[CANCER RESEARCH 54, 4899–4906, September 15, 1994]

Development and Reversion of Methionine Dependence in a Human Glioma Cell Line: Relation to Homocysteine Remethylation and Cobalamin Status[1]

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ABSTRACT

We investigated the biochemical changes which accompanied the development and reversion of methionine dependence in a human glioma cell line GaMg. This cell line attained a higher proliferation rate and more malignant morphology with increasing passages *in vitro*. Early passages (P10, P25, and P45) were able to grow in a methionine-deficient medium supplemented with homocysteine (Met^-Hcy^+), while a later passage (P60) had lost this ability, *i.e.*, it had become methionine-dependent. From P60 cells, a methionine-independent revertant (P60R) was established by exposing the cells to 5-aza-2-deoxycytidine, followed by culture in a Met^-Hcy^+ medium. In these genetically related cell lines, we investigated homocysteine remethylation and the functional state of cobalamin-dependent methionine synthase, the enzyme responsible for remethylation of homocysteine to methionine. The methionine synthase activity in cell extracts was similar in all cell sublines. Intact cell methionine biosynthesis and nitrous oxide-dependent homocysteine export reflect homocysteine remethylation in cells cultured in a Met^-Hcy^+ and methionine-containing (Met^+Hcy^-) medium, respectively. Both of these parameters, as well as the cellular content of the substrate 5-methyltetrahydrofolate, and the cofactor methylcobalamin, in addition to adenosylcobalamin, were high in P10, declined progressively in P45 and P60, and were restored in P60R. P25 cells had some unique features among the methionine-independent phenotypes because both homocysteine remethylation and the level of 5-methyltetrahydrofolate were low in Met^+Hcy^- medium. The maximal homocysteine export rate in the presence of nitrous oxide, which reflects the overall transmethylation rate, was high in P60 and even higher in P60R compared to the lower passages. The basis for development of methionine dependence during culture of this glioma cell line seems related to the combined effects of reduced methionine biosynthesis and an increased overall transmethylation rate. The single parameter which most closely correlated to the ability to use homocysteine for growth was methylcobalamin. These data support a model for methionine dependence, which implies impaired provision of cobalamin to methionine synthase.

INTRODUCTION

Met[3] is used for protein synthesis or is activated to AdoMet, which in turn functions in numerous AdoMet-dependent transmethylation reactions and serves as the aminopropyl donor in polyamine synthesis. Mammalian cells cannot use cysteine as a Met precursor; therefore, Met is an essential amino acid. However, in experiments with isolated cells as well as animal and human studies, the Met requirement can be met by supplying its immediate precursor, Hcy (Fig. 1; Ref. 1).

Met dependence denotes the inability of cells to grow *in vitro* when Met is replaced with Hcy in the culture medium (2, 3). The phenomenon has been demonstrated in a large number of malignant cell lines and in primary cultures of some rodent malignant tumors (3), and recently, also in 5 of 21 primary histocultures of human malignant tumors (4). With few exceptions (3), normal cells or tissues are Met independent (5). Therefore, Met dependence has been exploited in the design of experimental chemotherapeutic regimens (6–8).

Met dependence and its reversal in cancer cells has been studied in relation to characteristics of the malignant state (3, 9, 10). There is evidence that Met dependence occurs more frequently in highly metastatic cells (6, 11), but the reversion of Met dependence is not inevitably accompanied by loss of metastatic potential or the reversion to more benign phenotypes (9, 12).

The enzyme converting Hcy to Met, methionine synthase (5-methyltetrahydrofolate-homocysteine methyltransferase; EC 2.1.1.13), is a conceivable site of defect in Met-dependent cancer cells (13). This enzyme catalyzes a cobalamin-dependent reaction where remethylation of Hcy to Met is coupled to the formation of THF from 5-methyl-THF (Fig. 1; Ref. 1). Impaired function of methionine synthase has been demonstrated in some malignant cells (11, 13). However, several Met-dependent cells have high activity of methionine synthase, which has been determined both in cell free extract (14) as well as in intact cells incubated with high levels of Hcy (3, 15). Therefore, numerous studies have focused on impaired use of Met (3, 16, 17) or alterations in Met-dependent processes (18) as possible causes of Met dependence of cancer cells. Among these theories, an increased demand for AdoMet due to elevated overall transmethylation rate in Met-dependent cancer cells (3, 19, 20) has gained considerable support. More recent studies have shown that Met-dependent cells may have increased loss or disturbed use (11, 21) of cobalamins, emphasizing the importance of the methionine synthase reaction.

In the present study, we investigated five variants of the human glioma GaMg cell line. Preliminary experiments showed that this cell line was Met-independent at passage 10 but Met-dependent at passage 60. From the Met-dependent variant, we established a Met-independent revertant, P60R. With this panel of genetically related cell sublines, we investigated the relation between progression of the cell line, Met dependence, and the functional state of methionine synthase. The enzyme function was assessed by determination of intact cell remethylation, Hcy export rate, enzyme activity in cell-free extract, and the cellular content and distribution of cobalamin and folate. In some experiments, nitrous oxide was used as a pharmacological tool to inactivate methionine synthase (Fig. 1; Ref. 22).

MATERIALS AND METHODS

Chemicals

L-Met, cyanocobalamin, DL-5-methyl-THF (barium salt), dithioerythritol, and bovine serum albumin (deficient in cobalamin and cobalamin-binding

Received 5/9/94; accepted 8/4/94.

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[1] This work was supported by grants from the Norwegian Cancer Society and the Norwegian Research Council.

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[3] The abbreviations used are: Met, methionine; AdoMet, *S*-adenosylmethionine; Hcy, homocysteine; THF, tetrahydrofolate; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography; cblC, cblD, cblE and cblG mutants, cobalamin mutants of complementation classes C, D, E, and G, respectively; Met^-Hcy^+ medium, methionine-deficient cell culture medium supplemented with homocysteine; Met^+Hcy^- medium, cell culture medium containing methionine but not homocysteine.

