MOLECULAR PHARMACOLOGY, 25:418–424

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# Effects of Adenosine Dialdehyde on S-Adenosylhomocysteine Hydrolase and S-Adenosylmethionine-Dependent Transmethylations in Mouse L929 Cells

RONNDA L. BARTEL AND RONALD T. BORCHARDT

*Department of Biochemistry, Smissman Research Laboratories, University of Kansas, Lawrence, Kansas 66045*

Received October 28, 1983; Accepted January 30, 1984

SUMMARY

Adenosine dialdehyde (2′-*O*-[(*R*)-formyl(adenin-9-yl)methyl]-(*R*)-glyceraldehyde), which is formed by periodate oxidation of adenosine, was shown to be a potent inhibitor of *S*-adenosylhomocysteine hydrolase (AdoHcy hydrolase; EC 3.3.1.1) in mouse L929 cells. The inhibitory effects of adenosine dialdehyde on AdoHcy hydrolase were time-dependent, having a rapid onset with complete inhibition occurring within a 15-min incubation period. When mouse L929 cells were preincubated with adenosine dialdehyde for 15 min, then transferred to fresh medium, the AdoHcy hydrolase activity returned to control values within 16 hr. When cycloheximide, an inhibitor of protein synthesis, was included in the incubation medium, recovery of AdoHcy hydrolase activity was not prevented, suggesting that the recovery of enzyme activity was probably due to slow reversal of the inhibitor-enzyme complex. The inhibition of AdoHcy hydrolase by adenosine dialdehyde resulted in a time-dependent increase in endogenous AdoHcy levels. After an initial 15-min lag time, the concentration of AdoHcy continued to increase, reaching a maximum of 1200 pmoles/mg of protein in 12 hr. A slight increase in AdoMet levels was observed. Incubation of mouse L929 cells with adenosine dialdehyde also caused an inhibition of lipid methylation and protein carboxylmethylation which resulted from the compound's effect on AdoHcy hydrolase and the subsequent buildup of endogenous AdoHcy levels. Under the conditions that produce inhibition of AdoHcy hydrolase and AdoMet-dependent methyltransferases, adenosine dialdehyde had no effect on RNA or DNA synthesis. Therefore, adenosine dialdehyde appears to be a useful chemical probe with which to study the physiological role of AdoMet-dependent methylations.

## INTRODUCTION

Biological transmethylations, which utilize AdoMet[1] as a methyl donor, are involved in a number of important physiological processes, including the metabolism of biogenic amines (1); modification of macromolecules, e.g., proteins, nucleic acids, and phospholipids (2); bacterial (3, 4) and leukocyte chemotaxis (5, 6); and stimulus-secretion coupling (7–11). A common feature of most methyltransferases is the inhibition produced by the demethylated product, AdoHcy. It has been suggested that the product inhibition by AdoHcy together with the enzyme AdoHcy hydrolase (EC 3.3.1.1), which metabolizes AdoHcy in eukaryotes, constitute a biological regulatory mechanism for cellular methylations (12, 13).

This research was supported by United States Public Health Service Research Grant GM-29332.

[1] The abbreviations used are: adenosine dialdehyde, 2′-*O*-[(*R*)-formyl(adenin-9-yl)methyl]-(*R*)-glyceraldehyde; AdoHcy, *S*-adenosyl-L-homocysteine; AdoHcy hydrolase, *S*-adenosyl-L-homocysteine hydrolase; AdoMet, *S*-adenosyl-L-methionine; EBSS, Earle's balanced salts solution; Ara-A, adenine arabinoside.

0026-895X/84/030418-07$02.00/0
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The reaction catalyzed by AdoHcy hydrolase is the reversible hydrolysis of AdoHcy to adenosine and homocysteine. Although the equilibrium of the reaction favors the synthesis of AdoHcy, the rapid metabolic removal of adenosine and homocysteine *in vivo* pulls the reaction in the hydrolytic direction (13). The catalytic mechanism of AdoHcy hydrolase proposed by Palmer and Abeles (14) involves the NAD-dependent oxidation of the 3′-hydroxyl group of AdoHcy (hydrolytic direction) or adenosine (synthetic direction), resulting in the generation of 3′-keto-AdoHcy, 3′-ketoadenosine, and 3′-keto-4′,5′-dehydroadenosine as enzymatically bound intermediates. Adenosine dialdehyde, formed by periodate oxidation of adenosine, is structurally similar to these enzyme-bound intermediates and subsequently was tested and shown to be a potent inhibitor of AdoHcy hydrolase both *in vitro* and *in vivo* (15–18).

Here, we report the inhibitory effects of adenosine dialdehyde on AdoHcy hydrolase in mouse L929 cells and the resulting effects on cellular AdoHcy and AdoMet levels and AdoMet-dependent transmethylations, e.g., lipid methylation and protein carboxylmethylation.



