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# Disposition of Homocysteine in Rat Hepatocytes and in Nontransformed and Malignant Mouse Embryo Fibroblasts following Exposure to Inhibitors of *S*-Adenosylhomocysteine Catabolism[1]

Asbjørn M. Svardal,[2] Rune Djurhuus, Helga Refsum, and Per Magne Ueland

*Clinical Pharmacology Unit, Department of Pharmacology, University of Bergen, 5000 Bergen, Norway*

## ABSTRACT

*S*-Adenosylhomocysteine (AdoHcy) is catabolized to adenosine and homocysteine through the action of AdoHcy hydrolase, and this reaction is the only known source of L-homocysteine in vertebrates. The disposition of endogenously formed L-homocysteine was investigated in isolated rat hepatocytes and nontransformed and malignant C3H/10T½ mouse embryo fibroblasts exposed to 3-deazaaristeromycin or D-eritadenine, compounds which are potent inhibitors of AdoHcy hydrolase. Cells in suspension release large amounts of L-homocysteine into the extracellular medium whereas small amounts are retained within the intracellular compartment. The L-homocysteine egress is inhibited by 3-deazaaristeromycin or D-eritadenine in a manner which closely parallels the inhibitory effect on AdoHcy catabolism, suggesting that L-homocysteine egress may be coupled to its formation from AdoHcy. In liver cells, the accumulation of AdoHcy exceeded the inhibition of L-homocysteine egress, whereas in the fibroblasts inhibition of egress equalled the accumulation of AdoHcy. Inhibition of AdoHcy catabolism was associated with an increase in both free and protein bound L-homocysteine in liver cells, whereas depletion of intracellular L-homocysteine occurred in the mouse embryo fibroblasts under these conditions. These data suggest that some properties of nucleoside analogues may be related to their effects on L-homocysteine metabolism. Furthermore, L-homocysteine is exported into the extracellular medium in proportion to the formation from AdoHcy, and extracellular L-homocysteine may be a measure of the balance between L-homocysteine formation and utilization.

## INTRODUCTION

Several nucleoside analogues, some of which have cytostatic or antiviral properties, interact with AdoHcy[3] hydrolase (EC 3.3.1.1), the enzyme responsible for the metabolic degradation of the endogenous transmethylase inhibitor, AdoHcy. Some analogues competitively inhibit this enzyme, whereas others serve as irreversible inhibitors (inactivators) or as substrates. Cells exposed to these analogues accumulate AdoHcy and/or nucleosidylhomocysteine which in turn inhibit various AdoMet dependent transmethylation reactions. These aspects of the biological properties of nucleoside analogues have been studied in detail and are the subject of recent review articles (1–3).

Inhibitors of AdoHcy hydrolase block the conversion of AdoHcy to adenosine and L-homocysteine. This reaction is the only known source of L-homocysteine in vertebrates (2). Therefore, some of the biological effects caused by these inhibitors may be related to depletion of L-homocysteine relative to the metabolic demand. These aspects of nucleoside analogues have hitherto received little attention. Studies on the effects of inhibitors of AdoHcy catabolism on the disposition of L-homocysteine by cells seem warranted, because this thiol has been assigned a role in numerous pathological and metabolic processes, including the inherited disease, homocystinuria (4), methionine dependent cancer (5), atherosclerosis (6), side effects of antifolate therapy (7), methionine synthesis and interconversion of reduced folates (4).

No study has been made on cellular handling or metabolism of L-homocysteine following exposure to nucleoside analogues. This may be related to lack of a sensitive method required for the determination of the small amount of L-homocysteine in cells and tissues. We have recently developed a radioenzymic L-homocysteine assay which allows the determination of L-homocysteine in cells under physiological conditions and in the presence of high levels of AdoHcy following exposure to nucleoside analogues (8).

The aim of the present study was to investigate the effect of inhibitors of AdoHcy catabolism on the cellular level of L-homocysteine and on the distribution of L-homocysteine between the intracellular and extracellular compartment. Special features have been assigned to homocysteine metabolism in hepatic *versus* nonhepatic tissue (5) and normal *versus* malignant cells (4). We therefore investigated freshly isolated liver cells, nontransformed mouse embryo fibroblasts, C3H/10T½ Cl 8 (9), and a malignant clone, C3H/10T½ MCA Cl 16, obtained from Cl 8 cells by chemical transformation (10). 3-Deazaaristeromycin and D-eritadenine were used as inhibitors of AdoHcy catabolism, because these are metabolically stable compounds which have been shown to inhibit both isolated AdoHcy hydrolase and this enzyme in the intact cell (11, 12).

## MATERIALS AND METHODS

**Chemicals.** DL-Homocysteine, L-methionine, adenosine, AdoHcy, ATP, and DTE were obtained from Sigma Chemical Co., St. Louis, MO, and AdoMet was from Koch-Light Laboratories, Colnbrook, United Kingdom. D-Eritadenine was a gift from Dr. A. Holy, Institute of Organic Chemistry and Biochemistry, Praha, Czechoslovakia, and 3-deazaaristeromycin was kindly supplied by Dr. John Montgomery, Southern Research Institute, Birmingham, AL. [$^{14}$C]Adenosine (59 mCi/mmol) was purchased from the Radiochemical Centre, Amersham, United Kingdom.

**Preparation and Incubation of Isolated Rat Liver Cells.** The isolated rat hepatocytes were prepared by a collagenase perfusion method (13) and were incubated in an isotonic salt solution containing *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid buffer (pH 7.4), bovine serum albumin, glucose, salt, and antibiotics, as described elsewhere (14).

