JOURNAL OF CELLULAR PHYSIOLOGY 146:52–62 (1991)

Homocysteine Export From Cells Cultured in the Presence of Physiological or Superfluous Levels of Methionine: Methionine Loading of Non-Transformed, Transformed, Proliferating, and Quiescent Cells in Culture

BENEDICTE CHRISTENSEN,* HELGA REFSUM, OLAV VINTERMYR, AND PER MAGNE UELAND

The Clinical Pharmacology Unit, Department of Pharmacology and Toxicology (B.C., H.R., P.M.U.), and Cell Biology Research Group, Department of Anatomy (O.V.), University of Bergen, N-5021 Haukeland Hospital, Norway

Determination of the transient increase in plasma homocysteine following administration of excess methionine is an established procedure for the diagnosis of defects in homocysteine metabolism in patients. This so-called methionine loading test has been used for 25 years, but the knowledge of the response of various cell types to excess methionine is limited. In the present paper we investigated homocysteine export from various cell types cultured in the presence of increasing concentrations (15–1,000 μM) of methionine. For comparison of homocysteine export, the export rates per million cells were plotted versus cell density for proliferating cells, and versus time for quiescent cells. The homocysteine export from growing cells was greatest during early to mid-exponential growth phase, and then decreased as a function of cell density. The export rate was higher from phytohemagglutinin-stimulated than non-stimulated lymphocytes, and higher from proliferating than from quiescent fibroblasts. The hepatocytes showed highest export rate among the cell types investigated. The enhancement of homocysteine export by excess methionine ranged from no stimulation to marked enhancement, depending on cell type investigated, and three different response patterns could be distinguished: 1) quiescent fibroblasts and growing murine lymphoma cell showed no significant increase in homocysteine export following methionine loading; export from human lymphocytes was only slightly enhanced in the presence of excess methionine; 2) the homocysteine export from proliferating hepatoma cells and benign and transformed fibroblasts was stimulated three to eightfold by increasing the methionine concentration in the medium from 15 to 1,000 μM; and 3) the response to methionine loading was particularly increased (about 15-fold) in non-transformed primary hepatocytes in stationary culture. The results outline a potentially useful procedure for the comparison of homocysteine export during cell growth in the presence of various concentrations of methionine. The results are discussed in relation to the special feature of homocysteine metabolism in various cell types and tissues including liver, and to the possible source of plasma homocysteine following methionine loading in vivo.

The sulfur amino acid homocysteine (Hcy) is formed from methionine via S-adenosylhomocysteine (Ueland, 1982). Intracellular Hcy is either salvaged to methionine through remethylation or is converted to cysteine via the transsulfuration pathway. In most cells, the former reaction is catalyzed by the enzyme methionine synthase, which requires 5-methyltetrahydrofolate as a methyldonor, and methylcobalamin as cofactor. The first step in the transsulfuration pathway is catalyzed by the enzyme cystathionine β-synthase, which forms cystathionine. Cystathionine is then degraded to α-ketobutyrate and cysteine through the action of cystathionine γ-lyase. Both these reactions require vitamin B_6 as cofactor (Mudd et al., 1989).

Intracellular Hcy is probably exported into the extracellular medium when the rate of Hcy formation exceeds the metabolic capacity. This may occur either during enhanced Hcy formation and/or during inhibition of Hcy metabolism. Thus, such imbalance between production and utilization may increase the amount of

Received June 12, 1990; accepted September 7, 1990.

*To whom reprint requests/correspondence should be addressed.

Hcy in extracellular media like plasma or urine (Fig. 1) (Ueland and Refsum, 1989).

There is an increasing interest in plasma Hcy because the level increases during several diseases or deficiency states. Plasma Hcy seems to be a particularly useful parameter for the diagnosis and follow up of vitamin B_{12} and folate deficiency (Ueland and Refsum, 1989), and also of homocystinuria, an inborn error most commonly caused by lack of cystathionine β-synthase (Mudd et al., 1989). The moderate elevation observed in heterozygous homocystinuria and some other diseases may be of clinical significance, especially since moderate homocysteinemia seems to be an independent risk factor of vascular disease (Ueland and Refsum, 1989).

Plasma Hcy is usually determined as fasting level. However, plasma Hcy may be determined at certain time points after a standard oral dose of methionine. Methionine administration results in a marked raise in plasma Hcy within hours (Sardharwalla et al., 1974). This is the basis for the so-called methionine loading test (Ueland and Refsum, 1989), the metabolic basis of which is depicted in Fig. 1. This test may be particularly useful for the diagnosis of moderate defects in Hcy catabolism, like heterozygous cystathionine β-synthase deficiency (Boers et al., 1985a,b; Sardharwalla et al., 1974). It seems to be equally discriminatory as determination of enzyme activity in cultured fibroblasts from a skin biopsy (Boers et al., 1985a). The methionine loading test has also been used to reveal sex-related differences in the metabolism of methionine and Hcy (Boers et al., 1983), and to identify subjects at increased risk of cardiovascular disease (Brattström et al., 1988; Murphy-Chutorian et al., 1985).

Methionine loading in vivo obviously results in a pronounced enhancement of Hcy egress, at least from some cells. In these cells, methionine probably stimulates Hcy production through increased overall transmethylation rate. However, it is uncertain whether all or only some cell types export large amounts of Hcy in the presence of excess methionine.

In the present paper, we describe the experimental conditions and data treatment developed for the investigation of methionine loading of cells in culture. Cell types were selected based on diversity of origin, growth, and malignancy. Differences in response to methionine among cell types were observed, which were discussed in relation to their possible role in methionine homeostasis, special features of Hcy metabolism, and source of Hcy following methionine loading in vivo.

MATERIALS AND METHODS

Chemicals

L-methionine, L-homocystine, cyanocobalamin, folic acid, dithioerythritol (DTE), and *o*-phthaldialehyde were purchased from Sigma Chemical Co., St. Louis, MO. Sodium borohydride was from Fluka Chemie, AG, Switzerland and monobromobimane from Calbiochem, Behring Diagnostics, La Jolla, CA. Sodium metrizoate and Ficoll (Lymphoprep) was obtained from Nycomed AS, Oslo, Norway.

Cell lines

Human lymphocytes were isolated from the buffy coat from human blood according to the method of Böyum (Böyum, 1968), which includes density gradient centrifugation in a medium containing sodium metrizoate and Ficoll.

The murine lymphoma cell line R 1.1 WT (referred to as lymphoma cells) was supplied by Dr. Dennis Carson, Scripps Clinic, La Jolla, CA. This cell line arose spontaneously in a C58 mouse (Old et al., 1965), and shares antigenic and metabolic properties with normal thymocytes (Ralph, 1973).

The non-transformed murine fibroblast C3H/10$T_{1/2}$ Cl 8 cells (referred to as non-transformed or benign fibroblasts) (Reznikoff et al., 1973b) and the chemically transformed C3H/10$T_{1/2}$ MCA Cl 16 cells (transformed or malignant fibroblasts) (Reznikoff et al., 1973a) were obtained from Dr. J.R. Lillehaug, Department of Biochemistry, University of Bergen. Both cell types were derived from mouse embryo fibroblasts.

Primary hepatocytes (referred to as hepatocytes) were isolated by collagenase perfusion of liver from male Wistar rats (250 g) according to a slight modification (Christoffersen et al., 1984) of the method of Seglen (Seglen, 1976). The cell viability was assessed by the tryptophan dye exclusion test, and was higher than 90%. The hepatocytes were cultured on dilute collagen gel as described by Vintermyr and Døskeland (1987).

The clonal stain MH1C1 of rat hepatoma cells (referred to as hepatoma cells) derived from Morris hepatoma 7795 (Richardson et al., 1969) was obtained from The American Type Culture Collection (CCL 144). These cells are tumorigenic, but have retained various liver-specific functions (Gaudernack et al., 1973; Tashjian et al., 1970).

Culture conditions

The lymphocytes prepared from fresh human blood and the lymphoma cells taken from culture during exponential growth were washed in phosphate-buffered saline, resuspended, and seeded at a cell density of $5 \cdot 10^5$ and $5 \cdot 10^4$ cells/ml, respectively, in a methionine-deficient RPMI 1640 medium (Sigma). The medium was supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories, Irvine, Scotland), folic acid (4 μg/ml), cyanocobalamin (2 μg/ml), and methionine at concentrations indicated. These cells were grown in 5% CO_2/95% air at 37°C in 3.5 cm diameter dishes from Nunc (Roskilde, Denmark).

