, with a half-life of less than 10 to 30 min (Black and Tephly, 1983; Chanarin *et al.*, 1985; Koblin and Tomerson, 1990), whereas in humans, the inactivation is slower, and in human liver, reaches 50% after about 1 h (Koblin *et al.*, 1982; Royston *et al.*, 1988). In both animals (Brennt and Smith, 1989; Koblin and Tomerson, 1990; Xue *et al.*, 1986) and humans, variable residual

Received for publication January 9, 1992.
[1] This work was supported by grants from the Norwegian Cancer Society and from the AGA AB Medical Research Fund.
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ABBREVIATIONS: 5-methyl-THF, N^5-methyltetrahydrofolate; Hcy, homocysteine; PBS, phosphate-buffered saline; FD medium, RPMI medium containing 3 nM L-5-methyl-THF; LF medium, RPMI medium containing 10 nM L-5-methyl-THF; HF medium, RPMI medium containing 500 nM L-5-methyl-THF.

TABLE 1

Folate content in six cell lines cultured in a LF medium with low concentration (10 nM) of 5-methyl-THF

Data are given either as mean of duplicate determinations or mean of three to six determinations ± S.E.M.

Cell line	Duration	Folate Content
	days	*pmol/10⁶ cells*
Cl 8	0 = control	12.5 ± 1.9
	5	0.2
	10	<0.2
R 1.1	0	2.3 ± 0.4
	5	<0.2
	10	<0.2
HL-60	0	7.1 ± 0.9
	5	<0.2
	10	<0.2
KG-1a	0	9.1 ± 0.7
	5	0.7
	10	0.2
GaMg	0	14.9 ± 1.0
	5	1.9
	10	1.2
D-54MG	0	20.8 ± 2.1
	5	4.1
	10	2.8

TABLE 2

The activity of methionine synthase and nitrous oxide-induced inactivation of the enzyme in six cell lines

Cell Line	Culture Medium	Methionine Synthase Activity[a]	Initial Rate of Inactivation[b]	Residual Activity[b,c]
		nmol/h/mg protein	h^{-1}	*% of control*
Cl 8	FA	22.1 ± 0.8	0.08	30
	LF	11.2 ± 0.7	0.02	80
	HF	21.2 ± 0.7	0.07	35
R 1.1	FA	3.0 ± 0.3	0.05	45
	HF	13.0 ± 0.4	0.11	35
HL-60	FA	7.7 ± 0.9	0.11	15
	HF	9.9 ± 0.9	0.23	25
KG-1a	FA	8.3 ± 1.1	0.06	45
	LF	7.2 ± 0.4	<0.01	90
	HF	10.4 ± 0.2	0.09	40
GaMg	FA	8.5 ± 0.3	0.14	25
	LF	8.3 ± 0.2	0.02	45
	HF	9.6 ± 1.7	0.06	35
D-54MG	FA	21.7 ± 1.5	0.12	25
	LF	14.8 ± 0.5	0.03	40
	HF	18.9 ± 0.2	0.08	30

[a] Values are mean of four to six determinations ± S.E.M. The activities were determined in exponentially growing cells not exposed to nitrous oxide.

[b] Values are taken from experiments depicted in figures 1 to 6.

[c] Residual activity refers to activity remaining after 48 h of nitrous oxide exposure.

activity was observed, and the rate and extent of inactivation vary between tissues. In patients receiving nitrous oxide anesthesia, the methionine synthase activity in bone marrow was reduced to 50% after 6 h (Kano *et al.*, 1981). No significant effect on the enzyme activity in human placenta was observed after nitrous oxide exposure for 13 to 25 min (Landon and Toothill, 1986).

A convenient marker of nitrous oxide-induced inactivation of methionine synthase and insight into the inactivation process may improve the clinical and experimental use of nitrous oxide. Plasma Hcy is a promising marker because this parameter seems to be a responsive measure of nitrous oxide-induced inactivation of cobalamin in patients (Ermens *et al.*, 1991). The kinetics and determinants of the inactivation of methionine synthase may be critical for the side effects of nitrous oxide, and knowledge of such factors may forecast such effects (Nunn, 1987), including its ability to provoke toxic effects when interacting with the antifolate drug, methotrexate (Ueland *et al.*, 1986). Nitrous oxide potentiates the antileukemic effect of methotrexate and some other metabolites *in vivo* (Abels *et al.*, 1990), and exploitation of this interaction in the design of chemotherapeutic regimens (Abels *et al.*, 1990) will require detailed knowledge of the response of methionine synthase to nitrous oxide.

In the present work, we investigated the kinetics of inactivation of methionine synthase in cultured cells exposed to nitrous oxide. Both human and rodent cells were investigated, and both cells which thrive in a methionine-free medium supplemented with homocysteine and so-called methionine-dependent cells (Stern *et al.*, 1984) were included. The extracellular medium folate was investigated as a potential modulator of the inactivation, because incorporation of 5-methyl-THF, the physiological folate source (Kane and Waxman, 1989), into the cellular pool of reduced folates requires its demethylation catalyzed by methionine synthase (Finkelstein, 1990). The concurrent change in Hcy export rate was used as an indicator of Hcy remethylation under these conditions, and was evaluated as an indicator of methionine synthase inactivation.

Materials and Methods

Chemicals. L-Methionine, DL-homocysteine, S-adenosyl-L-methionine, cyanocobalamin, folic acid, DL-5-methyl-THF (barium salt) and dithioerythritol were purchased from Sigma Chemical Co. (St. Louis, MO). 2-Mercaptoethanol (p.a.) and methanol (Gradient Grade) were from Merck (Darmstadt, Germany), sodium borohydride and bis(3,5,5-trimethylhexyl)phthalate from Fluka Chemie AG, (Buchs, Switzerland) and monobromobimane from Molecular Probes (Eugene, OR). (±)-L-N^5-[*methyl*-^{14}C]methyltetrahydrofolate (54 mCi/mmol; barium salt) was purchased from Amersham (Buckinghamshire, England). (±)-L-N^5-[*methyl*-^{14}C]methyltetrahydrofolate was dissolved in 10 mM ascorbic acid and stored as 200-μl aliquots under nitrogen at −80°C until use. Nitrous oxide, supplied as a mixture of 50% N_2O, 25% N_2, 20% O_2 and 5% CO_2, and a mixture of 75% N_2, 20% O_2 and 5% CO_2, referred to as air, were obtained from AGA AB Norgas (Oslo, Norway). Bio-Rad AG 1-X8 resin (200–400 mesh, chloride form) was from Bio-Rad Laboratories (Richmond, CA). The material was slurry packed in 5-ml bed volume polypropylene columns [4.5 × 1.1 cm (i.d)] obtained from Pierce (Rockford, IL), and the material was covered with a polyethylene disc with pore size of 45 μm.

