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Disposition of Homocysteine and *S*-3-Deazaadenosylhomocysteine in Cells Exposed to 3-Deazaadenosine

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SUMMARY

The nucleoside analogue, 3-deazaadenosine (c^3-Ado), serves both as a substrate and as an inhibitor of *S*-adenosylhomocysteine (AdoHcy) hydrolase, and the ability of this compound to induce accumulation of intracellular AdoHcy and *S*-3-deazaadenosylhomocysteine (c^3-AdoHcy) in various cells and species has been widely documented. We here report on the effect of c^3-Ado on the disposition of homocysteine (Hcy) and c^3-AdoHcy in isolated rat hepatocytes and in non-transformed (Cl 8) and malignant (Cl 16) C3H/10T1/2 mouse embryo fibroblasts in culture. Both the liver cells and fibroblasts release large amounts of Hcy into the extracellular medium, whereas small amounts are retained within the cells. c^3-Ado (100–300 μM) nearly completely inhibits cellular Hcy egress. Intracellular Hcy in liver cells exposed to c^3-Ado is in fact increased in proportion to intracellular build-up of AdoHcy, whereas c^3-Ado nearly deprives the malignant Cl 16 cells of intracellular Hcy and decreases it markedly in Cl 8 cells. Adenosine exerts a similar effect as c^3-Ado on Hcy and AdoHcy in liver cells, but concentrations in the mM range are required, and the effect subsides within hours. In liver cells, c^3-Ado (300 μM) induces a higher level of c^3-AdoHcy than of AdoHcy. In the malignant (Cl 16) fibroblasts, c^3-AdoHcy content approaches the amount of AdoHcy whereas, in the non-transformed (Cl 8) fibroblasts, relatively small amounts of c^3-AdoHcy are formed. Notably, c^3-AdoHcy is released from all cell types in proportion to the intracellular amount, suggesting that c^3-AdoHcy is efficiently handled by the mechanism responsible for the cellular egress of nucleosidylhomocysteine. The possible role of Hcy and c^3-AdoHcy in the mechanism of action of c^3-Ado is discussed.

Several nucleoside analogues interact with AdoHcy hydrolase (EC 3.3.1.1.), the enzyme responsible for metabolic degradation of the endogenous transmethylase inhibitor, AdoHcy. These analogues may serve as a competitive inhibitor, inactivator, or substrate of this enzyme. Among these compounds, c^3-Ado acts both as a potent inhibitor and as a good substrate, and exhibits numerous interesting biological properties (1, 2).

The mechanism of action of c^3-Ado still remains an unsolved question. The concept of AdoHcy hydrolase as a target enzyme for nucleoside analogues (3, 4) led to numerous studies relating the ability of c^3-Ado to induce cellular build-up of AdoHcy and c^3-AdoHcy (3, 5) to the biological effects observed with this compound (1). However, recent studies (6) have shed doubt on AdoHcy hydrolase as the sole and primary molecular target of c^3-Ado.

There are two aspects of the interaction of c^3-Ado with AdoHcy hydrolase which have received no attention. 1) The AdoHcy hydrolase reaction is the only known source of Hcy in vertebrates (7), and inhibition of this enzyme may cause cellular depletion of Hcy. This effect may even be enhanced by trapping Hcy as c^3-AdoHcy by directing the AdoHcy hydrolase-catalyzed reaction in the synthetic direction. 2) Inhibition of AdoHcy catabolism causes build-up of intracellular AdoHcy, but the accumulation of AdoHcy is efficiently counteracted by export of large amounts of AdoHcy into the extracellular medium (8). Whether c^3-AdoHcy efflux occurs is unknown.

The present paper addresses these two points by investigating the disposition of endogenously formed Hcy and c^3-AdoHcy in cells following c^3-Ado exposure. Isolated hepatocytes as well as non-transformed and chemically transformed mouse embryo fibroblasts were used, since special features have been assigned to Hcy metabolism in liver cells versus other cell types (9), and in cancer cells versus normal cells (10).