The non-transformed and transformed fibroblasts and hepatoma cells were taken from exponentially growing cultures and seeded at a density of $3 \cdot 10^3$ cells/ml in a methionine-deficient EMEM medium supplemented with fetal calf serum, folic acid, and cyanocobalamin as described above, and 15 μM methionine. The cells were cultured in 3.5 cm diameter dishes (Nunc) in 5% CO_2/95% air at 37°C. After about 24 hours, the culture medium was removed, the cells were gently washed with phosphate-buffered saline, and fresh medium containing methionine at concentrations indicated was added.

The hepatocytes were seeded at $2 \cdot 10^5$ cells per ml in EMEM medium supplemented with fetal calf serum (2.5%), folic acid (4 μg/ml), cyanocobalamin (2 μg/ml), non-essential amino acids (0.33 mM), and methionine (15 μM). The medium was not supplemented with any

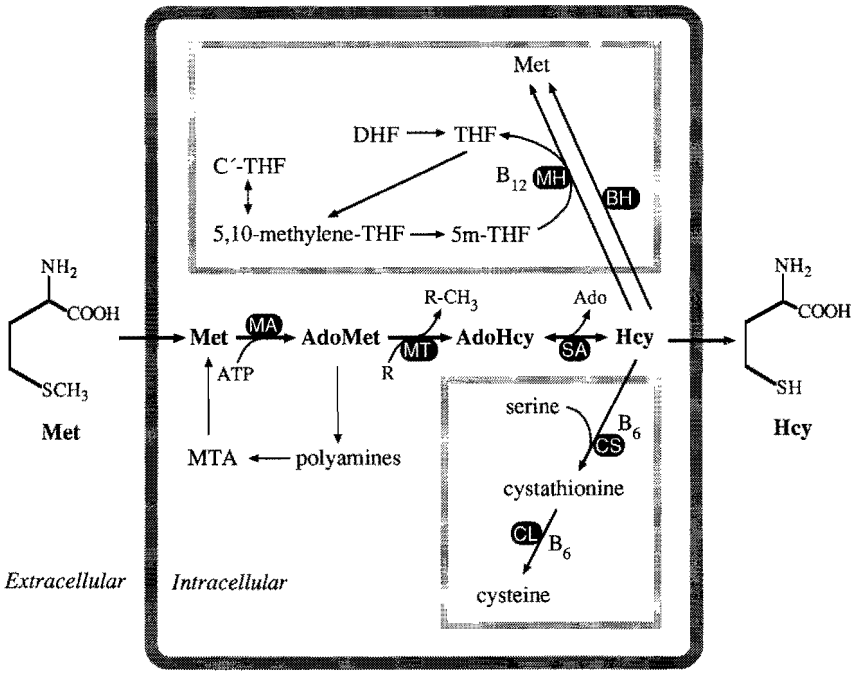

Fig. 1. Conversion of methionine to Hcy and the enzymes involved. Ado, adenosine; AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; BH, betaine-homocysteine methyltransferase; c'-THF, derivative of THF carrying one-carbon unit; CL, cystathionine lyase (γ-cystathionase); CS, cystathionine β-synthase; DHF, dihydrofolate; Hcy, homocysteine; MA, methionine adenosyltransferase; Met, methionine; MH, 5-methyltetrahydrofolate-homocysteine methyltransferase; MT, methyltransferase; MTA, 5'-methylthioadenosine; 5m-THF, 5-methyltetrahydrofolate; SA, S-adenosylhomocysteine hydrolase; THF, tetrahydrofolate.

growth factors. The hepatocytes were cultured in Multiwells (10 cm^2/dish; Nunc) covered with a dilute collagen gel prepared as described (Vintermyr and Døskeland, 1987). After incubation for 2 hours to allow plating, the medium and cells in suspension were removed, and fresh medium containing various concentrations of methionine was added.

Cell harvesting and counting

Upon harvesting, a sample from the medium was taken out from two parallel culture flasks or dishes in each group. The samples were centrifuged to remove cells in suspension, and the medium was frozen at −20°C until determination of Hcy and methionine. The corresponding cells were used for determination of cell density.

The fibroblasts and hepatoma cells were brought into suspension by trypsinization. These cells, and also the lymphoma cells and lymphocytes, were counted using a Coulter Counter Model 2M (Coulter Electronics Ltd., Luton, UK). The hepatocyte layer was washed with phosphate-buffered saline, and trichloroacetic acid added. The denatured protein was resuspended in 1 ml 0.3 N NaOH, and the protein determined according to the method of Bradford (1976).

Determination of Hcy in culture medium

Culture medium contains trace amounts (0.2–0.4 μM) of protein-bound Hcy probably derived from serum (10%). We avoided the interference from serum Hcy by determining free Hcy in medium from cells exporting small amounts of Hcy. Since Hcy is mostly trapped as soluble homocysteine-cysteine mixed disulfide, association of free Hcy with proteins occurs only under conditions of low levels of cysteine. Total Hcy was measured in the hepatocyte medium, since these cells consumed a significant amount of cysteine in the medium and exported large amounts of Hcy.

Free Hcy was determined with either a radioenzymic method (Svardal et al., 1986a; Ueland et al., 1984) or a modification of an automated fluorescence assay developed for the determination of total Hcy in plasma (Refsum et al., 1989). The correlation between these assays was excellent ($R > 0.95$). Total Hcy was determined using the automated fluorescence assay.

The fluorescence assay had the advantage of not being overranged when measuring the high concentrations of Hcy in the hepatocyte medium.

Determination of methionine in culture medium

Methionine was determined in the medium that was deproteinized with acid. The amino acid was derivatized with *o*-phthaldialdehyde (OPA) in the presence of mercaptoethanol in saturated borate buffer, pH 9.5 (Krishnamurti et al., 1984). The derivatization procedure and the injection into an HPLC column were carried out sequentially by a microprocessor controlled autosampler from Gilson, model 232. The methionine-OPA adduct was separated and quantitated by reversed-phase liquid chromatography and fluorescence detection (Krishnamurti et al., 1984).

Curve fitting and calculation of export rates

The accumulation of Hcy in the medium was determined in both proliferating and quiescent cells, cul-

tured in a medium containing different concentrations of methionine. The results are presented as export rate, which refers to net efflux, i.e., efflux less the minor uptake of Hcy under these conditions. For comparison of Hcy export from growing cells, we calculated the export rate per million cells. The calculations and presentation of data are as follows.

The cell number followed an exponential equation during the first part of the growth period, but deviated upon reaching confluence (the fibroblasts or hepatoma cells) or when the methionine level decreased markedly (lymphoma cells). Therefore, the growth curves were best fitted by a polynomial (usually trinomial) function. The data points for the growth and export were usually log-transformed, to obtain a better curve fit in the lower range of the curves. The cell number (N) was given by the equation:

$$\log N = a_0 + a_1 t + a_2 t^2 + a_3 t^3 + \ldots + a_n t^n \quad \text{(I)}$$

where a_0, a_1, a_2, a_3, and a_n are constants and t is time after addition of methionine.

The concentration (y) of Hcy in the medium increased as the Hcy accumulated as a function of time (t). These curves were also usually best fitted to a polynomial:

$$\log y = b_0 + b_1 t + b_2 t^2 + b_3 t^3 + \ldots + b_n t^n \quad \text{(II)}$$

In a few cases (export from lymphoma cells exposed to 15 and 30 μM methionine) the export curves were best fitted to the equation:

$$1/y = c_0 + c_1 t + c_2 t^2 + c_3 t^3 + \ldots + c_n t^n \quad \text{(III)}$$

The degree (n) of the polynomials, the constants (a_0, $a_1 \ldots a_n$; b_0, $b_1 \ldots b_n$; c_0, $c_1 \ldots c_n$) and the R-values were obtained using a curve-fitting program, Multifit, version 1.51 for the Apple Macintosh, from Day Computing, Cambridge, UK. Appropriate fitting of data included the criterion $R^2 > 0.98$.