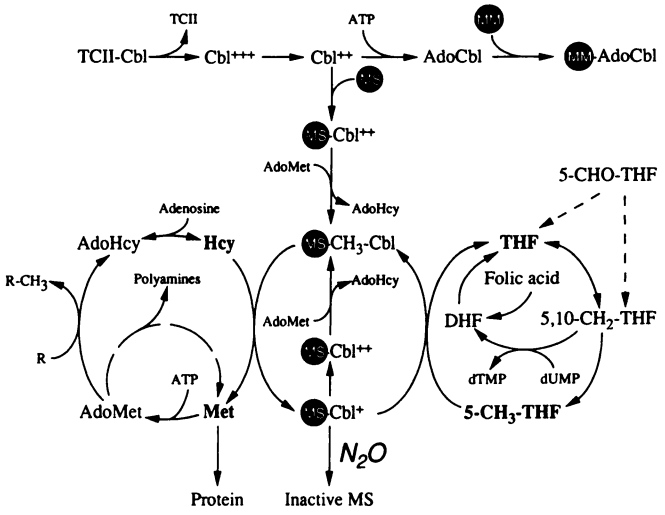

Fig. 1. Metabolism of substrates and cofactor involved in the methionine synthase reaction. *TCII*, transcobalamin II; *Cbl*, cobalamin; *AdoCbl*, adenosylcobalamin; *CH_3-Cbl*, methylcobalamin; *MM*, methylmalonyl CoA mutase; *MS*, methionine synthase; *AdoMet*, *S*-adenosylmethionine; *AdoHcy*, *S*-adenosylhomocysteine; *Hcy*, homocysteine; *Met*, methionine; *5-CHO-THF*, 5-formyltetrahydrofolate; *5-CH_3-THF*, 5-methyltetrahydrofolate; *5,10-CH_2-THF*, 5,10-methylenetetrahydrofolate; *THF*, tetrahydrofolate; *DHF*, dihydrofolate.

proteins) were obtained from Sigma Chemical Co. (St. Louis, MO). L-Hcy thiolactone, sodium borohydride, Epon 812, and bis(3, 5, 5-trimethylhexyl) phthalate were from Fluka Chemie AG, Buchs, Switzerland. 2-Mercaptoethanol (p.a.), methanol (gradient grade), polyvinylpyrrolidone, glutaraldehyde, and toluidine blue were from Merck (Darmstadt, Germany). Tetrabutylammonium hydroxide was from Janssen Chimica (Geel, Belgium). (±)-L-5-[*methyl*-^{14}C]methyl-THF (50 mCi/mmol; barium salt) and [^{57}Co]cyanocobalamin (0.3 Ci/μmol) were purchased from Amersham International (Buckinghamshire, United Kingdom). L-5-[3',5'-7(N)-^{3}H]formyl-THF (50 Ci/mmol) was delivered by Moravek Chemicals, Inc. (Brea, CA). DMEM was from GIBCO-BRL (Paisley, Scotland). Hog kidney hydrolase (10 mg protein/ml), prepared according to McMartin *et al.* (23), and salivary R-binder (24) were gifts from Drs. Ermens and van Kapel, both at Erasmus University, Rotterdam.

Cells

The GaMg cell line was established in 1984 from a glioblastoma multiforme tumor in a 42-year-old female (25). Variants of this cell line were obtained by repeated passages in DMEM containing 10% heat-inactivated newborn calf serum. The cell sublines were termed P10, P25, P45, and P60 according to the number of passages. From the Met-dependent variant P60, a Met-independent revertant (P60R) was established as follows: P60 cells were exposed to 5-aza-2-deoxycytidine (10 μM) for 2 days followed by culture in a Met-deficient DMEM supplemented with Hcy thiolactone (100 μM) for 2–3 weeks. The few cells that survived were cultured to mid-exponential growth phase in a Met-supplemented medium. Then the treatment with 5-aza-2-deoxycytidine (100 μM) was repeated twice. After 2 months of culture in a Met-deficient medium supplemented with Hcy thiolactone, the cells were able to proliferate.

Cell Culture Conditions, Cell Growth, and Harvesting

Cells were cultured in a custom-made DMEM (GIBCO-BRL), delivered as powder and identical in composition to the ordinary DMEM, except that it was without folic acid and Met. When not otherwise indicated, the medium (Met^+Hcy^-) contained 0.6 g/liter L-glutamine, 1.5 μM cyanocobalamin, 10 μM folic acid, and 100 μM Met and was supplemented with 10% heat-inactivated fetal calf serum and 330 μM of nonessential amino acids. The revertant P60R was maintained in a DMEM with the same composition, except that Met was replaced with 100 μM of Hcy thiolactone, and fetal calf serum was dialyzed against PBS for 24 h (Met^-Hcy^+ medium). The cells were cultured in an atmosphere of 5% CO_2/95% air at 37°C. The relative humidity was 98%.

Nitrous Oxide Exposure. Exposure of cells to air (controls) or nitrous oxide was performed by placing flasks or dishes in two modular incubator chambers (Billups-Rothenberg, Del Mar, CA). One chamber was flushed with nitrous oxide (50% N_2O, 25% N_2, 20% O_2, and 5% CO_2) and the other chamber with air (75% N_2, 20% O_2, and 5% CO_2). Both gases were moistened by passage through sterile water at 50°C and delivered at a rate of 5 liters/min for 10 min. The chambers were then kept at a temperature of 37°C.

Met Dependence. To assess the Met dependence, 10,000 cells were seeded in 3.5-cm dishes containing 3 ml Met^-Hcy^+ medium or Met^+Hcy^- medium, both supplemented with 10% of dialysed fetal calf serum. The growth was followed until maximal cell density was obtained in the Met^+Hcy^- medium.

Hcy Export and Methionine Synthase Activity. The Hcy export rate from cells was determined in a DMEM containing 50 μM Met. The cells were seeded in 25-cm^2 flasks (Nunc, Roskilde, Denmark) containing 10 ml medium and allowed to grow until mid-exponential phase. Then the medium was replaced with 5 ml fresh medium, and the flasks were placed in the incubator chambers and flushed with air or nitrous oxide. The cells were harvested after 0, 1.5, 3, 6, 12, 24, 36, and 48 h of gas exposure.

Folate Measurements. Cells were cultured to late exponential growth phase in a DMEM with 10 μM folic acid and then harvested for determination of total cellular folates. Alternatively, the cells were depleted of folate by culture for 14 days in a medium without folate but supplemented with hypoxanthine (40 μM) and thymidine (5 μM). Folate-depleted cells in mid-exponential growth phase were exposed for 24 h to fresh medium (2 ml) containing 42 nM L-5-[^{3}H]formyl-THF (50 Ci/mmol). Then the cells were transferred to a standard DMEM (containing 10 μM unlabeled folic acid as folate source). After 48 h of incubation to allow equilibration of the folate pool, the medium was collected, and cells were harvested for determination of folates.