Materials and Methods

Chemicals. DL-Homocysteine, L-methionine, adenosine, AdoHcy, and dithioerythritol (DTE) were obtained from Sigma Chemical Co., St Louis, MO, and AdoMet was from Koch-Light Laboratories, Colnbrook, UK. c^3-Ado was kindly supplied by Dr. John Montgomery,

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ABBREVIATIONS: AdoHcy, *S*-adenosylhomocysteine hydrolase; AdoMet, *S*-adenosylmethionine; c^3-Ado, 3-deazaadenosine; c^3-AdoHcy, *S*-3-deazaadenosylhomocysteine; Hcy, L-homocysteine; DTE, dithioerythritol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Southern Research Institute, Birmingham, AL. [^{14}C]Adenosine (0.59 Ci/mmol) was purchased from the Radiochemical Center, Amersham.

Preparation and incubation of isolated rat liver cells. The isolated rat hepatocytes were prepared by a collagenase perfusion method (11) and were incubated in an isotonic salt solution containing Hepes buffer, pH 7.4, bovine serum albumin, glucose, salt, and antibiotics, as described elsewhere (12).

Cell lines and culture conditions. Stock cultures of non-transformed, C3H/10T1/2 Cl 8 cells (13) and the chemically transformed C3H/10T1/2 MCA Cl 16 cells (14) 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 basal medium Eagle (Gibco, Paisley, Scotland), supplemented with 10% heat-inactivated fetal calf serum (Sera-Lab. Ltd., Sussex, England) at 37° in an atmosphere of 5% CO_2 in air, and a relative humidity of 95%.

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

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

Determination of free Hcy in cells. The cells were homogenized in 0.6 N perchloric acid. The precipitated protein was removed by centrifugation, and the acid was neutralized to pH 7.5. Hcy was assayed in the extract by a radioenzymic method based on the conversion of Hcy and its acid-soluble mixed disulfides to radioactive AdoHcy. Sufficient dilution of the extract ensures almost toal recovery of Hcy (15).

Determination of protein-bound Hcy 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 (16). The protein retained on the filter was dissolved in buffer, interfering purines were removed by charcoal, and protein-bound Hcy was released and condensed with [^{14}C]adenosine in the presence of DTE and AdoHcy hydrolase. Radioactive AdoHcy was then isolated by HPLC, and the radioactivity was determined by liquid scintillation counting. With this method, total recovery of Hcy added to the tissue extract is obtained, but a fraction of endogenous protein-bound Hcy may escape detection (16).

Determination of Hcy 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. The neutralized solution was supplemented with 10 mM DTE and treated (at 0°) twice with dextran-coated charcoal to remove inhibitors of AdoHcy hydrolase. Free Hcy was converted to [^{14}C]AdoHcy, which was isolated and quantified (15).

Determination of cellular content of AdoHcy, c^3-AdoHcy, and AdoMet. The cells were extracted in 0.6 N perchloric acid, and the supernatant was subjected to HPLC on a Nucleosil 10 SA column (0.46 × 25 cm). The flow rate was 1.5 ml/min. These compounds were analyzed in a single run by eluting the column with a stepwise gradient of ammonium formate, pH 3.5, in 20% methanol. The column was equilibrated with 300 mM; at 6 min the ammonium formate was increased to 600 mM and at 16.5 min it was increased to 1.23 M. The absorbance was recorded at 260 nm using a variable wavelength detector, Spectroflow model 773, from Kratos. At 24 min the detector received an auto zero signal from the autosampler (ISS 100 from Perkin Elmer). The retention times were 7.8 min (AdoHcy), 17.3 min (c^3-AdoHcy), and 26.7 min (AdoMet).

Determination of AdoHcy and c^3-AdoHcy in the extracellular medium. In addition to AdoHcy, another UV-absorbing material, which co-chromatographed with AdoHcy upon cation exchange chromatography, was exported from the cells. Therefore, AdoHcy in medium was determined by HPLC on a reverse phase column. The chromatographic system, which was developed for the Hcy assay (15), was used. c^3-AdoHcy in the medium was determined with the cation exchange system described in the preceding section.

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 gamma globulin was used as standard.

Results

Effect of c^3-Ado on Hcy egress. Hepatocytes and non-transformed (Cl 8) and malignant (Cl 16) fibroblasts release copious amounts of Hcy into the extracellular medium. The Hcy egress from these cells is almost linear with respect to time of incubation (Figs. 1A and 2, A and B).

