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RESPONSE OF THE METHIONINE SYNTHASE SYSTEM TO SHORT-TERM CULTURE WITH HOMOCYSTEINE AND NITROUS OXIDE AND ITS RELATION TO METHIONINE DEPENDENCE

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We compared the metabolic response of a methionine(Met)-dependent (P60) human glioma cell line with that of a Met-independent variant (P60H) when cultured in a homocysteine (Hcy) medium and exposed to N_2O. In Hcy medium (without Met), remethylation of Hcy in P60H cells was enhanced and supported growth, whereas remethylation was low in P60 cells, which failed to thrive under these conditions. Both cell types seemed to contain adequate amounts of folates and total cobalamin (Cbl). P60 cells showed increased total and methylcobalamin (CH_3Cbl) content after the shift to a Hcy medium, but the high, stable level of CH_3Cbl detected in P60H cells was not attained. Further metabolic differences were induced by N_2O exposure, which markedly reduced Met-synthase activity in cell-free extracts in both cell lines and completely blocked intact-cell Hcy remethylation in P60, whereas Hcy remethylation was only partly inhibited in P60H cells cultured in Met medium. The residual Hcy remethylation in P60H cells may be related to only a moderate depletion of CH_3Cbl. The resulting high CH_3Cbl level relative to Met-synthase activity during N_2O exposure was even higher in Hcy medium. These findings in P60H cells probably reflect increased provision of Cbl to support Hcy remethylation under metabolic strain. The inability of P60 to furnish CH_3Cbl to the enzyme may explain both the Met-dependent phenotype and the increased sensitivity of Hcy remethylation to N_2O exposure in these cells.
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Cancer cells grown both *in vivo* (Hoshiya *et al.,* 1995) and *in vitro* (Hoffman, 1985) often have a disturbed metabolism of methionine (Met). This defect becomes evident when cells are grown in a medium devoid of Met but supplemented with homocysteine (Hcy). In such a medium, ''Met-dependent'' cells cease to grow, whereas normal cells thrive. Met dependence, therefore, has been proposed as a target for cancer chemotherapy (Hoffman, 1985).

Met dependence is probably related to an imbalance between Met formation and consumption (Hoffman, 1985). Formation of Met in most cells is catalyzed by Met synthase (5-methyltetrahydrofolate-homocysteine methyltransferase; EC 2.1.1.13.), which requires methylcobalamin (CH_3Cbl) as a co-factor. In this reaction, Hcy receives a methyl group from 5-methyltetrahydrofolate (5-methyl-THF), producing Met and tetrahydrofolate (THF). The Met formed is then used for protein synthesis, adenosylmethionine (AdoMet)-dependent transmethylation reactions and polyamine formation (Finkelstein, 1990).

So far, there are only limited data (Hoffman, 1985) on how the Met-synthase system responds to culture in Hcy medium and the ability of the enzyme to increase the rate of Met formation. The aim of the present study was to compare the response of the Met-synthase system in a Met-independent (P60H) and -dependent (P60) variant of a human glioma cell line, GaMg, under conditions assumed to impose a metabolic strain on the Met-synthase system, *i.e.,* increased flux per unit of enzyme. In addition to culture in Hcy medium, cells were exposed to nitrous oxide (N_2O), which irreversibly inactivates Met synthase (Banerjee and Matthews, 1990).

MATERIAL AND METHODS

Chemicals

The various chemicals used in the cell-culture experiments and in the assays have been described (Fiskerstrand *et al.,* 1994). Cyanocobalamin, folic acid, L-Met, thymidine, hypoxanthine, bovine serum albumin (deficient in cobalamin [Cbl] and Cbl-binding proteins) were from Sigma (St. Louis, MO). L-Hcy thiolactone and bis(3,5,5-trimethylhexyl)phthalate were from Fluka (Buchs, Switzerland). Glacial acetic acid, phosphoric acid, 2-mercaptoethanol (p.a.) and polyvinylpyrrolidone were from Merck (Darmstadt, Germany); methanol (gradient grade) was a product of Labscan (Dublin, Ireland). L-[^{14}C]Hcy thiolactone (56 mCi/mmol), [^{57}Co]cyanoCbl (0.3 Ci/μmol) and (±)-L-5-[methyl-^{14}C]methyl-THF (50 mCi/mmol, barium salt) were purchased from Amersham (Aylesbury, UK). L-5-[3′,5′-7(N)-^{3}H]formyl-THF (25 Ci/mmol) was obtained from Moravek (Brea, CA). A custom-made powdered DMEM, identical to a standard DMEM but without folic acid and Met, was from GIBCO-BRL (Paisley, UK). Hog kidney hydrolase (10 mg protein/ml) was a gift from Dr. Ermens (Erasmus University, Rotterdam, The Netherlands).