The differential, dy/dt, is the export rate, which was given as (v) per unit cell mass (N):

$$v = dy/dt/N \quad \text{(IV)}$$

For the exponential growing cells, v was routinely plotted against the cell density on a logarithmic scale, whereas for the quiescent cells, v was plotted versus time.

RESULTS

Comparison of Hcy export rates at different cell densities

Preliminary experiments were performed to optimize the comparison of Hcy export from proliferating cells at different cell densities. In these experiments, lymphoma cells were seeded at three different cell densities, and the Hcy accumulating in the medium was determined during cell growth (Fig. 2A,B). The export rates per unit cell mass were plotted versus time after seeding. The most diluted cells showed the highest export rate (Fig. 2C). When the same data were replotted as export rates versus cell density, the graphs showed an upward curvature corresponding to an ini-

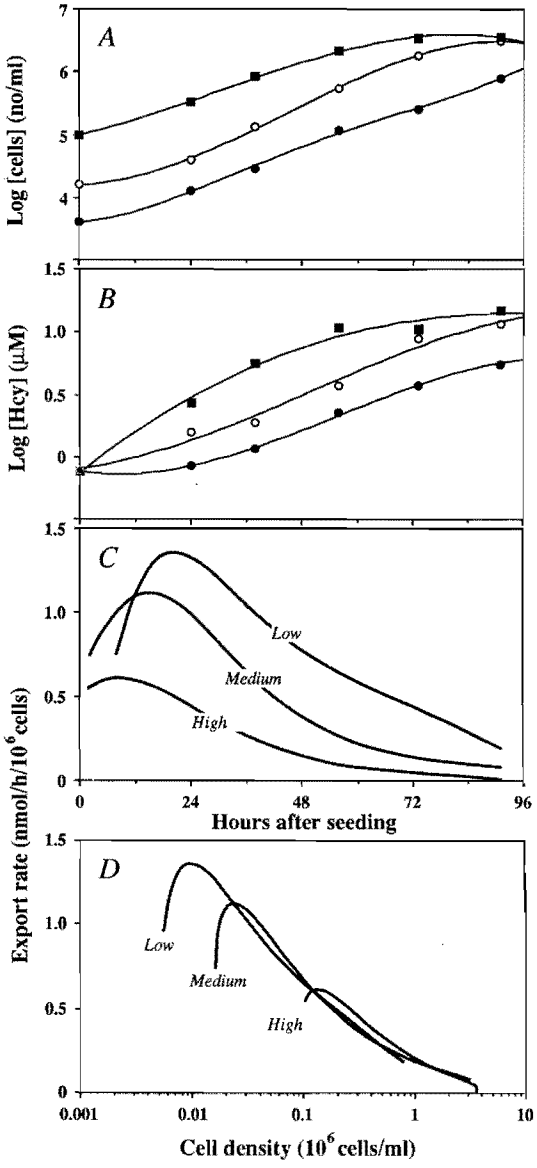

Fig. 2. The rate of homocysteine (Hcy) export as a function of time and cell density. Lymphoma cells were seeded at $4 \cdot 10^3$ (●), $1.6 \cdot 10^4$ (○), or $1 \cdot 10^5$ (■) cells/ml and the medium contained 200 μM methionine. The cell growth (A) and the amount of Hcy accumulating in the medium (B) were determined. The curves for growth and Hcy export were fitted to the following polynomial functions. **A:** Log $N = 4.99 + 1.72 \cdot 10^{-2}x + 3.04 \cdot 10^{-4}x^2 - 3.37 \cdot 10^{-6}x^3$, $R^2 = 0.999$ (■, growth of cells seeded at high density); Log $N = 4.20 + 2.60 \cdot 10^{-3}x + 7.75 \cdot 10^{-4}x^2 - 5.79 \cdot 10^{-6}x^3$, $R^2 = 1.000$ (○, growth of cells seeded at medium density); Log $N = 3.60 + 5.49 \cdot 10^{-3}x + 9.16 \cdot 10^{-4}x^2 - 1.37 \cdot 10^{-5}x^3 + 6.64 \cdot 10^{-8}x^4$, $R^2 = 1.000$ (●, growth of cells seeded at low density). **B:** Log $y = -0.13 + 3.04 \cdot 10^{-2}x - 2.15 \cdot 10^{-4}x^2 + 3.82 \cdot 10^{-7}x^3$, $R^2 = 0.990$ (■, export from cells seeded at high density); Log $y = -9.92 \cdot 10^{-2} + 5.71 \cdot 10^{-3}x + 2.08 \cdot 10^{-4}x^2 - 1.41 \cdot 10^{-6}x^3$, $R^2 = 0.985$ (○, export from cells seeded at medium density); log $y = -0.12 - 5.77 \cdot 10^{-3}x + 3.74 \cdot 10^{-4}x^2 - 2.26 \cdot 10^{-6}x^3$, $R^2 = 0.998$ (●, export from cells seeded at low density). The export rate (v) was determined as described in Materials and Methods (equation IV). **C:** Export rate plotted versus time after seeding. **D:** Export rate as a function of cell density.

tial incubation time of 12–24 hours, after which they were essentially superimposed (Fig. 2D). Based on these results, we decided to compare Hcy export rates from proliferating cells by plotting the export rate against cell density.

Export from non-proliferating cells was evaluated by a plot of export rate versus time.

Human lymphocytes

The lymphocytes were incubated for 69 hours in the presence of increasing concentrations (15–1,000 μM) of methionine in the culture medium. The experiment was performed both with and without addition of phytohemagglutinin (PHA) (Fig. 3).

The cell number and methionine concentrations were essentially constant throughout the experiment (data not shown).

The Hcy export was relatively low in these cells (< 0.03 nmol/h/10^6 cells), but increased markedly following stimulation with PHA. Notably, the export from human lymphocytes was only moderately ($< 40\%$) enhanced by excess methionine (Fig. 3).

Lymphoma cells

Lymphoma cells were incubated for 96 hours in the presence of increasing concentrations (15–1,000 μM) of methionine in the culture medium.

The growth rates were the same at different methionine concentrations up to $5 \cdot 10^5$ cells/ml. Then the growth of the cells seeded at 15 μM methionine levelled off, whereas cells seeded at 30 μM ceased to grow at higher cell density ($1–2 \cdot 10^6$ cells/ml). Proliferation proceeded to $3 \cdot 10^6$ cells/ml in the presence of high concentrations of methionine. The growth arrest of cells seeded at low methionine level was associated with consumption of a significant portion of methionine (Fig. 4, upper panels).

The Hcy export rate was maximal (0.55 nmol/h/10^6 cells at a cell density of $2 \cdot 10^5$ cells/ml) for lymphoma cells during early exponential growth, and then declined. The initial rate was the same for cells seeded at 30, 150, and 1,000 μM methionine, but the export dropped when methionine in medium was reduced to below ≈ 15 μM. Cells seeded at 15 μM did not obtain maximal Hcy export (Fig. 4).

Notably, Hcy export was more sensitive to methionine depletion than was cell growth.

Non-transformed and transformed fibroblasts

Non-transformed fibroblasts were grown in the presence of various concentrations of methionine (15–1,000 μM) until they reached confluence. The growth rate of these cells was not affected by the methionine concentrations, and the methionine consumption was moderate (Fig. 5, upper panels).

The Hcy export from the non-transformed fibroblasts was highest during exponential growth and decreased progressively as the cells reached confluence. The export rate was increased about threefold (from 0.12 to 0.40 nmol/h/10^6 cells, at a cell density of $4 \cdot 10^4$ cells/ml) when the methionine concentration was increased from 15 to 1,000 μM (Fig. 5, lower panel).

Proliferating transformed fibroblasts were methionine loaded, and similar results were obtained as with their non-transformed counterpart. The transformed cells reached a higher cell density, and consumed significant amounts of methionine. More importantly, the export rate seemed to be more dependent on extracellular methionine, and the export rate was stimulated fivefold, i.e., from 0.10 to 0.52 nmol/h/10^6 cells at a cell density of $2 \cdot 10^4$ cells/ml (Fig. 6).

When the non-transformed cells reach confluence, cell division is arrested (Reznikoff et al., 1973b). Non-transformed fibroblasts were grown until confluence, and then exposed to a medium containing from 15 to 1,000 μM methionine. In these quiescent fibroblasts, the methionine consumption was negligible, the Hcy export rate was low (0.05–0.09 nmol/h/10^6 cells) relative to that observed with growing fibroblasts, and the export rate was not influenced by the methionine concentration (data not shown).