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## MATERIALS AND METHODS

*Sources of chemicals.* Standard chemicals and supplies were purchased from the following commercial suppliers: calf intestinal adenosine deaminase, SP-Sephadex C-25, adenosine, 3-*O*-methyl-D-glucose, phloretin, and cycloheximide (Sigma Chemical Company, St. Louis, Mo.); Waymouth's 752/1 low calcium medium (KC Biologicals, Kansas City, Mo.); bovine calf serum (Hazelton Dutchland Laboratories, Aberdeen, Md.); 3a70 scintillation cocktail and [$^3$H]leucine (140 Ci/mmole) (Research Products International, Mt. Prospect, Ill.); Zorbax C-8 reversed phase HPLC column (DuPont, Wilmington, Del.); GF/C filters (Whatman, Clinton, N.J.); NSC tissue solubilizer (Amersham, Arlington Heights, Ill.); [*methyl*-$^3$H]thymidine (70 Ci/mmole), [2,8-$^3$H] adenosine (30 Ci/mmole), and [*methyl*-$^3$H]3-*O*-methyl-D-glucose (15 Ci/mmole) (ICN, Cleveland, Ohio); and [$^3$H]uridine (37 Ci/mmole) and [*methyl*-$^3$H]methionine (18 Ci/mmole) (New England Nuclear Corporation, Boston, Mass.).

[2,8-$^3$H]AdoHcy (10–12 mCi/mmole) was prepared by conversion of [2,8-$^3$H]adenosine to [2,8-$^3$H]5′-chloro-5′-deoxyadenosine followed by its condensation with L-homocysteine in sodium and liquid ammonia (19). Adenosine dialdehyde was prepared by paraperiodic acid oxidation of adenosine (19).

*Cell culture.* Mouse L929 cells were grown in suspension culture at 37° in Waymouth's 752/1 modified medium with low calcium and supplemented with 5% bovine calf serum. For convenience, some experiments were carried out in monolayer cultures. Methylation activities, AdoMet and AdoHcy levels, and AdoHcy hydrolase activity did not differ between suspension and monolayer cultures.

*AdoHcy hydrolase assay.* Mouse L929 cells ($1 \times 10^7$) were lysed in 400 μl of cold hypotonic buffer (10 mM $Na_2HPO_4$, 10 mM NaCl, 1.5 mM $MgAc_2$, pH 7.6), and the cell debris was removed by centrifugation in an Eppendorf Microfuge (8000 × *g*, 1 min). AdoHcy hydrolase activity in the supernatant was determined by a modified procedure of Chiang *et al.* (20). In a total volume of 500 μl, the incubation mixture contained 150 mM sodium phosphate (pH 7.6), 1.0 mM EDTA, 40 μM [2,8-$^3$H]AdoHcy, and four units of intestinal adenosine deaminase. The reaction was started by the addition of 320 μl of cell supernatant and then incubated for 60 min at 37°. The reaction was stopped by the addition of 100 μl of 5 N formic acid. The reaction mixture and a 500-μl wash were poured onto a column (1.2 × 4.0 cm) of SP-Sephadex C-25 equilibrated in 0.1 N formic acid. The column was eluted with 8.0 ml of 0.1 N formic acid, and the eluent containing [2,8-$^3$H]inosine (the product of the hydrolysis of [2,8-$^3$H]AdoHcy and the subsequent deamination of [2,8-$^3$H]adenosine) was collected. An aliquot (1.0 ml) of the eluent was added to 10 ml of 3a70 scintillation cocktail, and the level of radioactivity was determined by liquid scintillation spectrometry.

*Determination of endogenous AdoHcy concentrations.* Monolayer cultures ($6 \times 10^6$ cells) were incubated in serum-free Waymouth's 752/1 low-calcium medium with or without adenosine dialdehyde. The cells were washed with cold EBSS (21) and harvested by trypsinization. The cells were lysed in 125 μl of 0.4 N $HClO_4$, and the cell debris was removed by centrifugation. The pellet was solublized in 0.5 N NaOH, and the protein concentration was determined by the method of Lowry *et al.* (22). The AdoMet and AdoHcy concentrations in the supernatant were quantitated by HPLC. A 100-μl aliquot was injected into a Perkin-Elmer Series 3 HPLC equipped with a Zorbax C-8 reversed-phase column (25 cm × 4.6 mm). A two-step gradient program was used to elute the samples at a flow rate of 1.0 ml/min. Solvent A: acetonitrile; Solvent B: 50 mM $NaH_2PO_4$/10 mM heptane sulfonic acid, pH 3.3; Solvent program: 5–20% A, 0–15 min; 20–40% A, 15–25 min. Absorbance at 254 nm was monitored, and the peak areas were used to calculate the concentration of AdoMet and AdoHcy.