Harvesting. Samples of medium and cells were harvested from 2–3 parallel flasks in each group. Medium was stored at −20°C until analysis. Cells were brought into suspension by treating with trypsin (0.1 mg/ml; Bio Whittaker, MD) for 10 min; then the trypsin was inactivated with fresh medium. Finally, the cells were washed in PBS and centrifuged. The cell pellet was stored at −80°C until assayed for methionine synthase. Cell count was determined using a Coulter Counter Model ZM (Coulter Electronics Ltd., Luton, United Kingdom).

Invasion Assay. Glioma cell invasion into fetal brain cell aggregates was investigated in a three-dimensional cell coculture system. Rat brain aggregates were prepared from brains of rat fetuses. During culturing, the aggregates mature and then resemble normal adult rat brain tissue (26). Mature aggregates (150–200 μm in diameter) were confronted with tumor spheroids (150–200 μm) in a 96-multiwell dish for 96 h. The cocultures were then fixed for light microscopic examination and photography (26).

Biochemical Analyses

Intact Cell Assay. This assay for Met synthesis was performed by a modification (27) of the method developed by Watkins and Rosenblatt (28). The assay measures the incorporation of [^{14}C] methyl groups derived from 5-[^{14}C]methyl-THF into proteins of cells exposed to nitrous oxide or air for 18 h in a medium containing 0.2 mM L-Hcy thiolactone and 5 μM 5-[^{14}C]methyl-THF but lacking Met and folic acid.

Hcy Determination. Hcy in the medium was measured by a modification of an automated method developed for the determination of total Hcy in plasma (29, 30). The method includes reduction of all Hcy species with borohydride, followed by derivatization of reduced Hcy with monobromobimane and quantitation of the Hcy-bimane adduct by HPLC and fluorescence detection. The procedure was optimized for determination of total Hcy in culture medium by adding more concentrated monobromobimane (0.1 M) in a smaller volume (5 μl) and by increasing the injection volume from 20 to 100 μl. The within-day coefficients of variation (n = 10) of this modified version were 3.6, 1.2, 1.2, and 1.1% at Hcy concentrations of 0.4, 1.6, and 5.7 and 11.1 μM, respectively.

Methionine Synthase Activity. Determination of methionine synthase activity was performed by a modification (31) of the radioisotope assay described by Weissbach *et al.* (32). The assay measures the amount of radioactive Met formed from 5-[^{14}C]methyl-THF and Hcy.

Intracellular Cobalamins. Intracellular cobalamins were extracted using a slight modification of the method described by van Kapel *et al.* (24). Cells (2–6 × 10^6) from mid-exponential growth phase were trypsinized, washed four times with PBS containing albumin (0.1 mg/ml), and resuspended in

300–700 μl PBS. From this cell suspension, an aliquot of 160 μl was transferred to a 2-ml polypropylene screw-cap micro tube (Sarstedt, Nümbrecht, Germany) and mixed with 8 μl glacial acetic acid and 8 μl 1 M *N*-ethylmaleimide. The tube was then sealed and stored at −80°C until analysis. The cobalamins were extracted by diluting the thawed sample with distilled water to a final volume of 800 μl, followed by heating at 80°C for 30 min, and the insoluble cellular debris was removed by centrifugation at 1700 × *g* for 10 min at 4°C.

We slightly modified a previously described HPLC method (33) in order to obtain separation of methyl- and adenosylcobalamin from the other cobalamin forms. Four hundred μl of cell extract were injected into a 10 cm/3 μm octadecylsilane Hypersil column equilibrated with 50 mM sodium phosphate buffer, pH 3.0. The column was eluted at ambient temperature with a linear acetonitrile gradient (5–30% in 13.8 min). The flow rate was 1.3 ml/min. The retention times for the cobalamin standards were 10.5 min (hydroxycobalamin), 12 min (cyanocobalamin), 14 min (adenosylcobalamin), and 16 min (methylcobalamin). The column eluate was fractionated (fractions of 260 μl; 0.2 min) and collected into 5 ml polypropylene centrifuge tubes (Sarstedt) containing 25 μl 16.5% polyvinylpyrrolidone, using a Foxy model 200 automatic fraction collector.

Cobalamins in cell extract (100 μl) and HPLC fractions (285 μl) were determined by a radioisotope dilution assay developed by van Kapel *et al.* (24) using salivary R-binder as binding protein.

Extraction, Separation, and Determination of Radiolabeled 5-Methyl-THF. This was performed essentially as described by Ermens *et al.* (34). Cells ($1–2 \times 10^6$) precultured with radioactive 5-formyl-THF were trypsinized and then washed twice with PBS and albumin (0.1 mg/ml). The cell pellet was homogenized in 600 μl solution of 2% mercaptoethanol and 2% ascorbic acid (pH 6.0) supplemented with unlabeled folates (including 5-methyl-THF and 5-formyl-THF); the extract was heated for 5 min at 80°C and then cooled on ice for 5 min. Thereafter, folylpolyglutamate hydrolase (35 μl) was added to the extract, which was incubated for 1 h at 37°C, followed by heat-inactivation of the enzyme at 80°C for 5 min, cooling on ice for 5 min, and finally centrifugation for 5 min at 10,000 × *g*.

Culture medium (8 ml) was supplemented with mercaptoethanol and ascorbic acid (final concentrations, 2%); the medium was heated for 5 min at 80°C, and then centrifuged at 10,000 × *g* for 5 min. The supernatant was subjected to solid-phase extraction on 500 mg Bond Elute C_{18} columns (Varian, Harbor City, CA) equilibrated with 10 mM phosphate buffer containing 10 mM tetrabutylammonium hydroxide (pH 5.5). The column was washed with 10 ml of water, and the folates were eluted with 50% acetonitrile. The eluate was evaporated to dryness and dissolved in 500 μl of a solution containing 2% mercaptoethanol and 2% ascorbic acid.

