

# Folate Depletion Induced by Methotrexate Affects Methionine Synthase Activity and Its Susceptibility to Inactivation by Nitrous Oxide<sup>1</sup>

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## ABSTRACT

We compared the effects of methotrexate (MTX) and nitrous oxide on the methionine (Met) synthase system in two variants of a human glioma cell line. The cells were protected from cytotoxic effect of MTX by adding thymidine and hypoxanthine to the cell culture medium. MTX (0–1  $\mu$ M) was associated with a dose- and time-dependent reduction in 5-methyltetrahydrofolate (5-methyl-THF) in both cell lines. Already after 3 hr of exposure, 5-methyl-THF was reduced by 50% and after additional 48 hr, the level was undetectable. In addition to reduction in folate level, homocysteine (Hcy) remethylation in intact cells was markedly inhibited as judged by an increased export of Hcy from the cells, and Met synthase activity in cell extracts and level of cellular methylcobalamin (CH<sub>3</sub>Cbl) declined. MTX

reduced Hcy remethylation and CH<sub>3</sub>Cbl level more efficiently than nitrous oxide. In both cell variants, the inactivation of Met synthase by nitrous oxide was almost completely prevented in cells pre-exposed to MTX. This indicates that there is no catalytic turnover in cells exposed to MTX, and emphasizes the importance of the sequence of administration for synergistic effect of this drug combination. In conclusion, our data show that MTX through depletion of 5-methyl-THF reduces both the Met synthase activity and the cellular CH<sub>3</sub>Cbl level. Moreover, the effect of MTX on the Hcy remethylation is more pronounced than the inhibition caused by nitrous oxide. These observations should be taken into account in studies on MTX pharmacodynamics.

MTX is an antifolate drug that inhibits dihydrofolate reductase, the enzyme responsible for the regeneration of tetrahydrofolate from dihydrofolate. This effect is associated with depletion of cellular reduced folates, and thereby inhibition of numerous folate-dependent processes. Impeding of the thymidylate synthase and enzymes involved in purine biosynthesis impairs DNA synthesis, which probably explains the cytotoxicity of MTX (Bertino, 1993).

Met synthase (5-methyltetrahydrofolate-homocysteine methyltransferase, EC 2.1.1.13) catalyzes a folate-dependent reaction, in which 5-methyltetrahydrofolate (5-methyl-THF) functions as methyl donor, thereby converting Hcy to Met. Cobalamin (Cbl) serves as cofactor in this reaction. Because Met synthase operates at the point of convergence of folate, Cbl and sulfur amino acids (Finkelstein, 1990), impaired function of this enzyme may have secondary effects on many cellular processes, including S-adenosylmethionine-dependent methylation reactions and polyamine synthesis.

The anesthetic agent nitrous oxide is the only drug which

has been reported to directly inhibit Met synthase, and it probably oxidizes enzyme-bound cob(D)alamin formed during the catalytic cycle (Banerjee and Matthews, 1990). Notably, this effect of nitrous oxide may account for its diverse biological effects, including the megaloblastic changes in human bone marrow (Nunn, 1987; Amess *et al.*, 1978), antileukemic effect reported in patients (Ikeda *et al.*, 1989; Eastwood *et al.*, 1963) and experimental animals (Ermens *et al.*, 1989b; Abels *et al.*, 1990) and altered metabolism and plasma level of Hcy and related sulfur compounds (Nunn, 1987; Ermens *et al.*, 1991a; Christensen *et al.*, 1994).

The side effects reported for nitrous oxide (Nunn, 1987) point to significant biological consequences of Met synthase inhibition. Depletion of 5-methyl-THF by MTX (Bunni 1988, Ermens 1991b, Baram 1987) suggests that some effect of this antifolate drug may be related to inhibition of Met synthase. Furthermore, because the action of both nitrous oxide and MTX converges on 5-methyl-THF metabolism, one may expect a dynamic interaction between these two drugs (Ueland *et al.*, 1986b; Goldhirsch *et al.*, 1987; Ermens *et al.*, 1991b).

The aim of our work was to investigate changes of the Met synthase system secondary to the folate depletion induced by MTX, and to compare these changes to those caused by ni-

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**ABBREVIATIONS:** MTX, methotrexate; Met, methionine; THF, tetrahydrofolate; Hcy, homocysteine; Cbl, cobalamin; Thd, thymidine; Hx, hypoxanthine; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography.

trous oxide. First we confirmed that MTX depleted cellular 5-methyl-THF, and then we studied the relation between folate depletion and alteration in Met synthase, including the susceptibility of the enzyme to inactivation by nitrous oxide, the influence on intact cell Hcy remethylation and the content of Cbl cofactor. The study was performed with two genetically related human glioma cell lines, characterized by diverse functional state of the enzyme. The cells were cultured in the presence of Thd and Hx, to protect against MTX cytotoxicity.