**Cell Lines and Culture Conditions.** Stock cultures of nontransformed, C3H/10T½ Cl 8 cells (9) and the chemically transformed C3H/10T½ MCA Cl 16 cells (10) were obtained from the laboratory of Dr. J. R. Lillehaug, Department of Biochemistry, University of Bergen. Both cell types were grown on 10-cm plastic Petri dishes (Costar, Cambridge, MA) in Eagle's basal medium (Gibco, Paisley, Scotland), supplemented with 10% heat inactivated fetal calf serum (Sera-Lab, Ltd., Sussex, England) at 37°C in an atmosphere of 5% $CO_2$ in air, and a relative humidity of 95%.

Cl 8 cells were seeded at $6 \times 10^4$ cells/dish and MCA Cl 16 cells at $1.2 \times 10^5$ cells/ dish, so that both cell types just reached confluence at the time of initiation of drug exposure.

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[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; THF, tetrahydrofolate; MCA, 2-methylcholanthrene; Cl, clone; DTE, dithioerythritol; HPLC, high performance liquid chromatography.

The cells were harvested by removal of the culture medium, gently washing the cells twice with ice-cold phosphate buffered saline and immediately freezing the cells at −80°C. For determination of extracellular metabolites, samples of the medium were frozen at −20°C.

**Determination of Free L-Homocysteine in Cells.** The cells were separated from the medium and homogenized in 0.6 N perchloric acid. The precipitated protein was removed by centrifugation, and the acid was neutralized to pH 7.5. L-Homocysteine was assayed in the extract by a radioenzymic method described in detail previously (8).

**Determination of Protein Bound L-Homocysteine in Cells.** The cells were homogenized in ice-cold saturated ammonium sulfate, and the precipitated protein was immediately collected on Millipore filters and washed with ammonium sulfate, as described (15). The protein retained on the filter was dissolved in buffer, interfering purines were removed by charcoal, and protein bound L-homocysteine was released and condensed with adenosine in the presence of DTE and AdoHcy hydrolase. Unlabeled adenosine was used instead of radioactive adenosine (8) in the assay for protein bound L-homocysteine, because high blank values with radioactive adenosine. This was probably related to the formation of radioactive metabolites in the presence of native adenosine metabolizing enzymes, and this radioactive material increases the base-line radioactivity. AdoHcy was quantified by HPLC and UV detection, and the amount of AdoHcy not removed by charcoal treatment were determined and subtracted from total AdoHcy obtained after conversion of L-homocysteine to AdoHcy. Further details have recently been published (15).

**Determination of L-Homocysteine in the Extracellular Medium.** The medium was made 0.6 N in perchloric acid, the precipitated proteins were removed by centrifugation, and the acid was neutralized to pH 7.5, as described above. The neutralized solution was supplemented with 10 mM DTE and treated twice with dextran coated charcoal (at 0°C) to remove inhibitors of AdoHcy hydrolase (15). Free L-homocysteine was converted to [$^{14}$C]AdoHcy, which was isolated and quantified (8).

**Determination of Cellular Content of AdoHcy and AdoMet.** The cells were extracted in 0.6 N perchloric acid, and the supernatant was analyzed by HPLC on a Partisil 10 SCX column (0.46 x 25 cm). The flow rate was 1.5 ml. These compounds were analyzed in a single run by eluting the column with a stepwise gradient of ammonium formate, pH 3.5 (15).

**Determination of AdoHcy in the Extracellular Medium.** In addition to AdoHcy, another UV absorbing material, which cochromatographed with AdoHcy upon cation exchange chromatography, was exported from the cells. Therefore, AdoHcy in medium was determined by HPLC on a reversed phase column. The chromatographic system, which was developed for the L-homocysteine assay (8), was used.

**Determination of Cellular Content of ATP.** Samples (300 μl) from the cell suspension were mixed with two volumes of ice-cold 86% ethanol containing 10 mM EDTA. ATP was determined by a modification of a luciferin-luciferase method (16).

**Determination of Protein.** Cells were extracted with perchloric acid as described above and the precipitated protein was collected by centrifugation. The protein pellet was dissolved in 0.1 N NaOH, and the protein was determined by the method of Bradford (17), using the Bio-Rad protein assay kit. Bovine γ-globulin was used as standard.

## RESULTS

**Evaluation and Modification of the Assay for L-Homocysteine in Cells.** In the light of the paradoxical increase in intracellular L-homocysteine in hepatocytes following inhibition of its formation from AdoHcy (see below), we evaluated the possible interference with the L-homocysteine assay from high concentration of AdoHcy. AdoHcy (20 μM) was added to the homogenization medium used for the determination of free L-homocysteine (perchloric acid) and protein bound L-homocysteine (ammonium sulfate) in liver cells. Exogenous AdoHcy did not affect the amount of free L-homocysteine, and no AdoHcy was detected in the extract after the charcoal treatment. Exogenous AdoHcy or high level of AdoHcy formed in the cells resulted in trace amounts of AdoHcy tightly bound to proteins, and it was not removed by charcoal. This residual AdoHcy was routinely determined and corrected for. After this correction exogenous AdoHcy did not affect the amount of protein bound L-homocysteine (data not shown).

**Experimental Design.** L-Homocysteine egress was determined in cells exposed to two nucleoside analogues, D-eritadenine or 3-deazaaristeromycin, and in control cells and was related to the amount of L-homocysteine in the intracellular compartment. The inhibition of L-homocysteine egress ($\Delta$[L-homocysteine]$_e$) was compared to the total accumulation of AdoHcy in the cells and in the extracellular medium ([AdoHcy]$_t$).