Cell lines and stock cultures. Six different cell lines were used. The nontransformed C3H/10T1/2 Cl 8 cells (Cl 8 cells) are anchorage-dependent cells derived from mouse embryo fibroblasts (Reznikoff *et al.*, 1973a,b). The murine T-lymphoma cell line, R 1.1 (referred to as R 1.1 cells), arose spontaneously in a C58 mouse (Old *et al.*, 1965) and shares antigenic and metabolic properties with normal thymocytes (Ralph, 1973). The HL-60 promyelocytic cell line (HL-60 cells) was isolated from peripheral blood leukocytes from a patient with acute promyelocytic leukemia (Collins *et al.*, 1977). The acute myelogenous leukemia KG-1a cell line (KG-1a cells) is an undifferentiated variant of the KG-1 cell line which was isolated from bone marrow obtained from a patients with erythroleukemia that evolved into acute myelogenous leukemia (Koeffler *et al.*, 1980). The GaMg human glioma cell line (GaMg cells) was established from explanted biopsy specimens from a glioblastoma in a 42-year-old female (Akslen *et al.*, 1988). The D-54MG human glioma cell line (D-54MG cells) was established from a mixed anaplastic glioma removed by surgery in a 39-year-old female (Bigner *et al.*, 1981).

Stock cultures of KG-1a cells were grown in Iscove's modification of Dulbecco's medium (Flow Laboratories, Irvine, Scotland), Cl 8 cells in basal medium Eagle (Gibco Paistley, U.K.), the glioma cell lines GaMg and D-54MG in Dulbecco's modification of Eagle's medium (Flow

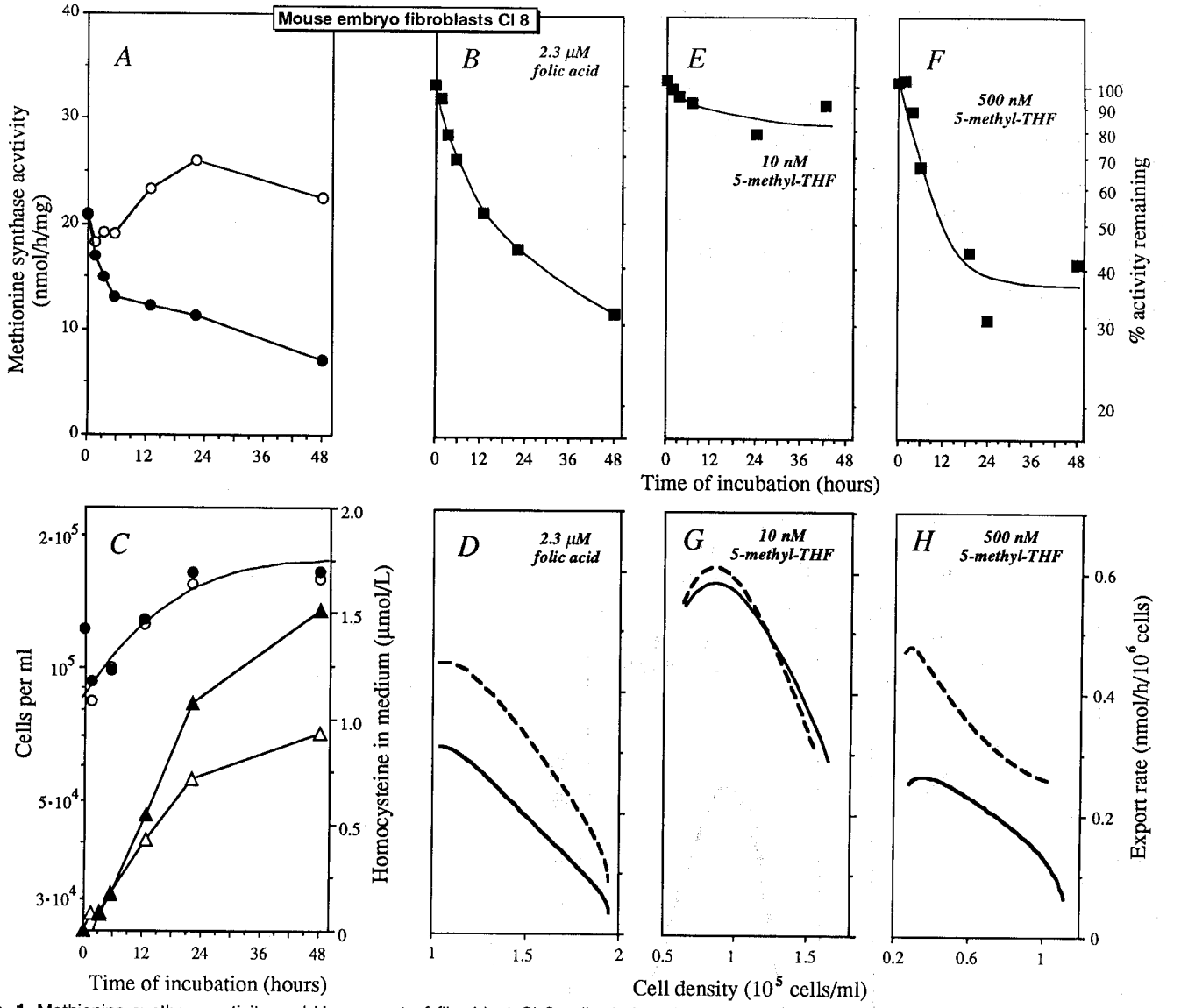

Fig. 1. Methionine synthase activity and Hcy export of fibroblast Cl 8 cells during nitrous oxide exposure. Cl 8 cells cultured in standard RPMI medium containing 2.3 μM folic acid were exposed to nitrous oxide or air (control cells) for 48 h. A) The methionine synthase in control cells (○) and treated cells (●); B) the percent activity remaining, which is the activity in the treated cells in percent of the activity of controls. C) The cell growth (○,●) and Hcy accumulating in the medium (△,▲) of cells exposed to nitrous oxide (●,▲) and of control cells (○,△). D) The data shown in C transformed into export rate curves, as described under "Materials and Methods." The broken line represents the export rate of cells exposed to nitrous oxide, whereas the solid line is the export of control cells. The same experiment was performed with Cl 8 cells cultured in LF medium (containing 10 nM 5-methyl-THF) and in HF medium (500 nM 5-methyl-THF), and the resulting inactivation curves (upper panels) and export rate curves (lower panels) are shown in "E-H". Each value is the average of duplicate measurements. The experiments were repeated three times, and a typical experiment is shown.

Laboratories) and the HL-60 cells and R 1.1 cells in RPMI 1640 medium (Flow Laboratories). All these media were supplemented with 10% fetal calf serum.