Cells

The human glioma cell line GaMg was established in 1984 from a glioblastoma multiforme tumor in a 42-year-old female (Akslen *et al.,* 1988). We have developed a number of variants from this parental line by repeated passages in culture media containing different forms and amounts of folate and Met or Hcy. In this study, 2 variants of GaMg were used. The Met-dependent P60 cells (Fiskerstrand *et al.,* 1994) were obtained by 60 passages in DMEM containing 200 μM Met. During growth in Met medium, P60 has lower levels of CH_3Cbl and adenosylcobalamin (AdoCbl) than Met-independent variants of GaMg (Fiskerstrand *et al.,* 1994). The Met-independent P60H cells (not previously described) have been subjected to 60 passages but have retained the Met independence of the parental cell line through culture in DMEM containing 50 μM of Hcy thiolactone without Met.

Cell-culture conditions and harvesting

Cells were cultured in an atmosphere of 5% CO_2, 95% air at 37°C with relative humidity of 98%. DMEM was used as medium in all experiments. The medium was supplemented with 0.6 g/l L-glutamine, 1.5 μM cyanoCbl, 10 μM folic acid, 5 μM thymidine, 40 μM hypoxanthine, 330 μM of non-essential amino acids, 10% heat-inactivated dialyzed FCS and 50 μM Met (Met medium) or 200 μM Hcy thiolactone (Hcy medium). Stem cultures were kept in Met medium (P60) or Hcy medium (P60H) but with non-dialyzed serum and without thymidine and hypoxanthine. P60H cells were cultured for one passage in Met medium prior to the experiments.

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For all assays, 3 or 4 dishes or medium samples from each group were harvested. Harvesting of cells used in the Cbl assay was performed in dim red light. Medium was stored at −20°C until analysis. Cells were trypsinized (0.1 mg/ml trypsin; Bio-Whittaker, Walkersville, MD) and the trypsin was inactivated with DMEM. Then, cells were washed with PBS and centrifuged (1–4 times), and samples were kept at −80°C until analysis. A Coulter Counter Model ZM (Coulter, Luton, UK) was used for cell counting.

Cell-culture experiments

Met synthase, Hcy export, Hcy incorporation and cellular Cbl. Cells were seeded in Met medium at approx. 1,000 cells/cm^2 in 6-cm dishes (for Met-synthase activity, Hcy export and Hcy incorporation) or 10-cm dishes (for Cbl assay). When cells reached the early log-growth phase, the medium was removed, cells were washed carefully with PBS and then Met or Hcy medium was added. Since Hcy export depends on cell density (Christensen *et al.,* 1991), the medium volume was adjusted so that the cell density was approximately 40,000 cells/ml. Methotrexate (MTX; 1 µM) was added to the Met medium in some Hcy export experiments. P60 cells exposed to MTX required 100 µM thymidine and 80 µM hypoxanthine to support growth. Hcy remethylation in intact cells was performed in Hcy medium using a modification of a method described by Watkins and Rosenblatt (1988). Hcy thiolactone was replaced with [^{14}C]Hcy thiolactone (200 µM), but otherwise the medium was identical to the Hcy medium described above.

After medium change, the dishes were first placed in the incubator for 3 hr, then transferred to modular incubator chambers (Billups-Rothenberg, Del Mar, CA), which were flushed for 10 min with either N_2O (50% N_2O, 25% N_2, 20% O_2 and 5% CO_2) or air (75% N_2, 20% O_2 and 5% CO_2). Gases were moistened by passage through sterile water at 50°C and delivered at a rate of 5 l/min. Chambers were kept at 37°C for the duration of the experiment, and the gas was replaced every 24 hr or after opening of the chamber. For determination of Hcy export rate and Hcy incorporation, cells were harvested immediately before the start and 6, 12, 24, 36, 48 and 72 hr after the start of gas exposure. Cells used for determination of Met synthase and intracellular Cbl were harvested immediately before and 48 hr after the start of gas exposure.