**L-Homocysteine Egress from Hepatocytes.** The release of L-homocysteine from the liver cells into the extracellular medium was linear with respect to time for at least 2.5 h. At this time point the amount of extracellular L-homocysteine (and its disulfides) was about 10 μM, which corresponds to about 2500 pmol/10$^6$ cells.

Both D-eritadenine and 3-deazaaristeromycin inhibit L-homocysteine egress from liver cells in a dose dependent manner, and D-eritadenine was most potent in this respect (Figs. 1 and 2). Notably, D-eritadenine completely arrested L-homocysteine egress after a lag period. This period was short in the presence of 30 μM D-eritadenine and long (about 60 min) in the presence of 3 μM D-eritadenine. In contrast, 3-deazaaristeromycin seemed to reduce the rate of L-homocysteine release immedi-

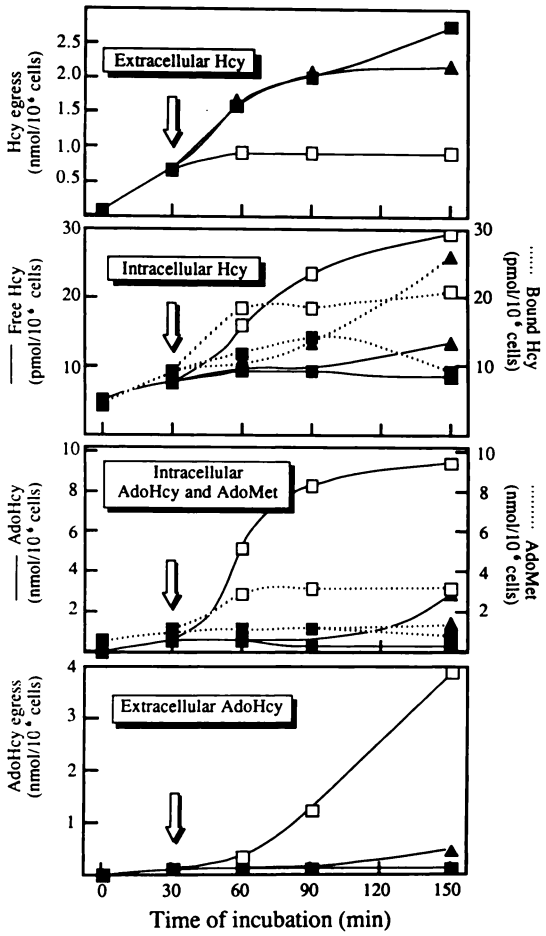


Fig. 1. Disposition of L-homocysteine (*Hcy*) and related compounds in hepatocytes exposed to D-eritadenine. Hepatocytes (4.9 × 10$^6$ cells/ml) were incubated in a medium supplemented with 200 μM methionine. After 30 min of incubation, the cell suspension was supplemented with no (■), 3 μM (▲), or 30 μM (□) D-eritadenine (*arrow*).

ately after addition to the cell suspension, no lag period was observed, and even in the presence of 100 μM 3-deazaaristeromycin there was a small but significant release of L-homocysteine into the medium (Fig. 2).

**Intracellular L-Homocysteine in Hepatocytes.** Intracellular L-homocysteine was low (less than 10 pmol/$10^6$ cells), and a portion was associated with proteins. Notably, both free and protein bound L-homocysteine increased in a dose and time dependent manner following inhibition of AdoHcy catabolism by either D-eritadenine or 3-deazaaristeromycin (Figs. 1 and 2).

**Disposition of AdoHcy and AdoMet by Hepatocytes.** D-Eritadenine (Fig. 1) and 3-deazaaristeromycin (Fig. 2) induced a dose dependent, massive accumulation of AdoHcy. In the presence of D-eritadenine, a lag period was observed before the build-up of AdoHcy occurred. The increase in intracellular AdoHcy was associated with the release of copious amounts of AdoHcy into the extracellular medium and a moderate elevation of intracellular AdoMet.

**ATP Content in Hepatocytes Accumulating AdoHcy.** It was conceivable that accumulation of large amounts of AdoHcy may induce ATP depletion by a mechanism similar to that described for cells accumulating *S*-adenosylethionine following ethionine exposure (18). Low ATP may in turn affect cellular transport. We therefore investigated ATP content in hepatocytes exposed to 100 μM 3-deazaaristeromycin for 2 h. ATP content in control cells was 7.9 ± 0.4 SE nmol/$10^6$ cells and decreased by less than 15% following drug exposure. Thus, accumulation of AdoHcy to amounts [about 9 nmol/$10^6$ cells (Figs. 1 and 2)] equalling the ATP level does not cause ATP depletion.

**Disposition of L-Homocysteine by Fibroblasts.** Both nontransformed C3H/10T½ Cl 8 (Cl 8) cells and their malignant counterpart, C3H/10T½ MCA Cl 16 (Cl 16) cells, exported large amounts of L-homocysteine into the medium. The concentration of extracellular L-homocysteine (and its disulfides) reached 3–8 μM within 96 h, corresponding to about 6000 pmol/$10^6$ cells. Small amounts of L-homocysteine (6–8 pmol/$10^6$ cells) were retained within the cells. In contrast to liver cells, most L-homocysteine associated with the fibroblasts was identified as free (acid soluble) L-homocysteine (Figs. 3 and 4), and only trace amounts of protein bound L-homocysteine were detected (data not shown).

D-Eritadenine (30 μM), which had a pronounced effect on the disposition of L-homocysteine and AdoHcy in liver cells (Fig. 1), only slightly reduced L-homocysteine egress from Cl 8 and Cl 16 cells and the amount of (free) L-homocysteine in these cells (Fig. 3). 3-Deazaaristeromycin (100 μM), on the other hand, nearly completely inhibited L-homocysteine egress and markedly reduced intracellular (free) L-homocysteine of both cell types (Fig. 4).