Cell culture conditions. All experiments were performed with cells transferred to and cultured in variants of the RPMI 1640 medium (Flow Laboratories). Four different folate supplementations were used: 1) Standard RPMI medium containing 2.3 μM of folic acid. 2) RPMI 1640 medium deficient of folic acid is referred to as FD medium. This medium contained 3 nM folate derived from the calf serum component. L-5-methyl-THF is the principle congener of serum folate in this species (Natsuhori *et al.*, 1991). 3) The FD medium was supplemented with 7 nM L-5-methyl-THF and the total amount of L-5-methyl-THF was 10 nM. This is called LF medium. 4) The FD medium supplemented with 497 nM L-5-methyl-THF is referred to as HF medium.

All these media contained 2 μg/ml of cyanocobalamin and 100 μM of methionine, and were supplemented with 10% heat-inactivated fetal calf serum.

Exponentially growing cells were washed with PBS and seeded in 50-ml (25 cm^2) tissue culture flasks (Nunc, Roskilde, Denmark) with 5 ml of tissue culture medium in an atmosphere of 5% CO_2 in air, when not otherwise indicated. The temperature was 37°C and the relative humidity 98%.

Exposure of cells to nitrous oxide. Immediately before nitrous oxide exposure, anchorage-dependent cells (Cl 8, GaMg and D-54MG) in midexponential growth received fresh medium. Likewise, the exponentially growing leukemic (R 1.1, HL-60 and KG1-a) cells in suspension were centrifuged and seeded in fresh medium at a density of 5 × 10^5 cells/ml before exposure. The culture flasks were carefully flushed for 1 min with either 50% nitrous oxide or air (control) delivered at a rate of 2 l/min *via* a sterile pasteur pipette. The gases passed through

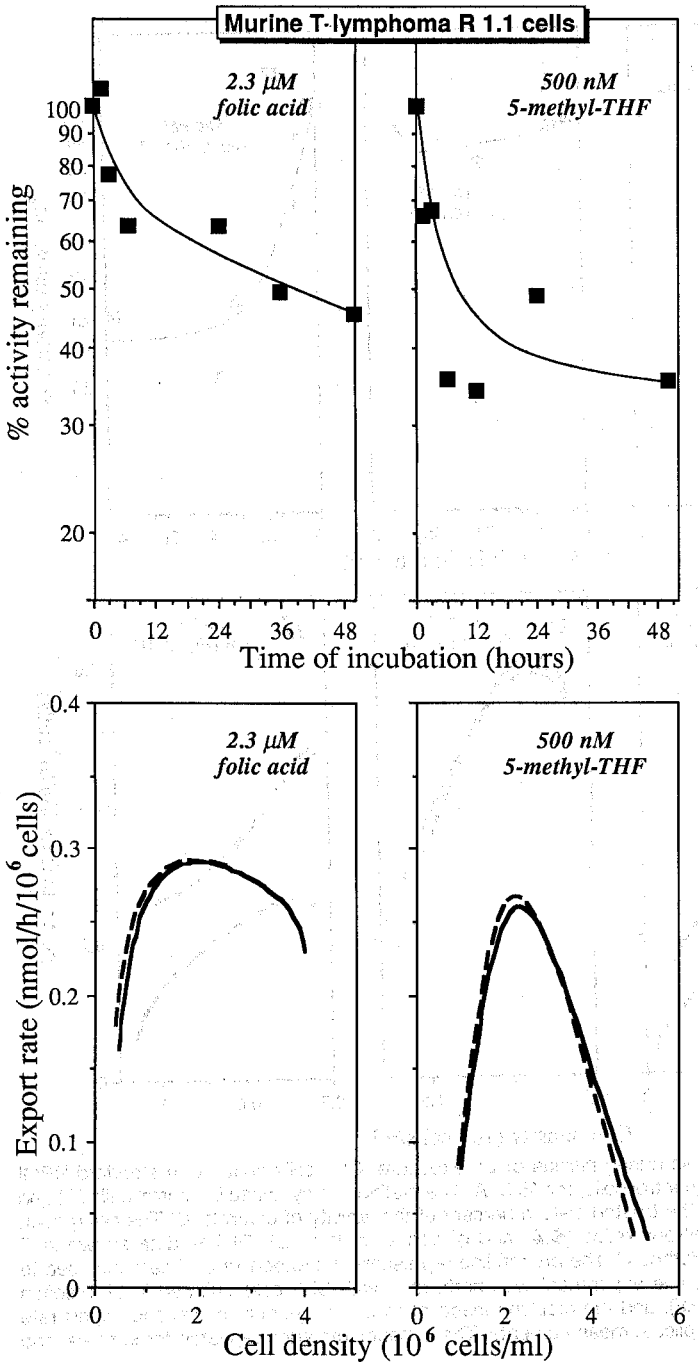

Fig. 2. Methionine synthase inactivation by nitrous oxide and the concurrent Hcy export rate of lymphoma R 1.1 cells. R 1.1 cells cultured in standard RPMI medium (2.3 μM folic acid) or in HF medium (500 nM 5-methyl-THF) were exposed to nitrous oxide or air (control cells). The upper panels show percent methionine synthase activity remaining (*i.e.*, the activity in the cells exposed to nitrous oxide in percent of the activity of control cells); the lower panels show Hcy export rates of treated cells (broken line) and control cells (solid line). Each value is the average of duplicate measurements. The experiments were repeated two times.