The Hcy efflux from hepatocytes was inhibited by about 75% in the presence of 100 μM c^3-Ado and almost completely inhibited by 300 μM c^3-Ado (Fig. 1A).

The Hcy efflux from both Cl 8 and Cl 16 cells was inhibited by c^3-Ado (300 μM) to about the same extent as the efflux from the hepatocytes (Fig. 2, A and B).

Effect of c^3-Ado on intracellular Hcy. Hcy in hepatocytes is almost equally distributed between a free (acid soluble) and a protein-bound fraction (16). Notably, both fractions increased

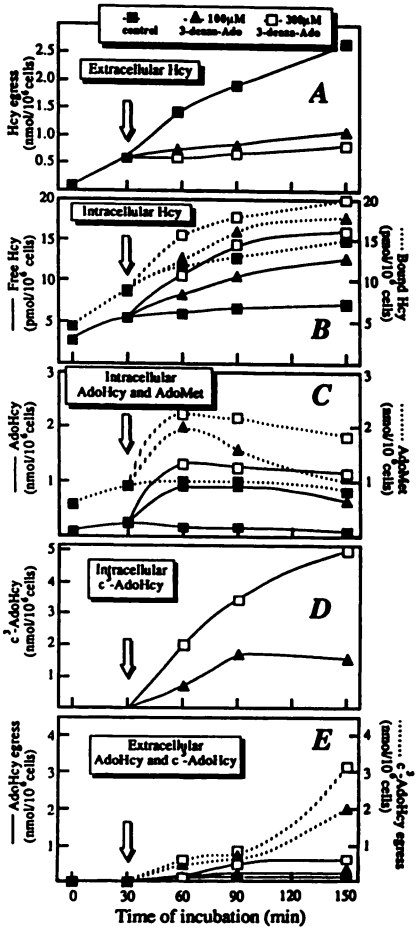

Fig. 1. Disposition of Hcy and related compounds in hepatocytes exposed to c^3-Ado. Hepatocytes ($4.9 \cdot 10^6$ cells/ml) were incubated in a medium supplemented with 200 μM methionine. After 30 min of incubation, c^3-Ado, at the concentrations indicated, was added to the cell suspension (*arrows*).