Hepatocytes and hepatoma cells

Non-transformed primary hepatocytes were allowed to attach to the collagen layer, and then subjected to methionine loading by replacing the medium with fresh medium containing 15–1,000 μM methionine. The hepatocytes were incubated under these conditions for 68 hours. The cell density ($2 \cdot 10^5$ cells/ml) remained essentially constant throughout this period.

There was a substantial decrease in the methionine in the medium during the experiment, especially at the lower concentrations. After 68 hours of incubation, about 60–75% of methionine was consumed by the cells supplemented with 15–150 μM, and 20% of methionine by the cells seeded in the presence of 1,000 μM. The log methionine concentrations versus time graph was linear at concentrations ≤ 150 μM (Fig. 7, lower left panel), showing that the methionine consumption under these conditions obeyed first order kinetics.

The Hcy export from the hepatocytes showed several unique features. At low concentrations of methionine (≤ 150 μM), the export rates were low during the first hours after medium shift, then increased, and showed a maximum after about 36 hours. Cells exposed to high methionine (1,000 μM) exported massive amounts almost immediately after loading. The exports rates were much higher (up to 25 nmol/h/10^6 cells) than for the cultured cells investigated, and were greatly (more than 15-fold) enhanced when the methionine concentration was increased from 15 to 1,000 μM. The stimulatory effect from methionine was particularly pronounced at high (> 150 μM) concentrations (Fig. 7).

After incubation for 68 hours, 60–100% of methionine consumed was recovered as Hcy in the medium (Fig. 7, lower left panel).

The hepatoma cells were cultured for 240 hours in the presence of increasing concentrations of methionine. The growth rate was exponential and essentially independent of extracellular methionine for about 144 hours, corresponding to $1 \cdot 10^5$ cells/ml. During this period, the methionine consumption was moderate. After this time point, the growth rate gradually declined, and for the cells supplemented with 15 or 30 μM methionine, there was a marked decrease in extracellular methionine (Fig. 8, upper panels).

The Hcy export from the hepatoma cells showed similarities with that observed with the anchorage-

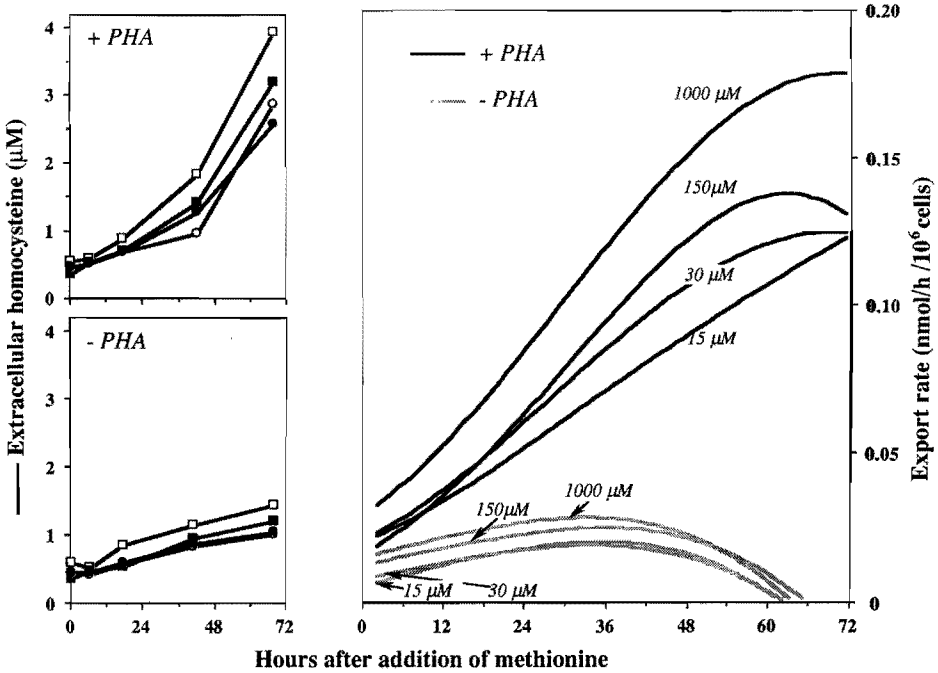

Fig. 3. Hcy export from human lymphocytes during methionine loading. Human lymphocytes were seeded at $5 \cdot 10^5$ cells/ml in the presence of 15 (●), 30 (○), 150 (■), and 1,000 μM (□) methionine. The experiment was performed with non-stimulated lymphocytes and with lymphocytes exposed to phytohemagglutinin (PHA) (9 μg/ml). The methionine consumption was less than 15%, and the cell counts were essentially constant throughout the 69 hour experiment. **Left:** Accumulation of Hcy in the medium in the absence and presence of PHA, respectively. These export curves were fitted to trinomial functions after log transformation (equation II). **Right:** Export rate per unit cell mass (v) obtained by a differential equation (equation IV), plotted versus cell density. Data for both non-stimulated and PHA stimulated lymphocytes are shown.

dependent fibroblasts. The export rate reached a maximum during early exponential growth, and then decreased progressively as cell density increased. The export rate was increased about eightfold (from 0.10 to 0.85 nmol/h/10^6 cells, at a cell density of $2 \cdot 10^4$ cells/ml) when the methionine concentrations were increased from 15 to 1,000 μM (Fig. 8, lower panel).

DISCUSSION

Background

Determination of plasma Hcy is useful in the diagnosis of a variety of disorders characterized by impaired Hcy metabolism. These include inborn enzyme defects like cystathionine β-synthase deficiency, and folate and vitamin B_{12} deficiencies. Moderate impairment seems to occur in postmenopausal women, and in patients with premature vascular disease (Ueland and Refsum, 1989).

Insufficient Hcy metabolism is diagnosed by determining fasting Hcy in plasma or the plasma level after ingestion of a standard methionine dose (Ueland and Refsum, 1989). The methionine loading test was introduced into clinical practice 25 years ago (Brenton et al., 1965; Fowler et al., 1971), but no experimental studies revealing the underlying cellular processes have been published, and basic understanding of the methionine loading test is still lacking.

Determination of cellular Hcy export in vitro requires a sensitive Hcy assay, and several such assays have been described during the last 6 years (Ueland and Refsum, 1989). The present study is based on two sensitive Hcy assays recently developed in our laboratory (Refsum et al., 1985, 1989). It is the first report characterizing the Hcy egress from cultured cells exposed to various concentrations of methionine.

Experimental design

The methionine concentrations (15, 30, 150, and 1,000 μM) were selected to mimic the conditions existing during a standard methionine loading test. The plasma level of methionine increases from physiological level (15–30 μM) (Gregory et al., 1986) to several hundred micromolar in patients subjected to this test (Ueland and Refsum, 1989).

The experimental conditions differed from those existing in vivo in at least two aspects. First, in the in vitro experiments the cell density is far less than in the intact organs in vivo, and in contrast to in vivo, the extracellular volume exceeds the intracellular space. Secondly, methionine loading of subjects results in a supraphysiological methionine concentration lasting for about 24 hours (Ueland and Refsum, 1989), whereas isolated cells were exposed to high concentrations of methionine for several days. These adaptations were necessary to achieve cell growth and Hcy accumulation in the medium sufficient for accurate determination.

