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# Modulation of glutathione content and the effect on methionine auxotrophy and cellular distribution of homocysteine and cysteine in mouse cell lines

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**The inability of cells in culture to grow in medium where methionine is replaced by its metabolic precursor, homocysteine, has been linked to neoplastic transformation and termed 'methionine dependence' or 'methionine auxotrophy'. The present investigation was undertaken to establish the influence of intracellular glutathione level on methionine auxotrophy in different mouse cell lines. A non-transformed, methionine-independent fibroblast cell line with essential normal growth rate in methionine-deficient, homocysteine-supplemented medium ($Met^-Hcy^+$), showed only a slight initial lag and then the same growth as control when glutathione was reduced to <5% by the glutathione synthesis inhibitor buthionine sulfoximine (BSO). Increasing cellular glutathione by cystamine in a completely methionine-dependent leukemia cell line did not stimulate the cells to proliferate in $Met^-Hcy^+$ medium. A partly methionine-dependent transformed fibroblast cell line with reduced capacity to proliferate in $Met^-Hcy^+$ medium showed increased growth potential when the cells were depleted of glutathione by a non-toxic concentration of BSO. An even higher growth potential of these cells in $Met^-Hcy^+$ medium was obtained by addition of a non-toxic concentration of cystamine, while only a transient increase of glutathione content was observed under these conditions. Both BSO and cystamine increased the fraction of protein-bound cysteine and homocysteine in the partly methionine-dependent cells. These metabolic alterations correlated with the increased ability of these cells to utilize homocysteine for growth. Our results suggest that methionine auxotrophy is a metabolic defect that is not related to the cellular glutathione status, but may be related to the intracellular distribution between free and protein-bound forms of other thiols as cysteine and homocysteine.**

## Introduction

The ability of cells in culture to grow in medium where methionine (Met*) is replaced by its metabolic precursor, homocysteine (Hcy), has been the subject of numerous investigations (1−4). Initial experiments (5,6) indicated that loss of the ability to utilize Hcy was linked to neoplastic transformation, and malignant cells were defined as 'methionine-dependent' (methionine auxotrophy). However, several investigations have revealed a more complex nature of this phenomenon since some normal cells have been shown to require Met for growth, and different malignant cells have been shown to be Met independent (3−9). These results indicate that even if Met dependence is a result of an oncogenic transformation process, it may not be an obligatory event for the neoplastic stage.

The biochemical basis for this metabolic defect is not well understood. No defects in enzymes involved in the metabolism of Met and Hcy have been demonstrated so far, but some reports indicate reduced ability to utilize endogenously formed Met in Met-dependent cells, low intracellular concentration of free Met and a low *S*-adenosylmethionine/*S*-adenosylhomocysteine ratio, suggesting decreased overall transmethylation rate in Met-deficient, Hcy-supplemented ($Met^-Hcy^+$) medium (1,10,11).

We have previously shown that the chemical form in which Hcy is administered to the cells is crucial. The reduced form of Hcy and of other thiols, including cysteine, is rather toxic to the cells (9,12−14), whereas the thiolactone form of homocysteine (Hcy-tl) is non-toxic at the concentration used in the growth experiments (9). Our recent report concerning co-culture of Met-independent fibroblasts with Met-dependent leukemia cells in $Met^-Hcy^+$ medium indicated that inability to utilize Hcy-tl for growth was not due to production and release of toxic factor(s) to the growth medium from the Met-dependent cells (15).

The alteration in metabolism of Hcy and Met found in many malignant cells may be a clue to the understanding of certain steps in the oncogenic process. Investigating such metabolic defects may also provide the necessary knowledge for designing effective chemotherapeutic regimens. Notably, co-culture experiments with normal and malignant cells in $Met^-Hcy^+$ medium have demonstrated selective growth of normal cells (15,16). A combination of cytostatic agents with selection for Met-independent cells has also been demonstrated to eliminate efficiently malignant cells from normal human cells (17).

In a previous report we demonstrated that a transformed cell line with reduced capacity to utilize Hcy-tl for growth showed altered glutathione (GSH) content when transferred to a medium containing Hcy-tl instead of Met (9). Glutathione is the most abundant non-protein thiol in the cells and is involved in cellular detoxication processes (18,19). Alteration of the GSH level may, therefore, reflect cellular response to toxic agents. On the other hand, alteration of the GSH level may affect the cellular sensitivity towards toxic agents, and it has been demonstrated that GSH partly protects the cells against the action of several cytostatic drugs. Compounds modulating glutathione level are therefore becoming increasingly important in cancer chemotherapy (19−21).

Several thiols, including Hcy, have been shown to elevate cellular glutathione content of mouse fibroblasts (22), possibly by increasing cysteine uptake (23). The elevation of cellular glutathione by cysteamine was accompanied by an increased Hcy export. Recent experiments revealed that cysteamine increased both GSH level and Hcy export by independent mechanisms (24) in these cells.