The intracellular water volume was determined according to the procedure of Kletzien *et al.* (23) and was used to calculate the micromolar concentration of AdoHcy in L929 cells.

*Lipid methylation assay.* Using the modified procedure of Hirata *et al.* (24), mouse L929 cells ($3 \times 10^6$) were incubated with adenosine dialdehyde in serum-free medium for varying times. The medium was then replaced with serum-free medium containing [*methyl*-$^3$H]methionine (25 μCi/ml, 1.4 μM). After 60 min at 37°, the cells were washed with 2 ml of EBSS and with 1.0 ml of a solution containing 20% trichloroacetic acid and 40 mM methionine, pH 7.5. The samples were centrifuged at 18,000 x *g* for 20 min at 4°. Each pellet was extracted with a mixture of 3.0 ml of 2:1 chloroform/methanol (v/v) and 2.0 ml of 0.1 M KCl in 50% methanol, and vortexed for 2 min. The phases were separated by centrifugation. The aqueous phase was removed, and the extraction was repeated with an additional 2.0 ml of 0.1 M KCl in 50% methanol. An aliquot (1.0 ml) of the organic phase was evaporated and counted for radioactivity in 10 ml of 3a70 scintillation cocktail. Apolar and polar lipids were not separated by this extraction.

*Protein carboxylmethylation assay.* Mouse L929 cells ($3 \times 10^6$) were incubated with adenosine dialdehyde in serum-free medium. At different times after adenosine dialdehyde was added, the medium was replaced with serum-free medium containing [*methyl*-$^3$H]methionine (80 μCi/ml, 4.4 μM). After a 60-min incubation at 37°, protein carboxylmethylation was determined by a modification of Diliberto *et al.* (7). After the cell pellet was washed with cold EBSS, 50 μl of bovine serum albumin (20 mg/ml) were added to the pellet as carrier protein, and 2.0 ml of cold trichloroacetic acid (10%) was added to precipitate the proteins. After centrifugation, the protein methyl esters in the trichloroacetic acid pellet were hydrolyzed in 750 μl of sodium borate (1.0 M, pH 11) containing 1% methanol (v/v) for 20 min at room temperature. The [$^3$H]methanol generated upon hydrolysis was extracted with 3.0 ml of toluene/isoamyl alcohol (3:2, v/vol). Two aliquots (1 ml) were transferred to scintillation vials. One aliquot was counted directly in 10 ml of 3a70 scintillation cocktail. The second aliquot was evaporated to dryness in a vacuum oven at 80° and then counted for radioactivity. The difference in radioactivity between the evaporated and non-evaporated samples was taken as a measure of protein carboxylmethylation.

*Assay of nucleic acid synthesis.* Mouse L929 cells ($3 \times 10^6$) in 500 μl of EBSS were incubated in the presence of adenosine dialdehyde and [$^3$H]thymidine or [$^3$H]uridine to measure DNA or RNA synthesis, respectively. After 60 min at 37°, the cells were washed and then precipitated with 1 ml of cold 10% trichloroacetic acid. The precipitates were collected on glass-fiber filters (GF/C) in a Millipore filtration manifold. The filters were air-dried, solubilized with 0.5 ml of NCS tissue solubilizer, and counted for radioactivity in 10 ml of 3a70 scintillation cocktail.

*Toxicity assay.* Mouse L929 cells were incubated at a density of $1 \times 10^6$ cells/ml in 25-ml spinner flasks in Waymouth's 752/1 low-calcium medium and 5% calf serum in the presence or absence of 5 μM adenosine dialdehyde. After 72 hr, [$^3$H]thymidine was added to a 1-ml aliquot of the cell suspension to a final concentration of 1 μCi/ml. After 3 hr at 37°, the cells were washed and precipitated with 10% trichloroacetic acid. The pellets were collected and counted as above.

*Reversibility with Tris buffer.* Mouse L929 cells ($1 \times 10^8$) were incubated with or without adenosine dialdehyde in 5 ml of EBSS for 15 min at 37°. The cells were lysed in 4 ml of cold hypotonic buffer (10 mM $Na_2HPO_4$/10 mM NaCl/1.5 mM $MgAc_2$, pH 7.6). Aliquots were assayed for AdoHcy hydrolase activity, before and after treatment with Tris buffer (1.0 M Tris/1.0 mM EDTA/5 mM dithiothreitol as described earlier (17).

*Reactivation of AdoHcy hydrolase.* Mouse L929 cells ($1.25 \times 10^8$) were preincubated in 10 ml of serum-free Waymouth's 752/1 medium with or without 0.5 μM adenosine dialdehyde at 37° for 15 min. After washing the cells with EBSS, they were resuspended in 125 ml of medium containing 5% calf serum with or without cycloheximide (5 μg/ml) and maintained in suspension culture for 24 hr. At various times, duplicate 10-ml aliquots (~$10 \times 10^6$ cells) were removed and the cells were washed with EBSS and lysed in 400 μl of hypotonic buffer. The lysate was assayed for AdoHcy hydrolase activity, as described above, and the protein concentration was determined according to the method of Lowry *et al.* (22).