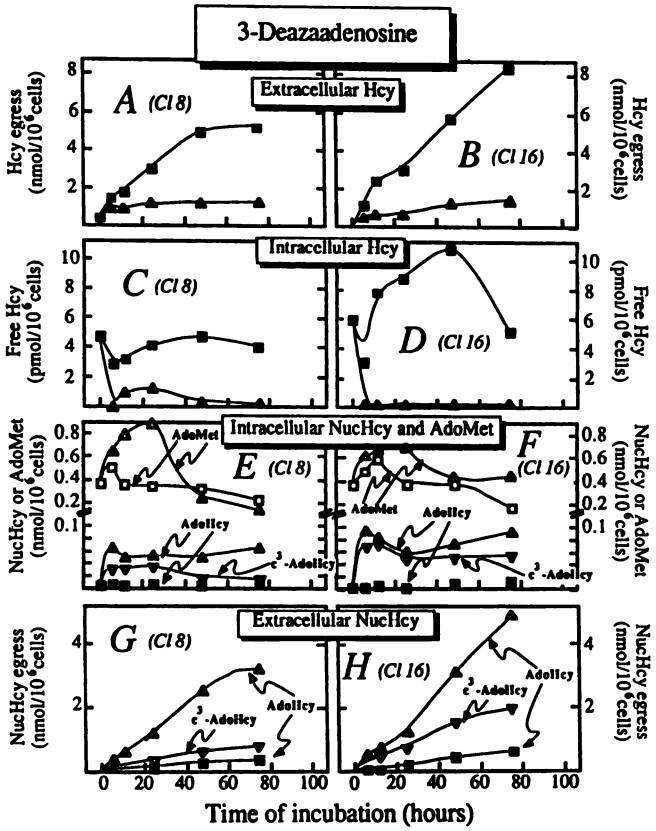

Fig. 2. Disposition of Hcy and related compounds in C3H/10T1/2 Cl 8 (*Cl 8*) cells and C3H/10T1/2 MCA Cl 16 (*Cl 16*) cells exposed to c^3-Ado. Cl 8 and Cl 16 cells were grown to confluence, which corresponds to $4.5 \cdot 10^6$ cells/dish for Cl 8 and $9 \cdot 10^6$ cells/dish for Cl 16 cells. The medium was then replaced with fresh medium which contained either no drug (control cells; ■, □) or 300 μM c^3-Ado (▲, △, ▽). Hcy, AdoHcy, c^3-AdoHcy, and AdoMet associated with the cells and Hcy, AdoHcy, and c^3-AdoHcy in the culture medium were determined as a function of the time of drug exposure.

rather than decreased following inhibition of AdoHcy catabolism by c^3-Ado (Fig. 1B).

Most intracellular Hcy in both Cl 8 and Cl 16 cells was free, and essentially no protein-bound Hcy was detected (data not shown). In the malignant Cl 16 cells, the Hcy content was somewhat higher than in Cl 8 cells. In contrast to the results obtained with hepatocytes, c^3-Ado reduced the Hcy content in Cl 8 cells by 80–90%, and in Cl 16 cells only trace amounts of Hcy were detected following c^3-Ado treatment (Fig. 2, C and D).

Intracellular c^3-AdoHcy, AdoHcy, and AdoMet. In accordance with data published previously (3, 18) c^3-Ado treatment of hepatocytes increased AdoHcy in a dose-dependent manner and also caused a moderate increase in AdoMet (Fig. 1C). In the fibroblasts, the elevation of AdoHcy content was far less than in the hepatocytes, and a moderate increase in AdoMet was observed (Fig. 2, E and F).

Hepatocytes exposed to 100 μM c^3-Ado accumulated about equal amounts of c^3-AdoHcy and AdoHcy, whereas intracellular c^3-AdoHcy increased linearly and was the most abundant nucleosidyl-Hcy within 1 hr after exposure to 300 μM c^3-Ado (Fig. 1D).

The c^3-AdoHcy accumulation induced by c^3-Ado was less in Cl 16 cell (Fig. 2F) than in the liver cells (Fig. 1D), but more pronounced in Cl 16 cells than in Cl 8 cells (Fig. 2, E and F).

Cellular c^3-AdoHcy egress. The c^3-AdoHcy egress from hepatocytes exposed to 100 μM c^3-Ado was greater than the AdoHcy egress, even though the intracellular levels of these two compounds were about the same. A moderate enhancement in c^3-AdoHcy egress was obtained when the c^3-Ado concentration was increased to 300 μM (Fig. 1E).

The c^3-AdoHcy egress was about half of the AdoHcy egress in Cl 16 cells exposed to c^3-Ado. Small amounts of c^3-AdoHcy relative to AdoHcy were released into the medium by Cl 8 cells (Fig. 2, G and H).

There was no export of AdoMet from the hepatocytes (12) or Cl 8 and Cl 16 cells (data not shown).

Effect of adenosine on the disposition of Hcy and AdoHcy by hepatocytes. Adenosine, a naturally occurring substrate and inhibitor of AdoHcy hydrolase (19), exerted an effect similar to that of c^3-Ado on the disposition of Hcy and AdoHcy by hepatocytes (Fig. 3), but some important differences were noted. High concentrations (in the mM range) were required to block Hcy egress from hepatocytes and induce AdoHcy accumulation in these cells. Furthermore, after about 1 hr following addition of 1 mM adenosine, the rate of Hcy efflux increased markedly. This increase was associated with a pronounced decline in intracellular AdoHcy. Thus, only a transient effect on Hcy was observed in the presence of adenosine (Fig. 3).