*Abbreviations: Met, methionine; Hcy, homocysteine; $Met^-Hcy^+$ medium, methionine-deficient, homocysteine-supplemented medium; Hcy-tl, homocysteine thiolactone; GSH, reduced glutathione; GSSG, oxidized glutathione; BSO, L-buthionine-(S,R)-sulfoximine; DTE, dithioerythritol; mBrB, monobromobimane; BME, basal medium Eagle; EMEM, Eagle's minimal essential medium; PE, plating efficiency.

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On the other hand, glutathione may be of vital importance in controlling and regulating the level of thiols like Hcy and maintaining the balance between the reduced and oxidized forms. Altered GSH metabolism, as in the partly Met-dependent malignant mouse fibroblasts (9), may affect the availability of reduced Hcy for Met synthesis.

The present investigation was undertaken to establish the influence of intracellular glutathione level on Met auxotrophy. Three different mouse cell lines with different ability to utilize Hcy-tl for growth were chosen: a non-transformed fibroblast cell line with approximately normal growth rate in $Met^-Hcy^+$ medium (Met-independent), a leukemia cell line completely unable to proliferate in $Met^-Hcy^+$ medium (Met-dependent) and a malignant fibroblast cell line with reduced proliferation capacity under these conditions (partly Met-dependent). The different cell lines were treated with L-buthionine-(*S*,*R*)-sulfoximine (BSO), a GSH synthesis inhibitor (25,26), or cystamine to modulate the intracellular GSH level. Toxicity studies were performed to select carefully concentrations of BSO and cysteamine/cystamine with pronounced effect on cellular glutathione content without inhibiting cell growth. The effect of GSH modulation on proliferation in $Met^-Hcy^+$ medium and on the intracellular distribution of glutathione, Hcy and cysteine between the reduced, oxidized and protein-bound forms is presented.

## Materials and methods

### *Chemicals*

L-Hcy-tl, L-Met, *S*-adenosylhomocysteine, dithioerythritol (DTE), L-cysteine, cysteamine and cystamine, vitamin $B_{12}$ (cyanocobalamin), folic acid, BSO, *N*-ethylmorpholine and reduced (GSH) and oxidized (GSSG) glutathione were obtained from Sigma Chemical Co., St Louis, MO. DMSO, hydrogen bromide, 5-sulfosalicylic acid (dihydrate), perchloric acid, acetic acid, orthophosphoric acid and methanol (for chromathography) were purchased from Merck AG, Darmstadt, FRG. Tetrabutylammonium hydroxide was obtained from Aldrich-Chemie, Steinheim, FRG. Sodium borohydride was from Fluka Chemie AG, Switzerland, and monobromobimane (mBrB) was from Calbiochem, Behring Diagnostics, La Jolla, CA.

Solution A refers to physiological salt solution containing 140 mM HBr and 44% DMSO.

### *Cell lines and culture conditions*

Stock cultures of non-transformed C3H/10T1/2 Cl 8 (27) and chemically transformed C3H/10T1/2 Cl T422 (28) mouse embryo fibroblasts were obtained from the laboratory of Dr J.R.Lillehaug, Department of Biochemistry, University of Bergen, and maintained as described previously (9).

The mouse T-lymphoma cell line R1.1 (29) was obtained from Dr Dennis A.Carson at Scripps Clinic and Research Foundation, La Jolla, CA Stock cultures of R1.1 were maintained in suspension culture as described previously (22).

Both cell lines were maintained at 37°C in an atmosphere of 5% $CO_2$ in air and a relative humidity of 95%.

### *Toxicity of BSO and cysteamine on mouse embryo fibroblasts*

*Growth inhibition.* Cells were seeded in tissue culture dishes (3.5 cm, Nunc, Denmark) in basal medium Eagle (BME, Flow Laboratories, Herts, UK) supplemented with 10% heat-inactivated fetal calf serum (Sera-Lab Ltd, Sussex, UK). In the exponential growth phase, the medium was replaced with fresh medium containing different concentrations of BSO or cystamine. At the times indicated, two parallel dishes from each group were removed, the cells trypsinized and counted using a Coulter Counter Model ZM (Coulter Electronics Ltd, Luton, UK).

*Plating efficiency (PE).* Two hundred cells were seeded per dish (6 cm, Nunc) in BME as above. One day later, the medium was replaced with fresh medium containing increasing concentrations of cysteamine (prepared immediately before use) or the corresponding disulfide, cystamine.

After 24 h the medium was replaced with fresh medium. Ten days after seeding, the medium was removed, the cells washed with 0.9% NaCl, fixed with methanol and stained with Giemsa. Colonies were scored and PE expressed as number of colonies in test group as a percentage of control. Each group consisted of at least five dishes.

### *Analysis of cell growth*

*Cl 8 and Cl T422 mouse fibroblasts.* Cells were seeded on tissue culture dishes (3.5 or 6 cm, Nunc) in $Met^-$ Eagle's minimal essential medium (EMEM, Flow Laboratories) supplemented with 1.5 μM vitamin $B_{12}$ (cyanocobalamin), 11.3 μM folic acid, 10% heat-inactivated, dialysed fetal calf serum (Sera-Lab Ltd) and either 100 μM L-Met ($Met^+Hcy^-$) or 100 μM L-Hcy-tl ($Met^-Hcy^+$). In all growth experiments the fibroblasts were seeded at a density of 2350 cells/$cm^2$. Two or three days after seeding the medium was replaced with fresh medium containing BSO or cystamine as indicated. At each time point two parallel dishes from each group were removed, the cells trypsinized and counted.