For the determination of protein synthesis, [$^3$H]leucine was added to duplicate 5-ml aliquots (~$5 \times 10^6$ cells) to a final concentration of 1

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μCi/ml and incubated for 60 min at 37°. The cells were washed in EBSS and precipitated with 1 ml of 10% cold trichloroacetic acid. The pellets were collected on GF/C filters in a Millipore manifold, and the radioactivity was determined as described above.

## RESULTS

When mouse L929 cells were incubated with adenosine dialdehyde, a concentration-dependent inhibition of AdoHcy hydrolase was observed (Fig. 1). Adenosine dialdehyde at concentrations of 1 μM or greater produced at least 95% inhibition of cellular AdoHcy hydrolase. When cells were incubated with 5 μM adenosine dialdehyde, greater than 80% inhibition of AdoHcy hydrolase activity was seen at zero time and greater than 95% inhibition at 2 min. Under these experimental conditions, the AdoHcy hydrolase was inhibited greater than 95% for up to 72 hr (Fig. 2). When lower concentrations of adenosine dialdehyde (1 μM) were employed, a time-dependent inhibition of AdoHcy hydrolase was observed (inset, Fig. 2).

The inhibition of AdoHcy hydrolase by adenosine dialdehyde (5 μM) resulted in a time-dependent increase in endogenous AdoHcy levels as shown in Fig. 3. A slight increase in AdoMet levels was also observed in the treated cells (data not shown). AdoHcy was also shown to accumulate in the medium of cells treated with 5 μM adenosine dialdehyde. At 72 hr after adenosine dialdehyde was added, AdoHcy reached 15 μM in the medium (74 nmoles/$10^6$ cells). No AdoMet was detected in the medium.

Incubation of mouse L929 cells with adenosine dialdehyde caused a concentration-dependent inhibition of lipid methylation and protein carboxylmethylation with maximal inhibition at 1 μM drug (data not shown). The maximal inhibition of lipid methylation and protein carboxylmethylation, however, was only partial, 50–60% and 30–40%, respectively. Table 1 shows that the inhibition of both methylation activities reached a maximum within the first 2 hr of adenosine dialdehyde treatment. The ratio of AdoHcy to AdoMet reached maximum at about 24 hr. Using cell lysates, adenosine dialdehyde was shown to have no direct inhibitory effects on the AdoMet-dependent lipid methyltransferases or protein carboxylmethyltransferase (data not shown). When exogenous AdoHcy is added to cell lysates, however, these methylations can be totally inhibited (data not shown).

When mouse L929 cells were treated with 5 μM adenosine dialdehyde and then fractionated by differential centrifugation, partial inhibition of lipid methylation and protein carboxylmethylation was observed in each of the subcellular fractions obtained (e.g., nuclei, mitochrondria, microsomes, and postmicrosomal supernatant), ruling out compartmentation as a reason for partial inhibition (data not shown). However, when exogenous AdoHcy and AdoMet was added to cell lysates at the concentrations observed in adenosine dialdehyde-treated cells, the same partial inhibition was seen (Table 2). Only when AdoHcy and AdoMet were added to the lysate at a ratio of 20:1 did the inhibition approach 100%.

To determine whether the effects of adenosine dialdehyde on AdoHcy hydrolase in mouse L929 cells are reversible, the lysate from cells that had been pretreated with adenosine dialdehyde were incubated with Tris buffer and then dialyzed. The results shown in Table 3 indicate that hydrolase activity could not be recovered under these experimental conditions. Treatment of the adenosine dialdehyde-inactivated bovine liver AdoHcy hydrolase under identical conditions resulted in partial recovery of hydrolase activity (18).

The inhibition of AdoHcy hydrolase caused by adenosine dialdehyde, however, can be reversed *in vivo* when cells are allowed to incubate over a 24-hr period. When mouse L929 cells treated with adenosine dialdehyde (0.5 μM) for 15 min and then transferred to fresh medium without inhibitor were incubated for 24 hr, the AdoHcy hydrolase activity returned to control values within 16 hr (Fig. 4). This recovery of the AdoHcy hydrolase activity might have been due to reversal of the enzyme-inhibitor complex or a result of synthesis of a new enzyme. To rule out the latter possibility, experiments similar to those described above were carried out in the presence of cycloheximide, an inhibitor of protein synthesis. Cycloheximide at a concentration of 5 μg/ml was shown to inhibit [$^3$H]leucine incorporation (>90%) without decreasing the cell number over a 24-hr incubation period. As shown in fig. 4, cycloheximide caused no loss in AdoHcy hydrolase in control samples, indicating that the enzyme has a very slow turnover rate. In adenosine dialdehyde-treated samples, cycloheximide did not prevent the recovery of enzyme activity, suggesting that the recovery of the enzyme activity was probably due to reversal of the enzyme-inhibitor complex.