For comparison of Hcy export from proliferating cells at different cell densities, we modified a previous

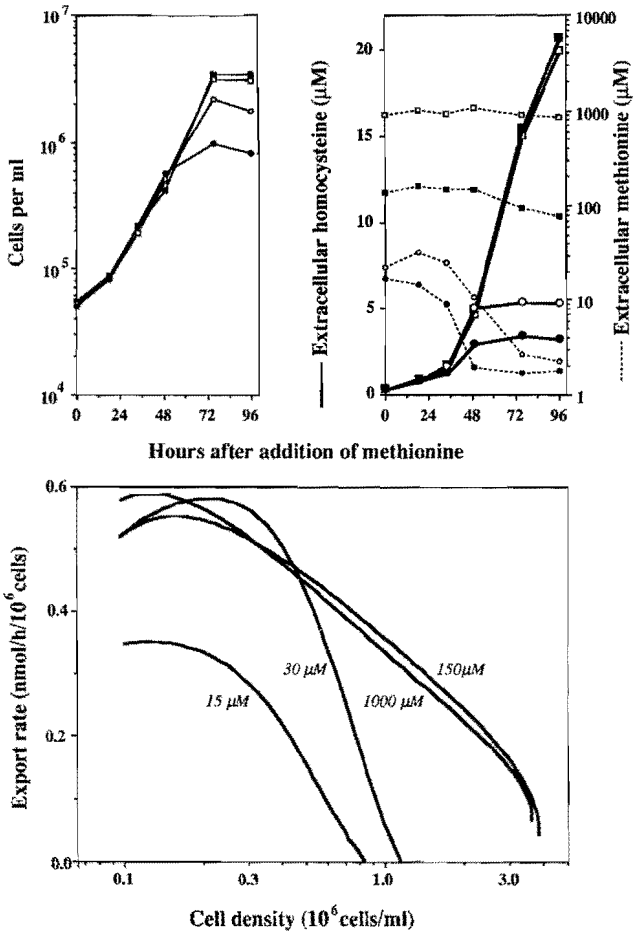

Fig. 4. Hcy export from lymphoma cells during methionine loading. Lymphoma cells were seeded at $5 \cdot 10^4$ cells/ml in the presence of 15 (●), 30 (○), 150 (■), and 1,000 μM (□) methionine. **Upper left:** Cell growth. **Upper right:** Accumulation of Hcy (solid lines) and methionine concentration (dashed lines) in the culture medium. The growth curves and the two export curves (initial methionine concentrations of 150 and 1,000 μM) were fitted to trinomial functions after log transformation (equations I and II), whereas the export curves obtained with cells seeded at 15 and 30 μM methionine were best fitted to a trinomial function of inversed values (equation III). **Bottom:** Export rate per unit cell mass (v) obtained by a differential equation (equation IV), plotted versus cell density.

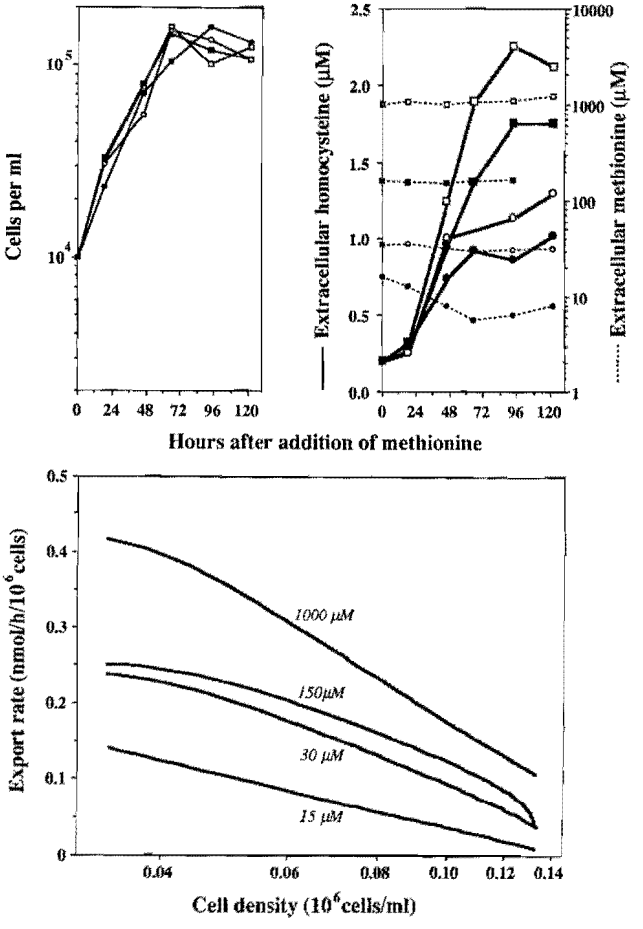

Fig. 5. Hcy export from proliferating non-transformed fibroblasts during methionine loading. Fibroblasts were grown to a density of $1 \cdot 10^4$ cells/ml and then transferred to a medium containing 15 (●), 30 (○), 150 (■), and 1,000 μM (□) of methionine. **Upper left:** Cell growth. **Upper right:** Accumulation of Hcy (solid lines) and methionine concentration (dashed lines) in the culture medium. The growth curves and the export curves were fitted to trinomial functions after log transformation (equations I and II). **Bottom:** Export rate per unit cell mass (v) obtained by a differential equation (equation IV), plotted versus cell density.

approach (Djurhuus et al., 1990) by plotting the export rates versus cell density. This presentation was chosen in the light of the experiments depicted in Fig. 2. However, the export-versus-time graph was useful for the comparison of export from stationary cells present at equal and essentially constant cell density.

Comparison of response of different cell types

Different cell types were tested to investigate the Hcy egress and the effect of methionine in anchorage-dependent and -independent cells and non-transformed and malignant cells. Hepatocytes were investigated because of unique features of Hcy metabolism in liver (Mudd et al., 1989). In the clinical setting, human lymphocytes are easily obtained.

For all proliferating cells (lymphoma cells, non-transformed and malignant fibroblasts, and hepatoma cells), the Hcy export was highest at low cell density during early exponential growth. The export from quiescent cells (confluent fibroblasts and non-stimulated human lymphocytes), except the hepatocytes, was low.

Methionine enhanced the Hcy export three- to eight-fold in some cultured cells (non-transformed and malignant fibroblasts and hepatoma cells), and did not stimulate (lymphoma cells, confluent fibroblasts) or slightly enhanced (human lymphocytes) export in other cells.

A unique response was observed with the non-transformed primary hepatocytes. The Hcy export from these cells was orders of magnitude higher than the other cell types, and the export rate was highly depen-

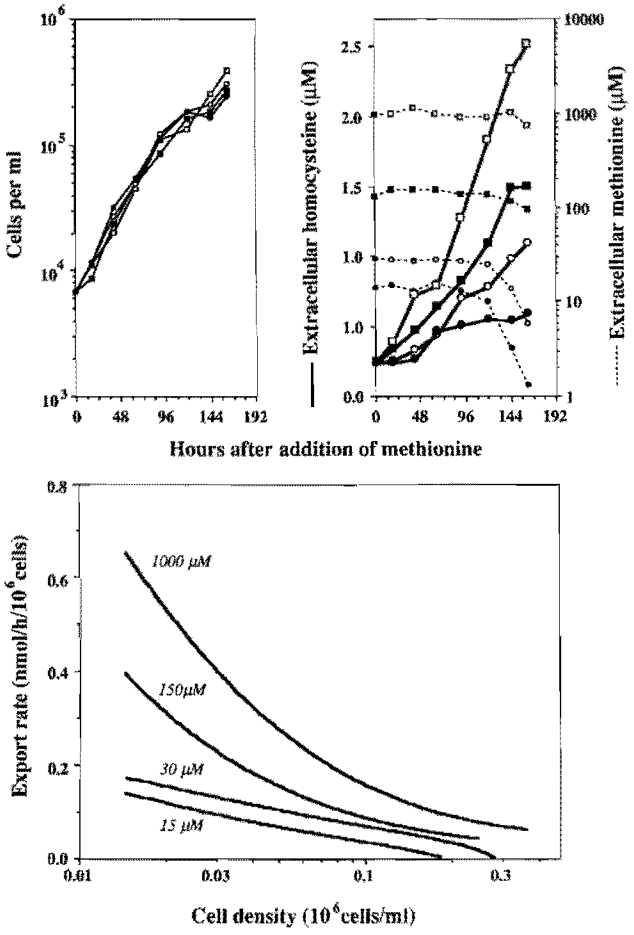

Fig. 6. Hcy export from transformed fibroblasts during methionine loading. Transformed fibroblasts were grown to a density of $7 \cdot 10^5$ cells/ml and then transferred to a medium containing 15 (●), 30 (○), 150 (■), and 1,000 μM (□) of methionine. **Upper left:** Cell growth. **Upper right:** Accumulation of Hcy (solid lines) and methionine concentration (dashed lines) in the culture medium. The growth curves and the export curves were fitted to trinomial functions after log transformation (equations I and II). **Bottom:** Export rate per unit cell mass (v) obtained by a differential equation (equation IV), plotted versus cell density.

dent on extracellular methionine. Notably, in these cells, the export rate was particularly enhanced at supraphysiological methionine concentrations (> 150 μM, Fig. 7). Furthermore, the hepatocytes were the only stationary cells showing export rates highly dependent on extracellular methionine.

