

# Co-ordinate variations in methylmalonyl-CoA mutase and methionine synthase, and the cobalamin cofactors in human glioma cells during nitrous oxide exposure and the subsequent recovery phase

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We investigated the co-ordinate variations of the two cobalamin (Cbl)-dependent enzymes, methionine synthase (MS) and methylmalonyl-CoA mutase (MCM), and measured the levels of their respective cofactors, methylcobalamin (CH<sub>3</sub>Cbl) and adenosylcobalamin (AdoCbl) in cultured human glioma cells during nitrous oxide exposure and during a subsequent recovery period of culture in a nitrous oxide-free atmosphere (air). In agreement with published data, MS as the primary target of nitrous oxide was inactivated rapidly (initial rate of 0.06 h<sup>-1</sup>), followed by reduction of CH<sub>3</sub>Cbl (to < 20%). Both enzyme activity and cofactor levels recovered rapidly when the cells were subsequently cultured in air, but the recovery was completely blocked by the protein-synthesis inhibitor, cycloheximide. During MS inactivation, there was a reduction of cellular AdoCbl and holo-MCM activity (measured in the absence of exogenous AdoCbl) to about 50% of pre-treatment levels. When the cells were transferred to air, both AdoCbl and holo-MCM activity

recovered, albeit more slowly than the MS system. Notably, the regain of the holo-MCM and AdoCbl was enhanced rather than inhibited by cycloheximide. These findings confirm irreversible damage of MS by nitrous oxide; hence, synthesis of the enzyme is required to restore its activity. In contrast, restoration of holo-MCM activity is only dependent on repletion of the AdoCbl cofactor. We also observed a synchronous fluctuation in AdoCbl and the much larger hydroxycobalamin pool during the inactivation and recovery phase, suggesting that the loss and repletion of AdoCbl reflect changes in intracellular Cbl homeostasis. Our data demonstrate that the nitrous oxide-induced changes in MS and CH<sub>3</sub>Cbl are associated with reversible changes in both MCM holoactivity and the AdoCbl level, suggesting co-ordinate distribution of Cbl cofactors during depletion and repletion.

**Key words:** adenosylcobalamin, cell culture, cobalamin metabolism, methylcobalamin, vitamin B<sub>12</sub>.

## INTRODUCTION

There are two cobalamin (Cbl)-dependent enzymes in mammalian cells, the mitochondrial L-methylmalonyl-CoA mutase (MCM; EC 5.4.99.2) and the cytosolic methionine synthase (MS, or 5-methyltetrahydrofolate-homocysteine methyltransferase; EC 2.1.1.13) [1]. MCM catalyses the conversion of L-methylmalonyl-CoA to succinyl-CoA, a reaction that requires adenosylcobalamin (AdoCbl) as a cofactor [2]. MS transfers a methyl group from 5-methyltetrahydrofolate to homocysteine. Tetrahydrofolate and methionine are formed in this reaction, in which methylcobalamin (CH<sub>3</sub>Cbl) is the secondary methyl donor (Scheme 1) [1].

MS is susceptible to inactivation by the anaesthetic gas nitrous oxide. Inactivation of MS has been demonstrated with purified enzyme [3], in cultured cells [4–7] and in experimental animals [8] as well as in humans [9–11]. This effect of nitrous oxide has been suggested as the mechanism behind the side effects from bone marrow and the central nervous system reported after prolonged exposure to this anaesthetic gas [12,13].

Nitrous oxide acts by oxidizing Cbl(I), which is formed as a transient intermediate during the catalytic cycle [14,15], thereby forming a hydroxyl radical that may react with the S-adenosylmethionine-binding domain of MS. This causes an irreversible