## Materials and Methods

**Chemicals.** The sources of various chemicals used in the cell culture experiments and in the assays have been reported previously (Fiskerstrand *et al.*, 1994). L-[<sup>14</sup>C]Hcy thiolactone (56 mCi/mmol), [<sup>57</sup>Co]Cbl (0.3 Ci/ $\mu$ mol) and ( $\pm$ )-L-5-[methyl-<sup>14</sup>C]methyl-THF (50 mCi/mmol, barium salt) were purchased from Amersham International (Buckinghamshire, UK). L-5-[3',5'-7(N)-<sup>3</sup>H]formyl-THF (25 Ci/mmol) was obtained from Moravek Chemicals, Inc. (Brea, CA). A custom-made powdered DMEM, identical to a standard DMEM but without folic acid and Met, was from GIBCO-BRL (Paisley, Scotland). Hog kidney hydrolase (10 mg protein/ml), prepared as described by McMartin *et al.* (1981), was a gift from Dr. Ermens, Erasmus University, Rotterdam.

**Cells and cell culture conditions.** The two variants of the human glioma cell line GaMg used in these experiments, have been described (Fiskerstrand *et al.*, 1994, 1997). The parent cell line was isolated in 1984 from a human glioblastoma multiforme tumor in a 42-yr-old woman (Akslen *et al.*, 1988). Both variants are passage number 60 of GaMg. The Met-dependent variant, P60, was developed by repeated passages in Met-containing medium (Fiskerstrand *et al.*, 1994), and the other variant, P60H, retained its Met independence by being passed in Hcy containing medium (Fiskerstrand *et al.*, 1997).

A custom made standard DMEM without folic acid and Met was used in all experiments. The medium was supplemented with 10% dialyzed fetal calf serum and contained 0.6 g/liter L-glutamine, 1.5  $\mu$ M CNCbl, 10  $\mu$ M folic acid, 330  $\mu$ M nonessential amino acids, and 50  $\mu$ M Met. The necessary amount of Thd and Hx to support growth in the presence of MTX was also added. The final concentrations were 5 and 100  $\mu$ M Thd, and 40 and 80  $\mu$ M Hx for P60H and P60, respectively.

Stem cultures of P60 were kept in Met medium, and P60H in Hcy medium. P60H had grown one passage in Met medium before start of experiments.

**Experimental designs.** For all experiments, cells were seeded in Met medium at a density of 1000 cells/cm<sup>3</sup> in 6-cm dishes (Nunc, Denmark) (most experiments), or in 10-cm dishes (Cbl determinations). When reaching log growth phase, the cells received fresh medium with 0 to 3  $\mu$ M MTX, and the medium volume was adjusted to 40,000 cells/ml (Fiskerstrand *et al.*, 1997). After 3 hr of incubation, the dishes were placed in modular incubator chambers (Billups-Rothenberg, Del Mar, CA) and exposed to air (75% N<sub>2</sub>, 20% O<sub>2</sub> and 5% CO<sub>2</sub>) or nitrous oxide (50% N<sub>2</sub>O, 25% N<sub>2</sub>, 20% O<sub>2</sub> and 5% CO<sub>2</sub>). The gas was moistened and tempered by passing through sterile H<sub>2</sub>O at 50°C, and then delivered to the chambers at a flow rate of 5 liters/min for 10 min.

Harvesting was at different time points, depending on the assay. Usually, three parallel dishes were trypsinized (0.1 mg/ml solution, Bio Whittaker, Walkersville, MD), the cells were washed several times and stored at -80°C until analysis. Medium was kept at -20°C until Hcy determination. Cells were counted using a Coulter counter (Coulter Electronics Ltd., Luton, UK).

Cells for Met synthase activity and Cbl measurements were harvested 48 hr after start of gas exposure, and for Hcy export after 0, 6, 12, 24, 36, 48 and 72 hr. Samples for folate (and simultaneous

enzyme activity) determinations were first depleted of folates by growing the cells for 10 days in medium without folate, but containing Hx/Thd. The cells were then seeded at the density given above, but medium was replaced after 24 hr with fresh medium containing either 42 nM 5-[<sup>3</sup>H]formyl-THF (for determination of folate distribution) or unlabeled 5-formyl-THF (for measurements of total folate or Met synthase activity). After incubation for 24 hr, medium was changed to standard DMEM, to allow saturation and equilibration of the folate pools. Medium was changed 36 hr later, and MTX was added. Dishes were incubated for 3 hr with this medium before gas exposure (air or nitrous oxide).

