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The Effect of Aliphatic Adenine Analogues on *S*-Adenosylhomocysteine and *S*-Adenosylhomocysteine Hydrolase in Intact Rat Hepatocytes

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SUMMARY

The aliphatic adenine analogues, D-eritadenine, L-eritadenine, L-threoeritadenine, and 9-(*S*)-(2,3-dihydroxypropyl)adenine [(*S*)DHPA] function as inhibitors/inactivators of purified *S*-adenosylhomocysteine (AdoHcy) hydrolase, but these compounds did not induce reduction of enzyme-bound NAD^+. D-Eritadenine, L-eritadenine, (*S*)DHPA, and L-*threo*-eritadenine inactivated AdoHcy hydrolase in hepatocytes, and the efficiency decreased in the order mentioned. Concurrently, there was an increase in the AdoHcy content. The accumulation of AdoHcy in the presence of (*S*)DHPA was more pronounced than would be expected from the inactivation of enzyme activity, suggesting that this compound may function as a reversible inhibitor as well. Furthermore, the inactivation of the intracellular enzyme by (*S*)DHPA is remarkable in the light of the fact that this compound induces no inactivation of purified AdoHcy hydrolase, but merely functions as an inhibitor of the enzyme. At low concentration of D-eritadenine (<6 μM), a distinct lag period could be demonstrated before accumulation of AdoHcy occurred. This suggests that the AdoHcy hydrolase activity must be decreased below a certain level to cause an increase in cellular AdoHcy. None of the analogues tested completely inactivated AdoHcy hydrolase and a residual enzyme activity was observed. The adenosine deaminase inhibitor, 2′-deoxycoformycin, did not potentiate the effect of these compounds on AdoHcy catabolism. The inactive enzyme formed in the presence of aliphatic adenine analogues was not reactivated under conditions where the inactivation induced by 9-β-D-arabinofuranosyladenine was reversible.

INTRODUCTION

AdoHcy[3] hydrolase (EC 3.3.1.1), the enzyme responsible for the metabolic degradation of the endogenous transmethylase inhibitor, AdoHcy, is a potential target enzyme for numerous adenosine analogues (1, 2). These compounds may serve either as an inhibitor, inactivator, or substrate for the enzyme, and thereby induce a massive accumulation of intracellular AdoHcy and/or the formation of *S*-nucleosidylhomocysteine, which in turn inhibit several transmethylation reactions (2).

The interaction of ara-A with AdoHcy hydrolase has been studied in detail. ara-A is an active site-directed agent inducing irreversible inactivation of AdoHcy hydrolase and a concurrent reduction of the enzyme bound NAD^+ (3, 4). ara-A also functions as an inactivator of AdoHcy hydrolase *in vivo*, and induces a massive accumulation of AdoHcy (5–7). In contrast, some of the most potent inactivators of isolated AdoHcy hydrolase yet discovered, as for example carbocyclic adenosine and 2-chloroadenosine (8), exert only a minimal effect on AdoHcy catabolism in intact cells.[4] This may be related to cellular handling of these adenosine analogues.[4]

Periodate-oxidized adenosine is a potent inhibitor of isolated AdoHcy hydrolase (9, 10) as well as AdoHcy catabolism in intact cells (10) and whole animals (11). The efficiency of this compound has been related to its structure, which resembles that of a postulated transient intermediate in the AdoHcy hydrolase reaction (9). Notably, both periodate-oxidized adenosine and the aliphatic adenine analogues investigated in the present study are acyclic adenosine derivatives modified in the sugar moiety.

Votruba, Holy, and coworkers have recently demon-

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[3] The abbreviations used are: AdoHcy, *S*-adenosylhomocysteine; ara-A, 9-β-D-arabinofuranosyladenine; (*S*)DHPA, 9-(*S*)-(2,3-dihydroxypropyl)adenine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; dCF, 2′-deoxycoformycin.

[4] J-S. Schanche, T. Schanche, P. M. Ueland, and J. Montgomery, submitted for publication.