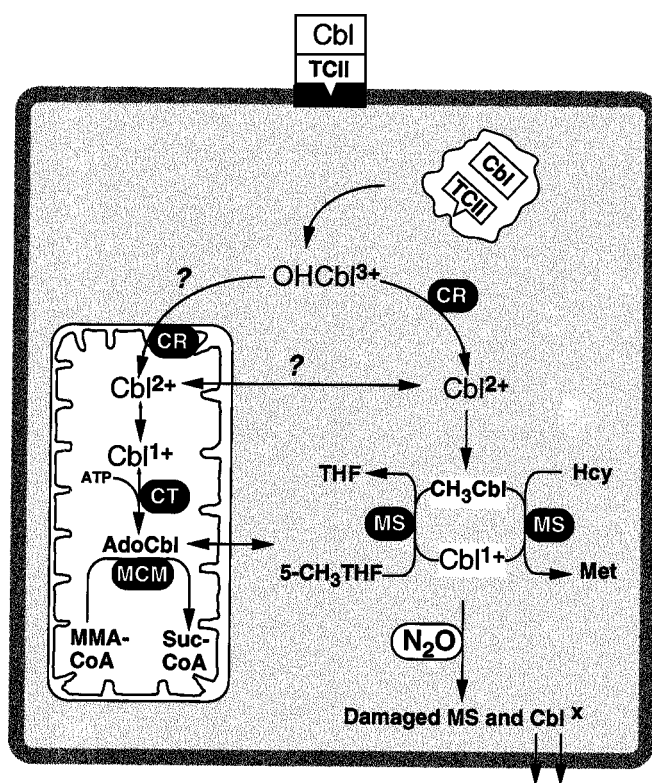
inactivation of the enzyme [16]. The oxidative damage is associated with the formation of Cbl analogues, which have been demonstrated in rats *in vivo* [8] and with the enzyme purified from *E. coli in vitro* [17].

Sparse data exist on the possible effects of nitrous oxide on the function of AdoCbl-dependent MCM. Holo-MCM activity in rats was reduced after prolonged exposure (2 weeks), which was explained by the formation of Cbl analogues and development of Cbl deficiency [8,18]. Surgical patients exposed to nitrous oxide for 24 h had increased urinary excretion of methylmalonic acid, suggesting inhibition of methylmalonyl-CoA metabolism [19]. Increased serum valine in 31 patients after only 3 h of nitrous oxide exposure has been interpreted as impaired MCM function [20]. Total MCM, however, was normal in white blood cells from patients subjected to nitrous oxide anaesthesia for 75–230 min [11].

In the present work, we investigated the changes of the MCM activity associated with inactivation of MS in human glioma cells during exposure to nitrous oxide and during the subsequent recovery phase after the cells had been transferred to air. We measured the holo-MCM activity, which is assayed in the absence of added AdoCbl and probably reflected the intracellular activity, and total MCM activity, which was obtained by saturating the enzyme with AdoCbl *in vitro*. These activities were related to

Abbreviations used: Cbl, cobalamin; AdoCbl, adenosylcobalamin; CH<sub>3</sub>Cbl, methylcobalamin; DMEM, Dulbecco's modified Eagle's medium; MS, methionine synthase; MCM, methylmalonyl-CoA mutase; OH-Cbl, hydroxycobalamin.

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**Scheme 1** Interference from nitrous oxide with Cbl metabolism and function

Abbreviations used: Cbl<sup>x</sup>, Cbl analogues; CH<sub>3</sub>Cbl, methylcobalamin; 5-CH<sub>3</sub>THF, 5-methyl-tetrahydrofolate; CR, cobalamin(III) reductase; CT, cobalamin(I) adenosyltransferase; Hcy, homocysteine; MMA-CoA, methylmalonyl-CoA; Suc-CoA, succinyl-CoA; TCII, transcobalamin II; THF, tetrahydrofolate.

changes in cellular AdoCbl and CH<sub>3</sub>Cbl levels. Our data suggest a co-ordinate distribution of Cbl cofactors between MS and MCM during inactivation and recovery.

## MATERIALS AND METHODS

### Chemicals

Sources of most reagents used have been given previously [21,22]. Cycloheximide, succinyl-CoA (sodium salt) and AdoCbl were purchased from Sigma, St. Louis, MO, U.S.A. D,L-Methylmalonyl-CoA was provided by Dr. Ruma Banerjee, University of Nebraska, Lincoln, NE, U.S.A. A custom-made powder of Dulbecco's modified Eagle's medium (DMEM), identical to a standard DMEM but without folic acid and methionine, was from Gibco-BRL (Paisley, Scotland).