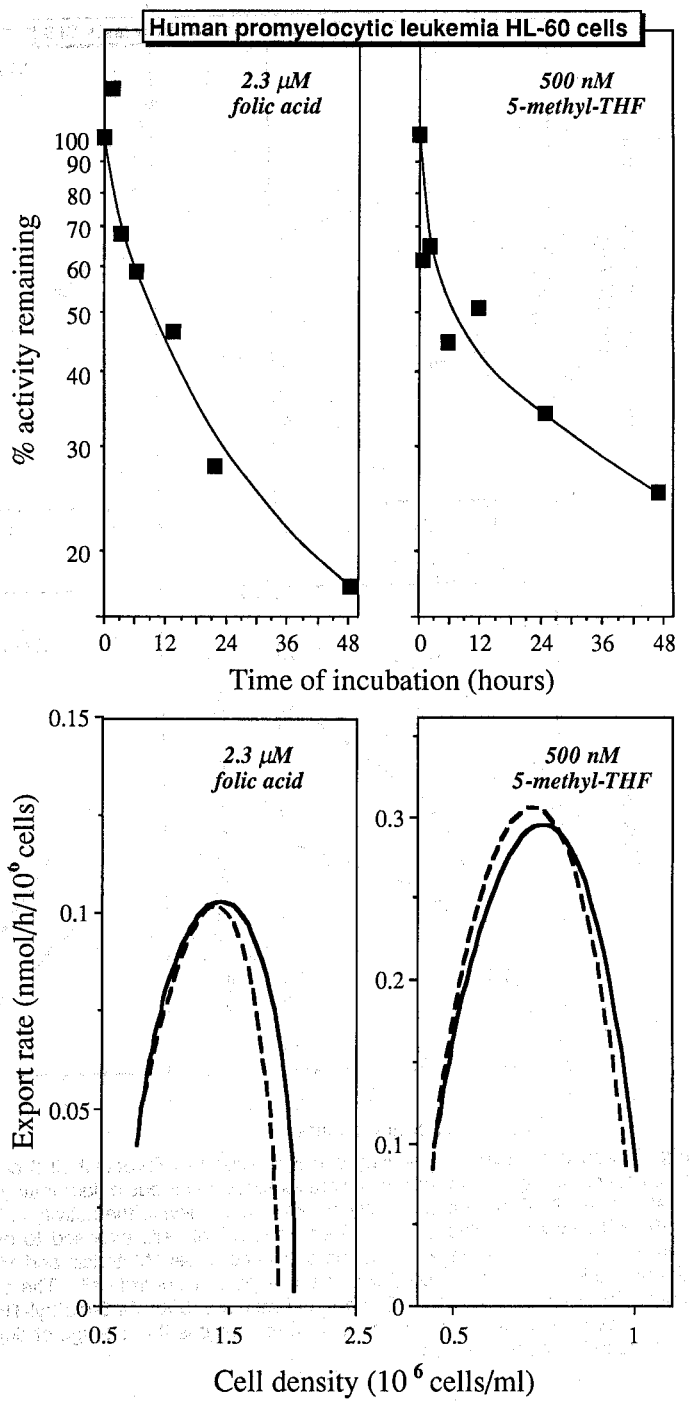

Fig. 3. Methionine synthase inactivation by nitrous oxide and the concurrent Hcy export rate of HL-60 cells. A detailed explanation is given in the legend to figure 2. The experiments were repeated two times.

a 0.2-μm Millex-FG_{50} gas filter (Millipore S.A., Molsheim, France) mounted between the pipette and the gas reservoir. Then the flasks were firmly capped and incubated at 37°C.

Cell harvesting. Samples of medium and the cells were harvested from two parallel flasks in each group, and the cells were protected from light during this process.

Medium was aspirated directly from the culture flasks with the anchorage-dependent cells. The suspension cells were removed by centrifugation before sampling of culture medium. Medium was stored at −20°C until analysis.

The anchorage-dependent cells were gently washed with PBS, trypsinized, resuspended and washed in PBS. The cells were finally collected by centrifugation, supernatant removed by aspiration and the pellet stored at −80°C until assayed for methionine synthase. The cells grown in suspension were washed and collected a similar way, and stored at −80°C.

Cell counts were determined using a Coulter Counter model ZM (Coulter Electronics Ltd., Luton, U.K.),

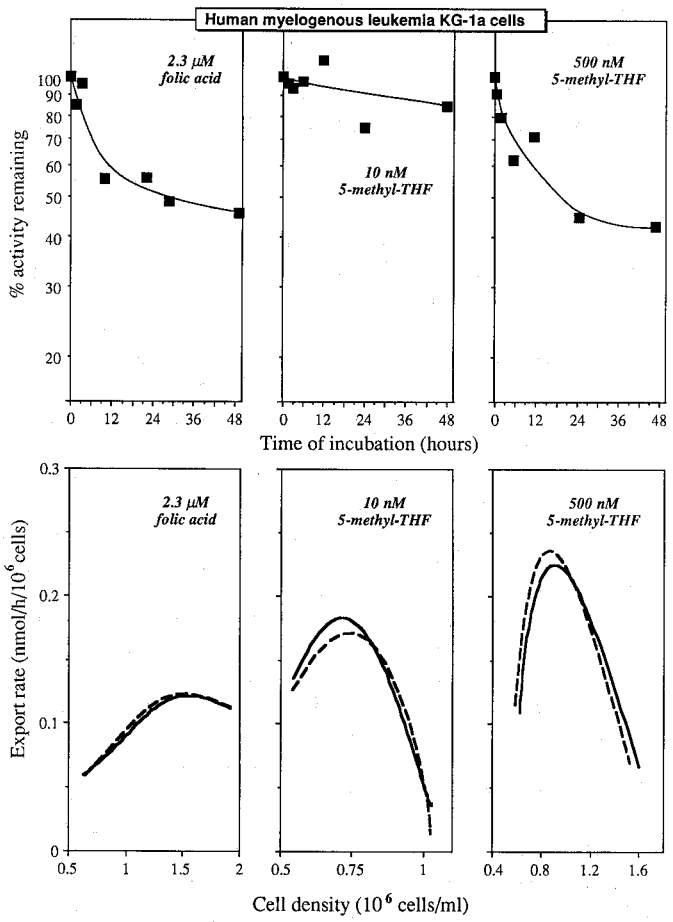

Fig. 4. Methionine synthase inactivation by nitrous oxide and the concurrent Hcy export rate of KG-1a cells. KG-1a cells cultured in standard RPMI medium (2.3 μM folic acid), in LF medium (10 nM 5-methyl-THF) or in HF medium (500 nM 5-methyl-THF) were exposed to nitrous oxide or air (control cells). A detailed explanation is given in the legend to figure 2. The experiments were repeated two times.

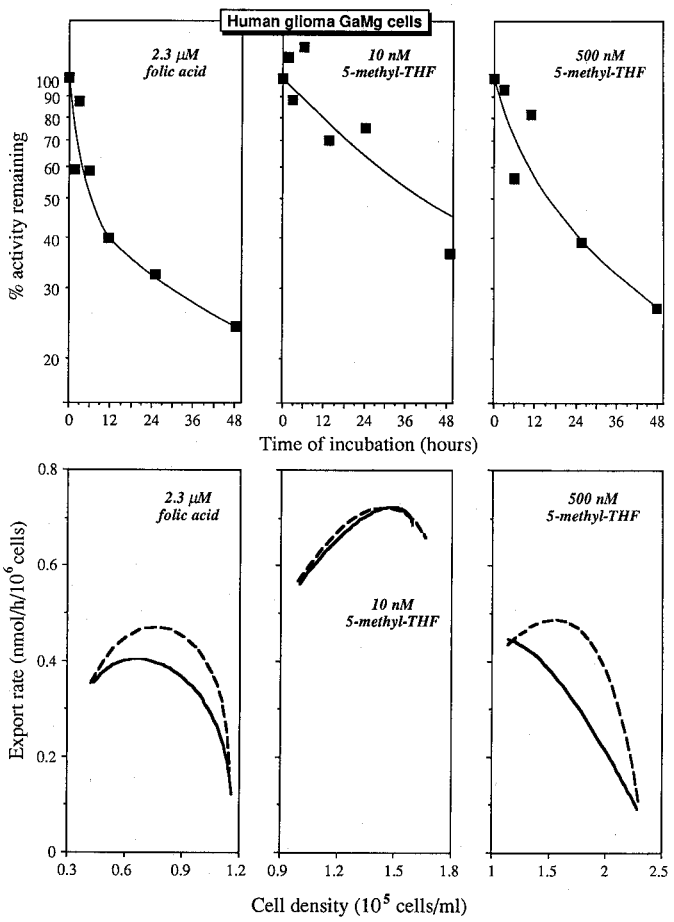

Fig. 5. Methionine synthase inactivation by nitrous oxide and the concurrent Hcy export rate of GaMg cells. Detailed explanations are given in legends to figures 2 and 4. The experiments were repeated two to three times, and a typical experiment is shown.