Possible mechanism

In general, net cellular Hcy export is a function of the balance between Hcy production from S-adenosylhomocysteine, and further metabolism via remethylation to methionine or catabolism via the transsulfuration pathway (Fig. 1). We have previously demonstrated that pharmacological inhibition of Hcy production (Svardal et al., 1986b,c) or remethylation (Ueland et al., 1986) affects Hcy export.

The Hcy export rate at physiological methionine levels was high during early exponential growth (Figs. 2, 4–6, and 8) and decreased markedly as anchorage-dependent cells reached confluence (Figs. 5, 6, and 8, Djurhuus et al., 1990). Similar results were obtained by Iizasa and Carson (1985), who showed that Hcy excretion from human lymphoblasts was proportional to the specific growth rate, suggesting a high overall transmethylation rate during cell division. This is in accordance with the finding of German et al. (1983) that stimulation of human peripheral mononuclear cells by PHA increased both S-adenosylmethionine utilization and Hcy export several-fold. Their findings agree perfectly with our observation that PHA enhanced the Hcy export from human lymphocytes, irrespective of methionine concentration in culture medium (Fig. 3).

Additional factors mediating the effects of proliferation and cell density on Hcy export should be considered. Proliferating cells may use reduced folates for purine and pyrimidine synthesis at the expense of 5-methyltetrahydrofolate, and therefore may insufficiently remethylate Hcy. Folate entering cancer cells may be channeled away from 5-methyltetrahydrofolate (Pheasant et al., 1983). Amino acid transport decreases markedly when cell density increases, and cell density may itself, independently of growth rate, regulate amino acid transport (Piedimonte et al., 1989) including Hcy egress.

High concentrations of extracellular methionine enhance Hcy egress from some cell types (Figs. 3, 5–8) probably by increasing the transmethylation rate and thereby Hcy formation. The observation that methionine administration increases the amount of S-adenosylmethionine in liver and also in tissues with a moderate to high transmethylation rate (Xue and Snoswell, 1986) supports this possibility. There are no data suggesting that methionine directly inhibits Hcy metabolism, but methionine may act via S-adenosylmethionine, which inactivates (Finkelstein and Martin, 1984a) or stimulates (Burke et al., 1971; Finkelstein et al., 1975) enzymes consuming Hcy.

Resting human lymphocytes (Fig. 3) and quiescent fibroblasts (data not shown) exported small amounts of Hcy, and the export from these cells was not or only marginally enhanced following methionine loading. This may reflect low transmethylation rate in nonproliferating cells (Iizasa and Carson, 1985), and the small amounts of Hcy formed do not exceed the metabolic capacity.

Lymphoma cells consumed a significant portion of methionine in the medium, and both growth arrest and inhibition of Hcy export were observed with lymphoma cells seeded at low methionine, probably due to methionine depletion (Fig. 4). No enhancement of Hcy export from the lymphoma cells in the presence of excess methionine suggests that Hcy formation is not increased under these conditions. The possibility that lack of response may be due to increased remethylation is unlikely, since the lymphoma cells are methionine auxotroph (Djurhuus and Ueland, 1989) and the Hcy remethylation in these cells, as in human lymphoblasts (German et al., 1983), is probably negligible.

No remethylation in the lymphoma cells suggests

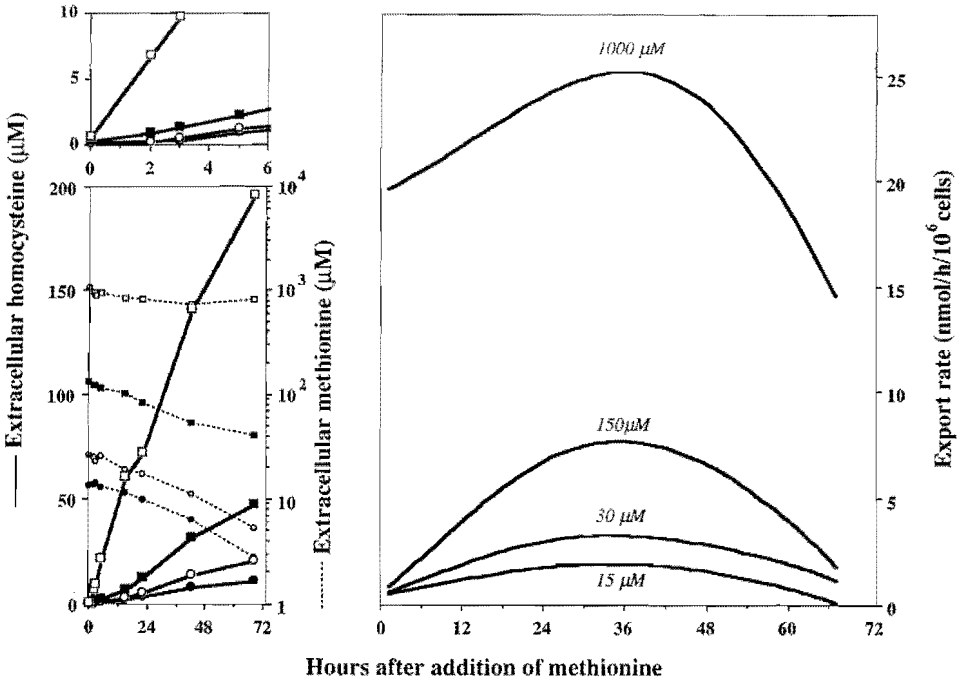

Fig. 7. Hcy export from primary hepatocytes during methionine loading. Hepatocytes were isolated by a collagenase perfusion method, seeded at a cell density of $2 \cdot 10^5$ cells/ml, and cultured for 2 hours in the presence of 15 μM methionine. At this time point, the medium was replaced with fresh medium containing 15 (●), 30 (○), 150 (■), and 1,000 μM (□) methionine, and incubated for a further 68 hours. The cell count was essentially constant throughout the experiment, as judged by the protein content of the hepatocyte layer extracted with acid. **Lower left:** Accumulation of Hcy (solid lines) and methionine concentration (dashed lines) in the culture medium. **Upper left:** Hcy export during the first 6 hours. The export curves were fitted to trinomial functions after log transformation (equations II). **Right:** Export rate per unit cell mass (v) obtained by a differential equation (equation IV), plotted versus time after adding methionine.

that enhancement of this pathway is not a mechanism of methionine conservation in these cells. This implies that the low Hcy export during methionine depletion may be due to decreased Hcy production via transmethylation. Thus, reduced Hcy export may be related to cellular conservation of methionine, and this methionine sparing effect may fully support growth during moderate methionine deficiency (Fig. 4). Notably, inhibition of Hcy export preceded reduction in cell proliferation, suggesting that methionine supply for protein or polyamine synthesis was given the highest priority.

Mechanism and physiological corollary of the hepatocyte response

Most enzymes involved in Hcy metabolism show particularly high activity in the liver. Liver possesses about 75% of the total-body capacity for transmethylation (Xue and Snoswell, 1986) and thereby for Hcy production. The enzyme catalyzing the alternate remethylation pathway of Hcy, betaine-Hcy methyltransferase (BH, Fig. 1), is confined to the liver, and only trace activities have occasionally been reported in other tissues. The liver is rich in all enzymes (MA, MT, SA, CS, and CL, Fig. 1) participating in the transsulfuration pathway, whereas other tissues lack all or some of these enzymes (Finkelstein et al., 1971). The remethylation of Hcy may be important for the maintenance of tissue methionine (Finkelstein and Martin, 1984b), whereas the transmethylation-transsulfuration pathway plays a role in the catabolism of excess methionine (Finkelstein and Martin, 1986). Such a catabolic sequence requires the utilization of a nonessential methyl acceptor forming an inert product. The glycine methyltransferase reaction has been suggested to serve such a function, and this enzyme is extremely abundant in liver (Cook and Wagner, 1981; Heady and Kerr, 1973). The data cited above unequivocally show that the liver holds a central position in methionine metabolism, conservation, and detoxification (Mudd et al., 1989), and the unique response of the hepatocytes to methionine loading should be considered in the light of this fact.