**Disposition of AdoHcy and AdoMet by Fibroblasts.** D-Eritadenine induced only a slight increase in intracellular AdoHcy and AdoMet and a minimal export of AdoHcy from both nontransformed (Cl 8) and malignant (Cl 16) cells (Fig. 3). Intracellular AdoHcy in Cl 8 and Cl 16 cells exposed to 3-deazaaristeromycin increased markedly for about 6–20 h and then leveled off, corresponding to a [AdoHcy]/[AdoMet] ratio of 0.5. These cells released large amounts of AdoHcy into the

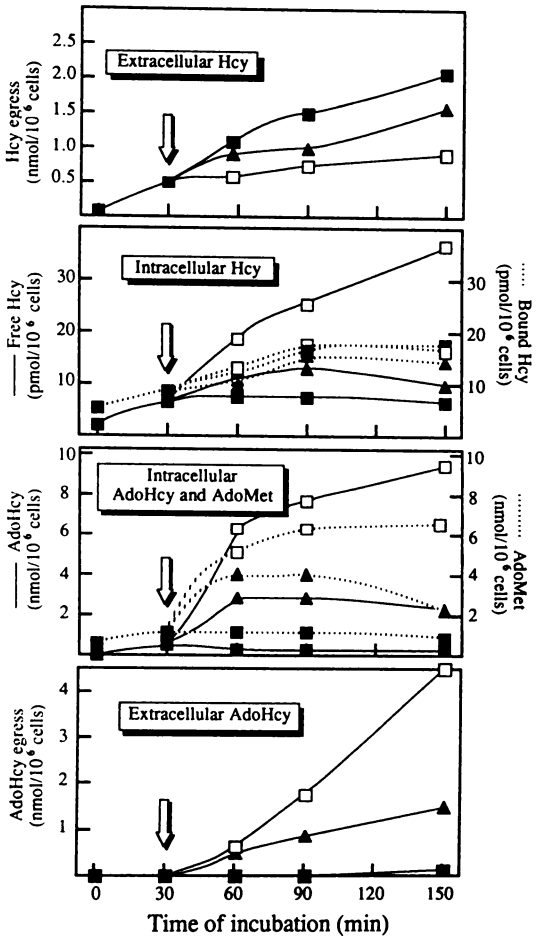


Fig. 2. Disposition of L-homocysteine (*Hcy*) and related compounds in hepatocytes exposed to 3-deazaaristeromycin. Hepatocytes (5.2 × $10^6$ cells/ml) were incubated in a medium supplemented with 200 μM methionine. After 30 min of incubation, the cell suspension was supplemented with no (■), 10 μM (▲), or 100 μM (□) 3-deazaaristeromycin (*arrow*).

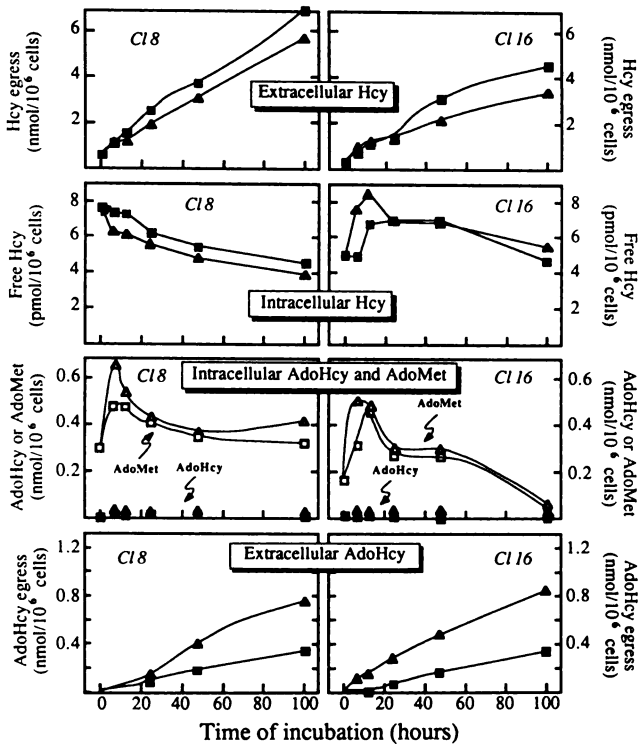


Fig. 3. Disposition of L-homocysteine (*Hcy*) and related compounds in C3H/10T½ Cl 8 (Cl 8) cells and C3H/10T½ MCA Cl 16 (Cl 16) cells exposed to D-eritadenine. Cl 8 and Cl 16 cells were grown to confluence, which corresponds to 3 × $10^6$ cells/dish for Cl 8 and 6 × $10^6$ cells/dish for Cl 16 cells. The medium was then replaced with fresh medium which contained either no drug (control cells, □, ■) or 30 μM D-eritadenine (△, ▲). L-Homocysteine, AdoHcy, and AdoMet associated with the cells and L-homocysteine and AdoHcy in the culture medium were determined as a function of the time of drug exposure.