### Cell line and culture conditions

We used a methionine-independent variant [23] (P60H) of a human glioma cell line, GaMg, which was established in 1984 from a glioblastoma multiforme tumour in a 42-year-old woman [24]. It is characterized by high activities of MS and MCM and their corresponding cofactors CH<sub>3</sub>Cbl and AdoCbl [23].

The P60H cells were cultured in DMEM supplemented with 0.6 g/l L-glutamine, 1.5 μM cyanocobalamin, 10 μM folic acid, 50 μM methionine, non-essential amino acids (each 330 μM; Bio Whittaker, Walkersville, MD, U.S.A.) and 10% (v/v) heat-

inactivated fetal calf serum (Biological Industries, Kibbutz Beth Haemek, Israel). If not otherwise indicated, the cells were cultured at 37 °C in an atmosphere of 5% CO<sub>2</sub>/95% air with a relative humidity of 98%.

### Experimental design

The cells were seeded in 9-cm dishes containing 7 ml of DMEM (for measurement of MCM and Cbl) or 6-cm dishes with 3 ml of DMEM (for measurement of MS) at a density of either 500 or 2000 cells/cm<sup>2</sup>, depending on the total culture time. After 3 days of culture, when the cells had reached early logarithmic growth phase, the medium was removed and fresh medium added so that the cell densities were either 15000 or 40000 cells/ml. Then the culture dishes were placed in modular incubator chambers (Billups-Rothenberg, Del Mar, CA, U.S.A.) and flushed with either air (75% N<sub>2</sub>/20% O<sub>2</sub>/5% CO<sub>2</sub>) or nitrous oxide (50% N<sub>2</sub>O/25% N<sub>2</sub>/20% O<sub>2</sub>/5% CO<sub>2</sub>). Both gases were moistened by passage through sterile water at 50 °C and delivered at a rate of 5 l/min for 10 min. The chambers were then closed and kept at 37 °C. Flushing with air or nitrous oxide was repeated every 24 h.

For the inactivation studies, cells were exposed to nitrous oxide or air (control) for up to 96 h. For the recovery studies, cells were cultured initially in the presence of nitrous oxide for 96 h, then in air for up to 24 h. Controls were exposed continuously to nitrous oxide or air for 120 h. Culture medium was not renewed.

In the recovery experiments, protein synthesis was inhibited by adding cycloheximide (3 μg/ml) to the culture medium. The inhibition was assessed by measuring the incorporation of [<sup>35</sup>S]methionine (> 1000 Ci/mmol; Amersham, Little Chalfont, Bucks, U.K.) into protein.

Cells were harvested from parallel dishes, and brought into suspension by incubation for 10 min with trypsin (0.1 mg/ml; Bio Whittaker), which was inactivated with fresh medium. The cells were then washed repeatedly in PBS, and centrifuged at 190 g for 5 min at 4 °C. Whenever possible, cells were kept on ice. Cell counting was performed with a Coulter Counter Model ZM (Coulter Electronics, Luton, U.K.). Samples were processed in dim red light, and stored at -80 °C until analysis.

### Enzyme activities

Cell pellets for the measurement of MCM activity were resuspended in 250 μl of 21% (v/v) glycerol containing thymidine (203 μmol/l) [22]. The assay is a fully automated HPLC technique that allows the unattended analysis of 56 samples per 24 h. Details on construction and performance have been published previously [22]. The assay measures the conversion of L-methylmalonyl-CoA to succinyl-CoA in the absence (holoactivity) or in the presence (total activity) of AdoCbl [22].

Determination of MS activity was performed by a modification [25] of the radioisotope assay described by Weissbach et al. [26]. The assay measures the amount of radioactive methionine formed from 5-[<sup>14</sup>C]methyltetrahydrofolate and homocysteine.

### Intracellular Cbl

Cbl was extracted from the cells by heating in the presence of glacial acetic acid and *N*-ethylmaleimide [21,27]. All procedures were carried out in dim red light.