The increased flux through the transmethylation-transsulfuration pathway in the presence of excess extracellular methionine probably results in elevation of intracellular Hcy. Elevation of Hcy by increasing methionine in the medium has been demonstrated with primary hepatocytes in suspension (Svardal et al., 1986a). The export rate is probably a function of intracellular Hcy concentration, but the possibility that methionine directly enhances Hcy export cannot be ruled out.

Our observations (Fig. 7) suggest that the Hcy export from hepatocytes is linked to the metabolic processes in vivo handling superfluous methionine. First, the

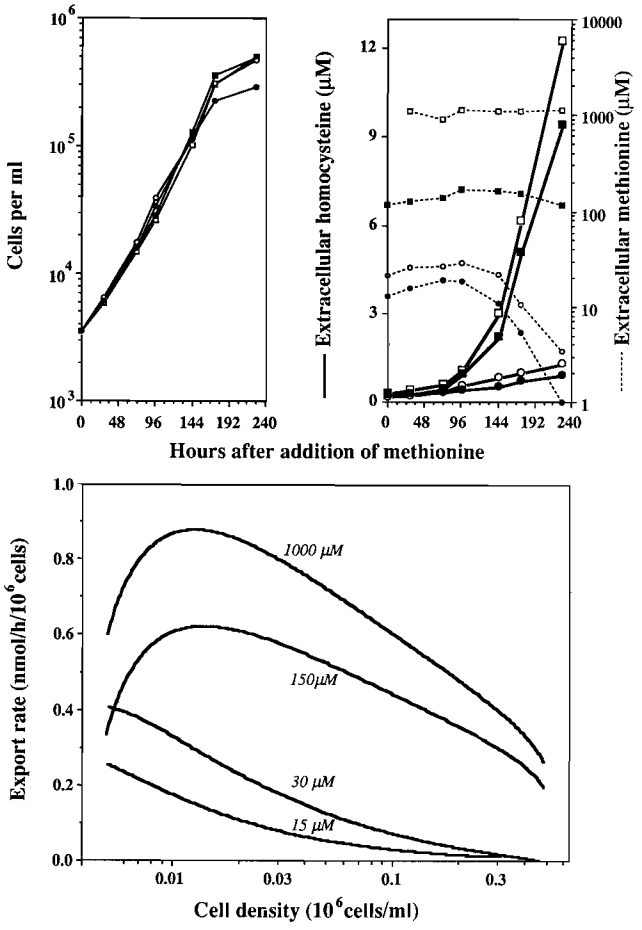

Fig. 8. Hcy export from hepatoma cells during methionine loading. Hepatoma cells were grown to a density of $3.3 \cdot 10^3$ cells/ml and then transferred to a medium containing 15 (●), 30 (○), 150 (■), and 1,000 μM (□) methionine. **Upper left:** Cell growth. **Upper right:** Accumulation of Hcy (solid lines) and methionine concentration (dashed lines) in the culture medium. The growth curves and the export curves were fitted to trinomial functions after log transformation (equations I and II). **Bottom:** Export rate per unit cell mass (v) obtained by a differential equation (equation IV), plotted versus cell density.

hepatocytes showed a much higher export rate than the other cells investigated, and after 3 days, a significant amount of methionine added to the medium (60–100%) could be recovered as Hcy. Secondly, the methionine consumption was a first order process, and such kinetics are suitable for a detoxification process. Finally, the Hcy egress was stimulated to the greatest extent at supraphysiological methionine concentrations.

Hcy export may be an important adaptive mechanism keeping the intracellular concentration below a certain level (Ueland et al., 1987), but whether it represents a quantitative important route of methionine disposal in the intact liver has not been evaluated. Such a mechanism requires metabolic handling or excretion of extracellular Hcy by other organs. Under physiological conditions and after methionine loading, the urinary excretion of Hcy represents only a minor portion of total amounts of Hcy formed (Ueland and Refsum, 1989). Plasma Hcy after methionine loading returns to normal within 24–48 hours, suggesting that reuptake and metabolism of Hcy occur.

CONCLUSIONS AND PERSPECTIVES

Hcy is exported from both normal, transformed, proliferating, and quiescent cells. The export rate and the enhancement by excess extracellular methionine differ between cell types, but for some cell types the rate and stimulation are maximal during early exponential growth. Hepatocytes are the only stationary cells that show Hcy export highly dependent on extracellular methionine, and the massive export from these cells suggests that the liver may be a major source of plasma Hcy during methionine loading of patients.

Here we describe data handling and presentation allowing the comparison of export rates also during cell proliferation. These principles may become useful in future studies on the Hcy export from cells with insufficient Hcy metabolism due to enzyme defects or following drug exposure. Determination of in vitro export rates may become an adjunct to enzyme determination for the diagnosis of inborn errors of Hcy metabolism.

ACKNOWLEDGMENTS

The technical assistance of Eli Gundersen, Gry Kvalheim, and Halvard Bergesen is highly appreciated. We are grateful to Drs. Thoralf Christoffersen and Johan R. Lillehaug for providing cell lines used in this study. B.C. is a fellow of the Norwegian Cancer Society. We are thankful to Dr. Lars Brattström for suggesting a study on methionine loading of cells in culture. This work was supported by grants from the Norwegian Cancer Society, and from Astri og Birger Torsteds legat til bekjempelse av kreft.

LITERATURE CITED

Boers, G.H., Smals, A.G., Trijbels, F.J., Leermakers, A.I., and Kloppenborg, P.W. (1983) Unique efficiency of methionine metabolism in premenopausal women may protect against vascular disease in the reproductive years. J. Clin. Invest., *72*:1971–1976.

Boers, G.H.J., Fowler, B., Smals, A.G.H., Trijbels, F.J.M., Leermakers, A.I., Kleijer, W.J., and Kloppenborg, P.W.C. (1985a) Improved identification of heterozygotes for homocystinuria due to cystathionine synthase deficiency by the combination of methionine loading and enzyme determination in cultured fibroblasts. Hum. Genet., *69*:164–169.

Boers, G.H.J., Smals, A.G.H., Trijbels, F.J.M., Fowler, B., Bakkeren, J.A.J.M., Schoonderwaldt, H.C., Kleijer, W.J., and Kloppenborg, P.W.C. (1985b) Heterozygosity for homocystinuria in premature peripheral and cerebral occlusive arterial disease. N. Engl. J. Med., *313*:709–715.

Böyum, A. (1968) Separation of leukocytes from blood and bone marrow. With special reference to factors which influence and modify sedimentation properties of hematopoietic cells. Scand. J. Clin. Lab. Invest., *21 (Suppl. 97)*:1–109.

Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., *72*:248–254.

Brattström, L., Israelsson, B., and Hultberg, B. (1988) Impaired homocysteine metabolism—a possible risk factor for arteriosclerotic vascular disease. In: Genetic Susceptibility to Environmental Factors a Challenge for Public Intervention. U. Smith, S. Eriksson, and F. Lindgärde, eds. Almqvist & Wiksell International, Stockholm, Sweden, pp. 25–34.

Brenton, D.P., Cushworth, D.C. and Gaull, G.E. (1965) Homocystinuria: metabolic studies on 3 patients. J. Pediat., *67*:58–68.

Burke, G.T., Magnum, J.H., and Brodie, J.D. (1971) Mechanism of mammalian cobalamin-dependent methionine biosynthesis. Biochemistry, *10*:3079–3085.

Christoffersen, T., Refsnes, M., Brønstad, G.O., Østby, E., J., H., Haffner, F., Sand, T.E., Hunt, N., and Sonne, O. (1984) Changes in hormone responsiveness and cyclic AMP metabolism in rat hepatocytes during primary culture and effects of supplementing the medium with insulin and dexamethasone. Eur. J. Biochem., *138*:217–226.

Cook, R.J., and Wagner, C. (1981) Measurement of folate binding protein from rat liver cytosol by radioimmunoassay. Arch. Biochem. Biophys., *208*:358–364.

Djurhuus, R., and Ueland, P.M. (1989) Growth kinetic studies of methionine dependence in co-culture of monolayer and anchorage independent mouse cell lines. Anticancer Res., *9*:1611–1616.

Djurhuus, R., Svardal, A.M., and Ueland, P.M. (1990) Growth state dependent increase of glutathione by homocysteine and other thiols, and homocysteine formation in glutathione depleted mouse cell lines. Biochem. Pharmacol., *39*:421–429.