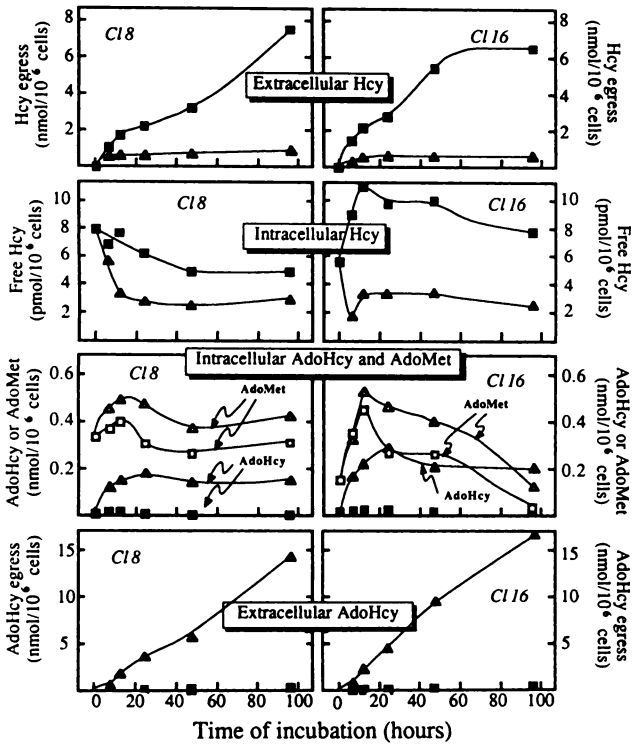


Fig. 4. Disposition of L-homocysteine (*Hcy*) and related compounds in C3H/10T½ Cl 8 (Cl 8) cells and C3H/10T½ MCA Cl 16 (Cl 16) cells exposed to 3-deazaaristeromycin. The experimental design was exactly as described in the legend to Fig. 3, except that D-eritadenine was replaced with 100 μM 3-deazaaristeromycin. □, ■, control cells; △, ▲, cells exposed to 3-deazaaristeromycin.

extracellular medium. Extracellular AdoHcy increased almost linearly and reached about 15,000 pmol/$10^6$ cells after about 96 h (Fig. 4).

**Relation between L-Homocysteine Egress and AdoHcy.** Figs. 1–4 show that a close relation exists between inhibition of L-homocysteine egress ($\Delta[Hcy]_e$) and accumulation of AdoHcy in the intra- and extracellular compartment ($[AdoHcy]_t$) following exposure to D-eritadenine or 3-deazaaristeromycin. This relation is summarized in Table 1. A striking difference was noted between hepatocytes and the fibroblasts. In hepatocytes, AdoHcy accumulation greatly exceeds inhibition of L-homocysteine egress, giving a $[AdoHcy]_t/\Delta[L\text{-homocysteine}]_e$ ratio of about 10, whereas in both Cl 8 and Cl 16 cells, this ratio was less than 3.

## DISCUSSION

**L-Homocysteine Egress under Various Conditions.** There have been recent reports demonstrating that L-homocysteine is exported from isolated cells into the extracellular medium, and small amounts are retained within the cells (15, 19–21). L-Homocysteine export from liver cells is stimulated by the presence of excess methionine and may be related to the metabolic flux through the AdoHcy hydrolase pathway (15). Inhibition of the 5-methyl-THF dependent salvage of L-homocysteine to methionine in C3H/10T½ MCA Cl 16 cells is associated with enhancement of L-homocysteine export (21, 22). These observations with isolated cells are in accordance with the finding of low L-homocysteine in tissues (8, 15) and high levels in extracellular media like plasma and urine (23). L-Homocysteine is markedly increased in plasma and urine from patients treated with methotrexate (21). On the basis of these findings the hypothesis has been made (21) that an export mechanism for L-homocysteine exists which ensures low intracellular L-homocysteine, which may be critical for some vital cellular function(s). Furthermore, extracellular L-homocysteine may be a measure of the balance between L-homocysteine formation and utilization.

**L-Homocysteine Egress and AdoHcy Hydrolase Activity.** The present paper describes the disposition of L-homocysteine in cells exposed to agents inhibiting its formation from AdoHcy, *i.e.*, nucleoside analogues, like D-eritadenine and 3-deazaaristeromycin.

D-Eritadenine, which induces a time dependent inactivation of isolated AdoHcy hydrolase and the enzyme in intact hepatocytes (12), completely inhibited L-homocysteine export after a lag period. The inhibition was associated with accumulation of intra- and extracellular AdoHcy (Fig. 1). The lag period probably reflects the time required for total inactivation of AdoHcy hydrolase to occur. Somewhat unexpected, D-eritadenine had only a slight effect on both L-homocysteine egress and AdoHcy in fibroblasts (Fig. 3).

3-Deazaaristeromycin reduced the rate of L-homocysteine egress and induced AdoHcy accumulation in a dose dependent manner in liver cells almost immediately after addition of the compound to the cell suspension (Fig. 2). Similar data were obtained with Cl 8 and Cl 16 cells (Fig. 4). Reduced rate of L-homocysteine export rather than total inhibition might be expected in the presence of a compound which acts as a competitive inhibitor of AdoHcy catabolism. 3-Deazaadenosine, which serves as a competitive inhibitor and substrate of AdoHcy hydrolase (1), has a similar effect on L-homocysteine egress (24).

The above data, taken together, suggest that inhibition of L-homocysteine egress and the AdoHcy accumulation are tightly linked processes. This possibility is further supported by a quantitative comparison of inhibition of L-homocysteine egress and AdoHcy accumulation (Table 1). In both liver cells and mouse embryo fibroblasts, the ratio between inhibition of L-homocysteine egress and AdoHcy accumulation is essentially constant for a particular cell type under different experimental conditions. These findings suggest that the rate of L-homocysteine export is proportional to L-homocysteine formation. One may speculate whether L-homocysteine egress is coupled to the AdoHcy hydrolase reaction.

Export of the most abundant thiol in the cells, glutathione, is perhaps a property of most cells. Glutathione export seems to be associated with the turnover of this compound and is inhibited by agents blocking glutathione formation (25). Thus, disposition of glutathione by cells may have some features in common with cellular handling of L-homocysteine.