**Met synthase assay.** The enzyme activity was determined according to a slight modification (Christensen *et al.*, 1992) of the assay described by Weissbach *et al.* (1963).

**Hcy export rates.** The concentration of Hcy in the medium was determined by an automated HPLC method (Refsum *et al.*, 1989; Fiskerstrand *et al.*, 1993), and the logarithm of the cell number and the concentration of Hcy in the medium was plotted against time of incubation. The equations obtained by curve fit to polynomial functions were used to calculate Hcy export rate, which describes change in Hcy concentration per hr per 10<sup>6</sup> cells. Export rates were plotted against time. The mathematic processing of data has been described in detail (Refsum *et al.*, 1991; Christensen *et al.*, 1991).

**Determination of folates.** This was based on a method developed by Ermens *et al.* (1991b), which was modified by us (Fiskerstrand *et al.*, 1994). Cells were trypsinized and then carefully washed twice with medium (without supplements). The cell pellet was dissolved in 600  $\mu$ l 2% ascorbic acid and 2% mercaptoethanol containing unlabeled folates as internal standard. Further processing included heat denaturation of proteins, enzymatic cleavage of polyglutamates by hog kidney hydrolase, heat inactivation of the hydrolase and centrifugation. Samples were injected on a 3- $\mu$ m octadecylsilane Hypersil column (10  $\times$  0.5 cm) equilibrated with 20 mM ammonium sulfate, 10 mM tetrabutylammoniumhydroxide at pH 6.5, and eluted with a nonlinear methanol gradient (12.5–27.5% in 35 min) at a flow of 1.5 ml/min. The retention times were 25 and 36 min for 5-formyl-THF and 5-methyl-THF, respectively. Fractions were collected by Foxy model 200 fraction collector, and radioactivity was counted.

Total folate was determined by the Quantaphase folate radioassay (Bio-Rad, Hercules, CA) which measures folic acid and 5-methyl-THF equally (Gregory, 1990).

**Determination of Cbl.** Extraction of Cbl was performed with slight modification (Fiskerstrand *et al.*, 1994) of the method described by van Kapel *et al.* (1983). After trypsination, cells were washed four times with PBS containing 0.1 mg/ml albumin, and 300  $\mu$ l cell suspension was stored at -80°C until analysis. After thawing, 8  $\mu$ l N-ethylmaleimide and 8  $\mu$ l acetic acid were added, and the volume was adjusted to 800  $\mu$ l with distilled water. Then, Cbl was extracted by heating at 80°C for 30 min.

The forms of Cbl were analyzed by HPLC (Jacobsen and Green, 1979; Fiskerstrand *et al.*, 1994). A sample volume of 400  $\mu$ l was injected into a 3- $\mu$ m octadecylsilane Hypersil column (10  $\times$  0.4 cm) equilibrated with 50 mM sodium phosphate buffer, pH 3.0. The Cbl forms were eluted at a flow of 1.3 ml/min with a linear acetonitrile gradient (5–30% in 13.8 min). The retention times were 10.5 min (OHCbl), 12 min (CNCbl), 14 min (adenosylCbl) and 16 min (CH<sub>3</sub>Cbl).

A radioisotope dilution assay described by van Kapel *et al.* (1983) was used to determine both total Cbl and the different Cbl forms in the HPLC fractions. Salivary R-binder was used as Cbl binding protein.

**Statistical analyses.** Results are given as mean and S.D. We used analyses of variance for comparison between different treatment groups. The significant levels were expressed as two-tailed.

## Results

**Cells and culture conditions.** The doubling time of P60H and P60 cultured in Met medium (with Thd/Hx) were 23 and 17 hr, respectively. Only the P60 cell line showed growth arrest when Hcy replaced Met (data not shown), which confirmed the Met-dependent phenotype of P60 cells (Fiskerstrand *et al.*, 1994), also in Thd/Hx-containing medium.

MTX inhibited the growth (IC<sub>50</sub> of 0.1–0.2  $\mu$ M) in a dose-dependent manner, and 1  $\mu$ M caused >90% growth inhibition of P60H and P60 cells. Both variants were rescued by including Thd and Hx in the cultured medium. With rescue, the growth of P60H cells was similar with or without MTX. A slight reduction in growth rate became apparent in P60 cells after 48 to 72 hr of MTX exposure (1  $\mu$ M).