Cellular folate. Cells used for the measurement of folate were initially depleted of folates by culture for about 10 days in a medium with thymidine (5 µM) and hypoxanthine (40 µM) but without added folic acid. Depleted cells were seeded in the same medium at approx. 1,000 cells/cm^2. After 2 days, the medium was replaced with a medium containing 42 nM labeled (5-methyl-THF determination) or unlabeled (total folate determination) 5-formyl-THF. After incubation for 18 hr, this medium was removed and Met or Hcy medium (containing 10 µM of folic acid) was added so that the cell density was approx. 40,000 cells/ml. Cells were harvested immediately before and 48 hr after the start of gas exposure.

Biochemical analyses

Intact cell assay. After thawing, cell pellets were dissolved in 150 µl H_2O. To remove protein-bound [^{14}C]Hcy, the extract was mixed with 150 µl 4 M $NaBH_4$ in 66 mM NaOH containing 33% dimethylsulfoxide, 50 µl 2 mM EDTA and 1.65 mM dithioerythritol, 10 µl 1-octanol and 100 µl 1.8 M/l HCl (Fiskerstrand *et al.,* 1993). After 5 min at room temperature, the protein was precipitated by adding 150 µl of 2 M sulfosalicylic acid. Following centrifugation, the supernatant was discarded and the protein precipitate was resuspended in 5 ml of 0.2 M sulfosalicylic acid. The precipitate was collected on a filter and the radioactivity counted as described (Fiskerstrand *et al.,* 1994).

Met-synthase activity in cell extract. Total Met synthase was determined using a modification (Christensen *et al.,* 1992) of the radioisotope assay developed by Weissbach *et al.* (1963). The assay measures the amount of radioactive Met formed from 5-[^{14}C]methyl-THF and Hcy, and enzyme activity of 1 U represents 1 nmol Met formed per hour.

Hcy determination. Determination of total Hcy was performed in medium from cells cultivated in Met medium. An automated HPLC method developed for the measurement of total Hcy in plasma (Refsum *et al.,* 1989; Fiskerstrand *et al.,* 1993) was optimized for the measurement of Hcy in cell-culture medium (Fiskerstrand *et al.,* 1994).

Intracellular Cbl. Cell extracts were prepared and the different forms of Cbl extracted according to a slight modification (Fiskerstrand *et al.,* 1994) of the method described by Van Kapel *et al.* (1983). Briefly, harvested cells were washed 4 times to remove extracellular cyanoCbl. The forms of Cbl were extracted in a solution containing glacial acetic acid and N-ethylmaleimide by heating at 80°C for 30 min, then the cell debris was removed by centrifugation at 14,000 *g* for 5 min. The supernatant was either used directly for analysis of total Cbl or injected into a reversed-phase column in order to separate the various Cbl forms (Fiskerstrand *et al.,* 1994). Cbl in the supernatant and the eluate fractions was determined using a radioisotope dilution assay (Van Kapel *et al.,* 1983).

Total folate and 5-methyl-THF. Extraction, treatment with hog kidney folylpolyglutamyl hydrolase, HPLC separation and determination of radiolabeled 5-methyl-THF were performed according to a slight modification (Fiskerstrand *et al.,* 1994) of the method described by Ermens *et al.* (1991). Total cell folates were determined with the Quantaphase folate radioassay (Bio-Rad, Hercules, CA), as described (Fiskerstrand *et al.,* 1994).

Hcy export rates and Hcy incorporation rates

The procedure for calculation of Hcy export rates has been published by Christensen *et al.* (1991). This assay is a measure of the amount of Hcy exported per cell per unit time. The amount of Hcy in medium and the cell number are log-transformed, and the values plotted *vs.* time of incubation. Curves are fitted using polynomial functions, and from the equations obtained, the change in Hcy concentration per hour is determined. The Hcy export rates, given in nmol/hr/million cells, were plotted against duration of gas exposure. The Hcy incorporation rate into proteins was determined using the same procedure, by replacing the amount of Hcy in the medium with the amount of [^{14}C]Hcy incorporated into proteins.

RESULTS AND DISCUSSION

Met dependence and growth characteristics

In Met medium, the doubling times of P60H and P60 cells were 28 hr and 18 hr, respectively. In Hcy medium, the growth rate of P60H cells was slightly reduced (doubling time, 38 hr), whereas the P60 cells did not thrive (Fig. 1). These data confirm the Met-dependent phenotype of the P60 cell line (Fiskerstrand *et al.,* 1994) and demonstrate the Met independence of P60H.