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strated that several aliphatic adenine analogues, the so-called eritadenines, are potent inhibitors of isolated AdoHcy hydrolase (12–15) and inhibitors of virus replication in host cells (16). To evaluate whether the biological effects of the eritadenines are mediated by inhibition of AdoHcy hydrolase, we investigated the effect of four aliphatic adenine derivatives, namely D-eritadenine, L-eritadenine, L-*threo*-eritadenine, and (*S*)DHPA (Table 1), on AdoHcy hydrolase and AdoHcy catabolism in intact cells.

MATERIALS AND METHODS

Chemicals. AdoHcy, DL-homocysteine, adenine, ara-A, Hepes, collagenase (type I), and adenosine deaminase (type I from calf intestinal mucosa) were obtained from Sigma Chemical Co., St. Louis, MO. dCF was a gift from Parke-Davis Research Laboratories, Ann Arbor, MI. The aliphatic adenine analogues, i.e., D-eritadenine, L-eritadenine, L-*threo*-eritadenine, and (*S*)DHPA, were synthesized according to published procedures (12, 13). [8-^{14}C]Adenosine (0.59 Ci/mmol) was from the Radiochemical Centre, Amersham, England.

Assay for AdoHcy hydrolase. The enzyme activity was determined by a radiochemical method described previously (17).

Inactivation of purified AdoHcy hydrolase. The rate of inactivation of purified mouse liver AdoHcy hydrolase by the aliphatic adenine analogues was determined by preincubation of the homogeneous enzyme (17) (30 μg/ml) in the presence of these compounds for various periods of time. The incubation buffer was 75 mM potassium phosphate buffer, pH 7.0, containing 2 mM dithiothreitol, and the temperature was 37°. Aliquots of 5 μl were transferred to the assay mixture (100 μl) containing 10 μM [8-^{14}C]adenosine and 3 mM DL-homocysteine.

Test for formation of adenine and reduction of enzyme-bound NAD$^+$. Possible formation of adenine from ara-A and the aliphatic adenine analogues in the presence of purified AdoHcy hydrolase was investigated by incubation of these compounds (5 μM) with high level of the enzyme (1.8 mg/ml) in 150 mM potassium phosphate buffer, pH 7.0, containing 2 mM dithiothreitol. The temperature was 37°. Adenine was quantified by HPLC on a cation exchange column (Partisil 10 SCX) eluted isocratically with 90 mM ammonium formate, pH 3.5, containing 1% isopropanol.

Reduction of enzyme-bound NAD$^+$ in the presence of ara-A and aliphatic adenine analogues was determined spectrophotometrically, by recording the increase in absorbance at 330 nm, resulting from the conversion of NAD$^+$ to NADH. The difference spectrum between 220 and 450 nm was recorded after the reaction had come to a halt. The same buffer was used as above, and the temperature was 20°. Details have been given elsewhere (4).

Preparation and incubation of isolated rat hepatocytes. The isolated hepatocytes were prepared by a collagenase perfusion method and were incubated in an isotonic salt solution containing Hepes buffer, pH 7.4, bovine serum albumin, glucose, salts, and antibiotics. The temperature was 37° (5).

Extraction of AdoHcy hydrolase from intact cells. The hepatocytes were incubated in the presence of potential inactivators of AdoHcy hydrolase.

Samples (250 μl) from the cell suspension were centrifuged through oil in precooled polyethylene tubes, and the pellet immediately was homogenized in ice-cold phosphate buffer containing DL-homocysteine (5). The homogenate was then frozen in liquid nitrogen.

Processing of the cellular extract. The extracts were either subjected to gel filtration on a Sephadex G-25 column (0.5 × 10 cm) or treated with dextran-coated charcoal for 20 min at 0–4°. This was carried out to remove purines (including aliphatic adenine analogues) existing free in solution or reversibly bound to AdoHcy hydrolase.

Determination of cellular content of AdoHcy and AdoMet. The hepatocytes were separated from the medium by centrifugation; the pellet was homogenized in 0.8 N perchloric acid. The acid-insoluble proteins were removed by centrifugation and the neutralized supernatant was subjected to HPLC on either a cation exchange or reversed-phase column, as described previously (5).