**Cellular folate content.** Total folate content of cells grown in a medium with Thd/Hx was about 35 pmol/10<sup>6</sup> cells in both cell lines. MTX induced a progressive depletion of total folate, and the folate content was reduced to 60% (P60H cells) and 75% (P60 cells) after 3 hr exposure to 1  $\mu$ M, and to 25% (P60H) and 8% (P60) after 48 hr (tables 1 and 2).

The amount of 5-methyl-THF was higher and accounted for a higher fraction of total folate in P60H cells than in P60 cells. MTX caused a time-dependent decrease in 5-methyl-THF in both cell lines. In cells exposed to 1  $\mu$ M MTX, the content was reduced to 50% after only 3 hr, to 20% after 12 hr and to undetectable levels after 48 hr (tables 1 and 2; fig. 3). In P60H cells, we determined a dose-response effect with an ID<sub>50</sub> value < 0.03  $\mu$ M MTX after 48 hr of exposure (table 1).

Nitrous oxide had marginal effects on total folate and 5-methyl-THF in both cells (tables 1 and 2), although there was a slight synergistic effect with MTX.

**Assessment of cellular remethylation by Hcy export rates.** Inhibition of Hcy remethylation is reflected by an increased concentration of Hcy extracellularly both *in vivo* and *in vitro* (Refsum *et al.*, 1997). Because the amount of Hcy released to the medium conceivably depends on both the number of cells and the duration of the experiment, we studied the changes in export of Hcy by determining the export rate, *i.e.*, the amount of Hcy exported from one million cell

per unit of time (Christensen *et al.*, 1991; Refsum *et al.*, 1991). We compared the Hcy export rate in the presence and absence of drug and assume that an increased difference in export rate reflected a more pronounced inhibition of Hcy remethylation.

In P60H cells, the Hcy export rate was increased by nitrous oxide (from 0.26 to 0.51 nmol/hr/10<sup>6</sup> cells), but even more by MTX (to 0.76 nmol/hr/10<sup>6</sup> cells) which exerted the same effect as the combination of MTX plus nitrous oxide (fig. 1; table 3). However, in P60 cells MTX, nitrous oxide and the combination of both increased Hcy export to the same extent. Thus, MTX alone seems to totally inhibit Hcy remethylation in both cell types, whereas nitrous oxide caused complete inhibition only in the P60 cells (fig. 1; table 3).

**Met synthase activity.** We first investigated the separate effect of MTX or nitrous oxide on Met synthase in P60H and P60 cells. MTX caused a dose-dependent reduction in enzyme activity in both cell lines with a half maximal effect at 0.2  $\mu$ M. After exposure to MTX (3  $\mu$ M) for 48 hr, the activity was reduced to 30 to 50% as compared to control cells (fig. 2A). Nitrous oxide inactivated the enzyme at an initial rate of 0.07 hr<sup>-1</sup> in P60 H and 0.12 hr<sup>-1</sup> in P60 cells (Fiskerstrand *et al.*, 1994), and after 48 hr, the remaining activity was ~ 20% of activity in cells not exposed to nitrous oxide (fig. 2B).

When P60H and P60 cells were treated with MTX before (for 3 hr) and during nitrous oxide exposure, MTX prevented in a dose-dependent manner the inactivation of the Met synthase (fig. 2B).

To investigate the possible role of folate depletion on the enzyme, we measured 5-methyl-THF and Met synthase activity in P60H cells exposed to nitrous oxide with and without MTX. Notably, the reduction of cellular content of 5-methyl-THF preceded the inhibitory effect of MTX on nitrous oxide-induced inactivation. This effect was moderate after 12 hr of nitrous oxide, but became substantial after 48 hr (fig. 3; table 1). Furthermore, the dose-response curves for the 5-methyl-THF depletion, both at 12 hr and 48 hr, were located to the left of curves for remaining enzyme activity (fig. 3). Similar data were obtained for the P60 cells (data not shown). These data suggest that the enzyme inactivation is fully prevented

TABLE 1  
Intracellular folate in P60H cells grown in MTX  $\pm$  nitrous oxide (N<sub>2</sub>O)