Flux of Hcy in intact cells

The level of Hcy in extracellular fluids is an established marker of Cbl and folate function, in both patients and cell-culture models (Ueland *et al.,* 1993). Since the amount of Hcy released from cells depends on the number of cells and the duration of the experiment, we prefer to study the export rate, *i.e.,* the amount of Hcy exported from one million cells per hour (Christensen *et al.,* 1991), rather than the medium concentration of Hcy. We assume that an increase in export rate indicates that Hcy remethylation becomes inhibited (Christensen *et al.,* 1992).

Hcy flux in Met medium. In Met medium, the maximal Hcy export rate of the Met-independent P60H cells increased from 0.26 to 0.51 nmol/hr/10^6 cells during N_2O exposure (Fig. 2, Table I). However, the export rate was further increased to 0.83 nmol/hr/10^6 cells by MTX (Table I), which probably inhibits Hcy remethylation through depletion of reduced folates (Bunni *et al.,* 1988). This unexpected finding suggests that Hcy export in P60H cells is only partly inhibited by N_2O. In P60, the basal Hcy export rate in Met medium was high (0.73 nmol/hr/10^6 cells) and increased to about 1

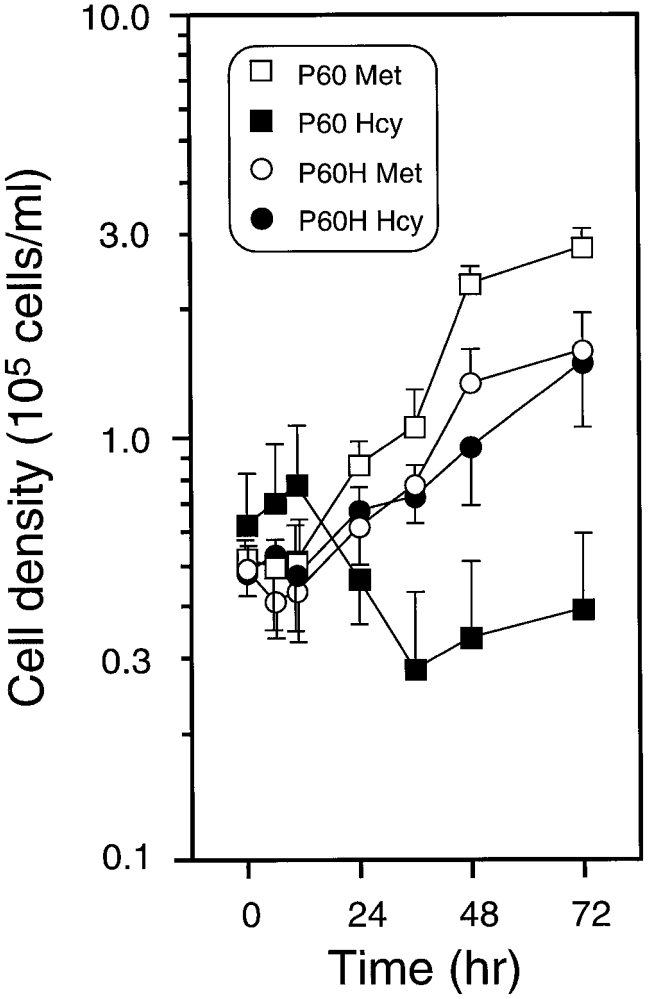

FIGURE 1 – Growth of GaMg human glioma variants P60 and P60H in Met medium (containing 50 μM Met) and in Hcy medium (200 μM Hcy thiolactone) devoid of Met. Data are means of 3 experiments ± SD.