*R1.1 mouse lymphoma cells.* Cells were seeded at a density of $2 \times 10^5$ cells/ml in tissue culture flasks (25 $cm^2$, Nunc) in $Met^-$ RPMI 1640 medium (Sigma) supplemented as above and containing 100 μM L-Met, 100 μM L-Hcy-tl and 20 μM cystamine as indicated. At each time point samples from two parallel flasks from each group were removed and counted.

### *Culture and processing of cells for analysis*

*Cl T422 transformed mouse fibroblasts.* Cells were seeded at a density of 2350 cells/$cm^2$ in tissue culture dishes (6 cm, Nunc, or 10 cm, Costar, USA) in $Met^-$ EMEM medium supplemented as above. Three or four days after seeding, the medium was replaced with fresh medium containing either 20 μM BSO or 25 μM cystamine. At the times indicated the cells were harvested by removing the medium from the dish, keeping the dish on ice, and the cells gently washed twice with 10 ml ice-cold PBS. The cells were then immediately extracted with ice-cold 5% sulfosalicylic acid containing 50 μM DTE and the cells scraped off the dish with a rubber policeman. After centrifugation the supernatant was used to determine the content of total free or reduced free glutathione, cysteine and Hcy employing procedures 1 or 2 (described below) respectively. The precipitated proteins were dissolved in 300 μl DMSO containing 50 μM DTE and analysed for protein-bound glutathione, cysteine and Hcy using procedure 3 below. The procedures used for the determination of the different forms of these sulfur compounds are based on a previously published method for glutathione in plasma (30).

For determination of cell number two parallel dishes from each group were harvested, the cells trypsinized and counted.

*R1.1 mouse lymphoma cells.* Exponentially growing cells ($2 \times 10^5$ cells/ml) were seeded in tissue culture flasks (25 $cm^2$, Nunc) in RPMI 1640 medium (Flow Laboratories) supplemented with 10% heat-inactivated fetal calf serum and either no additions or 20 μM cystamine (40 μM SH-equivalents). At the times indicated, samples of the cell suspension were harvested for analysis. The cell suspension was centrifuged at room temperature for 7 min at 500 *g*. The medium was then removed completely from the cell pellet, the cells resuspended in 5 ml ice-cold PBS and centrifuged at 4°C for 7 min at 500 *g*. The washing procedure was repeated once, the PBS removed and the cells immediately extracted with sulfosalicylic acid as described above. After centrifugation the acid supernatant was analysed for glutathione and cysteine according to procedures 1 and 2.

Two parallel samples of the cell suspension were removed at each time point for quantitation of cell numbers.

### *Analysis of glutathione, homocysteine and cysteine*

*Total free glutathione, homocysteine and cysteine (procedure 1).* To 30 μl of the protein-free (acid-treated) cell extract or medium described above, 30 μl of 1.4 M $NaBH_4$, 160 μl of solution A, 50 μl of 1.0 M *N*-ethylmorpholine (final pH 9.0) and 10 μl of 20 mM mBrB in 100% acetonitrile were added. After a 20 min incubation at room temperature (20°C) in the dark, 20 μl of 1.06 N perchloric acid was added.

*Reduced free glutathione, homocysteine and cysteine (procedure 2).* To 30 μl of the protein-free (acid-treated) cell extract, 30 μl of 5% sulfosalicylic acid/50 μM DTE, 160 μl of distilled water, 50 μl of 1.0 M *N*-ethylmorpholine (final pH 8.5) and 10 μl of 20 mM mBrB in 100% acetonitrile were added. After a 20 min incubation at room temperature (20°C) in the dark, 20 μl of 1.06 N perchloric acid was added.

*Protein-bound glutathione, homocysteine and cysteine (procedure 3).* To 30 μl of the DMSO dissolved protein described above, 30 μl of 2.0 M $NaBH_4$ and 15 μl 50% sulfosalicylic acid were added. After centrifugation, 145 μl of solution A, 50 μl of 1.0 M *N*-ethylmorpholine (final pH 9.0) and 10 μl of 20 mM mBrB in 100% acetonitrile were added. After a 20 min incubation at room temperature (20°C) in the dark, 20 μl of 1.06 N perchloric acid was added.

*Chromatography.* The HPLC system was programmed to inject 25 μl samples into a 150 × 4.6 mm column packed with 3 μm particles of ODS-Hypersil (Shandon Southern Ltd, Chesire, UK). The chromatography was performed at 25°C and at a flow rate of 1.5 ml/min. The elution solvent A was 0.25% acetic acid containing 10 mM tetrabutylammonium phosphate (pH 3.4), and solvent B was 20% acetonitrile containing 0.25% acetic acid and 10 mM tetrabutylammonium phosphate (pH 3.4). Solvent C was 75% acetonitrile. The elution was as follows: 0–13 min, 3–22% B linear gradient; 13.1–22 min, 45% B

isocratic and 22.1–28 min. 45–70% B linear gradient. The column was washed with solvent C for 5 min after each injection. The retention times for the mBrB derivatives of cysteine, Hcy and glutathione were 8.3, 13.1 and 27.3 min respectively.