Separation of CH<sub>3</sub>Cbl and AdoCbl from the other Cbl forms was performed by reversed-phase chromatography using a modification [21] of the method described by Jacobsen and Green [28]. Retention times for hydroxycobalamin (OHCbl), cyano-

cobalamin, AdoCbl and CH<sub>3</sub>Cbl were 10.5, 12, 14 and 16 min, respectively. The OHCbl, Cbl(I) and Cbl(II) species are measured collectively as OHCbl in this system. The column eluate was collected in 260- $\mu$ l fractions (0.2 min) into tubes containing 25  $\mu$ l of 16.5% polyvinylpyrrolidone. The concentrations of Cbl forms in cell extracts and HPLC fractions were determined by the radioisotope dilution assay developed by van Kapel et al. [27]. Salivary R-binder was used as binding protein.

The total Cbl levels in cell extracts and total Cbl values from the fractions observed after nitrous oxide exposure were essentially identical, suggesting that the cellular levels of Cbl analogues were insignificant.

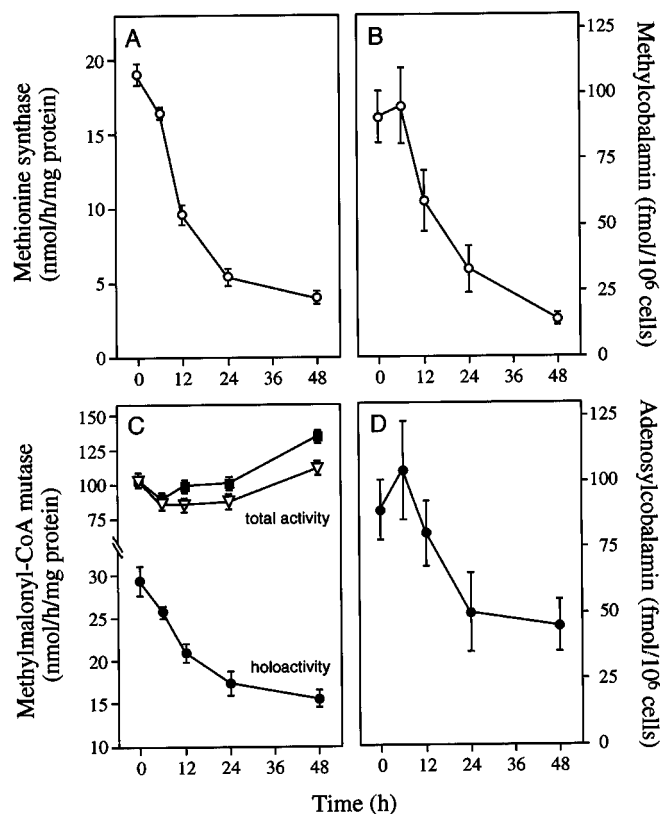
### Protein determination

Protein content was determined according to the method of Bradford [29], with bovine  $\gamma$ -globulin as a calibrator.

## RESULTS

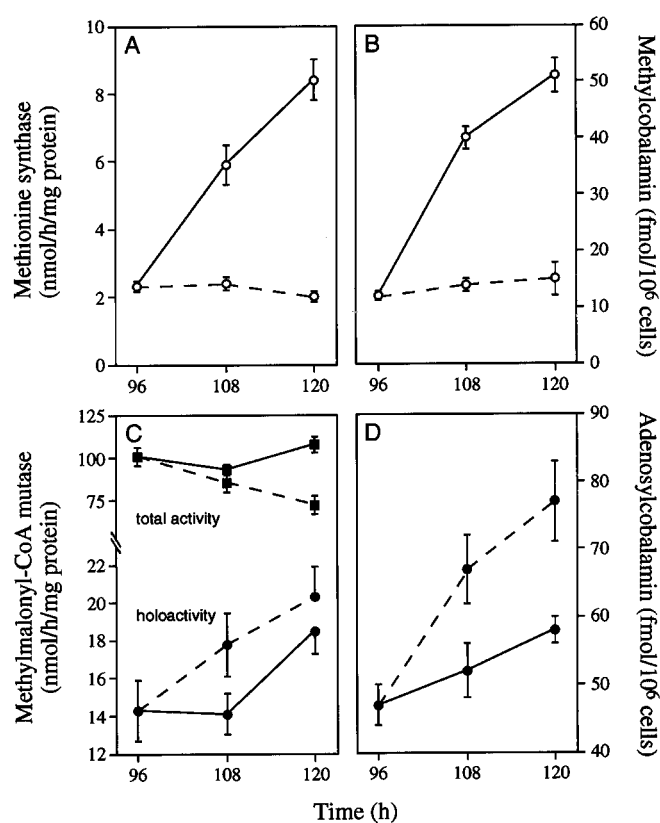
### Inactivation of MS and depletion of CH<sub>3</sub>Cbl