Cell extract and solid-phase extract of medium were subjected to HPLC using a modification of the method described by Ermens *et al.* (34). A volume of 400 μl was injected into a 10 cm/3 μm octadecylsilane Hypersil column equilibrated with 10 mM phosphate buffer containing 10 mM tetrabutylammonium hydroxide (pH 5.5). The folates were eluted by a nonlinear methanol gradient (12.5–27.5% in 35 min) at a flow rate of 1.5 ml/min. 5-Formyl-THF and 5-methyl-THF had retention times of 30 and 36 min, respectively. The whole eluate was collected into fractions of 750 μl using the Foxy model 200 fraction collector, and the radioactivity was determined by scintillation counting.

Amount of intracellular 5-methyl-THF was calculated from the total amount of cellular folate, based on the assumption that the radioactive tracer was equilibrated with all cellular folate species.

Determination of Total Cellular Folate. Cells ($1–6 \times 10^6$) were trypsinized and washed twice with serum-free medium without folic acid. The cell pellet was resuspended in 300 μl PBS, and then 100 μl was mixed with 500 μl 0.4% ascorbic acid and stored at −20°C until analysis of folate. The folate content was determined by the Quantaphase folate radioassay produced by Bio-Rad (Hercules, CA). The assay pH was adjusted to 9.2 so that folic acid and 5-methyl-THF could be measured equally (35).

Calculation of Export Rates

The Hcy export rate is a measure of the amount Hcy released from one million cells/h. The logarithm of the cell number (n) and the logarithm of the amount of Hcy accumulated in the medium (y) were plotted *versus* time (t) of incubation, and the curves were fitted to polynomial functions. The Hcy export rate (v) is obtained by calculating the change in Hcy concentration (Δy) during a short time interval (Δt), usually 1 h. The equation for v is:

$$v = \Delta y/\Delta t/n$$

n is the mean cell number during Δt:

$$n = \frac{n_t + n_{t-\Delta t}}{2}$$

The export rates were plotted against cell density. Details on the curve fitting and construction of export rate curves have been published (36).

RESULTS

Characteristics of Cell Sublines. The morphology changed gradually as the cells progressed in culture (Fig. 2, *left panel*). The early passages (P10 and P25) were long, bipolar cells with fibroblast-like appearance, which coalesced to form tightly packed monolayers. With increasing passage number, cellular processes and the cytoplasmic: nuclear ratio were reduced, the cells became hyperchromatic and epitheloid-like, and the P60 cells grew in multilayer aggregates. The revertant P60R attained a morphology resembling that of the earlier passages (Fig. 2, *left panel*).

All variants, including the revertant P60R, were invasive when tested in a coculture confrontation system (Fig. 2, *right panel*). The invasion of the early passages, P10 and P25, was characterized by intermingling of tumor cells with the brain tissue, and the confrontation with the P10 cells was leaving only a thin brim of intact brain aggregate. The higher passages and P60R showed a different invasion pattern. No single tumor cells were found in the brain tissue. There was a diffuse confrontation zone with dissolution of the brain tissue, and the tumor cells reached the cell-rich core of the brain aggregate (Fig. 2, *right panel*).

The growth rate increased with increasing passage number. In a Met^+Hcy^- medium, the doubling time was about 41 h for P10, 27 h for P25 and P45, and 18 h for P60. The maximal cell density increased in the same order. The revertant P60R had a doubling time of 27 h, with a maximal cell density in-between that of P45 and P60 cell (data not shown). Similar growth properties were observed when these cells were cultured in a Met^+Hcy^- medium that contained dialysed fetal calf serum (Fig. 3, *left panel*).

Met Dependence. The early passages P10, P25, and P45, and the revertant P60R grew in a Met-deficient medium supplemented with Hcy (Met^-Hcy^+). The doubling times were 90 h for P10, 39 h for P25 and P45, and 43 h for P60R (Fig. 3, *right panel*), corresponding to a relative grow rate of 45–70% of the rate observed in the Met^+Hcy^- medium (Fig. 3, *left panel*). Notably, P60 cells did not proliferate in the Met^-Hcy^+ medium (Fig. 3, *right panel*) and represent a Met-dependent phenotype.

Intact Cell Remethylation. All variants incorporated significant amounts of radioactivity from 5-[^{14}C]methyl-THF into proteins (Table 1). The incorporation was highest for the P10 cell subline (about 300 pmol/10^6 cells) and intermediate for the P25, P45, and P60R (80–200 pmol/10^6 cells); P60 incorporated only trace amounts (15 pmol/10^6 cells).

Nitrous oxide, which inactivates methionine synthase, markedly reduced the intact cell Hcy remethylation (to 16–24%) in P10, P25, and P45 cells and only moderately (to 60–70%) reduced the Hcy remethylation in P60 and P60R cells (Table 1).

Hcy Export Rates. We determined the Hcy export rates of the glioma cells, which were cultured in a medium containing 50 μM of Met and exposed to nitrous oxide or air (Fig. 4; Table 2). Low Met favors Hcy remethylation, and the difference in Hcy export with and