RESULTS

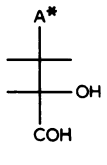
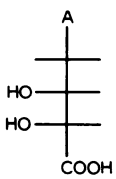
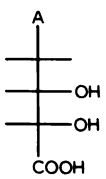
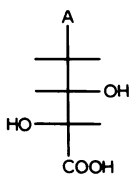
Interaction of aliphatic adenine analogues with purified mouse liver AdoHcy hydrolase. In accordance with published data on purified AdoHcy hydrolase from rat liver (13), mouse leukemia (14), or tobacco tissue cells (15) we observed that D-eritadenine was an extremely potent inactivator of AdoHcy hydrolase from mouse liver and was far more effective than L-eritadenine (Fig. 1) and ara-A (5; data not shown) in this respect. The initial rate constant of the inactivation could not be determined at high concentrations (0.15 μM) of D-eritadenine, but was higher than 1 min^{-1}. (*S*)DHPA (50 μM) did not induce a time-dependent decrease in enzyme activity (Fig. 1).

Adenine was not liberated from the aliphatic adenine analogues when these compounds (5 μM) were incubated with a high level (1.8 mg/ml) of mouse liver AdoHcy hydrolase. Furthermore, these compounds did not induce reduction of enzyme-bound NAD$^+$ as judged by difference spectrophotometry. Both adenine formation and NAD$^+$ reduction were observed in the presence of ara-A in parallel experiments (data not shown).

Inactivation of AdoHcy hydrolase in intact liver cells. D-Eritadenine rapidly inactivated AdoHcy hydrolase in rat hepatocytes, and this compound was by far the most potent inactivator among the aliphatic adenine analogues tested (Fig. 2).

TABLE 1

Aliphatic adenine analogues tested and their structural formulae

Structural formulae:				
Name:	(S)-Dihydroxy propyladenine	D-Eritadenine	L-Eritadenine	L-Threoerit-adenine

*A = Adenine

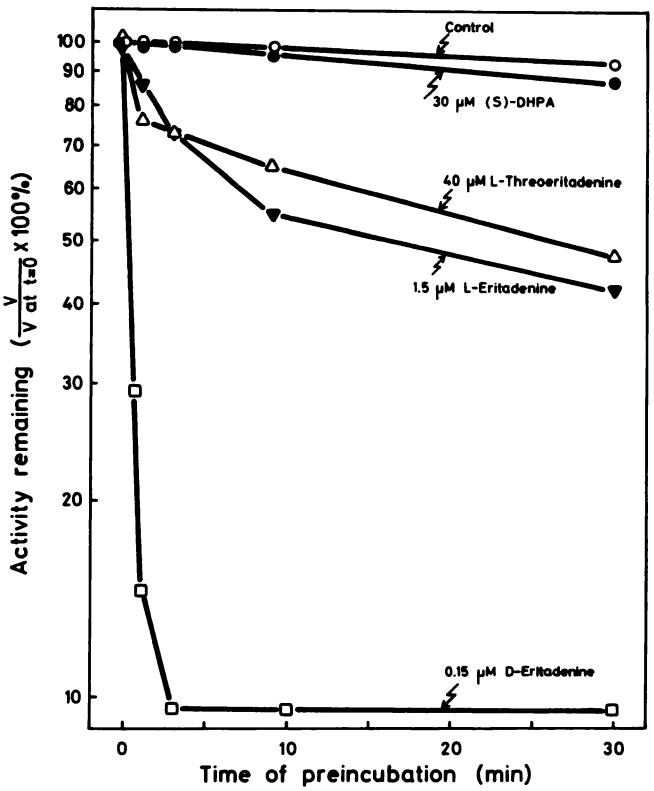


FIG. 1. *Inactivation of homogeneous mouse liver AdoHcy hydrolase in the presence of some aliphatic adenine analogues*

Purified liver AdoHcy hydrolase was preincubated with 0.15 μM D-eritadenine, 1.5 μM L-eritadenine, 30 μM (*S*)DHPA, or 40 μM L-*threo*-eritadenine. The temperature was 37°.