Exposure of P60H cells to nitrous oxide caused a rapid inactivation of MS, proceeding at an initial rate of 0.06/h ( $t_{1/2}$  = 12 h). The CH<sub>3</sub>Cbl content declined at a similar rate after an initial lag phase of about 6 h. Thus the enzyme inactivation preceded the CH<sub>3</sub>Cbl depletion by 6–12 h (Figures 1A and 1B).



**Figure 1** Reduction of the activities of MS (A) and MCM (C) and the concentrations of the respective cofactors, CH<sub>3</sub>Cbl (B) and AdoCbl (D) in human glioma cells during nitrous oxide exposure

Human glioma cells were exposed to nitrous oxide for 48 h as described in the Materials and methods section. For comparison, total MCM activity in cells exposed to air ( $\nabla$ ) is indicated in (C). Values are means  $\pm$  S.E.M. of 3–4 determinations.



**Figure 2** Increase in the activities of MS (A) and MCM (C) and the concentrations of CH<sub>3</sub>Cbl (B) and AdoCbl (D) during recovery after nitrous oxide exposure of human glioma cells

After 96 h of nitrous oxide exposure, cells were cultured in air in the absence (solid line) or presence (dashed line) of cycloheximide. Values are means  $\pm$  S.E.M. of 3–6 determinations.

After 48 and 96 h, the residual MS activities were  $18 \pm 2$  (Figure 1A) and  $13 \pm 0.5\%$  (results not shown), respectively. The corresponding values for CH<sub>3</sub>Cbl levels were  $15 \pm 3$  (Figure 1B) and  $13 \pm 3\%$  (results not shown), indicating that a stable plateau was obtained.

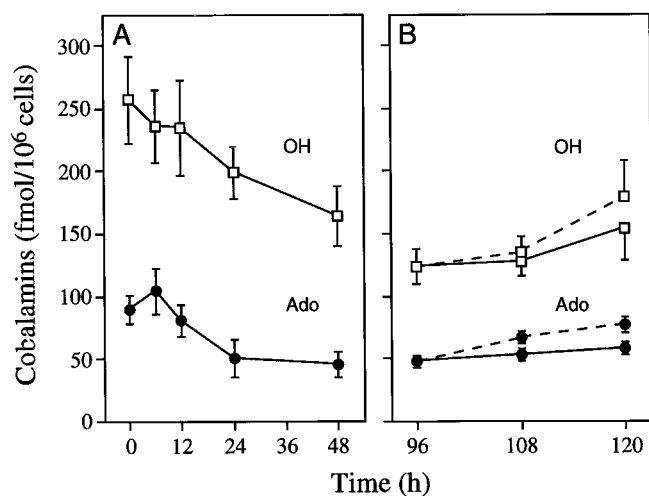
### Holo- and total MCM activities and AdoCbl content

Holo-MCM activity is assayed in the absence of added AdoCbl and is probably limited by cellular AdoCbl. The activity decreased at a rate of 0.02/h ( $t_{1/2}$  = 34 h) during nitrous oxide exposure and, after 48 h, the residual activity was  $65 \pm 4\%$  (Figure 1C). The total MCM activity, assayed with added AdoCbl, is a measure of holo- and apo-MCM. Total activity increased during nitrous oxide exposure (Figure 1C). Thus the ratio between holo- and total activity, denoted activity ratio, decreased at a rate of 0.03/h ( $t_{1/2}$  = 23 h). Within 48 h, the activity ratio decreased from  $0.29 \pm 0.023$  to  $0.13 \pm 0.012$  (results not shown).