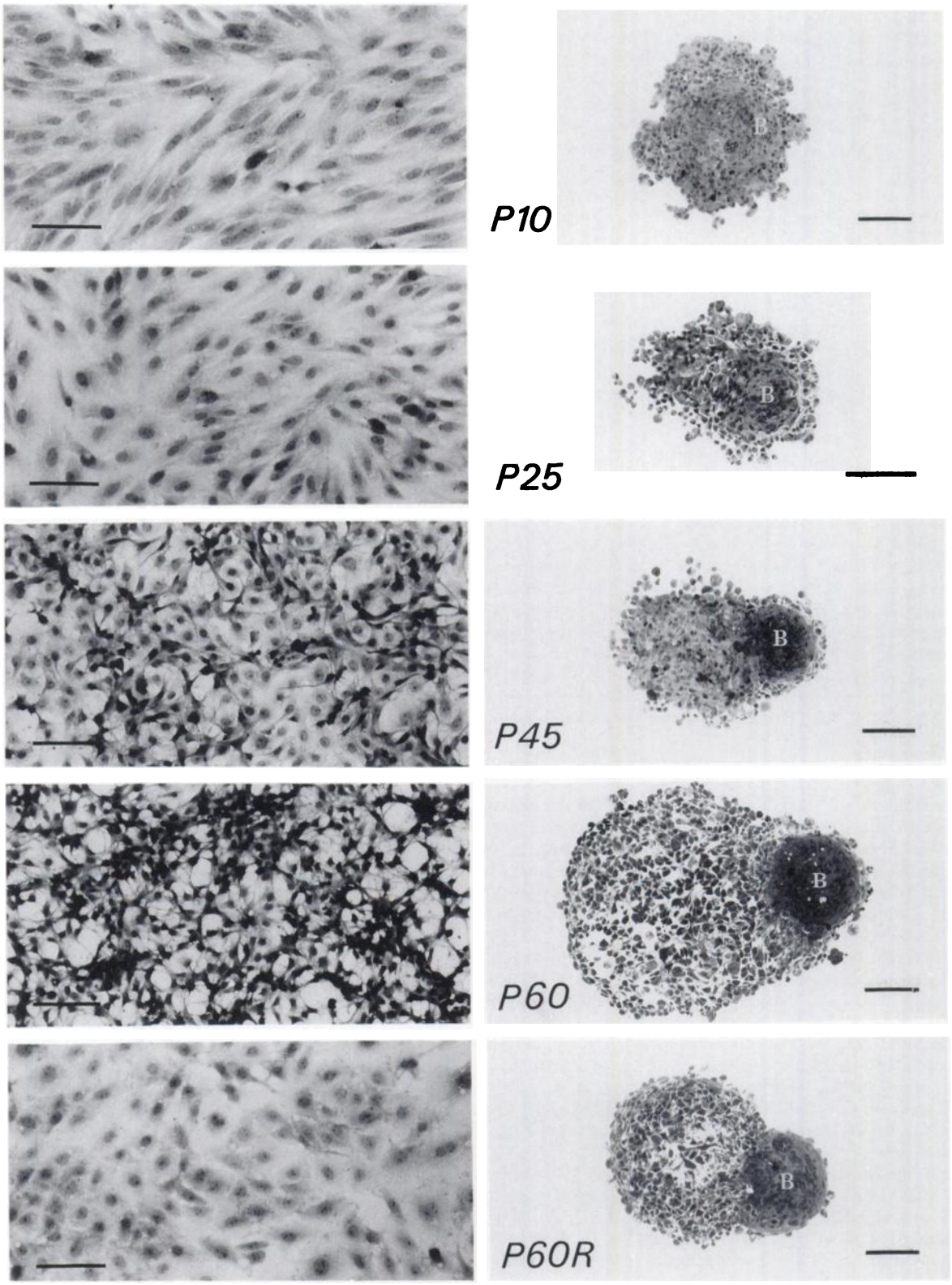

Fig. 2. Morphology (*left panels*) and invasive properties (*right panels*) of variants of the GaMg human glioma cell line. The *left panels* show confluent monolayer cultures of P10, P25, P45, P60, and P60R. From P10 to P60, there is a gradual progression towards more aggressive phenotypes. The revertant P60R resembles the earlier passages. The *right panels* show the invasion of tumor cells into aggregates of normal rat brain cells (*B*) after 4 days of coculture. In P10 and P25, there is intermingling of tumor cells and normal brain tissue. In cocultures of P60 and P60R, the confrontation zone is diffuse, with severe destruction of brain tissue. *Bars*, 100 μm.

without nitrous oxide is an extracellular measure of flux through methionine synthase (27, 37).

The Hcy export rate of all cell sublines increased immediately after seeding, then reached a maximum, and declined when the cell density approached confluence. In cells exposed to air, the mean maximal Hcy export rate was highest for P60 (about 0.8 nmol/h/10^6), intermediate for P25, P45, and P60R (0.35–0.5 nmol/h/10^6), and lowest for P10 cells (about 0.15 nmol/h/10^6). When these cells were exposed to

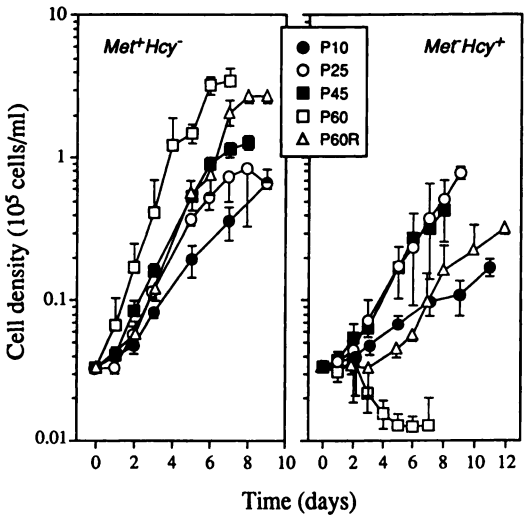

Fig. 3. Growth of variants of GaMg human glioma cell line in a medium containing 100 μM Met (*left panel*) and in a Met-deficient medium supplemented with 100 μM of Hcy (*right panel*). Data shown are means of three experiments ± SD.

Table 1 *Intact cell Hcy remethylation in variants of GaMg human glioma cell line incubated in the absence and presence of nitrous oxide*

Cells in exponential growth phase were incubated in medium containing 5 μM 5-[^{14}C]methyl-THF and 200 μM Hcy for 18 h and exposed to either air or nitrous oxide, as described in "Materials and Methods." Values are the mean of 3-4 experiments ± SD.

	Air	N_2O	
Variant	pmol/10^6 cells	pmol/10^6 cells	% of air
P10	310 ± 33	49 ± 7	16 ± 4
P25	214 ± 19	37 ± 2	18 ± 3
P45	177 ± 20	42 ± 5	24 ± 8
P60	15 ± 3	10 ± 3	68 ± 9
P60R	80 ± 12	47 ± 2	58 ± 9

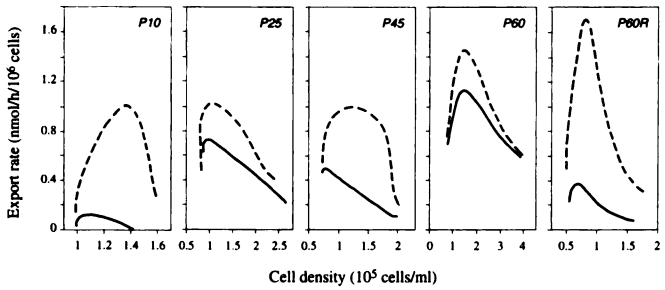

Fig. 4. Hcy export rates of variants of GaMg human glioma cell line in the absence and presence of nitrous oxide. The cells were exposed either to air (*solid line*) or nitrous oxide (*broken line*), as described in "Materials and Methods." The experiment was repeated three to four times, and typical data are shown.

nitrous oxide, Hcy export increased, and P10, P25, and P45 cells attained almost the same maximal export rate of about 0.75 nmol/h/10^6 (Fig. 4; Table 2). In P60 and P60R, the highest maximal export rate was about 1–1.2 nmol/h/10^6 cells. Thus, the nitrous oxide-dependent Hcy export, which is the difference in Hcy export rate of cells cultured with and without nitrous oxide, was high in P10 and P60R, moderate in P45, and low in P25 and P60. (Fig. 4; Table 2).