Determination of methionine synthase activity. This was performed by a modification of the method described by Weissbach *et al.* (1963). The cell pellet was thawed and homogenized in 200 μl of 50 mM potassium phosphate buffer, pH 7.4, containing 100 mM NaCl, 10 mM dithioerythritol and 0.05% Triton X-100. The incubation mixture (final volume of 100 μl in 0.6-ml polyethylene tubes) contained 400 μM DL-homocysteine, 500 μM (±)-L-N^5-[*methyl*-^{14}C]methyltetrahydrofolate (2 μCi/μmol), 50 μM cyanocobalamin, 300 μM *S*-adenosyl-L-methionine, 125 mM 2-mercaptoethanol, 50 mM potassium phosphate buffer, pH 7.4, and 50 μl of cell extract. The incubation was started by addition of the extract, and the incubation mixture was overlayered with 50 μl of bis(3,5,5-trimethylhexyl)phthalate to protect the assay mixture from air (Garras *et al.*, 1991). The incubation was carried out at 37°C in the dark, and was terminated by adding 400 μl of ice-cold water to the incubation mixture. The resulting solution was immediately applied to a Bio-Rad AG 1-X8 column equilibrated with water. One ml of water was added to the column and the eluate discarded. [^{14}C]Methionine was then eluted with 2.0 ml of water and collected into scintillation vials. After addition of 5 ml of scintillation fluid, the radioactivity was determined in a Packard Tri-Carb liquid scintillation counter.

Determination of Hcy in the culture medium. Hcy in the culture medium was determined by a modification of an automated procedure developed for the determination of total Hcy in plasma (Refsum *et al.*, 1989). The method measures both the major free fraction of Hcy and the small amounts of protein-bound Hcy in the culture medium.

The medium was treated with borohydride and then derivatized with monobromobimane. The reduction, derivatization and the injection into the high-performance liquid chromatography column were carried out sequentially by a microprocessor controlled autosampler from Gilson, model 232-401 (Refsum *et al.*, 1989). The Hcy-monobromobimane adduct was separated on a 15-cm 3-μm ODS Hypersil column eluted with an acetonitrile gradient (0–10% in 11.5 min) in 58 mM ammonium nitrate/40 mM ammonium formate buffer, pH 3.7. The flow rate was 2 ml/min. The effluent was monitored by fluorescence detection. The adduct showed a retention time of 11 min in this system.

Curve fitting and calculation of export rates. The logarithm of the cell number (N) and the amount of Hcy accumulated in the medium (y) were plotted *vs.* time of incubation, and the curves were fitted to polynomial functions. The Hcy export rate (v), given as the amount of Hcy exported per unit time and cell mass, was calculated from the equation

$$v = dy/dt/N$$

The export rates were plotted against cell density (Christensen *et al.*, 1991).

Details on the curve fitting and construction of export rate curves have been published (Christensen *et al.*, 1991; Refsum *et al.*, 1991).

Determination of folate. Folate in the cells or serum was assayed using the Quantaphase folate radioassay produced by Bio Rad (Hercules, CA). We measured medium folate and cellular folate with different procedures, optimized for the determination of serum folate and erythrocytes folate, respectively. The method is a radioligand displacement assay using ^{125}I-labeled folic acid and folate binding protein from bovine milk coupled to polymer beads as binding protein. The assay

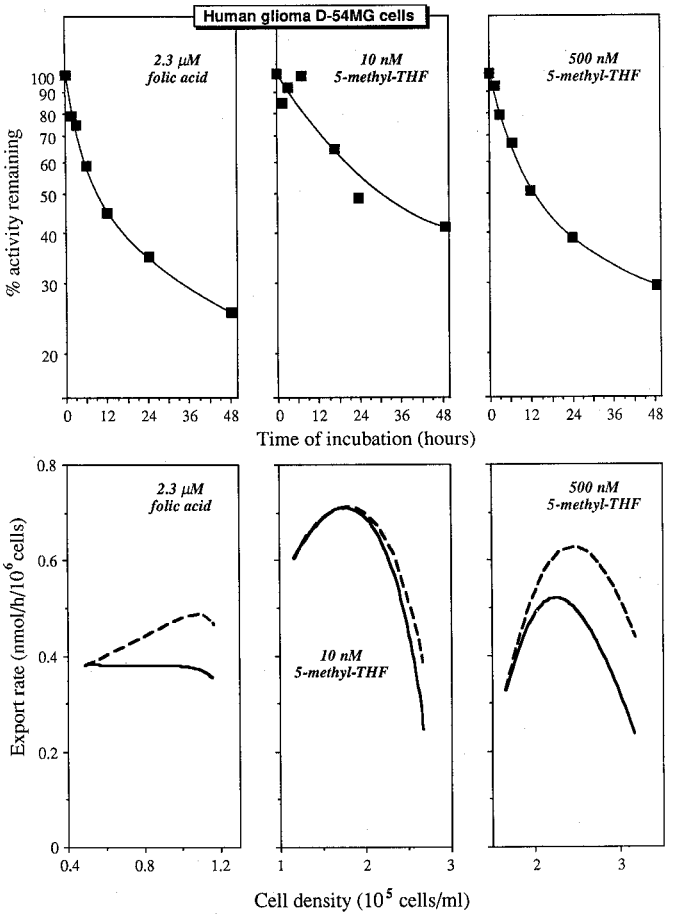

Fig. 6. Methionine synthase inactivation by nitrous oxide and the concurrent Hcy export rate of D-54MG cells. Detailed explanations are given in legends to figures 2 and 4. The experiments were repeated two times.

pH was adjusted to 9.2 so that folic acid and 5-methyl-THF are measured equally (Gregory, 1990).

The cells (about $30 \cdot 10^6$) were washed in ice-cold PBS, centrifuged and resuspended in 100 to 300 μl of 0.4% ascorbic acid for determination of intracellular folate.

Determination of protein. Protein was determined by the method of Bradford (1976) using bovine γ-globulin as standard.