**Intracellular and Extracellular L-Homocysteine.** Conceivably, L-homocysteine export is a function of the amount intracellular L-homocysteine available to the mechanism responsible for the L-homocysteine export, and inhibition of L-homocysteine egress by nucleoside analogues may be related to low concentration of intracellular, free, L-homocysteine. This possibility was not supported by the data obtained with hepatocytes, showing that nucleoside analogues increased rather than decreased both free and protein-bound L-homocysteine in these cells (Figs. 1 and 2). A similar unexpected observation has recently been made with whole animals given injections with the drug combination 9-β-D-arabinofuranosyladenine plus 2′-deoxycoformycin (8). This paradoxical effect on intracellular L-homocysteine in hepatocytes may be explained as follows. Different compartments of intracellular L-homocysteine exist, and only a minor L-homocysteine compartment is available for L-homocysteine

Table 1 *Relationship between S-adenosylhomocysteine accumulation in the extra- and intracellular compartment and inhibition of Hcy[a] egress in hepatocytes and the C3H/10T½ Cl 8 and Cl 16 cells exposed to nucleoside analogues*

Total amounts of intra- and extracellular AdoHcy ($[AdoHcy]_t$), the inhibition of Hcy egress, *i.e.*, the difference between extracellular Hcy in the absence and presence of inhibitor ($\Delta[Hcy]_e$), and the ratio between $[AdoHcy]_t$ and $\Delta[Hcy]_e$ are determined from data presented in Figs. 1–4.

| Cell type | Inhibitor | Time of exposure (h) | Total AdoHcy ($[AdoHcy]_t$) (pmol/$10^6$ cells) | Inhibition of Hcy egress ($\Delta[Hcy]_e$) (pmol/$10^6$ cells) | $[AdoHcy]_t/\Delta[Hcy]_e$ ratio |
|---|---|---|---|---|---|
| Hepatocytes | D-Erit-A (3 μM) | 1 | 342 | | |
| | | 1.5 | 384 | | |
| | | 2.5 | 3,298 | 387 | 8.5 |
| | D-Erit-A (30 μM) | 1 | 5,883 | 540 | 10.9 |
| | | 1.5 | 9,350 | 979 | 9.5 |
| | | 2.5 | 13,060 | 1,768 | 7.4 |
| | c³Ari (10 μM) | 1 | 3,664 | | |
| | | 1.5 | 4,068 | 417 | 9.7 |
| | | 2.5 | 3,703 | 644 | 5.7 |
| | c³Ari (100 μM) | 1 | 6,777 | 285 | 23.8 |
| | | 1.5 | 9,160 | 687 | 13.3 |
| | | 2.5 | 13,911 | 1,362 | 10.2 |
| C3H/10T½ Cl 8 | D-Erit-A (30 μM) | 24 | 170 | 547 | 0.31 |
| | | 48 | 418 | 708 | 0.59 |
| | | 100 | 760 | 709 | 1.07 |
| | c³Ari (100 μM) | 6 | 714 | 480 | 1.5 |
| | | 12 | 1,875 | 1,243 | 1.5 |
| | | 24 | 3,104 | 1,798 | 1.7 |
| | | 48 | 5,977 | 2,813 | 2.1 |
| | | 100 | 14,925 | 6,581 | 2.3 |
| C3H/10T½ Cl 16 | D-Erit-A (30 μM) | 12 | 186 | 245 | 0.76 |
| | | 24 | 296 | 302 | 0.98 |
| | | 48 | 445 | 812 | 0.55 |
| | | 100 | 823 | 990 | 0.83 |
| | c³Ari (100 μM) | 6 | 978 | 1,056 | 0.9 |
| | | 12 | 2,198 | 1,703 | 1.3 |
| | | 24 | 4,595 | 2,266 | 2.0 |
| | | 48 | 9,416 | 4,803 | 2.0 |
| | | 100 | 17,748 | 6,051 | 2.9 |

[a] Hcy, L-homocysteine; D-Erit-A, D-eritadenine; c³Ari, 3-deazaaristeromycin.

export. The major fraction of L-homocysteine may participate in an equilibrium between L-homocysteine and AdoHcy, catalyzed by enzyme(s) metabolizing these compounds. This possibility is supported by the parallel increase in intracellular L-homocysteine and AdoHcy (Figs. 1 and 2). Inhibition of L-homocysteine egress under conditions of elevated intracellular L-homocysteine is compatible with coupling of AdoHcy hydrolase reaction and L-homocysteine export.

In both nontransformed and malignant C3H/10T½ cells, inhibition of AdoHcy catabolism leads to a marked reduction of intracellular L-homocysteine. In these cells the major fraction of intracellular L-homocysteine may be available for export.

**Metabolism of L-Homocysteine and AdoHcy in Hepatocytes and in Nontransformed and Malignant Mouse Embryo Fibroblasts.** In hepatocytes exposed to nucleoside analogues, the L-homocysteine egress and the inhibition of L-homocysteine egress was only about 10% of intra- and extracellular AdoHcy accumulation (Table 1). In Cl 8 and Cl 16 cells incubated with 3-deazaaristeromycin, the inhibition of L-homocysteine egress was of the same order of magnitude as the total amount of AdoHcy.