Methionine Synthase Activity in Cell-Free Extract. The specific activities of methionine synthase in the extract of all passages, including the revertant P60R, were similar and in the range of 13–20 nmol/h/mg protein (Table 3).

Methionine synthase in all cell types cultured in DMEM with 50 μM Met was rapidly inactivated by nitrous oxide. The initial rate of inactivation was in the range of 0.046–0.1/h but was highest for P60 and P60R cells, intermediate for P25 and P45, and lowest for P10 (Table 3). The residual enzyme activity after 24–48 h of exposure was 10–20% and essentially the same for all cell types (Table 3).

Folates. We determined the total cell folate and the amount of 5-methyl-THF in the glioma cells to investigate whether impaired function of methionine synthase may affect distribution of the intracellular folate species according to the methyl folate trap hypothesis (38).

The total folate content of glioma cells grown in Met$^+$Hcy$^-$ medium (containing 10 μM of folic acid) was 38–85 pmol/10^6 cells (Table 4). In cells depleted and then repleted with folate, the folate content was reduced by 20–50% (Table 4). More importantly, in P10 cells, 5-methyl-THF accounted for 40% of the total folate pool, whereas in P25, P45, and P60, the percentage was 4–11%. In the revertant P60R, 5-methyl-THF level was 16% of the total amount of folate (Table 4). Thus, particularly low levels of 5-methyl-THF were detected in P25 and P60 cells. We found no 5-methyl-THF in the

Table 2 *Maximal Hcy export rate of variants of GaMg human glioma cell line incubated in the absence and presence of nitrous oxide*

Cells in exponential growth phase were transferred to culture medium containing 50 μM Met and exposed to either air or nitrous oxide for 0-48 h. Hcy export rate curves were constructed as described in "Materials and Methods" and shown in Fig. 4. The maximal export rate from three separate experiments was determined from the graphs. Values are the mean of 3-4 experiments ± SD.

	Air	N_2O	N_2O-dependent export[a]	
Variant	nmol/h/10^6 cells	nmol/h/10^6 cells	nmol/h/10^6 cells	% of N_2O
P10	0.14 ± 0.04	0.70 ± 0.21	0.56 ± 0.23	79 ± 8
P25	0.51 ± 0.16	0.70 ± 0.25	0.19 ± 0.10	25 ± 7
P45	0.42 ± 0.10	0.81 ± 0.23	0.39 ± 0.13	47 ± 6
P60	0.81 ± 0.18	1.01 ± 0.25	0.20 ± 0.08	19 ± 5
P60R	0.35 ± 0.12	1.22 ± 0.45	0.87 ± 0.39	71 ± 8

[a] Difference between Hcy export rate in cells exposed to N_2O and air.

Table 3 *Methionine synthase activity in extract from variants of GaMg human glioma cell line cultured in the absence and presence of nitrous oxide*

Cells in exponential growth phase were exposed to either air or nitrous oxide for 0-48 h. Values are the mean of 3-4 experiments ± SD.

Variant	Enzyme activity[a] nmol/h/mg protein	Initial rate of inactivation (h^{-1})	Residual activity[b] % of air
P10	19.6 ± 4.4	0.046	13.8 ± 3.5
P25	19.8 ± 11.3	0.075	21.5 ± 4.6
P45	13.1 ± 5.8	0.064	19.2 ± 4.4
P60	13.0 ± 6.8	0.100	15.8 ± 2.3
P60R	13.9 ± 5.4	0.100	11.5 ± 1.1

[a] Total methionine synthase activity in cells exposed to air for 24 h.
[b] Activity after 24 h of nitrous oxide exposure.

Table 4 *Total folate and 5-methyl-THF in variants of GaMg human glioma cell line*

In experiment 1, total folate was determined in exponentially growing cells cultured in DMEM with 10 μM folic acid. In experiment 2, the cells were depleted of folate and then incubated in medium containing 42 nM 5-[^{3}H]formyl-THF for 24 h. The folate pool was then repleted and equilibrated by transferring the cells to DMEM containing 10 μM folic acid and cultured under these conditions for 2 days. Cells were harvested in exponential growth phase, and total folate and 5-methyl-THF were determined, as described in "Materials and Methods."

	Experiment 1[a]	Experiment 2[b]		
Variant	Total folate pmol/10^6 cells	Total folate pmol/10^6 cells	5-methyl-THF pmol/10^6 cells	5-methyl-THF % of total
P10	69 ± 14	41	16.0	39.0
P25	38 ± 8	29	1.3	4.5
P45	53 ± 7	44	4.8	10.9
P60	56 ± 19	33	1.3	3.9
P60R	85 ± 16	44	6.8	15.5

[a] Values are the mean of three determinations ± SD.
[b] Values are the mean of two determinations.

Table 5 *Total cobalamin and different cobalamin forms in variants of GaMg human glioma cell line*

Variant	Cellular cobalamin							
	Total fmol/10^6 cells	OH[a] fmol/10^6 cells (%)[b]		CN fmol/10^6 cells (%)		Ado fmol/10^6 cells (%)		CH_3 fmol/10^6 cells (%)	
P10	1084 ± 241	303 ± 113	(27)	490 ± 184	(44)	111 ± 18	(11)	138 ± 12	(13)
P25	507 ± 180	159 ± 44	(33)	183 ± 81	(36)	68 ± 42	(13)	79 ± 55	(14)
P45	631 ± 62	185 ± 58	(30)	239 ± 96	(37)	82 ± 12	(13)	103 ± 21	(17)
P60	477 ± 264	158 ± 54	(36)	280 ± 199	(56)	14 ± 6	(3)	16 ± 5	(4)
P60R	727 ± 254	300 ± 122	(41)	285 ± 116	(39)	74 ± 16	(11)	59 ± 7	(9)

[a] OH, hydroxycobalamin; CN, cyanocobalamin; Ado, adenosylcobalamin; CH_3, methylcobalamin.
[b] Mean percentage of total cellular cobalamin level.

culture medium. These results suggest that folates are not trapped as 5-methyl-THF in the Met-dependent P60 cells.