Finkelstein, J.D., and Martin, J.J. (1984a) Inactivation of betaine-homocysteine methyltransferase by adenosylmethionine and adenosylethionine. Biochem. Biophys. Res. Commun., *118*:14–19.

Finkelstein, J.D., and Martin, J.J. (1984b) Methionine metabolism in mammals. Distribution of homocysteine between competing pathways. J. Biol. Chem., *259*:9508–9513.

Finkelstein, J.D., and Martin, J.J. (1986) Methionine metabolism in mammals. Adaptation to methionine excess. J. Biol. Chem., *261*:1582–1587.

Finkelstein, J.D., Kyle, W.E., and Harris, B.J. (1971) Methionine metabolism in mammals. Regulation of homocysteine methyltransferases in rat tissue. Arch. Biochem. Biophys., *146*:84–92.

Finkelstein, J.D., Kyle, W.E., Martin, J.J., and Pick, A.M. (1975) Activation of cystathionine synthase by adenosylmethionine and adenosylethionine. Biochem. Biophys. Res. Commun., *66*:81–87.

Fowler, B., Sardharwalla, I.B., and Robins, A.J. (1971) The detection of heterozygotes for homocystinuria by oral loading with L-methionine. Biochem. J., *122*:23p–24p.

Gaudernack, G., Rugstad, H.E., Hegna, I., and Prydz, H. (1973) Synthesis of serum proteins by a clonal strain of rat hepatoma cells. Exp. Cell Res., *77*:25–30.

German, D.C., Bloch, G.A., and Kredich, N.M. (1983) Measurements of S-adenosylmethionine and L-homocysteine metabolism in cultured human lymphoid cells. J. Biol. Chem., *258*:10997–11003.

Gregory, D.M., Sovetts, D., Clow, C.L., and Scriver, C.R. (1986) Plasma free amino acid values in normal children and adolescents. Metabolism, *35*:967–969.

Heady, J.E., and Kerr, S.J. (1973) Purification and characterization of glycine N-methyltransferase. J. Biol. Chem., *248*:69–72.

Iizasa, T., and Carson, D.A. (1985) Differential regulation of polyamine synthesis and transmethylation reactions in methylthioadenosine phosphorylase deficient mammalian cells. Biochim. Biophys. Acta, *844*:280–287.

Krishnamurti, C.R., Heindze, A.M., and Galzy, G. (1984) Application of reversed-phase high-performance liquid chromatography using pre-column derivatization with o-phthaldialdehyde for the quantitative analysis of amino acids in adult and fetal sheep plasma, animal feeds and tissues. J. Chromatogr., *315*:321–331.

Mudd, S.H., Levy, H.L., and Skovby, F. (1989) Disorders of transsulfuration. In: The Metabolic Basis for Inherited Diseases. C.R. Scriver, A.L. Beadet, W.S. Sly, and D. Valle, eds. McGraw-Hill, New York, pp. 693–734.

Murphy-Chutorian, D.R., Wexman, M.P., Grieco, A.J., Heininger, J.A., Glassman, E., Gaull, G.E., Ng, S.K.C., Feit, F., Wexman, K., and Fox, A.C. (1985) Methionine intolerance: A possible risk factor for coronary artery disease. J. Am. Coll. Cardiol., *6*:725–730.

Old, L., Boyse, E., and Stockert, E. (1965) The G (Gross) leukemia antigen. Cancer Res., *25*:813–819.

Pheasant, A.E., Bates, J., Blair, J.A., and Nayyiz-Mazhir, R. (1983) Investigations of tissue folates in normal and malignant tissues. Br. J. Cancer, *47*:393–398.

Piedimonte, G., Baginski, I., Silvotti, L., Petronini, P.G., and Borghetti, A.F. (1989) Density-dependent regulation of amino acid transport in a Burkitt lymphoma cell line. Cancer Lett., *47*:121–125.

Ralph, P. (1973) Retention of lymphocyte characteristics by myelomas and q^+-lymphomas: Sensitivity to cortisol and phytohemagglutinin. J. Immunol., *110*:1470–1475.

Refsum, H., Helland, S., and Ueland, P.M. (1985) Radioenzymic determination of homocysteine in plasma and urine. Clin. Chem., *31*:624–628.

Refsum, H., Ueland, P.M., and Svardal, A.M. (1989) Fully automated fluorescence assay for determining total homocysteine in plasma. Clin. Chem., *35*:1921–1927.

Reznikoff, C.A., Bertram, J.S., Brankow, D.W., and Heidelberger, C. (1973a) Quantitative and qualitative studies of chemical transformation of cloned C3H mouse embryo cells sensitive to postconfluence inhibition of cell division. Cancer Res., *33*:3239–3249.

Reznikoff, C.A., Brankow, D.W., and Heidelberger, C. (1973b) Establishment and characterization of a clone of C3H mouse embryo cells sensitive to postconfluence inhibition of cell division. Cancer Res., *33*:3231–3238.

Richardson, U.I., Tashjian, J.A.H., and Levine, L. (1969) Establishment of a clonal strand of hepatoma cells which secretes albumin. J. Cell Biol., *40*:236–247.

Sardharwalla, I.B., Fowler, B., Robins, A.J., and Komrower, G.M. (1974) Detection of heterozygotes for homocystinuria. Study of sulphur-containing amino acids in plasma and urine after L-methionine loading. Arch. Dis. Child., *49*:553–559.

Seglen, P.O. (1976) Preparation of isolated rat liver cells. Methods Cell Biol., *13*:29–83.

Svardal, A., Refsum, H., and Ueland, P.M. (1986a) Determination of in vivo protein binding of homocysteine and its relation to free homocysteine in the liver and other tissues of the rat. J. Biol. Chem., *261*:3156–3163.

Svardal, A.M., Djurhuus, R., Refsum, H., and Ueland, P.M. (1986b) Disposition of homocysteine in rat hepatocytes and in nontransformed and malignant mouse embryo fibroblasts following exposure to inhibitors of S-adenosylhomocysteine catabolism. Cancer Res., *46*:5095–5100.

Svardal, A.M., Djurhuus, R., and Ueland, P.M. (1986c) Disposition of homocysteine and S-3-deazaadenosylhomocysteine in cells exposed to 3-deazaadenosine. Mol. Pharmacol., *30*:154–158.

Tashjian Jr, A.H., Bancroft, F.C., Richardson, U.I., Goldlust, M.B., and Rommels, F.A., and Ofner, P. (1970) Multiple differentiated functions in an unusual clonal strand of hepatoma cells. In Vitro, *6*:32–45.

Ueland, P.M. (1982) Pharmacological and biochemical aspects of S-adenosylhomocysteine and S-adenosylhomocysteine hydrolase. Pharmacol. Rev., *34*:223–253.

Ueland, P.M., Helland, S., Broch, O.-J., and Schanche, J.-S. (1984) Homocysteine in tissues of the mouse and rat. J. Biol. Chem., *259*:2360–2364.

Ueland, P.M., and Refsum, H. (1989) Plasma homocysteine, a risk factor for vascular disease: Plasma levels in health, disease, and drug therapy. J. Lab. Clin. Med., *114*:473–501.

Ueland, P.M., Refsum, H., Male, R., and Lillehaug, J.R. (1986) Disposition of endogenous homocysteine by mouse fibroblast C3H/10T1/2 Cl 8 and the chemically transformed C3H/10T1/2MCA Cl 16 cells following methotrexate exposure. J. Natl. Cancer. Inst., *77*:283–289.

Ueland, P.M., Refsum, H., Svardal, A.M., Djurhuus, R., and Helland, S. (1987) Perturbation of homocysteine metabolism by pharmacological agents in experimental and clinical use. In: Tumor Cell Differentiation. Biology and Pharmacology. J. Aarbakke, P.K. Chiang, and H.P. Koeffler, eds. Humana Press, Clifton, NJ, pp. 269–278.

Vintermyr, O.K., and Døskeland, S.O. (1987) Cell cycle parameters of adult rat hepatocytes in a defined medium. A note on the timing of nucleolar DNA replication. J. Cell. Physiol., *132*:12–21.

Xue, G.-P., and Snoswell, A.M. (1986) Quantitative evaluation and regulation of S-adenosylmethionine-dependent transmethylation in sheep tissues. Comp. Biochem. Physiol., *85*:601–608.