	MTX	Air			N <sub>2</sub> O	
		0 hr	12 hr	48 hr	12 hr	48 hr
	$\mu$ M	pmol/10 <sup>6</sup> cells (% of unexposed cells)				
Total folate	0.0	44 $\pm$ 7 (100)	33 $\pm$ 2 (100)	33 $\pm$ 8 (100)	31 $\pm$ 1 (94)	23 $\pm$ 1 (70)
	0.03	33 $\pm$ 2 (75)	27 $\pm$ 1 (82)	13 $\pm$ 1 (39)	33 $\pm$ 5 (100)	10 $\pm$ 0 (30)
	0.1	39 $\pm$ 5 (87)	25 $\pm$ 2 (76)	11 $\pm$ 1 (33)	24 $\pm$ 1 (73)	10 $\pm$ 1 (30)
	0.3	24 $\pm$ 4 (55)	25 $\pm$ 0 (76)	10 $\pm$ 2 (30)	17 $\pm$ 4 (52)	9 $\pm$ 0 (27)
	1.0	26 $\pm$ 3 (59)	16 $\pm$ 1 (36)	8 $\pm$ 1 (24)	11 $\pm$ 1 (33)	8 $\pm$ 1 (24)
P		.02	.002	.004	.0007	.0001
5-Methyl-THF	0.0	36 $\pm$ 2 (100)	27 $\pm$ 2 (100)	30 $\pm$ 2 (100)	27 $\pm$ 1 (100)	24 $\pm$ 1 (80)
	0.03	28 $\pm$ 1 (78)	22 $\pm$ 2 (82)	12 $\pm$ 2 (40)	32 $\pm$ 2 (119)	9 $\pm$ 1 (30)
	0.1	31 $\pm$ 1 (86)	20 $\pm$ 0 (74)	9 $\pm$ 1 (30)	23 $\pm$ 2 (85)	8 $\pm$ 1 (27)
	0.3	19 $\pm$ 2 (53)	19 $\pm$ 1 (70)	1 $\pm$ 0 (3)	13 $\pm$ 0 (48)	1 $\pm$ 0 (3)
	1.0	18 $\pm$ 1 (50)	5 $\pm$ 1 (19)	0 $\pm$ 0 (0)	2 $\pm$ 1 (7)	0 $\pm$ 0 (0)
P		.0001	.0001	.0001	.0001	.0001

Cells were depleted of folates and incubated with either 42 nM [<sup>3</sup>H] 5-formyl-THF (for determination of 5-methyl-THF) or 42 nM 5-formyl-THF (for determination of total folate) for 24 hr. Then the cells received fresh standard DMEM to deplete and equilibrate the folate pool. After 36 hr, the medium was changed and MTX (0–1  $\mu$ M) was added. The dishes were preincubated for 3 hr and then exposed to air or N<sub>2</sub>O for 0, 12 or 48 hr before harvesting. Values are mean  $\pm$  S.D. of two to three determinations, and P values indicate significance between treatment groups (MTX concentrations) as revealed by analysis of variance.

TABLE 2  
Intracellular folate in P60 cells grown in MTX ± N<sub>2</sub>O

	MTX μM	Air			N <sub>2</sub> O	
		0 hr	12 hr	48 hr	12 hr	48 hr
		pmol/10 <sup>6</sup> cells (% of unexposed cells)				
Total folate	0.0	19 ± 3 (100)	28 ± 2 (100)	36 ± 7 (100)	21 ± 1 (75)	34 ± 12 (94)
	1.0	14 ± 6 (74)	15 ± 5 (54)	3 ± 2 (8)	8 ± 1 (29)	2 ± 1 (6)
5-Methyl-THF	0.0	13 ± 5 (100)	20 ± 2 (100)	16 ± 1 (100)	20 ± 0 (100)	27 ± 2 (169)
	1.0	7 ± 2 (54)	3 ± 1 (18)	0 ± 0 (0)	2 ± 1 (10)	0 ± 0 (0)

Cells were depleted of folates and incubated with either 42 nM [<sup>3</sup>H] 5-formyl-THF (for determination of 5-methyl-THF) or 42 nM 5-formyl-THF (for determination of total folate) for 24 hr. Then the cells received fresh standard DMEM to replete and equilibrate the folate pool. After 36 hr, the medium was changed and MTX (0–1 μM) was added. The dishes were preincubated for 3 hr and then exposed to air or N<sub>2</sub>O for 0, 12 or 48 hr before harvesting. Values are mean ± S.D. of two to three determinations.