Results

Folate content and viability of cells cultured in medium with low folate. The folate content in different cells cultured in the standard RPMI medium was between 7 and 20 pmol/10^6 cells, except for the R 1.1. cells which contained lower amount of folate. When the cells were transferred and cultured in the medium with 10 nM 5-methyl-THF (LF medium), the folate content was drastically reduced within 5 to 10 days (table 1). After 10 days, the folate content was below the detection limit in R 1.1 cells, HL-60 cells and Cl 8 cells (table 1).

Cell viability, determined with dye exclusion test, was higher than 96% for all cell types cultured in the standard RPMI and HF medium, and for Cl 8 cells, KG-1a cells, GaMg and D-54MG cells cultured in LF medium. The R 1.1 cells and HL-60 cells did not thrive in LF medium, and the viability declined to <40% within 5 days (data not shown).

Based on these results, the experiments with cells cultured in medium with low folate (FD, LF medium) were restricted to the Cl 8, KG-1a, GaMg and D-54MG cells.

Methionine synthase activity and Hcy export rate. Methionine synthase activity for cells grown in the ordinary standard RPMI medium was highest for the Cl 8 and D-54MG cells (about 22 $nmol \cdot mg^{-1} \cdot h^{-1}$), intermediate for the HL-60, KG-1a and GaMg cells, and lowest for the R 1.1 cells (about 3 $nmol \cdot mg^{-1} \cdot h^{-1}$) (table 2)

No marked alteration in methionine synthase activity was observed when the cells were cultured in media with different folate content, except for the R 1.1 cells, where the enzyme activity increased about 4-fold upon transfer from the standard RPMI medium to the HF medium (table 2).

The Hcy export rate for all cells investigated increased after seeding, then reached a maximum, after which the export rate declined. The maximal export rate for cells grown in the standard RPMI medium varied in the range from 0.1 (HL-60 cells) to 0.4 nmol/h/10^6 cells (the glioma cells) (figs. 1–6). The pronounced decline in export rate as a function of the moderate increase in cell count (figs. 1–6) is related to the high cell density (Christensen *et al.*, 1991), which was required to simultaneously detect methionine synthase activity.

The Hcy export from the Cl 8 cells, GaMg cells and D-54MG cells was highest when these cells were grown in a medium with low folate (figs. 1, 5, 6 and 7). Such a relation was not found for the KG-1a cell line (figs. 4, 7).

Kinetics of methionine synthase inactivation induced by nitrous oxide. Methionine synthase activity was determined in the six cell lines cultured in the standard RPMI medium and the HF medium, before and during 48 h of nitrous oxide exposure. The Cl 8, KG-1a, GaMg and D-54MG cells were also tested in the LF medium.

Nitrous oxide induced inactivation of methionine synthase in all six cell types cultured in the standard RPMI medium. The initial rate of inactivation varied slightly (between 0.05 and 0.14 h^{-1}). Then, the inactivation leveled off, and there was a significant residual activity after 48 h of exposure (figs. 1–6). This residual activity was relatively high (about 45%) for the R 1.1 and KG-1a cells (figs. 2 and 4), intermediate for the Cl 8 cells (fig. 1) and lowest (15–25%) for the HL-60 (fig. 3) and the glioma GaMg and D-54MG cells (figs. 5 and 6).

The kinetics of the inactivation of methionine synthase in cells cultured in the HF medium (containing 500 nM 5-methyl-THF) were similar to that observed in the standard RPMI medium (figs. 1–6).

The rate and extent of methionine synthase inactivation were markedly inhibited when the cells (Cl 8, KG-1a and the glioma GaMg and D-54MG cells) were transferred and cultured in the LF medium containing only 10 nM 5-methyl-THF (figs. 1, 4, 5 and 6). Under this condition, the initial rate was reduced to 15 to 25% of the rate observed when the cell were cultured in the standard RPMI medium (table 2). The residual activity was between 80% (Cl 8 cells, fig. 1; KG-1a cells, fig. 4) and 40% (glioma GaMg and D-54MG cells, figs. 5 and 6) (table 2).

Hcy export rates during nitrous oxide exposure. We measured the concurrent Hcy export from cells exposed to nitrous oxide and in control cells. When the cells were cultured in standard RPMI and HF media rich in folate, nitrous oxide induced a marked increase in the export rate of Cl 8 cells (fig. 1) and a moderate increase in the two glioma GaMg and D-54MG cells (figs. 5 and 6), but did not affect the export rate of the leukemic KG-1a, R 1.1 and HL-60 cells (figs. 2, 3 and 4). The export rates of the Cl 8, GaMg and D-54MG cells were higher when these cells were cultured in FD medium containing