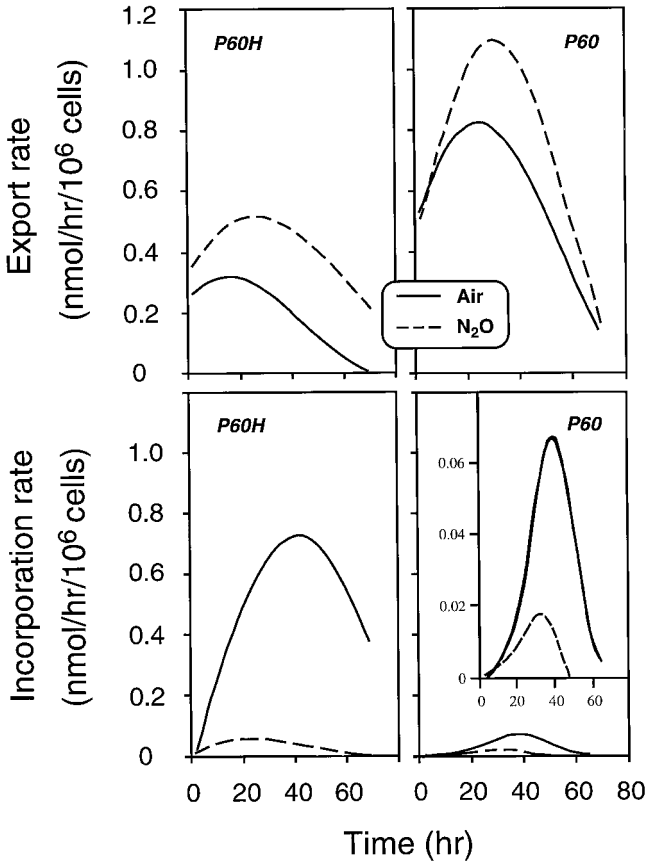

FIGURE 2 – Hcy export rates (top) and incorporation rates (bottom) of GaMg human glioma variants P60 and P60H in the absence and presence of N_2O. Cells were exposed either to air (solid line) or to N_2O (broken line), as described in "Material and Methods". The experiment was repeated 3 or 4 times, and the data shown are typical.

nmol/hr/10^6 cells during N_2O exposure alone or combined with MTX (Fig. 2, Table I). These data indicate that P60 cells have a lower Hcy remethylation rate than P60H cells. Moreover, in P60, N_2O seems to completely inhibit Hcy remethylation. These findings point to different responses of the 2 cell lines to N_2O of the Met-synthase system.

Hcy flux in Hcy medium. To study remethylation in Hcy medium, we measured incorporation of radioactivity into proteins of cells incubated with [^{14}C]Hcy thiolactone. We and others (Watkins and Rosenblatt, 1988; Fiskerstrand *et al.,* 1994) have used a similar assay for estimation of intact-cell remethylation, based on incorporation of radioactivity derived from 5-[^{14}C]methyl-THF. We prefer the assay using [^{14}C]Hcy because the medium used was identical to the Hcy medium. Moreover, the values obtained were almost 100-fold higher than the incorporation from 5-[^{14}C]methyl-THF in otherwise identical conditions (data not shown). This is explained by the fact that the specific activity of intracellular 5-[^{14}C]methyl-THF declines due to dilution with unlabeled 5-methyl-THF formed by regeneration.

Figure 2 demonstrates that P60H cells incorporated [^{14}C]Hcy at a high rate (0.73 nmol/hr/10^6 cells). This is an under-estimate of Hcy remethylation since AdoMet-dependent transmethylation is not accounted for, but it actually exceeded the remethylation in Met medium, as measured by the increase in Hcy export rate induced by N_2O and MTX (0.57 nmol/hr/10^6 cells) (Table I). In contrast, the Met-dependent P60 cells incorporated only trace amounts (0.07 nmol/hr/10^6 cells) (Fig. 2, Table I), consistent with low Hcy remethylation in these cells. Thus, compared with Met medium, culture in Hcy medium more clearly demonstrates a defect in Hcy remethylation of P60 cells relative to P60H cells.

Folate status

In Met medium, P60H and P60 cells contained essentially the same amount of total folate (Fig. 3). In P60H cells, 5-methyl-THF accounted for 60% of total folate, whereas the fraction was lower (30%) in P60 cells. Notably, in both cell lines, 5-methyl-THF was the major folate species (data not shown), an observation that has been made in cells cultured in folic acid-rich media (Bunni *et al.,* 1988; Ermens *et al.,* 1991).

Both cell lines responded to the Hcy medium and N_2O exposure (in both medium types) with an increase in 5-methyl-THF. In P60, total folate also increased in response to Hcy (Fig. 3). A possible explanation for the high 5-methyl-THF in Hcy medium is stimulation of methylene-THF reductase by low AdoMet (Finkelstein, 1990). In addition, N_2O may increase the 5-methyl-THF level according to the methylfolate-trap hypothesis (Finkelstein, 1990). Regardless of the mechanism, the high 5-methyl-THF level observed in both P60H and P60 cells during culture in Hcy medium suggests sufficient supply of this co-substrate for the Met-synthase reaction, and lack of 5-methyl-THF seems an unlikely cause of Met dependence in P60 cells.