*Instrumentation.* The instrumentation used was as described previously (30).

*Determination of protein*

Protein was determined according to Bradford (31) using the Bio-Rad Protein Assay Kit. Bovine γ-globulin was used as protein standard.

## Results

*Met-independent cells*

C3H/10T1/2 Cl 8 mouse fibroblasts have previously been shown to be Met independent since they grow well in $Met^{-}Hcy^{+}$ medium, although at a slightly slower rate than in $Met^{+}Hcy^{-}$ medium (9). BSO at a concentration of 20 μM reduces the glutathione content to <5% of control with minimal toxicity against these cells (22). Depletion of glutathione in Cl 8 cells by BSO did not influence the growth in $Met^{+}Hcy^{-}$ medium, and had only a slight lag-producing effect on the growth in $Met^{-}Hcy^{+}$ medium (Figure 1).

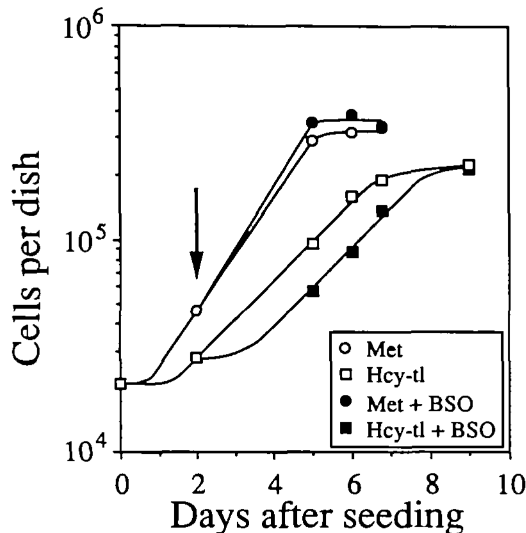


**Fig. 1.** Depletion of glutathione and effect on utilization of Hcy-tl by Met-independent Cl 8 mouse fibroblasts. Cells were seeded (21 000 cells/dish; 3.5 cm) in $Met^{-}$ medium supplemented with either 100 μM L-Met or 100 μM L-Hcy-tl. Two days after seeding (arrow) the medium was removed and replaced with fresh medium as above containing either no additions or 20 μM BSO.

*Met-dependent cells*

R1.1 mouse lymphoma cells have previously been shown to be completely Met dependent since they do not proliferate in $Met^{-}Hcy^{+}$ medium (15). Since both cystamine and β-mercaptoethanol elevate glutathione levels in C3H/10T1/2 Cl 8 cells (22), these compounds were used to increase glutathione levels in R1.1 cells in an attempt to alter the ability to utilize Hcy-tl for growth. Neither cysteamine nor β-mercaptoethanol at concentrations ranging from 2 to 50 μM had any effect on the lack of proliferation of R1.1 cells in $Met^{-}Hcy^{+}$ medium, while only minor growth inhibitory effects at the highest concentrations could be observed in medium containing Met (data not shown). Cystamine is less toxic than the reduced form, but has the same ability to increase glutathione in Cl 8 cells (22), and 20 μM cystamine increased glutathione up to 1.4-fold in R1.1 cells (Table I). However, increased glutathione level did not cause these cells to proliferate in $Met^{-}Hcy^{+}$ medium (Figure 2).

It should be noted that the intracellular increase in glutathione was accompanied by an increase in cysteine content, while no alteration in the distribution between the reduced and the oxidized form of cysteine was observed (Table I).

*Partly Met-dependent cells*

C3H/10T1/2 Cl T422 transformed mouse fibroblasts have previously been shown to be partly Met dependent since they proliferate in $Met^{-}Hcy^{+}$ medium, but at a much slower rate than in $Met^{+}Hcy^{-}$ medium. To investigate whether modulation of GSH status had any effect on the ability of Hcy-tl to support growth, the cells were treated with BSO or cystamine and analyzed for growth and content of glutathione, homocysteine and cysteine.