L-Eritadenine, (*S*)DHPA, and L-*threo*-eritadenine also induced a time-dependent decrease in AdoHcy hydrolase activity in the rat liver cells, and the efficiency decreased in the order mentioned (Fig. 2). The enzyme activity was not recovered by gel filtration of the extract or treatment of the extract with charcoal.

AdoHcy hydrolase was not completely inactivated even after prolonged incubation of the liver cells in the presence of D-eritadenine and the other adenine derivatives tested in this work. Thus, a residual enyme activity (2–5%) was observed (Fig. 2) as previously reported for cells and tissues exposed to ara-A (5–7).

The rate and extent of inactivation of AdoHcy hydrolase in liver cells in the presence of the aliphatic adenine analogues were not affected by adding the adenosine deaminase inhibitor, 2′-deoxycoformycin to the cell suspension (data not shown).

Cellular content of AdoHcy and AdoMet. A dose-dependent increase in AdoHcy content in hepatocytes was observed in the presence of D-eritadenine, L-eritadenine, and (*S*)DHPA, and the potency decreased in the order mentioned. L-*threo*-eritadenine induced only a slight increase in AdoHcy content. (*S*)DHPA was far more potent than would be expected from the degree of inactivation of AdoHcy hydrolase induced by this compound. This may be attributed to the fact that (*S*)DHPA functions mainly as reversible inhibitor (12) of intracellular AdoHcy hydrolase.

There was a lag period before accumulation of AdoHcy occurred in cells exposed to D-eritadenine, and this was especially pronounced at low concentrations of the compound (Fig. 2). 2′-Deoxycoformycin did not enhance the effect of aliphatic adenine analogues on AdoHcy content in hepatocytes (data not shown).

Accumulation of AdoHcy in cells exposed to aliphatic adenine analogues was associated with AdoHcy egress into the extracellular medium (5; data not shown).

The amount of AdoMet increased up to 2-fold in cells treated with aliphatic adenine analogues, and a correlation between elevation of AdoMet and accumulation of AdoHcy was observed (data not shown).

Test for reactivation of AdoHcy hydrolase in rat hepatocytes. We have previously shown that AdoHcy hydrolase activity gradually recovered when liver cells exposed to ara-A were transferred to a fresh medium containing adenosine deaminase (18). Enhancement of the reactivation by extracellular adenosine deaminase may either be related to degradation of the inactivator, ara-A, or an endogenous substrate for adenosine deaminase, i.e. adenosine (18). Because the aliphatic adenine analogues tested in the present study are not substrates for adenosine deaminase (13, 19, 20), test for reactivation of inactive enzyme formed after treatment with hepatocytes with these compounds may serve to differentiate between these two possibilities.

The liver cells were preincubated for 30 min with D-eritadenine, (*S*)DHPA, L-eritadenine, or ara-A, centrifuged and then resuspended in fresh medium containing adenosine deaminase. The enzyme activity in cells treated with ara-A gradually recovered, whereas no increase in activity was observed in cells exposed to the aliphatic adenine analogues (Fig. 3). In fact, a further decrease in enzyme activity was observed after cells treated with D-eritadenine were transferred to a fresh medium. The inactivation proceeded at an almost constant rate for at least 4 hr, and there was a concurrent accumulation of AdoHcy (Fig. 3). In these cells, the profound inactivation of AdoHcy hydrolase probably caused accumulation of AdoHcy in amounts which exceeded that exported into the extracellular medium. In contrast, in cells exposed to L-eritadenine or (*S*)DHPA, there was a decrease in the amount of AdoHcy after the cells were transferred to a fresh medium (Fig. 3). This suggests that larger amounts of AdoHcy are exported from and degraded by these cells than the amount formed as a product of transmethylation reactions.