Cobalamins. The total intracellular cobalamin content was highest for the P10 cells (1084 fmol/10^6 cells), and the values were 507, 631, 727, and 477 fmol/10^6 cells for P25, P45, P60R, and P60 cells, respectively (Table 5). The most notable differences were observed for the cellular content of methylcobalamin and adenosylcobalamin. The concentrations of these cobalamin forms were substantially higher in the Met-independent variants of the GaMg glioma cell line, including the revertant P60R, than in the Met-dependent P60 cells, which contained low concentrations of both species (Fig. 5; Table 5).

DISCUSSION

Met Dependence. Met dependence has been linked to malignant or transformed cells (3). In a recent study, it was shown that only about 25% of primary histocultures of fresh human tumors exhibit Met dependence (4), whereas a substantial fraction of permanent cell lines is Met dependent (3). This may indicate that Met dependence is a late event in the oncogenic process. However, in patients with familial colon cancer or Gardner's syndrome, Met dependence has been demonstrated in fibroblasts (early passages) obtained several years before the malignant disease became apparent (39), suggesting that Met dependence represents a phenotypic expression of a genotype related to oncogenic transformation (39).

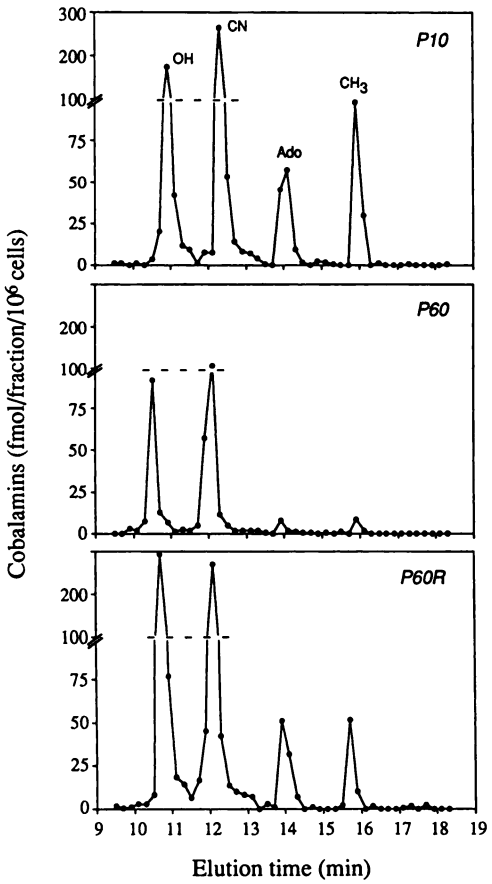

Fig. 5. HPLC elution profile for various cobalamin forms in extracts from variants of the GaMg human glioma cell line. *OH*, hydroxycobalamin; *CN*, cyanocobalamin; *Ado*, adenosylcobalamin; *CH_3*, methylcobalamin.

In the present study, we have used variants of the human GaMg glioma cell line (25), which are genetically closely related. The primary tumor was invasive *in vivo* (25), and all variants from P10 to P60 exhibited invasive properties *in vitro* (Fig. 2, *right panel*), reflecting the malignant character of the cell line. With increasing passages, the cells successively acquired a higher proliferation rate, diminished contact inhibition, a more malignant morphology, and a change in the pattern of invasion (Fig. 2). A similar phenotypic instability *in vitro* has been reported for several other cell lines (40).

In GaMg, Met dependence became apparent after 60 passages (Fig. 3) but could not be related to any particular change in morphology or growth behavior (Figs. 2 and 3). The Met-independent revertant (P60R) had a morphology resembling the lower passages but retained the invasive pattern of P60 (Fig. 2). Our data are consistent with previous reports that Met dependence is an important but not essential event in oncogenic progression (9, 10, 12) and that some (3, 10) but not all (9) revertant cell lines have different transformation-associated properties from their Met-dependent origin.

Intact Cell Remethylation. Incorporation of radioactivity from 5-[^{14}C]methyl-THF into protein is an established assay for Hcy remethylation in intact cells (3, 10, 28). The experiment is usually performed in Met^-Hcy^+ medium that can change growth, metabolism, and methionine synthase activity in cells previously cultured in a medium containing Met (14, 41). Furthermore, the cellular content of 5-methyl-THF and uptake of 5-[^{14}C]methyl-THF may vary during cell transformation (42, 43). These factors may lead to a marked difference in specific activity of intracellular 5-[^{14}C]methyl-THF in different cell lines, thereby making comparison of data difficult.

We found that Met-independent cells, especially P10, P25, and P45, had high Met biosynthesis. The marked suppression (>80%) caused by nitrous oxide in the P10, P25, and P45 cells (Table 1) also supports a high Hcy remethylation rate in these cells. The incorporation was moderate in P60R and low in P60, and nitrous oxide only reduced incorporation by 30–40% (Table 1). This indicates that these cells remethylate Hcy at a lower rate than the early passages, at least during culture in a Met^-Hcy^+ medium.

Hcy Export Rate. Determination of Hcy export rate in cells is an alternative method to study the functional state of methionine synthase in intact cells (31, 36, 37). An advantage is that this assay can be performed without changing composition of the medium with respect to Met and folate, thus maintaining stable growth and metabolic conditions. The information obtained is two-fold: (*a*) the nitrous oxide-dependent Hcy export reflects Hcy remethylation (27); and (*b*)

because Hcy is formed stoichiometrically during AdoMet-dependent transmethylation (1), the Hcy export in the presence of nitrous oxide is a measure of the overall transmethylation rate (44–46).