Under the conditions which produce total inhibition

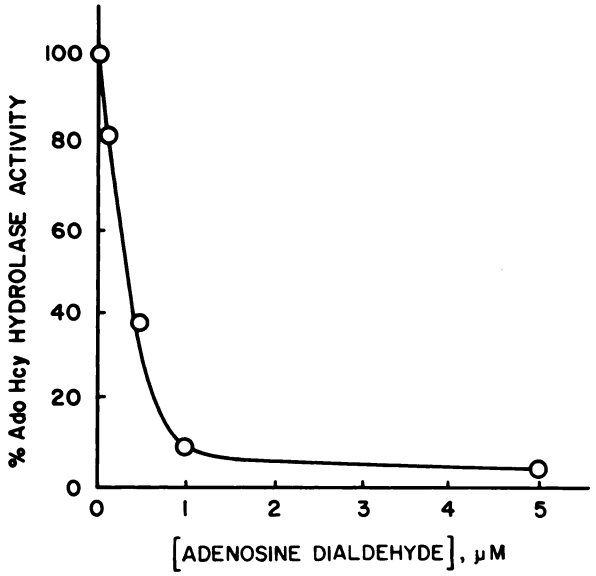


FIG. 1. *Inhibition of mouse L929 cell AdoHcy hydrolase by adenosine dialdehyde*

Mouse L929 cells were incubated for 15 min at 37° with adenosine dialdehyde in EBSS. The cells were washed with EBSS and then lysed in cold hypotonic buffer (10 mM $Na_2HPO_4$/10 mM NaCl/1.5 mM $MgAc_2$, pH 7.6). The cell debris was removed by centrigutation, and AdoHcy hydrolase activity was determined in the supernatant using [2,8-$^3$H] AdoHcy as the substrate as described under Materials and Methods. One hundred per cent AdoHcy hydrolase activity was 24,000 dpm/1 × $10^7$ cells. Each value represents four determinations.

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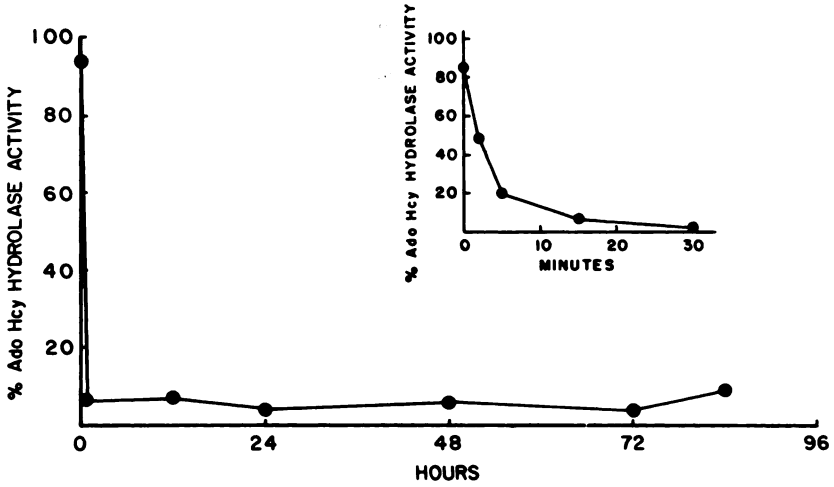


FIG. 2. *Time course of AdoHcy hydrolase inhibition by adenosine dialdehyde*

Monolayer cultures of mouse L929 cells ($1 \times 10^7$ cells) were incubated with 5 μM adenosine dialdehyde in serum-free Waymouth's 752/1 low-calcium medium for the indicated times. The monolayers were washed with EBSS and harvested by trypsinization. The cells were lysed and assayed for AdoHcy hydrolase activity as described under Materials and Methods. One hundred per cent AdoHcy hydrolase activity was 28,000 dpm/$1 \times 10^7$ cells. Each value represents four determinations.

Inset. Time dependency of the inhibition of AdoHcy hydrolase. Monolayer cultures were incubated with 1 μM adenosine dialdehyde and AdoHcy hydrolase activity was determined.

of AdoHcy hydrolase and partial inhibition of AdoMet-dependent methyltransferases, adenosine dialdehyde (1 μM) was shown to have no effect on RNA or DNA synthesis (data not shown). However, 5 μM adenosine dialdehyde did show slight cytostatic activity. During the first 48 hr of adenosine dialdehyde treatment (5 μM), the rate of cell growth was significantly decreased without any effect on cell viability (data not shown).