DISCUSSION

Introductory experiments were carried out to characterize the interaction of the aliphatic adenine analogues with homogeneous AdoHcy hydrolase purified from mouse liver (Fig. 1). We were able to confirm the data obtained with AdoHcy hydrolase isolated from various sources (12–15), including rat liver (12, 13), showing that D-eritadenine, L-eritadenine, and L-*threo*-eritadenine induce a time-dependent inactivation of the enzyme with decreasing efficiency in the order mentioned. (*S*)DHPA was not an inactivator (Fig. 1). The latter compound functions as a competitive inhibitor of the rat liver enzyme (12). These data indicate that the aliphatic ad-

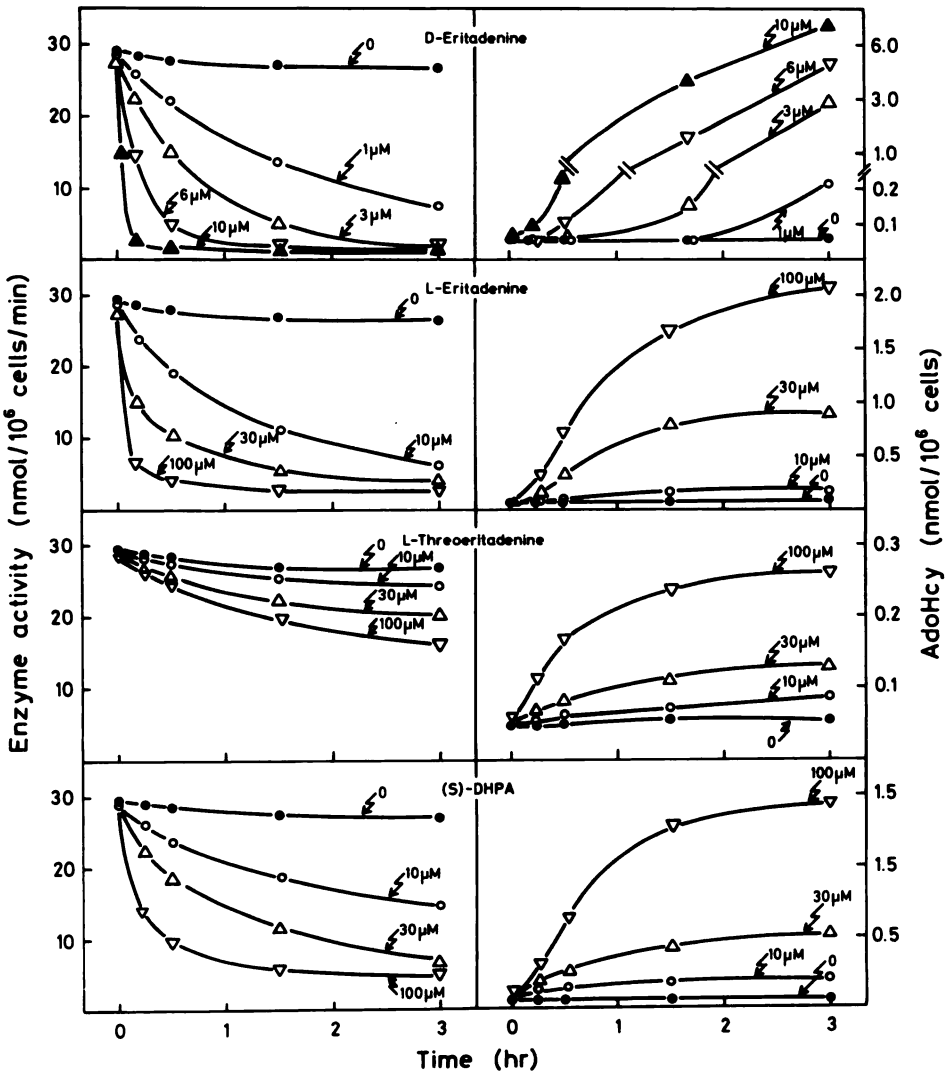


FIG. 2. *Inactivation of AdoHcy hydrolase and accumulation of AdoHcy in hepatocytes exposed to aliphatic adenine analogues*

Rat hepatocytes (6 × 10^6 cells/ml) were incubated with increasing concentrations of D-eritadenine, L-eritadenine, L-*threo*-eritadenine, or (*S*)DHPA. The panels to the left show the rate of inactivation of the enzyme, whereas the panels to the right show the cellular content of AdoHcy. Values represent mean for duplicate determinations. A typical experiment is shown. The experiment was repeated five times.

enine analogues exert a similar effect on AdoHcy hydrolase from different sources.