TABLE I – FLUX OF HCY IN GLIOMA CELLS

	P60H cells (nmol/hr/10^6 cells)		P60 cells (nmol/hr/10^6 cells)	
	Hcy export	HCY incorporation	Hcy export	Hcy incorporation
Air	0.26 ± 0.08	0.73 ± 0.13	0.73 ± 0.13	0.07 ± 0.03
N_2O	0.51 ± 0.15	0.08 ± 0.02	0.97 ± 0.14	0.02 ± 0.01
N_2O + MTX	0.83 ± 0.08		0.92 ± 0.20	
N_2O-dependent increase[1]	0.25		0.24	
N_2O + MTX-dependent increase[2]	0.57		0.19	

Cells were grown in Met medium (Hcy export) or Hcy medium (Hcy incorporation), exposed to air or N_2O and harvested after 6, 12, 24, 36, 48 and 72 hr. In one set of experiments, cells grown in Met medium were exposed to 1 μM MTX. The export and incorporation curves were constructed as described in ''Material and Methods''. The maximum export and incorporation rates were determined from the graphs. Data from 3 or 4 experiments are given as mean ± SD.–[1]Difference in export rate between cells exposed to N_2O and cells exposed to air.–[2]Difference in export rate between cells exposed to N_2O + MTX and cells exposed to air.

Met synthase in cell-free extracts and changes in Cbl status

Met-synthase activity in cell-free extracts is determined in the presence of a reducing system and high concentrations of Cbl, Hcy and 5-methyl-THF (Weissbach *et al.,* 1963). In Met medium, the Met-synthase activity in P60 cells was 50% lower than in P60H cells (Fig. 3). This could explain the difference in Hcy remethylation in these 2 variants, but we and others have shown that the standard Met-synthase assay possibly reflects the cellular concentration of total enzyme but not the catalytic turnover in intact cells (Rosenblatt *et al.,* 1984; Fiskerstrand *et al.,* 1994). This hypothesis is supported by the finding of increased Hcy remethylation despite decreased Met-synthase activity in P60H cells transferred to Hcy medium (Fig. 3, Table I).

Met synthase was maximally inactivated by N_2O within 24 hr in both cell lines irrespective of culture medium (data not shown). There was a residual activity of 17–40%, with the highest remaining activity in P60 cells in Hcy medium. Residual activity of 10–30% after N_2O exposure has been observed *in vivo* (Kondo *et al.,* 1981), in cell cultures (Fiskerstrand *et al.,* 1994; Christensen *et al.,* 1992; Rosenblatt *et al.,* 1984) and *in vitro* (Drummond and Matthews, 1994).

The apparent paradox that Hcy remethylation in P60H cells in Met medium is only partly inhibited by N_2O despite marked inactivation of Met synthase (Table I, Figs. 2, 3) points to higher catalytic activity of the remaining enzyme. A possible mechanism is that a higher fraction of Met synthase has bound Cbl, *i.e.,* that it exists as a holo-enzyme. However, holo-enzyme activity, determined by excluding Cbl from the assay, did not correlate to Hcy remethylation in intact cells (data not shown). Notably, the holo-enzyme exists with various oxidation states of Cbl, of which only CH_3Cbl can methylate Hcy in the intact cell (Chen *et al.,* 1995). We therefore measured cellular forms of Cbl and calculated the amount of CH_3Cbl per enzyme unit. We found that >90% of CH_3Cbl is protein-bound (data not shown). The level of CH_3Cbl may reflect the catalytic capacity of intracellular Met synthase and the ratio between CH_3Cbl and (total) enzyme activity, the functional state of the enzyme.

In P60H cells cultured in Met or Hcy medium, total Cbl was essentially the same, and CH_3Cbl accounted for 10–16% of total Cbl (Fig. 3). There was a slight reduction of both CH_3Cbl and Met-synthase activity when these cells were transferred to Hcy medium, but the concomitant increase in Hcy remethylation (Table I) suggests the presence of excess functional holo-enzyme. Irrespective of medium type, N_2O exposure was associated with a reduction in the CH_3Cbl level, a finding which should be related to inactivation of Met synthase and consequent loss of CH_3Cbl (Kondo *et al.,* 1981). However, compared with the degree of enzyme inactivation, the CH_3Cbl level remained high, especially in Hcy medium (Fig. 3). These data may indicate that P60H cells, in conditions that impose a strain on Met synthase, convert a higher fraction of the enzyme into a functional holo-enzyme in order to maintain Hcy remethylation.