To establish suitable, non-toxic concentrations of BSO and cystamine, toxicity experiments were performed. A concentration of 20 μM BSO had no significant effects on the growth of Cl T422 cells, while 100 μM BSO had a slight growth inhibitory effect 4 days after addition (Figure 3). The reduced and oxidized form of cysteamine differed in toxicity against these cells (Figure 4). From the toxicity experiments the $LD_{90}$ for cysteamine (–SH) was estimated to be 270 μM, while for

**Table I.** Intracellular content of free cysteine and glutathione in R1.1 cells

| Hours after addition | Addition | Cysteine | | Glutathione | |
|---|---|---|---|---|---|
| | | Total | Reduced | Total | Reduced |
| 0 | no addition | 0.115 ± 0.005 | 0.020 ± 0.002 | 2.739 ± 0.050 | 1.951 ± 0.115 |
| 6 | no addition | 0.155 ± 0.052 | 0.035 ± 0.010 | 4.000 ± 0.073 | 3.876 ± 0.162 |
| | 20 μM cystamine | 0.218 ± 0.026 | 0.045 ± 0.006 | 5.496 ± 0.249 | 5.207 ± 0.043 |
| 24 | no addition | 0.191 ± 0.017 | 0.035 ± 0.003 | 3.313 ± 0.239 | 2.991 ± 0.174 |
| | 20 μM cystamine | 0.257 ± 0.023 | 0.051 ± 0.004 | 4.172 ± 0.047 | 3.774 ± 0.225 |
| 48 | no addition | 0.095 ± 0.004 | 0.015 ± 0.002 | 1.722 ± 0.092 | 1.535 ± 0.042 |
| | 20 μM cystamine | 0.131 ± 0.019 | 0.023 ± 0.003 | 1.926 ± 0.234 | 1.813 ± 0.092 |
| 72 | no addition | 0.078 ± 0.008 | 0.011 ± 0.001 | 0.941 ± 0.022 | 0.878 ± 0.032 |
| | 20 μM cystamine | 0.087 ± 0.01 | 0.014 ± 0.002 | 1.008 ± 0.017 | 0.900 ± 0.040 |

Cells were grown in $Met^{+}Hcy^{-}$ medium containing either no additions or 20 μM cystamine. The results are expressed as equivalents of reduced form (nmol/$10^6$ cells) and represent the mean of four determinations ± SD.

cystamine (−SS−) the $LD_{90}$ was >1000 μM SH-equivalents (corresponding to 500 μM cystamine).

Since we have previously shown that both forms had the same ability to increase glutathione in C3H/10T1/2 Cl 8 cells (22), we preferred cystamine for the following experiments. A concentration of 50 μM cystamine (100 μM SH-equivalents) had a distinct growth inhibiting effect on these cells, while 16 μM cystamine (32 μM SH-equivalents) had no effect (Figure 5). Concentrations of 320 and 1000 μM SH equivalents of cystamine completely inhibited the growth of Cl T422 cells.

Based on these findings, we used 20 μM BSO and 25 μM cystamine (50 μM SH-equivalents) to modulate the GSH level of Cl T422 cells. The results demonstrate that neither BSO nor cystamine at these concentrations had any significant effect on the cell growth in $Met^{+}Hcy^{-}$ medium (Figure 6A). On the contrary, distinct effects were observed in $Met^{-}Hcy^{+}$ medium. BSO had a slight lag-producing effect, but then stimulated the cells to grow at a rate similar to the cells receiving Hcy-tl alone, but to an essentially higher density (Figure 6A). Remarkably, the glutathione content in these cells was reduced to 1.3% of control at day 7, and this low glutathione level persisted throughout the experiment (Figure 6B).

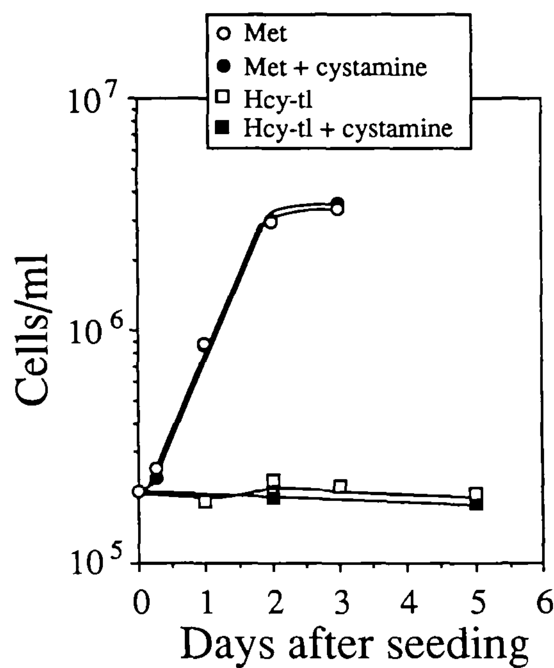


**Fig. 2.** Elevation of glutathione by cystamine and effect on utilization of Hcy-tl by Met-dependent R1.1 mouse leukemia cells. Two hundred thousands cells/ml were seeded in $Met^{-}$ medium supplemented with either 100 μM L-Met or 100 μM L-Hcy-tl and with or without co-addition of 20 μM cystamine. Duplicate samples from each group were used to determine the number of cells/ml.