Active site-directed inactivation of AdoHcy hydrolase by ara-A as well as other nucleosides is often associated with reduction of enzyme-bound NAD^+ and liberation of the purine base from the nucleoside (3, 4, 21). The aliphatic adenine analogues tested in the present work did not induce a reduction of NAD^+ tightly bound to AdoHcy hydrolase, and liberation of adenine from these compounds was not observed. Nevertheless, D-eritadenine is an extremely potent inactivator of AdoHcy hydrolase both *in vitro* (13) (Fig. 1) and *in vivo* (Fig. 2) and is probably one of the most effective inhibitors of AdoHcy catabolism in intact cells yet discovered. (*S*)DHPA and L-eritadenine also induced a massive accumulation of AdoHcy in liver cells. These data show that irreversible reduction of NAD^+ tightly bound to AdoHcy hydrolase is not a prerequisite for effectiveness in intact liver cells.

The reservation should be made that lack of NAD^+ reduction and adenine formation has been demonstrated only with enzyme purified from mouse liver. Other adenosine analogues interact differently with AdoHcy hydrolase from different mammalian species (22, 23). It is therefore possible that the inactivation or inhibition of the enzyme from sources other than mouse liver by aliphatic adenine analogues may be associated with reduction of NAD^+ and/or conversion of the analogue to adenine.

The finding that (*S*)DHPA induces an irreversible inactivation of intracellular AdoHcy hydrolase (Fig. 2) is remarkable in the light of the fact that (*S*)DHPA functions as a reversible inhibitor of the isolated enzyme (12, 13) (Fig. 1). The inactivation of AdoHcy hydrolase observed with intact cells is not an artifact caused by cellular accumulation of AdoHcy, because no inactivation of AdoHcy hydrolase was observed with cells exposed to the potent AdoHcy hydrolase inhibitor, 3-deazaaristeromycin.[4] The latter observation also suggests that the inactivation of intracellular AdoHcy hydrolase by (*S*)DHPA is not related to inhibition of metabolic