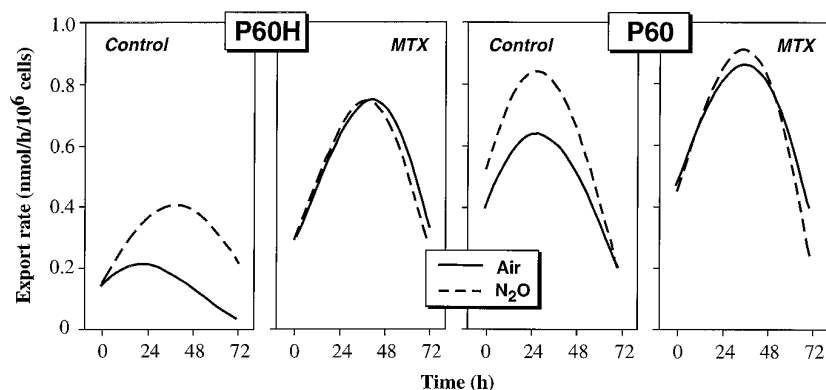


Fig. 1. Hcy export rate from cells exposed to air (solid line) or nitrous oxide (broken line), in the presence or absence of 1 μM MTX. Cells were preincubated for 3 hr with or without 1 μM MTX, before exposure to either air or nitrous oxide (± MTX). The construction of Hcy export curves is described in "Materials and Methods." The experiment was repeated two to three times, and typical data are shown.

TABLE 3  
Hcy export in cells grown in MTX ± N<sub>2</sub>O

	P60H Cells		P60 Cells	
	Hcy export		Hcy export	
	nmol/hr/10 <sup>6</sup> cells			
Air	0.26 ± 0.08	0.59 ± 0.10		
N <sub>2</sub> O	0.51 ± 0.15	0.83 ± 0.04		
MTX	0.76 ± 0.05	0.89 ± 0.25		
MTX + N <sub>2</sub> O	0.83 ± 0.08	1.02 ± 0.24		
P*	.0001	.04		

Cells were cultured in a medium ± 1 μM MTX, and after 3 hr, they were exposed to air or N<sub>2</sub>O. Three parallels were harvested at 0, 6, 12, 24, 36, 48 and 72 hr after start of gas exposure. The export curves were constructed as described in "Materials and Methods," and maximal export rate obtained after 18 to 36 hr are given. Values are mean ± S.D. of three to four experiments.

\* P values indicate significance between treatment groups as revealed by analysis of variance.

only when 5-methyl-THF is reduced to nearly undetectable levels, *i.e.*, to less than 5 pmol/10<sup>6</sup> cells.

**Cellular Cbl content.** We confirmed (Fiskerstrand *et al.*, 1994, 1997) that total Cbl content was essentially the same in the two cell lines, and the level of CH<sub>3</sub>Cbl was higher in P60H (83 fmol/10<sup>6</sup> cells) than in P60 (30 fmol/10<sup>6</sup> cells) (table 4). Nitrous oxide markedly reduced the level of CH<sub>3</sub>Cbl (to 20–40%) in both cell lines. Notably, MTX reduced CH<sub>3</sub>Cbl (to 25%) equally or even more efficiently than nitrous oxide. In MTX-exposed cells, nitrous oxide caused essentially no further reduction of CH<sub>3</sub>Cbl (table 4).

## Discussion

**Aim and study design.** We have studied the effect of MTX and nitrous oxide in two cell lines characterized by

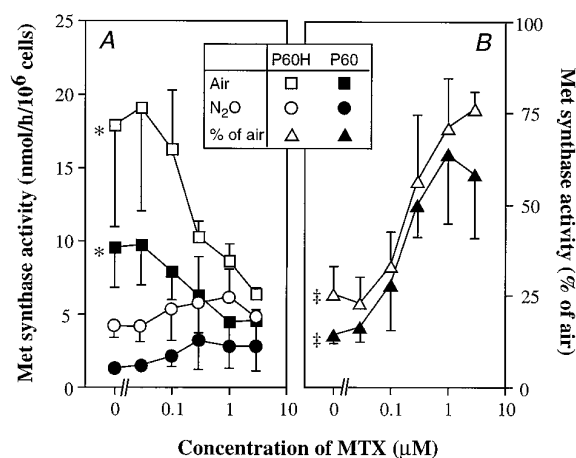
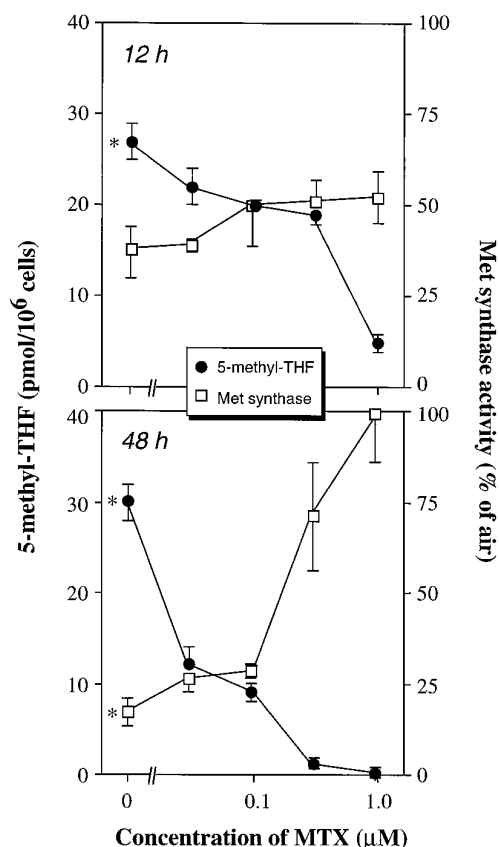