## DISCUSSION

In recent years, AdoMet-dependent methyltransferases have become important targets for the design of potential therapeutic agents (1, 2, 25). Two general approaches have been employed in the design of inhibitors of AdoMet-dependent methylations (2). One general approach aims to develop inhibitors that would function directly in a particular methyltransferase, e.g., analogues of the methyl-acceptor substrate, the methylated product, AdoMet or AdoHcy. The second approach aims to design inhibitors that would function indirectly by initially inhibiting AdoMet biosynthesis or AdoHcy metabolism. The AdoHcy metabolism approach has attracted considerable attention in recent years, in part because of the wealth of biochemical information (26) that has emerged concerning the properties of AdoHcy hydrolase, its mechanism of catalysis, and its role in AdoHcy metabolism.

On the basis of its structural similarities to the various

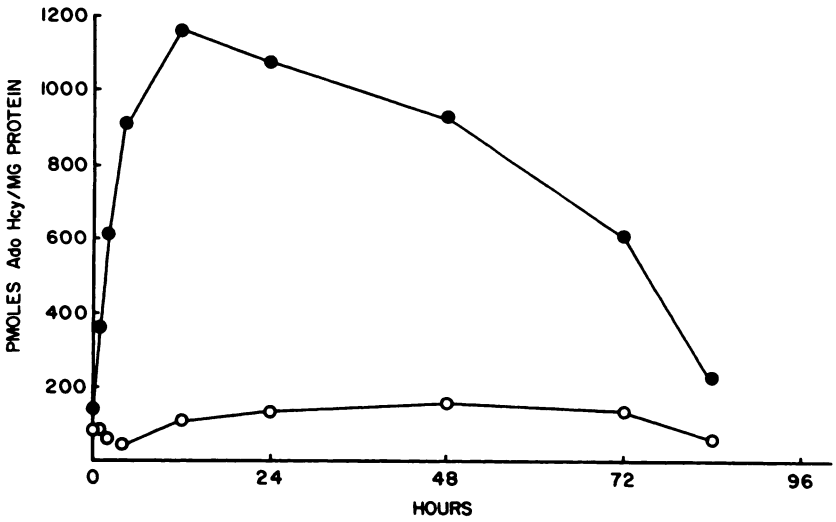


FIG. 3. *Time course of changes in endogenous AdoHcy concentrations after treatment of L929 cells with adenosine dialdehyde*

Monolayer cultures were incubated with (●) or without (○) 5 μM adenosine dialdehyde in serum-free medium. At various times, the cells were harvested and the protein and AdoHcy concentrations were quantitated as described under Materials and Methods. Each value represents four determinations.

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TABLE 1

*Inhibition of L929 cell lipid methylation and protein carboxylmethylation after treatment with adenosine dialdehyde*

Monolayer cultures were incubated with or without 5 μM adenosine dialdehyde at 37°. At the indicated times, the cells were washed with EBSS and labeled with [*methyl*-$^3$H]methionine for 60 min at 37°. Lipid methylation, protein carboxylmethylation, and AdoHcy and AdoMet levels were quantitated as described under Materials and Methods. Each value represents four determinations.

| Time | Control | | Adenosine dialdehyde-treated | | | |
|---|---|---|---|---|---|---|
| | AdoHcy | AdoHcy/AdoMet ratio | AdoHcy | AdoHcy/AdoMet ratio | % Inhibition[a] | |
| | | | | | Lipid methylation | Protein carboxylmethylation |
| *hr* | *μM* | | *μM* | | | |
| 0 | 4 | 0.14 | 9 | 0.28 | 17 | 15 |
| 1 | 9 | 0.14 | 44 | 0.53 | 47 | 37 |
| 2 | 8 | 0.10 | 74 | 0.71 | 58 | 34 |
| 4 | 6 | 0.07 | 109 | 0.83 | 56 | 32 |
| 24 | 13 | 0.13 | 108 | 1.09 | 53 | 25 |
| 48 | 10 | 0.11 | 86 | 0.94 | 56 | 13 |

[a] Values for 100% activity for lipid methylation and protein carboxylmethylation were 30,000 dpm/3 × $10^6$ cells and 5,000 dpm/3 × $10^6$ cells, respectively.

3′-keto nucleoside intermediates generated by AdoHcy hydrolase, Hoffman (15, 16) examined the inhibitory effects of adenosine dialdehyde toward this enzyme and found it to be a potent inhibitor *in vitro* and *in vivo*. More recently, our laboratory (17, 18) has studied the effects of a series of nucleoside dialdehydes on bovine liver AdoHcy hydrolase and elucidated the mechanism by which they produce inactivation of the enzyme *in vitro*. The nucleoside dialdehydes apparently inactivate bovine liver AdoHcy hydrolase by reacting with a crucial active-site amino acid residue, probably a lysine residue, forming an inhibitor-enzyme complex linked by a Schiff-base bond. Under appropriate experimental conditions *in vitro*, e.g., incubation with a nitrogenous base such as Tris, this complex can be dissociated with recovery of the enzyme activity. The inhibitor can only be irreversibly linked to the enzyme by exposure of the inhibitor-enzyme complex to reducing agents, e.g., sodium borohydride.