The AdoCbl content declined concomitantly with the holo-MCM activity but at a slightly higher rate (0.04/h,  $t_{1/2}$  = 18 h). After 48 h,  $54 \pm 7\%$  remained (Figure 1D).

### Recovery of MS activity and repletion of CH<sub>3</sub>Cbl

Cells were exposed to nitrous oxide for 96 h, and both MS activity and CH<sub>3</sub>Cbl content were reduced to less than 20% under these conditions. The cells were then cultured in air for an



**Figure 3** Co-variation of OHcbl and AdoCbl

Parallel changes in the concentrations of OHcbl and AdoCbl in glioma cells during nitrous oxide exposure (A) and recovery (B) after prior nitrous oxide exposure for 96 h. The recovery experiments were performed in the absence (solid line) and presence (dashed line) of cycloheximide. Values are means  $\pm$  S.E.M. of 3–6 determinations.

additional 24 h, and both the enzyme and its cofactor showed a parallel increase (Figures 2A and 2B), which reached  $58 \pm 1$  and  $53 \pm 3\%$ , respectively, of levels detected in cells exposed to air for 96 h. Notably, cycloheximide (used at a concentration titrated not to induce cell death) completely blocked the restoration of both, demonstrating that the recovery of enzyme function was dependent on protein synthesis (Figures 2A and 2B).

#### Increase in MCM holoactivity and AdoCbl content during the recovery phase

During the recovery period of 24 h, holo-MCM activity and AdoCbl content increased slowly by 20–30% of the trough values, and reached 70–80% of the levels detected in cells cultured in air for 96 h. In the absence of cycloheximide, the increase of holo-MCM showed a lag period for the first 12 h. This lag was abolished by cycloheximide, which enhanced the recovery of MCM and AdoCbl (Figures 2C and 2D). Thus comparison of recoveries of holo-MCM activity and AdoCbl content (Figures 2C and 2D) shows a positive relation between these two processes, indicating that holo-MCM activity depends on cellular AdoCbl.

MCM total activity decreased in cells treated with cycloheximide, whereas the activity remained essentially unchanged in the absence of this inhibitor (Figure 2C). Accordingly, the recovery of the activity ratio of MCM was fastest when protein synthesis was inhibited (results not shown).

#### Relation between various Cbl species during inactivation and recovery

Nitrous oxide exposure induced a marked reduction in cellular  $\text{CH}_3\text{Cbl}$  (to  $< 20\%$ ) and a moderate (50%) reduction in AdoCbl (Figure 1). Notably, the changes in AdoCbl during nitrous oxide treatment and the recovery phase closely paralleled the changes in the much larger OHcbl pool (Figure 3).

## DISCUSSION

### Experimental system and design

We studied the interrelation between MCM and MS activities and the Cbl cofactors in a human glioma cell line during nitrous oxide exposure and subsequent incubation in air. This glioma cell line, P60H, is characterized by high levels of Cbl cofactors (Figure 4A) and MCM and MS activities (Figure 1, [21,30]), which make it suitable for studies on Cbl metabolism and function. The cells were cultured in a medium with excess cyanocobalamin, and resupplementation of the medium was not required to obtain restoration of enzyme activities. By using isolated cells, we avoided the influence from tissue distribution of nitrous oxide, the possible regional difference in Cbl homeostasis and redistribution of Cbl species between tissues. In addition, blocking protein synthesis with cycloheximide allowed us to differentiate between recovery caused by reactivation of the cofactor–enzyme complex and recovery due to synthesis of new enzyme.

### Inactivation and recovery of MS and concurrent changes in $\text{CH}_3\text{Cbl}$ levels

MS is regarded as the primary target for nitrous oxide [15]. We observed a time-dependent inactivation of the enzyme activity (Figure 1A), as has been consistently reported *in vivo* and *in vitro* [3,9,11,31–34]. The kinetics of the inactivation equalled that previously observed for the enzyme in cultured cells [4–6,21,23]. After a prolonged exposure for 120 h, the residual activity was only 12%.