Fig. 2. MTX decreases Met synthase activity (A) and prevents inactivation of the enzyme by nitrous oxide (B). A shows the Met synthase activity in cells exposed to air and nitrous oxide as a function of MTX concentration, and B shows the remaining activity after exposure to nitrous oxide. Cells were preexposed to different concentrations of MTX for 3 hr followed by exposure to nitrous oxide or air for 48 hr (± MTX). Values are mean ± S.D. of three experiments. Symbols denote significant changes (\*P < .05; †P < .001) as a function of MTX concentrations as revealed by analysis of variance.

different ability to use Hcy instead of Met for growth. To prevent growth arrest and toxicity induced by MTX, we supplied the medium with Thd/Hx. Interestingly, these culture conditions increased the level of 5-methyl-THF and CH<sub>3</sub>Cbl in both cell lines compared to medium with no (Fiskerstrand *et al.*, 1994) or lower Thd/Hx concentration (Fiskerstrand *et al.*, 1997). Despite this folate sparing effect of Thd/Hx, P60



**Fig. 3.** 5-Methyl-THF depletion by MTX and its relation to the prevention of Met synthase inactivation by nitrous oxide. P60H cells were exposed to different concentrations of MTX (0–1  $\mu$ M) for 3 hr, followed by exposure to air or nitrous oxide  $\pm$  MTX for 12 or 48 hr. Remaining activity of Met synthase after nitrous oxide exposure and intracellular amount of 5-methyl-THF were determined. Values are mean  $\pm$  S.D. of three determinations. \*Significant changes ( $P = .0001$ ) as a function of MTX concentrations as revealed by analysis of variance.

**TABLE 4**  
**Intracellular content of total Cbl and CH<sub>3</sub>Cbl**

	P60H Cells		P60 Cells	
	Total Cbl	CH <sub>3</sub> Cbl	Total Cbl	CH <sub>3</sub> Cbl
	<i>fmol/10<sup>6</sup> cells</i>			
Air	509 $\pm$ 79	83 $\pm$ 17	576 $\pm$ 80	28 $\pm$ 3
N <sub>2</sub> O	448 $\pm$ 119	32 $\pm$ 6	518 $\pm$ 185	5 $\pm$ 2
MTX	478 $\pm$ 42	20 $\pm$ 8	456 $\pm$ 22	6 $\pm$ 2
MTX + N <sub>2</sub> O	456 $\pm$ 98	15 $\pm$ 7	433 $\pm$ 22	5 $\pm$ 0
P*	.77	.0001	.39	.0001

Cells were preincubated for 3 hr (in air) with 1  $\mu$ M MTX and further exposed to this drug plus air or N<sub>2</sub>O for 48 hr. Values are mean  $\pm$  S.D. of three to four experiments.

\* P values indicate significance between treatment groups as revealed by analysis of variance.

cells remained Met dependent, and MTX still caused an extensive and rapid depletion of 5-methyl-THF.

**Folate depletion and inhibition of Hcy remethylation.** MTX induced a marked time- and concentration-dependent reduction of cellular 5-methyl-THF (tables 1 and 2; fig. 3). Such an MTX effect on folate distribution has also been reported by others both *in vitro* (Bunni *et al.*, 1988; Baram *et al.*, 1987; Ermens *et al.*, 1991b) and *in vivo* (Bunni *et al.*, 1994; Selhub *et al.*, 1991), and it has been suggested that the

depletion of 5-methyl-THF may be partly explained by an inhibition of methylene-THF reductase (Chabner *et al.*, 1985).

Extracellular Hcy is a marker of intracellular folate and Cbl status, and several clinical and cell culture studies have demonstrated that both MTX and nitrous oxide lead to increased extracellular Hcy level (Ueland *et al.*, 1986a; Refsum *et al.*, 1986; Ermens *et al.*, 1991a; Christensen *et al.*, 1992; Refsum *et al.*, 1997). We showed that MTX is an equal (P60 cells) or even stronger (P60H cells) inhibitor of Hcy remethylation than nitrous oxide (fig. 1; table 3). The increased sensitivity to nitrous oxide in Met-dependent P60 cells compared to P60H cells is probably related to low CH<sub>3</sub>Cbl content in P60 cells (Fiskerstrand *et al.*, 1997).