The difference in L-homocysteine disposition in hepatocytes *versus* fibroblasts should be related to unique features of L-homocysteine and AdoHcy metabolism in liver cells. In liver (and kidney) L-homocysteine is efficiently salvaged to methionine by two enzymes, requiring betaine or 5-methyl-THF as methyl donor, respectively (26), whereas in other cells this reaction is catalyzed by a single enzyme (4) which many contribute insignificantly to the overall L-homocysteine metabolism (20). L-Homocysteine is efficiently metabolized to cysteine via the transsulfuration pathway in hepatocytes (27), whereas in other cell lines the activity of this pathway is either low or absent (28–30). Furthermore, in the liver the bulk of AdoHcy is a product of and is dependent on a single transmethylation reaction, namely the AdoMet dependent methylation of guanidoacetic acid (31). In most other cells, AdoHcy is formed from multiple methyl transfer reactions, some of which may be more sensitive than guanidoacetate methyltransferase towards the feedback inhibition of AdoHcy. The special features of L-homocysteine and AdoHcy metabolism in liver cells *versus* most other cell types should be taken into account when using L-homocysteine egress (20) or AdoHcy accumulation (32, 33) as a measure of the overall transmethylation rate.

Most malignant cells are methionine dependent and do not thrive in a medium where methionine is replaced with L-homocysteine. Nontransformed cells grow well under these conditions (5, 32). The Cl 16 cells are an example of malignant cells where L-homocysteine does not fully support growth, whereas the growth rate of Cl 8 cells is about the same in presence of L-homocysteine or methionine.[4] The methionine dependence of cancer cells has been related to an increase in the overall transmethylation rate relative to that of normal cells (32). We observed that L-homocysteine egress from Cl 8 cells equalled that from Cl 16 cells (Figs. 3 and 4). The inhibition of L-

[4] R. Djurhuus, A. Svardal, J. R. Lillehaug, and P. M. Ueland, unpublished results.

homocysteine egress by 3-deazaaristeromycin and the initial rates of accumulation of intra- and extracellular AdoHcy in the presence of this inhibitor were only slightly higher for Cl 16 cells than Cl 8 cells. Thus, our data did not support the possibility of higher transmethylation rate in malignant Cl 16 cells compared with their normal counterpart, Cl 8 cells.

**Possible Consequences of Cellular L-Homocysteine Depletion.** Indirect evidence has been provided that the cytostatic effects of 3-deazaaristeromycin are caused by inhibition of the L-homocysteine dependent conversion of 5-methyl-THF to THF (34). Cantoni *et al.* suggested that inhibition of AdoHcy hydrolase by 9-β-D-arabinofuranosyladenine may result in severe deficiency in L-homocysteine, which in turn would reduce methionine biosynthesis. They also suggested that if 9-β-D-arabinofuranosyladenine is given in combination with methotrexate, the rescue therapy with 5-formyl-THF would fail because the conversion to THF is blocked (35). Inhibition of methionine synthesis by 2′-deoxyadenosine and 9-β-D-arabinofuranosyladenine in lymphoblasts has recently been demonstrated (36). These findings, taken together, suggest that the possible interference with L-homocysteine formation and thereby methionine synthesis in the presence of nucleoside analogues should be further investigated.