Consistent with Hoffman's results using adenosine dialdehyde in mouse liver (16), we found in this study that the compound is a potent inhibitor of AdoHcy hydrolase in mouse L929 cells. Adenosine dialdehyde at nanomolar concentrations causes a rapid and complete inactivation of AdoHcy hydrolase in mouse L929 cells. The inhibition of this AdoHcy metabolic pathway leads to a coincident increase in cellular AdoHcy levels and partial inhibition of AdoMet-dependent methylations, e.g., lipid methylations and protein carboxylmethylation. The increases in AdoHcy caused by adenosine dialdehyde follow a time course that is comparable to that produced by Ara-A, another inhibitor of AdoHcy hydrolase, in L929 cells (27, 28).

The inhibition of mouse L929 cellular AdoHcy hydrolase by adenosine dialdehyde cannot be reversed by treating the isolated inhibitor-enzyme complex *in vitro* with Tris buffer. This observation is in contrast to the adenosine dialdehyde-bovine liver AdoHcy hydrolase complex, which can be reversed by treatment with Tris,

TABLE 2

*Effect of AdoHcy/AdoMet ratio on lipid methylation and protein carboxylmethylation in vitro*

Mouse L929 cells (1 × $10^8$) were lysed in 2.0 ml of 10 mM $K_2HPO_4$, pH 7.3. Aliquots (0.1 ml) of the lysate were incubated with [*methyl*-$^3$H]AdoMet and AdoHcy for 60 min at 37°. Lipid methylation and protein carboxylmethylation were quantitated as described under Materials and Methods. Each value represents four determinations.

| AdoHcy | AdoMet | % Inhibition[a] | |
|---|---|---|---|
| | | Lipid methylation | Protein carboxylmethylation |
| *μM* | *μM* | | |
| 2 | 40 | 2 | 3 |
| 4 | 40 | 10 | 13 |
| 20 | 40 | 33 | 24 |
| 40 | 40 | 55 | 38 |
| 800 | 40 | 89 | 90 |

[a] Values for 100% activity for lipid methylation and protein carboxylmethylation were 3,500 dpm/l × $10^6$ cells and 3,300 dpm/l × $10^6$ cells, respectively.

TABLE 3

*Effect of Tris buffer and dialysis on adenosine dialdehyde-inactivated AdoHcy hydrolase from mouse L929 cells*

Mouse L929 cells were incubated with or without adenosine dialdehyde (0.5 μM) in EBSS at 37° for 15 min. The cells were washed with EBSS and then lysed in cold hypotonic buffer (10 mM $Na_2HPO_4$/10 mM NaCl/1.5 mM $MgAc_2$, pH 7.6). The cell debris was removed by centrifugation, and the supernatant was diluted with an equal volume of either 0.2 M Tris or 1.0 M Tris buffer. After incubation at 28° for 4 hr, the lysates were dialyzed against the same buffer at 4° for 12 hr. AdoHcy hydrolase activity was determined using [2,8-$^3$H]AdoHcy as described under Materials and Methods. Each value represents six determinations.

| Predialysis treatment | % AdoHcy hydrolase activity[a] | |
|---|---|---|
| | 0.2 M Tris | 1.0 M Tris |
| None | 21 | 44 |
| Tris buffer, 28°, 4 hr | 11 | 36 |
| Tris buffer, 28°, 4 hr, and dialysis, 4°, 12 hr | 8 | 37 |

[a] Values for 100% AdoHcy hydrolase activity was 115 pmoles of [$^3$H] inosine formed/min·mg of protein.

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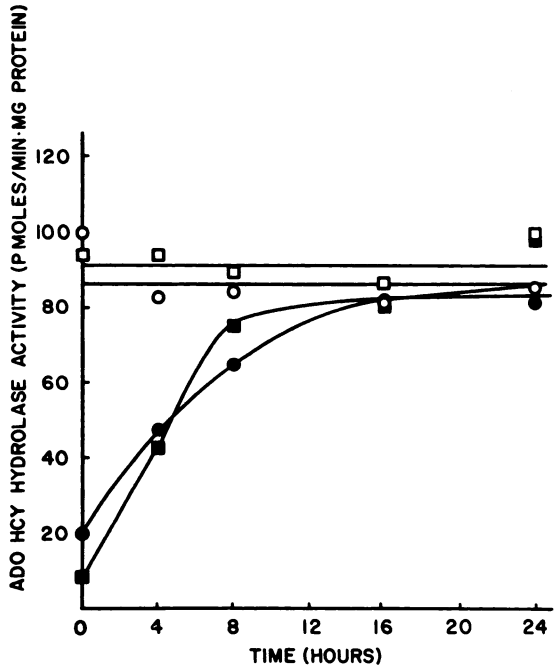