In P10 and P60R, the nitrous oxide-dependent export was 70–80% of maximal Hcy export rate after nitrous oxide exposure, indicating that these cell sublines remethylate most of the Hcy formed. Both P25 and P60 had low Hcy remethylation, as judged by nitrous oxide-dependent Hcy export. The apparently low Hcy remethylation in P25 cells and high Hcy remethylation in P60R (Fig. 4;, Table 2) are in contrast to the results from intact cell remethylation (Table 1). This suggests that medium components may affect intracellular Hcy remethylation. Hcy, Met, and the source of folate are likely candidates.

The maximal Hcy export rate in the presence of nitrous oxide was essentially the same for P10, P25, and P45 cells. For P60 and P60R cells, 50% higher maximal Hcy export was detected, suggesting a higher transmethylation rate in these cells. Thus, the progression of the GaMg glioma cell line is associated with an increased transmethylation rate, which may perturb the balance between Met synthesis and use. However, this does not fully explain Met dependence in P60 because P60R, which had an even higher transmethylation rate, was able to grow in a $Met^{-}Hcy^{+}$ medium. Thus, the unique feature of P60 seems to be a high overall transmethylation rate combined with reduced salvage of homocysteine to methionine, causing an imbalance between methionine production and consumption. Notably, the difference in transmethylation rate in these variants of GaMg is moderate compared to that observed in Met-independent and -dependent cells in other systems (3, 20).

Enzyme Level in Cell Extracts. The data on Hcy remethylation in intact cells indicate that Met dependence in GaMg may be due to low methionine synthase activity. Methionine synthase in cell-free extract is determined in the presence of supraphysiological concentrations of substrates, excess cobalamin and AdoMet, and a reducing system (32). The activity probably reflects the total amount of apoenzyme plus holoenzyme in the extract but not necessarily the activity in intact cells, as demonstrated for the cblE mutant (37, 47). In the different variants of GaMg, we observed that the methionine synthase activities were similar (Table 3). Thus, impaired Hcy remethylation in some variants (Tables 1 and 2) is not explained by low methionine synthase expression in these cells but rather points to inadequate provision of substrates or cofactor for the enzyme reaction.

Substrate and Cofactor. Low intact cell remethylation (in P60 cells; Table 1) or low nitrous oxide-dependent export (in P25 and P60 cells; Table 2) cannot be attributed to insufficient Hcy supply, since the former assay is carried out with high concentration of Hcy in the medium, and P60 cells form (and export; Table 2) more Hcy than the P10 cells. Lack of 5-methyl-THF is unlikely during intact cell remethylation, which is carried out in a medium with excess 5-methyl-THF. However, determination of Hcy export rate is performed in a medium containing folic acid. Since uptake of folates may change during transformation (42) and the level of 5-methyl-THF declines in rapidly proliferating cells (48), a lack of 5-methyl-THF should be considered.

There were marked differences in the folate status of the different variants of GaMg (Table 4). The Met-dependent P60 had low 5-methyl-THF, but more unexpected was the finding that both total cell folate and 5-methyl-THF were low in P25. Notably, the level of 5-methyl-THF in the different variants of GaMg showed an inverse correlation with the Hcy export rate for cells cultured in air (Fig. 4; Table 2), and confirms the role of 5-methyl-THF in the methionine synthase reaction (1). The low level of 5-methyl-THF may contribute to the Met dependence of P60 but does not have this effect in P25. This points to additional metabolic defect(s) in P60.

Methylcobalamin and adenosylcobalamin are cofactors of methionine synthase and methylmalonyl CoA mutase, respectively (Fig. 1), and these enzymes are the main intracellular cobalamin-binding proteins (49, 50). Since methylcobalamin is a catalytic intermediate in the methionine synthase reaction (22), the cellular level is probably related to the catalytic activity of this enzyme.

As the cells progressed from P10 to P60, there was a parallel decline in the level of both methylcobalamin and adenosylcobalamin (Table 5; Fig. 5). This may point to the sites of metabolic deletion, *i.e.*, processes responsible for provision of cobalamin to both the cytosolic methionine synthase and the mitochondrial methylmalonyl CoA mutase (Fig. 1). Such possible sites are transcobalamin-dependent uptake of cobalamin, demonstrated for Met-dependent human lymphocytes (21) and transformed glial cells (51). Furthermore, intracellular trafficking of cobalamin (52) may be affected, as suggested in cblC and cblD disorders (47), and recently proposed to be responsible for the Met dependence of human melanoma cell lines (11). Alternatively, a constant ratio between the amount of these two cobalamin species may reflect a coordinate regulation of these two enzymes.

Nitrous Oxide Effect. We primarily used nitrous oxide as a pharmacological tool to investigate the role of methionine synthase in intact cell remethylation and Hcy export. The nitrous oxide effect on methionine synthase in various human glioma cell sublines (Tables 1–3; Fig. 4) confirms that there are differences in the functional state of the enzyme in these cells. Moreover, the nitrous oxide response serves to differentiate the defect in the Met-dependent P60 from that in cblE and cblG mutants, because methionine synthase in mutant fibroblasts are not (cblE; Ref. 47) or only slightly (cblG) inactivated by nitrous oxide (37).

Studies in a cell-free system indicate that only catalytically active enzyme is inactivated by nitrous oxide (22). This hypothesis concurs with our results obtained in the Met-independent variants of GaMg and several other cell types investigated (31, 47). However, the observation that methionine synthase in extract from the Met-dependent P60 cells is extensively inactivated, and at a higher rate than the enzyme from P10 and P45 cells (Table 3), is apparently in conflict with the prevailing model (22). This raises exiting questions regarding the mechanism of action of nitrous oxide and the functional state of the intracellular enzyme.

In conclusion, Met-dependence of P60 cells is probably due to low methionine biosynthesis combined with a high overall transmethylation rate, whereas glioma cells with an isolated low homocysteine remethylation rate (P25) or high transmethylation rate (P60R) represent methionine-independent phenotypes. Thus, in this glioma cell line, the development of methionine dependence during *in vitro* passaging is probably related to more than one metabolic alteration. Of these, low methylcobalamin level seems to be the single parameter which most closely correlates with methionine dependence. Studies on uptake and cellular trafficking of cobalamins may yield important information about Met dependence.

ACKNOWLEDGMENTS

We thank Eli Gundersen, Elfrid Blomdal, Gry Kvalheim, Halvard Bergesen, and Tor Christensen for expert technical assistance.

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