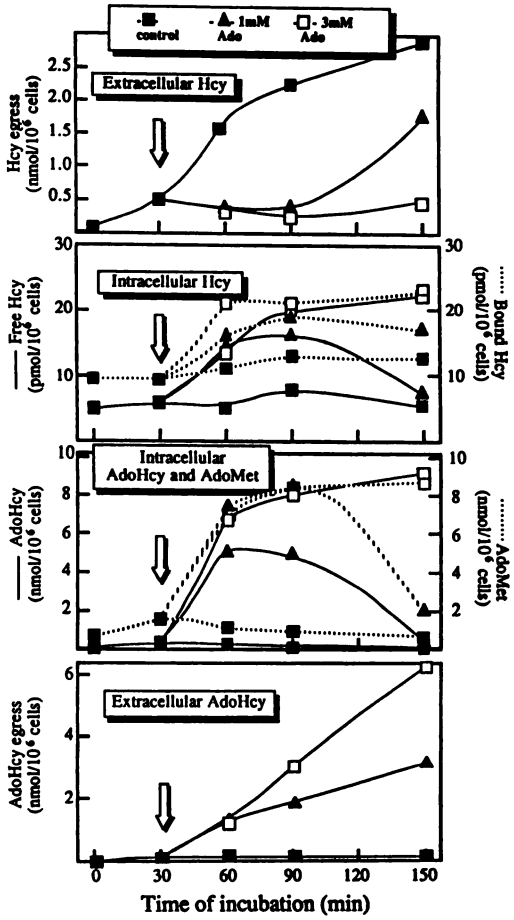

Fig. 3. Disposition of Hcy and related compounds in hepatocytes exposed to adenosine. Hepatocytes ($4.9 \cdot 10^6$ cells/ml) were incubated in a medium supplemented with 200 μM methionine. After 30 min of incubation, adenosine, at the concentrations indicated, was added to the cell suspension (*arrows*).

Discussion

Effect of c^3-Ado on Hcy metabolism. The amount of Hcy in tissues and cells is low (15, 16), and Hcy egress may be an important mechanism for the maintenance of Hcy within certain limits (8, 20). The present paper demonstrates that c^3-Ado nearly completely blocks Hcy egress from both hepatocytes and fibroblasts (Figs. 1A and 2, A and B). c^3-AdoHcy and AdoHcy are accumulating in proportion to the inhibition of Hcy egress (Figs. 1 and 2). This finding suggests that the inhibitory effect of c^3-Ado is mediated by AdoHcy hydrolase and that the mechanism responsible for the Hcy egress may in some way be coupled to the activity of this enzyme. Formation of c^3-AdoHcy shows that c^3-Ado, in addition to being an inhibitor of AdoHcy hydrolase, serves as a substrate of this enzyme and condenses with endogenous Hcy. Conceivably, this may be an efficient mechanism for the removal of cellular Hcy. This possibility is supported by the finding that c^3-Ado (Figs. 1 and 2) is equally or even more efficient than 3-deazaaristeromycin as an inhibitor of Hcy egress, even though the latter agent induces a higher level of intra- and extracellular nucleosidyl-Hcy (unpublished data).

Adenosine is the natural substrate of the AdoHcy synthase reaction catalyzed by AdoHcy hydrolase and is an equally good substrate of this enzyme as c^3-Ado (19). In spite of this, adenosine is less efficient than c^3-Ado as an inhibitor of Hcy egress and AdoHcy catabolism, and induces only a transient effect on these parameters (Fig. 3). This can be explained by the fact that adenosine, in contrast to c^3-Ado, is a metabolically labile compound which is rapidly cleared from the intracellular compartment (21). In addition, the pronounced increase in Hcy egress upon relief of the inhibitory effect of adenosine on AdoHcy catabolism (Fig. 3) supports the idea that a close relation exists between AdoHcy hydrolysis and Hcy egress.

We have previously communicated that 3-deazaaristeromycin increases rather than decreases intracellular Hcy (8). c^3-Ado has a similar effect on Hcy content in hepatocytes (Fig. 1B). The increase in intracellular Hcy in some cells (Figs. 1B, 2B, and 3) and tissues following inhibition of AdoHcy catabolism (15) is remarkable. It may suggest that a major portion of intracellular Hcy varies as a function of AdoHcy. An enzyme-catalyzed equilibrium between these two compounds may explain such a relation. Furthermore, this finding also supports the possibility that Hcy egress is not a function of intracellular Hcy, but rather the rate of formation of this compound.