The changes in Cbl status of the P60 cells were markedly different. Total Cbl content in the Met medium was about the same as in its Met-independent counterpart, P60H, but, as reported (Fiskerstrand *et al.,* 1994), P60 contained only trace amounts of CH_3Cbl. Notably, there was a massive increase in total Cbl content when these cells were transferred to a medium containing Hcy. This was associated with an increase in the level of CH_3Cbl, but the level was still markedly lower than in P60H cells. In response to N_2O, the level of CH_3Cbl (Fig. 3) was reduced proportionally to the decline in Met-synthase activity.

Thus, there are 2 marked differences in the Met-synthase system of these GaMg variants. First, in Met medium, P60H has a higher CH_3Cbl level than P60. This may explain why P60H cells efficiently remethylate Hcy and, therefore, can be transferred to Hcy medium without the pronounced metabolic changes observed in P60 cells. Second, the ability to increase the level of CH_3Cbl per unit enzyme is substantial in P60H but almost absent in P60. This probably represents a metabolic advantage for the Met-independent variant during conditions when the enzyme is strained.

Hcy medium and Cbl metabolism

Our data suggest that the transfer from Met to Hcy medium can affect Cbl uptake and distribution (Fig. 3). This is supported by a consistent increase in AdoCbl content in P60H after replacing Met with Hcy, which became more pronounced in the presence of N_2O (Table II). The Cbl co-factors have a common route of synthesis (Shevell and Rosenblatt, 1992), and the increase in AdoCbl combined with the ability to increase the level of CH_3Cbl per unit enzyme under metabolic strain (Hcy and N_2O) (Fig. 3) point to a higher flux of Cbl through this pathway. These data indicate that high cellular Hcy or low Met may activate processes involved in the regulation of Cbl trafficking. This mechanism may not operate properly in P60 cells, which respond to strain on Met synthase by increased total Cbl but minimal changes in CH_3Cbl or AdoCbl (Fig. 3 and Table II).

N_2O effect, Met dependence and Cbl metabolism

Our data suggest that Hcy remethylation in the Met-independent cell line P60H in Met medium is partly resistant to inhibition by N_2O, whereas Hcy remethylation in P60 is almost completely blocked. Some studies also suggest that there is a stronger effect of N_2O in Cbl deficiency and in malignant cells. Met synthase has been shown to be inactivated faster by N_2O in Cbl-deficient patients (Landon *et al.,* 1992). In cancer cells, which are often Met-dependent (Hoffman, 1985) and may have a disturbed Cbl metabolism (Liteplo, 1990), N_2O causes growth inhibition of some, but not all, cell types (Kano *et al.,* 1983). Ermens *et al.* (1989) have shown in a rat model that N_2O is more toxic to leukemic than to