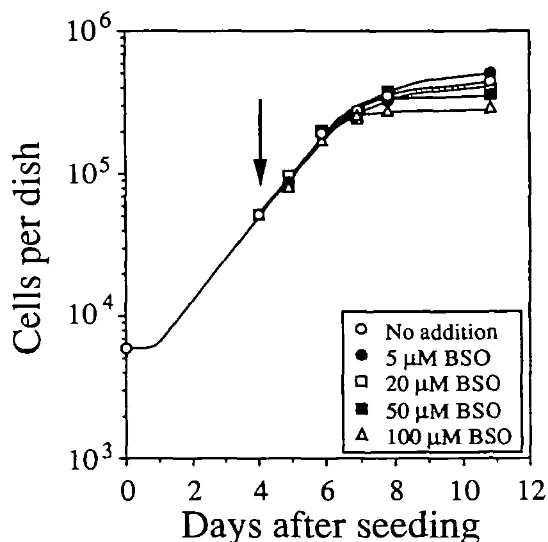


**Fig. 3.** Dose-dependent effect of BSO on growth of Cl T422 cells. Cells (6000/dish; 3.5 cm) were seeded in $Met^{-}$ medium supplemented with 100 μM L-Met, and 4 days after seeding (arrow) the medium was replaced by fresh medium containing BSO at the concentrations indicated. Duplicate dishes were used for determination of cell number.

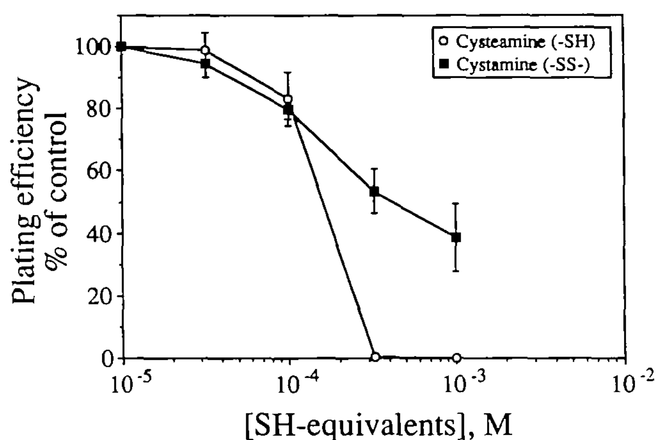


**Fig. 4.** Toxicity of cysteamine on Cl T422 cells. Two hundred cells were seeded per dish (6 cm) and treated for 24 h with cysteamine (reduced form) or cystamine (disulfide form) as indicated. Ten days after seeding, colonies were stained and scored. Results are expressed as number of colonies as percentage of control (relative plating efficiency). Each point represents the average of at least five dishes.

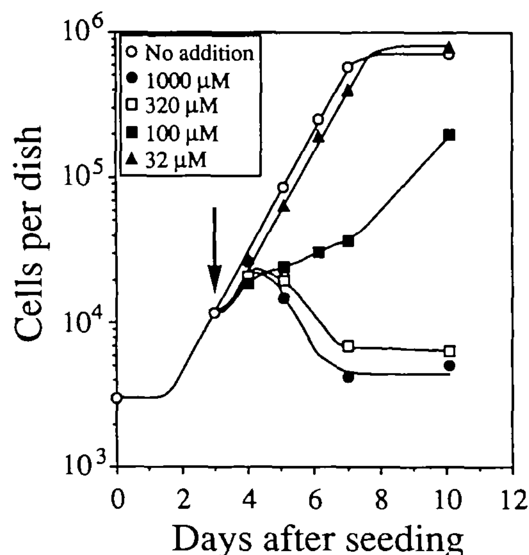


**Fig. 5.** Effect of cystamine on growth of Cl T422 cells. Three thousand cells were seeded per dish (3.5 cm) in Met-containing medium, and 3 days after seeding (arrow) the medium was replaced by fresh medium containing cystamine at the concentrations indicated. Concentrations are given as sulfhydryl (SH) equivalents. Duplicate dishes were used for determination of cell number.

Addition of cystamine did not increase the growth rate of these cells but they grew to a higher density than both the BSO-treated and the untreated control cells (Figure 6A).

The effect of BSO or cystamine on the intracellular contents of cysteine, homocysteine and glutathione is shown in Table II. No essential difference in free glutathione content in cells treated with cystamine compared to control was observed 3 or 7 days after addition of cystamine. Notably, there was a 2- to 3-fold increase in protein-bound glutathione after addition of cystamine to the cells. BSO increased the total content of free cysteine, while Hcy was less affected. Both BSO and cystamine increased the fraction of protein-bound cysteine and Hcy (Table II).