There was a temporal and quantitative relationship between decrease in MS activity and loss of  $\text{CH}_3\text{Cbl}$ , as demonstrated in rat liver *in vivo* [8], but enzyme inactivation seemed to be slightly precedent (Figures 1A and 1B). This is in accordance with a model where oxidative modification of the protein structure is the primary event, which in turn impairs the reductive methylation of the prosthetic group [17].

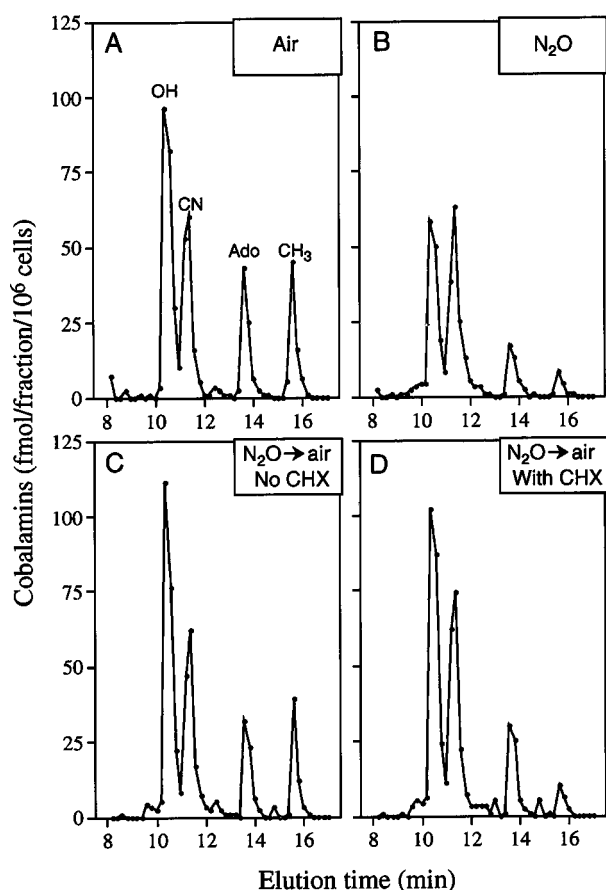
Recovery of MS activity occurred in parallel with  $\text{CH}_3\text{Cbl}$  repletion, and both processes were blocked completely under conditions of protein-synthesis inhibition (Figures 2A and 2B). This demonstrates that there was no repair or reactivation of damaged enzyme, but that the recovery was dependent on new synthesis of enzyme.

Finally, both inactivation and recovery data (Figures 1 and 2) demonstrate a close relationship between the MS activity and  $\text{CH}_3\text{Cbl}$  content. This observation adds support to the notion that  $\text{CH}_3\text{Cbl}$  levels reflect the catalytic turnover of MS [7,23].

Our data on recovery of MS activity are consistent with previous work in mice, pigs and rats exposed to nitrous oxide, demonstrating a gradual recovery of enzyme activity for several days after removal of the gas [8,31,35,36]. The authors suggested that regain of MS activity requires new synthesis of enzyme [8,35,36], which is supported by the observation of there being no reversal of the nitrous oxide effect on isolated bacterial MS after re-equilibration in air [3].

### MCM activity and AdoCbl content

During nitrous oxide exposure of glioma cells, the reduction in  $\text{CH}_3\text{Cbl}$  preceded the slower decline in AdoCbl responsible for the moderate decrease in MCM holoactivity (Figure 1). This sequence of events probably reflects the initial MS inactivation and the subsequent Cbl redistribution, which eventually leads to low AdoCbl content. The inhibition in MCM holoactivity is explained by reduction in AdoCbl cofactor level, since sim-



**Figure 4** HPLC elution profile of OHCbl (OH), cyanocobalamin (CN), AdoCbl (Ado) and  $\text{CH}_3\text{Cbl}$  ( $\text{CH}_3$ ) in glioma cells exposed to nitrous oxide and during recovery after nitrous oxide exposure

(A) The elution profile for cells cultured in air; (B) cells exposed to nitrous oxide for 96 h; (C) cells exposed to nitrous oxide for 96 h and then cultured in air for additional 24 h; and (D) the same experiments as in (C) but in the presence of cycloheximide (CHX).

ultaneous measurement of MCM activity in the presence of added AdoCbl demonstrated normal total activity (Figure 1C).