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## REFERENCES

1. Ueland, P. M. Pharmacological and biochemical aspects of *S*-adenosylhomocysteine and *S*-adenosylhomocysteine hydrolase. Pharmacol. Rev., *34:* 223–253, 1982.
2. Cantoni, G. L., and Chiang, P. K. The role of *S*-adenosylhomocysteine and *S*-adenosylhomocysteine hydrolase in the control of biological methylation. *In:* D. Cavallini, G. E. Gaull, and V. Zappia (eds.), Natural Sulfur Compounds: Novel Biochemical and Structural Aspects, pp. 67–80. New York: Plenum Publishing Corp., 1980.
3. Chiang, P. K. *S*-Adenosylhomocysteine hydrolase. Measurement of activity and use of inhibitors. Methods Pharmacol., *6:* 127–145, 1985.
4. Mudd, S. H., and Levy, H. L. Disorders of transsulfuration. *In:* J. B. Standbury (ed.), Metabolic Basis of Inherited Diseases, pp. 522–559. New York: McGraw-Hill, 1983.
5. Hoffman, R. M. Altered methionine metabolism, DNA methylation and oncogenic expression in carcinogenesis. Biochim. Biophys. Acta, *73:* 49–87, 1984.
6. McCully, K. S. Homocysteine theory of arteriosclerosis: development and current status. Atherosclerosis Rev., *11:* 157–246, 1983.
7. Barak, A. J., Tuma, D. J., and Beckenhauer, H. C. Methotrexate hepatotoxicity. J. Am. Coll. Nutr., *3:* 93–96, 1984.
8. Ueland, P. M., Helland, S., Broch, O-J., and Schanche, J-S. Homocysteine in tissues of the mouse and rat. J. Biol. Chem., *259:* 2360–2364, 1984.
9. Reznikoff, C. A., Brankow, D. W., and Heidelberger, C. Establishment and characterization of a clone of C3H mouse embryo cells sensitive to postconfluence inhibition of cell division. Cancer Res., *33:* 3231–3238, 1973.
10. Reznikoff, C. A., Bertram, J. S., Brankow, D. W., and Heidelberger, C. 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, 1973.
11. Montgomery, J. A., Clayton, S. J., Thomas, H. J., Shannon. W. M., Arnett, G., Bodner, A. J., Kion, I-K., Cantoni, G. L., and Chiang, P. K. Carbocyclic analogue of 3-deazaadenosine. A novel antiviral agent using *S*-adenosylhomocysteine hydrolase as a pharmacological target. J. Med. Chem., *25:* 626–629, 1982.
12. Schanche, J-S., Schanche, T., Ueland, P. M., Holy, A., and Votruba, I. The effect of aliphatic adenine analogues on *S*-adenosylhomocysteine and *S*-adenosylhomocysteine hydrolase in intact rat hepatocytes. Mol. Pharmacol., *26:* 553–558, 1984.
13. Berry, M. N., and Friend, D. S. High-yield preparations of isolated rat liver parenchymal cells. J. Cell Biol., *43:* 506–520, 1969.
14. Helland, S., and Ueland, P. M. Inactivation of *S*-adenosylhomocysteine hydrolase by 9-β-D-arabinofuranosyladenine in intact cells. Cancer Res., *42:* 1130–1136, 1982.
15. Svardal, A., Refsum, H., and Ueland, P. M. 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, 1986.
16. Steen, V. M., and Holmsen, H. Synergism between thrombin and epinephrine in human platelets: different dose-response relationship for aggregation and dense granule secretion. Thromb. Haemostasis, *54:* 680–683, 1985.
17. Bradford, M. 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, 1976.
18. Shull, K. H., McConomy, J., Vogt, M., Castillo, A., and Faber, E. On the mechanism of induction of hepatic adenosine triphosphate deficiency by ethionine. J. Biol. Chem., *241:* 5060–5070, 1966.
19. German, D. C., Bloch, G. A., and Kredich, N. M. Measurements of *S*-adenosylmethionine and L-homocysteine metabolism in cultured human lymphoid cells. J. Biol. Chem., *258:* 10997–11003, 1983.
20. Iizasa, T., and Carson, D. Differential regulation of polyamine synthesis and transmethylation reactions in methylthioadenosine phosphorylase deficient mammalian cells. Biochim. Biophys. Acta, *844:* 280–287, 1985.
21. Ueland, P. M., Svardal, A., Refsum, H., Lillehaug, J. R., Schanche, J-S., and Helland, S. Disposition of endogenous *S*-adenosylhomocysteine and homocysteine following exposure to nucleoside analogues and methotrexate. *In:* R. T. Borchardt, C. R. Creveling, and P. M. Ueland (eds.), Biological Methylation and Drug Design. Experimental and Clinical Roles of *S*-Adenosylmethionine, pp. 263–274. Clifton, NJ: Humana Press, Inc., 1986.
22. Ueland, P. M., Refsum, H., Male, R., and Lillehaug, J. R. Disposition of endogenous homocysteine by mouse fibroblast C3H/10T½ Cl 8 and the chemically transformed C3H/10T½ MCA Cl 16 cells following methotrexate exposure. J. Natl. Cancer Inst., in press, 1986.
23. Refsum, H., Helland, S., and Ueland, P. M. Radioenzymic determination of homocysteine in plasma and urine. Clin. Chem., *31:* 624–628, 1985.
24. Svardal, A. M., Djurhuus, R., and Ueland, P. M. Disposition of homocysteine and *S*-3-deazaadenosylhomocysteine in cells exposed to 3-deazaadenosine. Mol. Pharmacol., in press, 1986.
25. Dethmers, J. K., and Meister, A. Glutathione export by human lymphoid cells: depletion of glutathione by inhibition of its synthesis decreases export and increases sensitivity to irradiation. Proc. Natl. Acad. Sci. USA, *78:* 7492–7496, 1981.
26. Finkelstein, J. D., and Martin, J. J. Methionine metabolism in mammals. Distribution of homocysteine between competing pathways. J. Biol. Chem., *259:* 9508–9513, 1984.
27. Beatty, P. W., and Reed, D. J. Involvement of cystathionine pathway in the biosynthesis of glutathione by isolated rat hepatocytes. Arch. Biochem. Biophys., *204:* 80–87, 1980.
28. Brodie, A. E., Potter, J., Ellis, W. W., Evenson, M. C., and Reed, D. J. Glutathione biosynthesis in murine L5178 lymphoma cells. Arch. Biochem. Biophys., *210:* 437–444, 1981.
29. Brodie, A. E., Potter, J., and Reed, D. J. Unique characteristics of rat spleen lymphocytes, L1210 lymphoma and HeLa cells in glutathione biosynthesis from sulfur-containing amino acids. Eur. J. Biochem., *123:* 159–164, 1982.
30. Fowler, B. Transsulphuration and methylation of homocysteine in control and mutant human fibroblasts. Biochim. Biophys. Acta, *721:* 201–207, 1982.
31. Im, Y. S., Chiang, P. K., and Cantoni, G. L. Guanidoacetate methyltransferase. Purification and molecular properties. J. Biol. Chem., *254:* 11047–11050, 1979.
32. Hoffman, R. M., and Stern, P. H. Cancer, methionine and transmethylation. *In:* R. T. Borchardt, C. R. Creveling, and P. M. Ueland (eds.), Biological Methylation and Drug Design. Experimental and Clinical Roles of *S*-Adenosylmethionine, pp. 215–226. Clifton, NJ: Humana Press, Inc., 1986.
33. Hoffman, J. L. The rate of transmethylation in mouse liver as measured by trapping *S*-adenosylhomocysteine. Arch. Biochem. Biophys., *205:* 132–135, 1980.
34. Kim, I-K., Aksamit, R. R., and Cantoni, G. L., Mechanism of the cytostatic activity of 3-deazaaristeromycin, an inhibitor of *S*-adenosylhomocysteine hydrolase. J. Biol. Chem., *257:* 14726–14729, 1982.
35. Cantoni, G. L., Aksamit, R. R., and Kim, I-K. Methionine biosynthesis and vidarabine therapy. N. Engl. J. Med., *307:* 1079, 1982.
36. Boss, G. R., and Pilz, R. B. Decreased methionine synthesis in purine nucleoside-treated T and B lymphoblasts and reversal by homocysteine. J. Clin. Invest., *74:* 1262–1268, 1984.