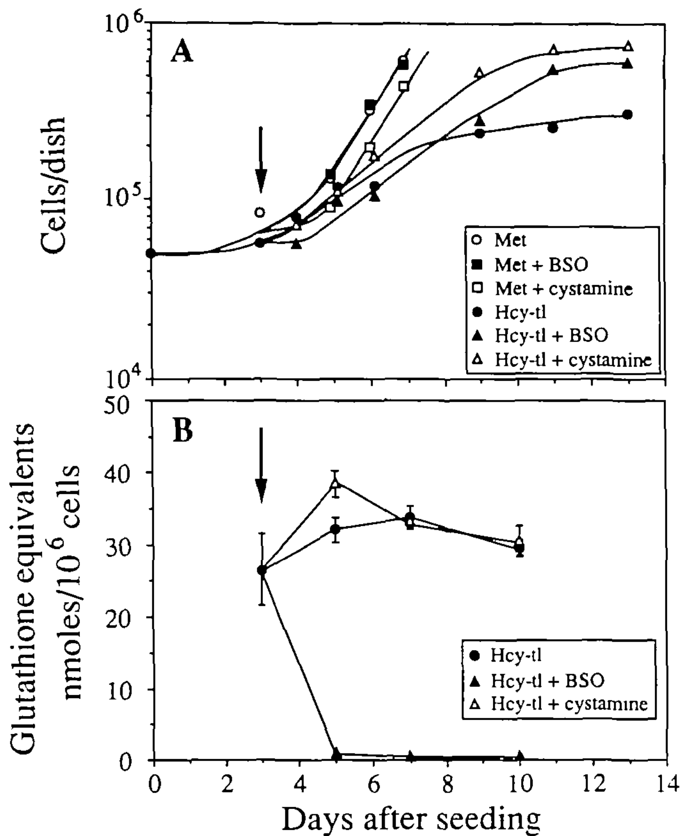


**Fig. 6.** Modulation of glutathione and effect on utilization of Hcy-tl by partly Met-dependent Cl T422 mouse fibroblasts. Cells were seeded (50 000 cells/dish; 6 cm) in Met⁻ medium supplemented with either 100 μM L-Met or 100 μM L-Hcy-tl. Three days after seeding (arrow) the medium was removed and replaced with fresh medium as above containing either no additions, 20 μM BSO or 25 μM cystamine. (A) Cell growth. Duplicate dishes were used for determination of cell number. The growth curves are representative examples of three separate experiments. (B) Cellular content of total glutathione in cells grown in Hcy-tl supplemented medium. Results are expressed as equivalents of reduced glutathione and represent the average of four determinations ± SD.

## Discussion

The aim of the present investigation was to study the influence of glutathione status on Met dependence. The results clearly demonstrate that depleting Met-independent cells of glutathione by BSO had no effect on the ability to utilize Hcy for growth (Figure 1). The initial lag produced by BSO in $Met^-Hcy^+$ medium may indicate that the cells need some time to adapt to the low level of cellular glutathione. One might speculate if this lag period represents breakdown of BSO or re-establishing GSH levels via other mechanisms. However, these possibilities are rejected by the facts that further addition of BSO to the non-transformed Cl 8 cells after they had resumed growth in $Met^-Hcy^+$ medium had no effect on growth (data not shown). Furthermore, the malignant Cl T422 cells resumed growth 24 h after BSO treatment despite the persisting low level of GSH (Figure 6).

In contrast to the Cl 8 cells, the R1.1 leukemia cells are completely Met dependent (15). Cystamine increased both intracellular glutathione and cysteine in these cells (Table I), but had no effect on growth in $Met^-Hcy^+$ medium (Figure 2). Addition of 500 μM L-2-oxothiazolidine-4-carboxylate, which is a non-toxic cysteine precursor and a potent enhancer of intracellular GSH level (32,33), did not initiate proliferation in $Met^-Hcy^+$ medium (data not shown). Depleting the R1.1 cells of glutathione by 20 μM BSO may slightly decrease the Hcy export rate (22), but had no effect on the cells' inability to grow in $Met^-Hcy^+$ medium (data not shown).

The malignant Cl T422 cells represent an intermediate between the non-transformed Cl 8 cells and the leukemic R1.1 cells with respect to growth in $Met^-Hcy^+$ medium (9,15). The cells are partly Met dependent and are therefore especially suited for modulating glutathione levels and determining the effect on the ability to utilize Hcy-tl for growth.

The Cl T422 cells seemed to be slightly less sensitive to the cytotoxic effects of BSO than Cl 8 cells. Concentrations >20 μM had a slight growth-inhibitory effect on Cl 8 cells 48 h after addition (22), while no effects on growth of Cl T422 cells could be observed at concentrations up to 100 μM 48 h after addition (Figure 3).

We have previously shown that homocysteine in the reduced

**Table II.** Intracellular content of cysteine, homocysteine and glutathione in C3H/10T1/2 Cl T422 cells

| Days after addition | Addition | Cysteine | | | Homocysteine | | | Glutathione | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Free | | Protein-bound | Free | | Protein-bound | Free | | Protein-bound |
| | | Total | Reduced | | Total | Reduced | | Total | Reduced | |
| 3 | no addition | 0.360 ± 0.025 | ND | 0 024 ± 0.005 | 0.082 ± 0.012 | ND | 0.016 ± 0.002 | 20 04 ± 1.40 | 18.46 ± 1.29 | 0.092 ± 0.014 |
| | 20 μM BSO | 0 757 ± 0.053 | 0.057 ± 0.004 | 0.071 ± 0.036 | 0 131 ± 0.020 | ND | 0.052 ± 0.031 | 1.496 ± 0.105 | 1.218 ± 0.085 | 0.028 ± 0.002 |
| | 25 μM cystamine | 0.399 ± 0.028 | 0.039 ± 0.003 | 0.052 ± 0.016 | 0.113 ± 0.017 | ND | 0.046 ± 0.014 | 20.29 ± 1 42 | 19 48 ± 1.36 | 0.181 ± 0.013 |
| 7 | no addition | 0.412 ± 0 029 | 0.027 ± 0.002 | 0.032 ± 0.005 | 0.078 ± 0.012 | ND | 0.026 ± 0.003 | 23.71 ± 1.66 | 20.75 ± 1.45 | 0.195 ± 0.014 |
| | 20 μM BSO | 0.559 ± 0.039 | 0.061 ± 0.004 | 0.086 ± 0.026 | 0.053 ± 0.008 | ND | 0.059 ± 0.041 | 0.873 ± 0.061 | 0.714 ± 0.050 | 0.022 ± 0.002 |
| | 25 μM cystamine | 0.260 ± 0.018 | 0.030 ± 0.002 | 0.091 ± 0.014 | 0.040 ± 0.006 | ND | 0.040 ± 0.003 | 27.31 ± 1.91 | 25.41 ± 1.78 | 0.556 ± 0.039 |