The data from the recovery experiment complete this picture: the AdoCbl and MCM holoactivity increased in parallel during inhibition of protein synthesis, which is consistent with recovery from reversible enzyme inhibition. Notably, the increase in AdoCbl content in particular, but also in MCM holoactivity, is actually enhanced by the protein-synthesis inhibitor (Figures 2C and 2D), which may be explained by no competition for available Cbl by the regenerating MS system.

The increase in total MCM activity in glioma cells during nitrous oxide exposure (Figure 1C) indicates that the expression of this enzyme increased despite a negative Cbl homeostasis. Some, but not all, studies have shown similar findings. Cardinale et al. [37] reported that Cbl deficiency significantly increased MCM apoenzyme activity in rat and pig liver, whereas Kennedy et al. [38,39] could not demonstrate a similar effect in the livers of sheep and cattle. Watanabe et al. [40] even found a significant decrease in total MCM activity in the livers of Cbl-deficient rats.

#### Cbl redistribution, metabolism and retention

The kinetics of Cbl depletion and repletion (Figures 1 and 2) were determined under conditions of sufficient extracellular Cbl

to restore Cbl status. Thus provision of Cbl cofactors to the two enzymes, MS and MCM, was rate-limited by intracellular processes involving Cbl distribution or metabolism.

The first step leading to cellular Cbl depletion during nitrous oxide exposure is probably irreversible damage of Cbl bound to MS, which may lead to formation of biologically inactive Cbl analogues [8,17]. We did not detect Cbl analogues after nitrous oxide exposure in our cells (Figure 4B). Thus damaged Cbl is likely to be excreted into the culture medium leading to the depletion of total Cbl pools (Scheme 1). The decrease in OHCbl has also been observed by Quadros et al. [41].

Several mechanisms for the reduction in cellular AdoCbl content should be considered. Low AdoCbl may be related to inactivation of the AdoCbl-forming enzyme, mitochondrial cobalamin(I) adenosyltransferase (EC 2.5.1.17) [42,43], or depletion of its co-substrate ATP. However, inhibition of the cobalamin(I) adenosyltransferase is unlikely, since no decrease in AdoCbl was detected in glioma cells exposed to the combination of nitrous oxide and methotrexate (T. Fiskerstrand, unpublished work), conditions under which MS is protected against inactivation due to folate depletion [7]. The AdoCbl-lowering effect of nitrous oxide is probably secondary to reduction of the much larger cellular OHCbl pool. This is supported by the observation (Figures 3 and 4) that the decrease and recovery of AdoCbl content closely paralleled variations in OHCbl content. Thus the AdoCbl fluctuations induced by nitrous oxide may reflect variations in overall intracellular Cbl homeostasis.

Reduction of the AdoCbl levels during nitrous oxide exposure (Figures 1D and 3B) is in contrast with the data of Kondo et al. [8]. These authors demonstrated loss of  $\text{CH}_3\text{Cbl}$  and a proportionate increase in AdoCbl in the liver of rats exposed to nitrous oxide. This discrepancy may reflect differential Cbl metabolism in brain cells [44] versus liver cells, but may also be related to no apparent retention of Cbl analogues in the human glioma cells (Figure 4B), as opposed to the rat liver [8]. Conceivably, glioma cells may release possible Cbl analogues into the culture medium.

#### Conclusion

Nitrous oxide oxidizes the Cbl cofactor of MS. This process damages the protein structure, and thereby irreversibly inactivates the enzyme. Low catalytic turnover of MS leads to impaired  $\text{CH}_3\text{Cbl}$  formation, whereas AdoCbl depletion probably reflects reduction of major cellular Cbl pools. Low AdoCbl is responsible for low holo-MCM activity, and MCM is not covalently modified. In line with this, the recovery of MS requires new synthesis of enzyme, whereas the recovery of MCM activity is only dependent on repletion of cofactor level. Our data confirm the close relationship between  $\text{CH}_3\text{Cbl}$  levels and the catalytic activity of MS, and suggest a slow co-ordinated Cbl distribution between cytosolic MS and mitochondrial MCM under conditions of variable Cbl status.

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