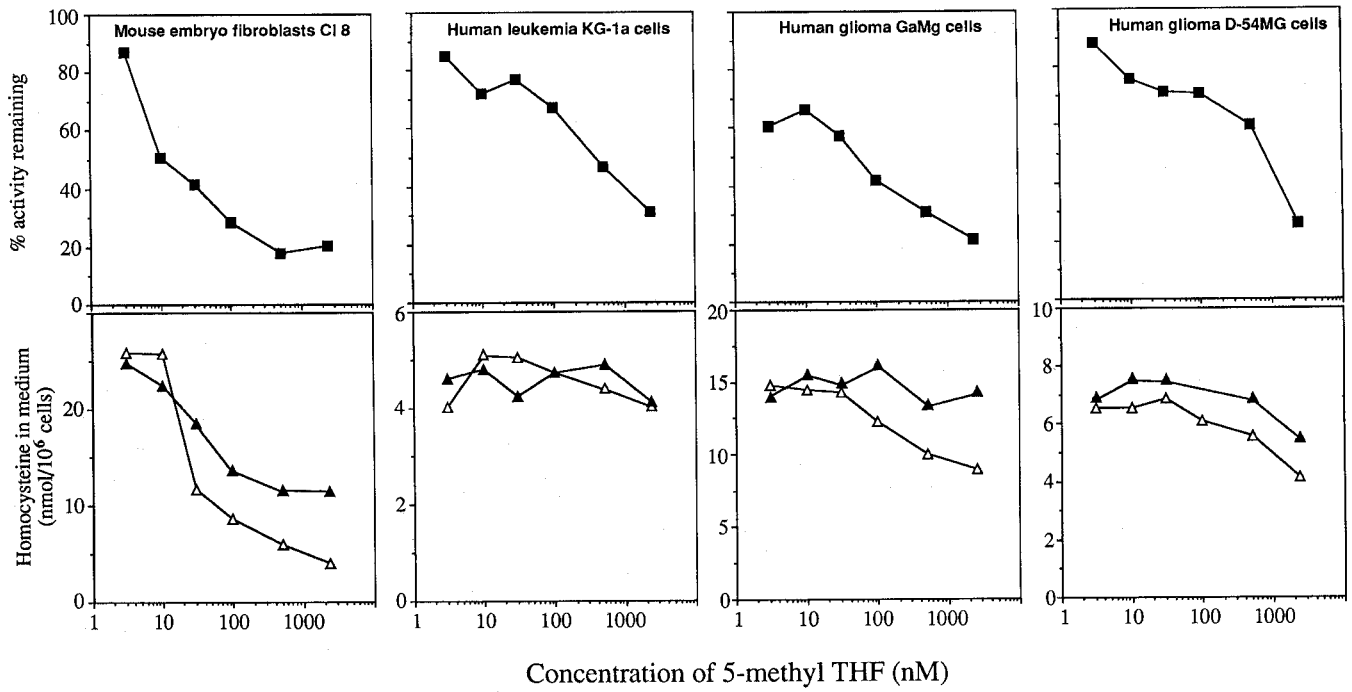

Fig. 7. Effect of the concentration of 5-methyl-THF in the culture medium on nitrous oxide-induced inactivation of methionine synthase and Hcy export of four cell lines. The cells (Cl 8, KG-1a, GaMg and D-54MG) were 5/18-109ax p-2542grown in a LF medium (10 nM 5-methyl-THF) for 10 days, and then for a further 5 days in a folate-deficient FD medium. At the start of the experiment, the cells were transferred to a fresh medium containing 3 to 2300 nM 5-methyl-THF. The cell densities were 2×10^4 cells/ml (Cl 8 cell), 1×10^5 cells/ml (KG-1a cells), 3.5×10^4 cells/ml (GaMg cells) and 3.5×10^4 cells/ml (D-54MG cells), and increased by 40 to 200% during the experimental period. The cells were exposed for 24 h either to nitrous oxide or air (control cells). The upper panels show the percent methionine synthase activity remaining (*i.e.*, the activity in the cells exposed to nitrous oxide in percent of the activity of control cells); the lower panels show the Hcy export given as Hcy accumulated in the medium divided by the average cell density during the experimental period. ▲, cells exposed to nitrous oxide; △, control cells. Each value is the average of duplicate measurements. The experiments were repeated two times.

low folate compared with the standard RPMI and HF media containing high folate, but the export was not further enhanced by nitrous oxide (figs. 1, 5 and 6).

5-Methyl-THF in culture medium, methionine synthase inactivation and Hcy export. The cells (Cl 8, KG-1a, GaMg and D-54MG) which thrived in the LF medium with 10 nM 5-methyl-THF, were transferred to and cultured in a folate-deficient FD medium for 5 days. We then investigated the methionine synthase inactivation and the concurrent Hcy export during 24 h of nitrous oxide exposure of the cells cultured in media supplemented with increasing concentrations (3–2300 nM) of 5-methyl-THF (fig. 7).

In the four cell types tested, the residual methionine synthase activity after the nitrous oxide exposure decreased as a function of the increasing 5-methyl-THF concentration (fig. 7, upper panels).

The Hcy export was determined as the amount accumulating in the culture medium during the 24-h period. In the Cl 8 cells and both glioma (GaMg and D-54MG) cell lines, the Hcy export was reduced as a function of increasing amounts of 5-methyl-THF in the medium. Nitrous oxide partly attenuated this reduction and thus stimulated the Hcy export at high medium folate (fig. 7, lower panels). Notably, neither medium folate nor nitrous oxide altered the Hcy export from the KG-1a cells (fig. 7). This finding is in accordance with the data presented in figure 4.

Discussion

Experimental system and design. Methionine synthase inactivation has been extensively studied *in vivo* (Black and Tephly, 1983; Chanarin *et al.*, 1985; Koblin and Tomerson, 1990; Koblin *et al.*, 1982; Royston *et al.*, 1988), and in some detail *in vitro* in cell-free systems (Banerjee and Matthews, 1990; Frasca *et al.*, 1986). Only sparse data exist on nitrous oxide exposure of isolated or cultured cells (Boss, 1985; Rosenblatt *et al.*, 1984), and the conditions affecting the susceptibility of the intracellular enzyme to nitrous oxide has not been investigated.

Using intact cells preserves the physiological reducing systems and enzyme concentration, and these are factors critical for methionine synthase activity and the susceptibility of the enzyme to nitrous oxide, at least *in vitro* (Banerjee and Matthews, 1990; Taylor, 1982). We investigated six different murine and human cell lines. All cell lines were transferred to a RPMI medium (with different folate) before the experiment, because medium components may affect methionine synthase activity, as shown for methionine, folate and vitamin B_{12} (Kamely *et al.*, 1973; Tautt *et al.*, 1982; Taylor and Hanna, 1975). In addition, the methionine content is a modulator of Hcy export of several cell lines (Christensen *et al.*, 1991). Conceivably, the medium composition may affect the susceptibility of methionine synthase to nitrous oxide, but this question has not previously been addressed. Thus, standardization of the culture conditions may allow the comparison between the responses to nitrous oxide of different cell types.

We measured the effect of nitrous oxide on the target enzyme methionine synthase, but also the cellular export of Hcy. The methionine synthase activity was measured in cell-free extracts, whereas the changes in Hcy export may reflect the inhibition

of metabolic flux through the methionine synthase pathway in the intact cell. The Hcy export was presented as graphs of export rates *vs.* cell density using computer-assisted construction of export rate curves. This presentation allows comparison of Hcy export between cell types during growth and at different growth rates and cell densities (Christensen *et al.*, 1991; Refsum *et al.*, 1991).

Methionine synthase inactivation and Hcy disposition. Nitrous oxide induced a time-dependent inactivation of methionine synthase in six murine and human cell lines cultured in the presence of high medium folate, but the effect was associated with increased Hcy export in only the nonleukemic cells (*i.e.*, fibroblasts Cl 8 and the two glioma cell lines, GaMg and D-54MG) (figs. 1–7). Likewise, the antifolate drug methotrexate increases the Hcy export from Cl 8 fibroblasts, but not from the lymphoma R 1.1 cells (Refsum *et al.*, 1991). This may indicate essentially no folate-dependent remethylation of Hcy in these malignant hematological cells, as demonstrated for cultured human lymphoid cells by German *et al.* (1983). This explanation is in accordance with the observations that fibroblast Cl 8 is a methionine-independent cell line (Djurhuus *et al.*, 1988), the human glioma cells grow slowly in the methionine-deficient medium supplemented with Hcy (T. Fiskerstrand, unpublished), whereas the leukemic cells investigated are essentially methionine dependent (Djurhuus and Ueland, 1989; Kano *et al.*, 1982). These indications of low Hcy remethylation in some cells are puzzling because there are both significant methionine synthase activity (table 2) and significant Hcy formation (figs. 2, 3 and 4) in such cells. Functional compartmenation of Hcy and methionine metabolism should be considered (Stern *et al.*, 1984).