Intracellular Hcy is reduced to trace amounts in malignant Cl 16 cells and is markedly reduced, albeit to a lesser degree, in Cl 8 cells, following c^3-Ado exposure (Fig. 2, C and D). This correlates with accumulation of higher amounts of c^3-AdoHcy in Cl 16 cells than in Cl 8 cells (Fig. 2, E–H.). The reduction of intracellular Hcy in these cells by 3-deazaaristeromycin was less pronounced, in spite of accumulation of large amounts of AdoHcy in the presence of this agent (unpublished data). These data are in accordance with the suggestion that trapping of Hcy as c^3-AdoHcy may be an efficient mechanism leading to depletion of cellular Hcy content.

Possible consequences of Hcy depletion. Lack of intracellular Hcy following c^3-Ado exposure may inhibit *de novo* methionine synthesis from Hcy and thereby cause starvation of cells for methionine. Notably, inhibition of methionine biosynthesis has recently been demonstrated in lymphoblasts exposed to purine nucleosides (22). Conceivably, profound inhibition of methionine biosynthesis may alter the disposition of exogenous methionine. This possiblity should be related to the finding that c^3-Ado inhibits the incorporation of radioactive sulfur of methionine into specific proteins (23).

Another detrimental effect of Hcy depletion, which has recently been pointed out by several workers (22, 24, 25), is inhibition of the conversion of 5-methyltetrahydrofolate to tetrahydrofolate. In this reaction Hcy serves as methyl acceptor, and lack of Hcy may trap reduced folates like 5-methyltetrahydrofolate.

Egress of c^3-AdoHcy. There are consistent reports that inhibition of AdoHcy catabolism leads to export of copious amounts of AdoHcy into the extracellular medium. This has been demonstrated for several cell types and may represent an important mechanism relieving the cells of the metabolic effects of AdoHcy (8).

In the present work we demonstrate that c^3-AdoHcy formed in the intracellular compartment is exported at a rate comparable to that of AdoHcy. In the liver cells, c^3-AdoHcy seems to be more efficiently exported than AdoHcy when the intracellular concentrations of c^3-AdoHcy and AdoHcy are similar (Fig. 1). In the fibroblasts, no obvious preference for either compound could be demonstrated (Fig. 2).

Export of c^3-AdoHcy has some implications. First, this process counteracts the intracellular effects of c^3-AdoHcy. Second, c^3-AdoHcy egress makes this compound available to the cell surface. Membrane acceptors interacting with c^3-AdoHcy have been demonstrated on the outer surface of the cell membrane (26), and these acceptors may mediate biological effects of exogenous c^3-AdoHcy and related compounds (27).

Different metabolic response to c^3-Ado among cell types. In hepatocytes, the accumulation of nucleosidyl-Hcy following c^3-Ado (300 μM) exposure (10 nmol/10^6 cells after 2.5 hr) greatly exceeds the inhibition of Hcy egress (2 nmol/10^6 cells) (Fig. 1), whereas in both non-transformed and malignant fibroblasts these parameters were of the same order of magnitude (4 (Cl 8 cells)–7 (Cl 16 cells) nmol/10^6 cells after 75 hr) (Fig. 2). This may be related to the unique features of Hcy and AdoHcy metabolism in liver cells. In the liver two separate enzymes are responsible for the conversion of Hcy to methionine (9). In addition, a single transmethylation reaction, the methylation of guanidoacetate, is responsible for the bulk of AdoHcy (28), and this reaction may be less sensitive than most transmethylation reactions in fibroblasts to the inhibitory effect of AdoHcy.

In hepatocytes, c^3-AdoHcy is the predominating nucleosidyl-Hcy whereas, in fibroblasts, more AdoHcy than c^3-AdoHcy is formed. Similar differences in metabolic response to c^3-Ado have been demonstrated between other tissues (5), and have been explained by different properties of AdoHcy hydrolase (29).

The view is prevailing that Hcy metabolism and the overall transmethylation rate are different in cancer cells and normal cells (10). The only obvious differences between Cl 16 cell and Cl 8 cells observed in the present study are greater accumulation of intra- and extracellular c^3-AdoHcy and a more pronounced depletion of Hcy in Cl 16 cells than in Cl 8 cells following c^3-Ado exposure. The biological significance of this observation remains to be defined.

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