**Effects of MTX and nitrous oxide on Cbl metabolism.** Cbl deficiency eventually leads to deprivation of the intracellular folate pool, in accordance with the folate trap hypothesis (Shane and Stokstad, 1985). The data on the influence of folates on Cbl metabolism are however sparse (Quadros *et al.*, 1976; Quadros and Jacobsen, 1995). We found that MTX reduced the CH<sub>3</sub>Cbl content to the same extent (P60 cells), or more (P60H) than nitrous oxide (table 4). This effect of MTX is probably secondary to insufficient supply of 5-methyl-THF for Hcy remethylation, and adds strong support to the notion (Fiskerstrand *et al.*, 1997) that the amount of cellular CH<sub>3</sub>Cbl reflects the catalytic activity of Met synthase. Our data may therefore provide some insight into the mechanisms behind low serum or erythrocyte Cbl after MTX treatment or during folate deficiency. Leeb *et al.* (1995) demonstrated normal serum Cbl and low Cbl in erythrocytes from patients receiving low dose MTX therapy. A related phenomenon is low serum Cbl in patients with folate deficiency and no overt Cbl deficiency, which was reported by Mollin and Ross in 1957 (Mollin *et al.*, 1962) and confirmed in later studies (Van der Weyden *et al.*, 1972). We recently observed that hyperhomocysteinemic subjects with the C677T mutation in the methylene-THF reductase gene and low plasma folate (*i.e.*, low 5-methyl-THF) often had a low serum Cbl level (Guttormsen *et al.*, 1996).

**MTX effects on Met synthase activity.** We could distinguish two effects of cellular exposure to MTX on enzyme activity in cell free extract.

Conceivably, the decreased level of Met synthase in the presence of MTX (figs. 2 and 3) is induced either by folate depletion (tables 1 and 2; fig. 3) or accumulation of MTX polyglutamates. The former possibility agrees with published reports (Christensen *et al.*, 1992; Quadros and Jacobsen, 1995) demonstrating effects on Met synthase activity from medium folate.

MTX antagonized the enzyme inactivation induced by nitrous oxide. Our data (fig. 3; table 1) suggest that this effect is related to a substantial reduction of 5-methyl-THF. This agrees with a previous observation that inactivation of intracellular Met synthase by nitrous oxide is prevented when the cells are cultured in low folate medium (Christensen *et al.*, 1992). According to the prevailing model (Banerjee and Matthews, 1990), catalytic turnover is a major determinant of the Met synthase inactivation rate, and MTX probably acts by decreasing the availability of 5-methyl-THF and thereby decreases the catalytic turnover of the enzyme.

**Interaction between MTX and nitrous oxide.** Synergism between MTX and nitrous oxide has been reported,

including their effect on folate distribution (Kano *et al.*, 1981; Ermens *et al.*, 1991b), on proliferation of human bone marrow cells (Kano *et al.*, 1981) and on the antileukemic effect in a rat model (Kroes *et al.*, 1986; Abels *et al.*, 1990). However, the synergistic effect of this combination is probably critically dependent on sequence of administration (Ermens *et al.*, 1989a). This may be explained by our data which showed that MTX administered before N2O protected the enzyme from inactivation by nitrous oxide, and in line with this, the Hcy export rate did not increase further by the combination of the two agents, than by MTX given alone.

**Conclusions and perspectives.** The implications of our findings are 3-fold.

MTX inhibits the Met synthase reaction and may therefore have remote effects related to Hcy accumulation as well as Met depletion. Low Met leads to reduced S-adenosylmethionine formation. MTX may in this way inhibit the transmethylation reactions, which may be partly responsible for the antiinflammatory effect of this drug (Cronstein, 1992; Nesher *et al.*, 1991).

MTX severely decreases cellular CH<sub>3</sub>Cbl level, and this may mirror an early event in the development of Cbl deficiency reported in patients with folate deficiency (Mollin *et al.*, 1962) or patients receiving MTX therapy (Van der Weyden *et al.*, 1972). Thus, impaired Cbl function may represent an additional component of MTX pharmacodynamics.

Pretreatment with MTX prevents irreversible inactivation of methionine synthase by nitrous oxide. This may explain published data on the toxicity of the drug combination being dependent on the sequence of the administration (Ermens *et al.*, 1989a), and should guide strategies to obtain synergy or prevent side-effects.

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