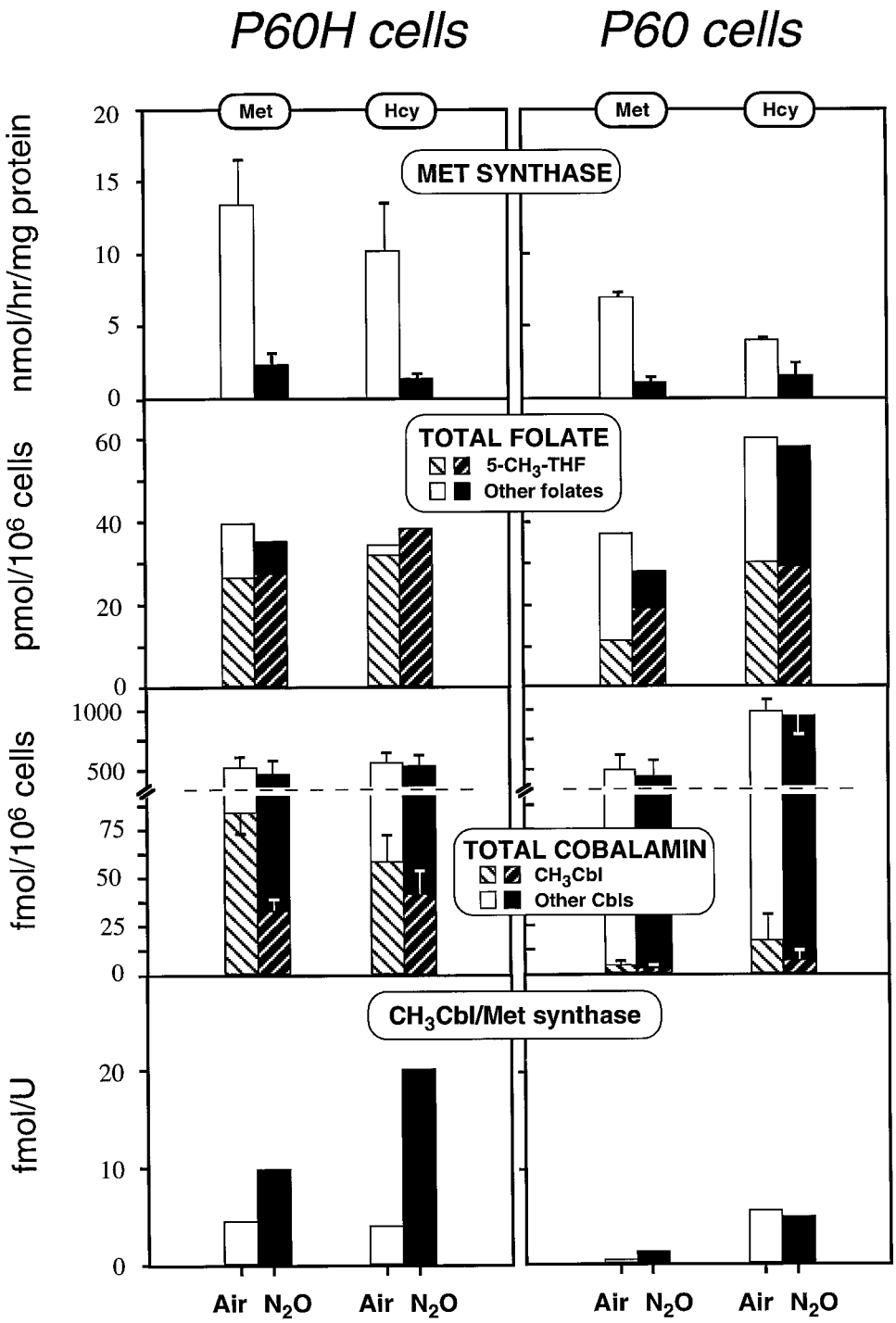

FIGURE 3 – Met-synthase activity and its relation to the distribution of folates and cobalamins in P60H and P60 cells. Cell lines were exposed to N_2O or transferred to Hcy medium or both. Culture in Hcy medium and/or N_2O exposure was associated with lower cell number and low cobalamin, especially in P60, and in these experiments the number of dishes harvested was routinely increased to obtain detectable levels of enzyme activity and cobalamin. (The data are presented as mean values of either 2–3 or 3–4 parallel experiments; bars indicate SD.)

normal bone-marrow cells, and clinical studies in the 1960s showed that N_2O could induce remission in patients with leukemia (Eastwood *et al.*, 1963). One possible explanation for the differential effect of N_2O on various cell types is that susceptible cells with deficient or disturbed Cbl function, such as the Met-dependent P60 cells in this study, are unable to circumvent the effect of N_2O on Met synthase by increasing the level of functional holo-enzyme. This also may explain the observation in humans and in rats that adverse effects of N_2O are more pronounced in Cbl deficiency (Louis-Ferdinand, 1994; O'Leary *et al.*, 1985).

TABLE II – INTRACELLULAR CONTENT OF CH_3Cbl AND AdoCbl

Culture conditions		P60H cells (fmol/10^6 cells)		P60 cells (fmol/10^6 cells)	
		CH_3Cbl	AdoCbl	CH_3Cbl	AdoCbl
Met	Air	83 ± 17	97 ± 27	2 ± 3	17 ± 7
Met	N_2O	32 ± 6	66 ± 12	1 ± 1	12 ± 8
Hcy	Air	57 ± 21	123 ± 27	17 ± 18	20 ± 12
Hcy	N_2O	41 ± 17	144 ± 66	6 ± 7	18 ± 13

Cells were cultured in Met or Hcy medium and harvested after exposure to air or N_2O for 48 hr. All values are means of 3 or 4 experiments.

CONCLUSION

Culture in Hcy medium and/or exposure to N_2O induce changes in Cbl and folate metabolism. The Met-independent P60H cells have higher Hcy remethylation in Hcy medium than in Met medium, possibly due to increased provision of Cbl for co-factor synthesis. In P60 cells, the low cellular level of CH_3Cbl and limited ability to increase CH_3Cbl per unit Met synthase probably explain both the observed Met dependence and the efficient inhibition of Hcy remethylation by N_2O. A possible role of Hcy or low Met in the regulation of Cbl metabolism deserves further investigation.

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