Cells were grown in $Met^-Hcy^+$ medium containing either no additions, 20 μM BSO or 25 μM cystamine. The results are expressed as equivalents of reduced form (nmol/$10^6$ cells) and represent the mean of five determinations ± SD.
ND, not detectable.

form is toxic to C3H/10T1/2 cells, while the thiolactone or disulfide of homocysteine is non-toxic at 100 μM (9). In accordance with this, the present results clearly demonstrate the increased toxicity caused by the thiol group of cysteamine at concentrations >100 μM (Figure 4). Cystamine has excellent solubility properties and elevates glutathione content with about the same efficiency as the reduced counterpart in these cells (22). Cystamine was therefore preferred in the present experiments. As shown, 25 μM cystamine (50 μM SH-equivalents) had only a minor, if any, growth-inhibitory effect on Cl T422 cells (Figure 5).

In accordance with our previous results (9), the Cl T422 cells proliferated in $Met^-Hcy^+$ medium, but at a distinctly lower rate than in the presence of Met (Figure 6A). Forty-eight hours after addition of cystamine, the glutathione content was only slightly elevated (Figure 6B), but no effects on growth in the $Met^-Hcy^+$ medium had been observed. In contrast, there was a significant promotion of growth in $Met^-Hcy^+$ medium 4–5 days after exposure to cystamine and at a point where glutathione content was not different from that of control cells (Figure 6A).

Even more remarkable were the results obtained when the cells were depleted of glutathione by BSO. After the small initial lag, the cells started to proliferate at a rate similar to the cells receiving Hcy-tl only. In agreement with previous results (22), BSO reduced the glutathione content of Cl T422 cells to <3% of control within 48 h after addition (Figure 6). Two days later, the glutathione content was only 1.3% of control, and at this stage growth of the control cells levelled off, while the glutathione-depleted cells continued to proliferate, reaching a level just below that of the cystamine-treated cells. It is noteworthy that the cells were able to grow for an extended period of time at a GSH level of 1.3% of control, indicating that high levels of glutathione are not required to keep Hcy utilized for Met synthesis in reduced form. This also implies that methionine synthetase is active during severe glutathione depletion, despite the fact that this enzyme requires an efficient reducing system for activity *in vitro* (34).

Previously we have shown that cysteamine caused a transient increase in glutathione level in C3H/10T1/2 Cl 8 cells (24), and the same pattern is recognized in the Cl T422 cells (Figure 6B, Table II). Notably, there was a 2- to 3-fold increase in protein-bound glutathione 3 and 7 days after addition of cystamine to the cells (Table II), which may indicate that protein-bound glutathione is more stable than free glutathione.

BSO markedly increased the total content of free cysteine, while Hcy was less affected (Table II). The increase in cysteine may be due to reduced consumption of cysteine for GSH synthesis, or to increased uptake of cystine after BSO treatment as suggested by others (35). Both BSO and cystamine increased protein-bound cysteine or Hcy, and also the fraction of the protein-bound form compared to the total amount of these thiols (free reduced + free oxidized + protein-bound) increased after treatment with BSO or cystamine (Table II). BSO probably acts by reducing the cellular reduction potential thus making protein thiol groups more susceptible to mixed disulfide formation. The effect of cystamine could be related to a possible role in the formation of protein disulfide bonds. It has been suggested that cystamine provides disulfide for a thiol–disulfide exchange reaction as a intermediate step in protein disulfide bond formation in the presence of high levels of cellular glutathione and glutathione reductase (36).

The alterations in the distribution between the protein-bound and the free forms of cysteine and Hcy correlated with the increased growth potential of the Cl T422 cells in $Met^-Hcy^+$ medium (Figure 6A). These findings point to the possibility that utilization of Hcy for growth may be affected by protein thiolation, which has been suggested to play an important role in metabolic regulation (3).

In conclusion, our results indicate that the ability to utilize Hcy-tl for growth is not dependent on the intracellular glutathione status, but may be related to the intracellular distribution between free and protein-bound forms of other thiols such as cysteine and Hcy. The biochemical basis and consequences of this defect on Hcy or Met metabolism should be further investigated.

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