Extracellular folate source. Most culture media contain supraphysiological concentration (2–10 μM) of folic acid, which contrasts to the normal serum level of 5-methyl-THF (5–20 nM) *in vivo* (Kane and Waxman, 1989).

We cultured six cell lines in LF medium with low folate for up to 10 days, and a marked folate depletion was observed in all cell lines (table 1). The cells were not cultured in low folate for an extended period of time to avoid the selection of phenotypes (Jansen *et al.*, 1990). The marked folate depletion (table 1) agrees with consistent reports on reduction of cellular folate upon transfer of cultured cells to medium containing low folate (Kamen and Capdevila, 1986; Kane *et al.*, 1986), and reflects the strict dependence of cellular folate content on the concentration of folate in the medium (Bunni *et al.*, 1988; Rhee *et al.*, 1990). Increasing attention has been paid to the concentration of folate in cell culture media because this may be an important determinant of several folate-dependent processes (Jansen *et al.*, 1990; Kamen and Capdevila, 1986; Rhee *et al.*, 1990; Van der Laan *et al.*, 1991).

We tested various concentrations of folic acid or 5-methyl-THF added to the cultured medium. There are consistent reports that 5-methyl-THF supports the growth of cells in culture (Fujii *et al.*, 1981; Kano *et al.*, 1986; Taylor and Hanna, 1975), and for some cell types is more efficient than folic acid (Taylor and Hanna, 1975). However, folic acid is stable in culture medium, whereas 5-methyl-THF has been found to lose about 30% of its biological activity measured by microbiological assays after 48 h of incubation in RPMI medium (Kano *et al.*, 1986). Thus, reservation should be made regarding the extracellular levels of 5-methyl-THF, which may be somewhat overestimated.

Methionine synthase inactivation and extracellular folate. A high concentration of folate in the medium enhanced the nitrous oxide-induced inactivation of methionine synthase in all cell types. Medium folate and nitrous oxide affected the Hcy export rate only in the three nonleukemic cell lines (figs. 1–7). This may reflect that significant metabolic flux through methionine synthase is confined to these cell lines, and further highlight changes in Hcy export rate as an indicator of the functional state of the methionine synthase in intact cells.

Intake of high doses of folic acid reduces plasma Hcy, even in subjects without overt folate deficiency (Brattström *et al.*, 1988). This observation mirrors reduction in Hcy export rate by increasing the 5-methyl-THF concentration in the culture medium (fig. 7).

Possible mechanisms and kinetics of the inactivation. Nitrous oxide inactivates methionine synthase by oxidation of cobal(I)amin, which is transiently formed during catalytic turnover (Chanarin *et al.*, 1985). This hypothesis is supported by results obtained with isolated enzyme showing that inactivation of the enzyme from rat liver and *Escherichia coli* occurs only in the presence of all components required for catalytic turnover, including Hcy and 5-methyl-THF. A relation exists between moles of methionine formed and the amount of enzyme inactivated (Frasca *et al.*, 1986). It is conceivable that the increased cellular pools of reduced folates, including 5-methyl-THF (Bunni *et al.*, 1988), observed in cells cultured in media rich in folate (table 1) may increase the flux through methionine synthase. The resulting high catalytic turnover may explain the enhancement of methionine synthase inactivation observed in cells cultured in the presence of high concentrations of folate (table 2). Decreased Hcy export from some cells at high extracellular folate (fig. 7) is in accordance with this explanation.

Methionine synthase is also inactivated in cells (R 1.1, HL-60 and KG-1a) in which neither folate, the antifolate MTX (Refsum *et al.*, 1991) nor nitrous oxide (figs. 2, 3, 4 and 7) affect the Hcy export, suggesting essentially no Hcy remethylation in these cells. This interpretation seems difficult to reconcile with the model for methionine synthase inactivation cited above, and suggests that the model does not strictly apply to the enzyme in intact cells.

Inactivation of methionine synthase *in vivo* is faster in rodents than in humans (Black and Tephly, 1983; Chanarin *et al.*, 1985; Koblin and Tomerson, 1990; Koblin *et al.*, 1982; Royston *et al.*, 1988). We observed no marked difference between rate of inactivation on murine (Cl 8, R 1.1) and human (HL-60, KG-1a, D-54MG and GaMg) cell lines. The different inactivation kinetics (Black and Tephly, 1983; Chanarin *et al.*, 1985; Koblin and Tomerson, 1990; Koblin *et al.*, 1982; Royston *et al.*, 1988) may not reflect different properties of methionine synthase, but rather a higher metabolic flux through methionine synthase in rodents than in humans.

Conclusion and perspectives. We have shown that the folate supply and thereby the cellular folate status is a determinant of the rate and extent of methionine synthase inactivation induced by nitrous oxide in six cell types. The inactivation was associated with increased Hcy export in three cells carrying out significant Hcy remethylation, and in these cells, high extracellular folate alone reduces the export. This response may serve as a model for the elevation of plasma Hcy observed in patients exposed to nitrous oxide (Ermens *et al.*, 1991), and for the suppression of plasma Hcy after folic acid intake (Brattström *et al.*, 1988). The detection of significant methio-

nine synthase activity responsive to nitrous oxide but without a concurrent enhancement of Hcy export in three leukemic cell lines (figs. 2, 3 and 4) presents intriguing questions regarding the functional state of the enzyme in these cells and its possible relation to cellular methionine dependence.

The present study points to administration of folic acid, Leucovorin (formyltetrahydrofolate) or antifolate drugs as possible means to modulate the nitrous oxide-induced inactivation of methionine synthase. Formyltetrahydrofolate has been used with differing results to protect patients against the deleterious effect of nitrous oxide (Nunn, 1987), but the timing of administration may be a critical factor which has been overlooked. Conceivably, coadministration of nitrous oxide and formyltetrahydrofolate may enhance the cobalamin inactivation, whereas supplementing folate after the nitrous oxide exposure may have a beneficial clinical effect. Our finding that methionine synthase is protected against inactivation by low folate suggests that antifolate drugs may have a similar effect. The results from the interaction between nitrous oxide and antifolate drugs may therefore depend on the sequence of exposure of the agents, as has recently been demonstrated in rats given methotrexate and nitrous oxide (Ermens *et al.*, 1989). These exciting aspects should stimulate further investigation of the methionine synthase inactivation under various metabolic conditions.

Acknowledgments

We thank Eli Gundersen and Gry Kvalheim for technical assistance. The D-54MG cells were kindly provided by Dr. Darell D. Bigner at the Duke University Medical Center, Department of Pathology. The GaMg cell line was a gift from Dr. Rolf Bjerkvig at the Gade Institute, Department of Pathology.

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