FIG. 4. *Reactivation of adenosine dialdehyde-inactivated AdoHcy hydrolase in mouse L929 cells*

Mouse L929 cells were preincubated with (●, ■) or without (○, □) adenosine dialdehyde (0.5 μM) in serum-free 752/1 medium for 15 min at 37°. The cells were then washed and resuspended in 125 ml of 5% calf serum medium ($1 \times 10^6$ cells/ml) with (■, □) or without (●, ○) cycloheximide (5 μg/ml). The cells were incubated for 24 hr at 37°. At the indicated times, duplicate aliquots (10 ml) were removed and the cells lysed were in hypotonic buffer. The supernatant was assayed for AdoHcy hydrolase activity, and protein concentration was determined as described under Materials and Methods. The level of protein synthesis was also determined by following [$^3$H]leucine incorporation as described under Materials and Methods. The 5 μg/ml concentration of cycloheximide caused more than 90% inhibition of [$^3$H]leucine incorporation without significant cell toxicity during the 24-hr duration of the experiment.

resulting in recovery of 80–90% of the enzyme activity (18). At this time, we are unable to explain the different responses of the inhibitor-bovine liver AdoHcy hydrolase complex and the inhibitor-mouse L929 cell AdoHcy hydrolase complex to Tris buffer. This different behavior may reflect structural differences in the two enzymes and therefore differences in the nature of the inhibitor-enzyme complexes.

It was interesting, however, that if mouse L929 cells were pretreated with adenosine dialdehyde for 15 min, then incubated in inhibitor-free medium for 24 hr, the AdoHcy hydrolase activity returned to control values with 16 hr. This *in vivo* regeneration of enzyme activity is apparently not due to synthesis of new enzyme, since the rate of recovery of AdoHcy hydrolase activity is the same in the presence or absence of the protein synthesis inhibitor cycloheximide. In fact, in control cultures treated only with cycloheximide, no changes in AdoHcy hydrolase activity were observed, suggesting that the enzyme turnover is very slow in mouse L929 cells. The mechanism by which the inhibitor-AdoHcy hydrolase complex is reversed *in vivo* is unknown, but most likely it involves simple dissociation of the ligand from the enzyme. A similar phenomenon may be responsible for reactivation of Ara-A-inactivated AdoHcy hydrolase, because Helland and Ueland (27) found that regeneration of the AdoHcy hydrolase activity occurs only when extracellular adenosine deaminase is added to hepatocytes or L929 cells.

Another interesting observation was that, even at concentrations of adenosine dialdehyde that produced maximal inhibition of AdoHcy hydrolase activity, we could only obtain partial inhibition of AdoMet-dependent methylations. It appears that a significant portion (40–60%) of the mouse L929 cell lipid methylation and protein carboxylmethylation is resistant to the drastic increases in cellular AdoHcy (up to 100 μM). Even though the AdoHcy concentration in adenosine dialdehyde-treated cells is 1 order of magnitude greater than the reported $K_i$ values for the lipid methyltransferases and protein carboxylmethyltransferase (29), the AdoMet concentration remains high enough to allow methylation to continue. This fact may make adenosine dialdehyde useful in studying the inhibition of methyltransferases with low $K_i$ values for AdoHcy, such as mRNA guanine 7-methyltransferase (29).

Consistent with an earlier report where Ara-A was used to inhibit AdoHcy hydrolase (28), mouse L929 cells begin to release copious quantities of AdoHcy into the extracellular medium after prolonged treatment with adenosine dialdehyde. This may represent an alternative cellular mechanism to relieve the accumulation of AdoHcy caused by blocking AdoHcy hydrolase activity. Helland and Ueland (28) have examined the effects of Ara-A on mouse L929 cells. Both Ara-A and adenosine dialdehyde show similar effects on AdoHcy hydrolase and AdoHcy accumulation. However, Ara-A is also metabolized by adenosine kinase and adenosine deaminase, so that 100-fold higher concentrations must be used to produce the same effects as adenosine dialdehyde on AdoHcy metabolism. Other inhibitors, such as 3-deazaadenosine, are substrates as well as inhibitors of AdoHcy hydrolase.

Although adenosine dialdehyde has a drastic effect on AdoHcy metabolism in mouse L929 cells, at concentrations of 1 μM or less it has no effect on AdoMet synthesis, nucleic acid synthesis, or cell viability. Therefore, adenosine dialdehyde may be a useful probe with which to study the physiological role of AdoMet-dependent methylation reactions (30).

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**Send reprint requests to:** Dr. Ronald T. Borchardt, Department of Pharmaceutical Chemistry, Room 3006 Malott, University of Kansas, Lawrence, Kan. 66045.

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