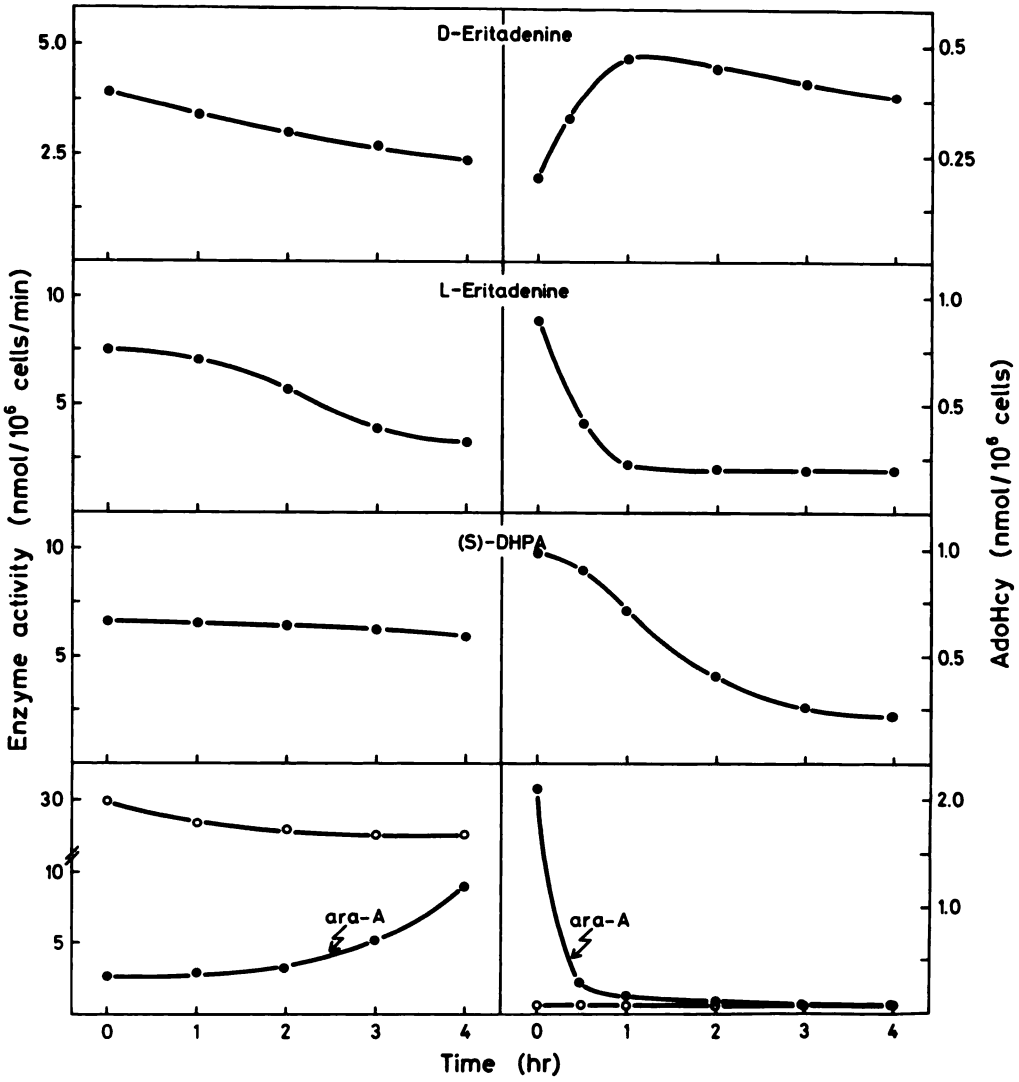


FIG. 3. *Test for reactivation of AdoHcy hydrolase in hepatocytes exposed to aliphatic adenine analogues*

Rat hepatocytes (6×10^6 cells/ml) were incubated with 10 μM D-eritadenine, 100 μM L-eritadenine, 30 μM (*S*)DHPA, and 30 μM ara-A for 3 min, and then transferred to a fresh medium containing adenosine deaminase (16 units/ml). The panels to the left show the AdoHcy hydrolase activity in the liver cells, and the right panels show the amount of AdoHcy in these cells. ○, control; ●, treated cells. Values represent mean for duplicate determinations. A typical experiment is shown. The experiment was repeated four times.

flux through the AdoHcy hydrolase pathway. Furthermore, (*S*)DHPA did not increase the amount of adenosine in hepatocytes,[5] showing that the AdoHcy hydrolase inactivation was not induced by this nucleoside.

The disparity between the effect of (*S*)DHPA on isolated AdoHcy hydrolase (12, 13) (Fig. 1) and on the enzyme in intact cells (Fig. 2) could be explained by modification of the enzyme during isolation or alterations in the microenvironments of the enzyme, resulting in changes in some factor(s) critical for the interaction of the enzyme with nucleoside analogues.

The inactivation of AdoHcy hydrolase in hepatocytes exposed to low concentrations of D-eritadenine lasted for several hours (Figs. 2 and 3). This indicates that D-eritadenine is not consumed within the duration of the experiments. Furthermore, there was no accumulation of AdoHcy before the enzyme activity was reduced to a small fraction of the activity present in nontreated liver cells. Then an abrupt increase in AdoHcy content was observed (Fig. 2). Similar observations have been made previously (6, 24). For example, treatment of mice with 2′-deoxycoformycin markedly reduced AdoHcy hydrolase activity in several tissues, but induced essentially no increase in AdoHcy content (24). Thus, the AdoHcy hydrolase activity *in vivo* probably exceeds that required to handle the amount of AdoHcy offered by the transmethylation reactions.

The AdoHcy hydrolase activity in hepatocytes exposed to D-eritadenine and other aliphatic adenine analogues was not completely inactivated (Fig. 2). A residual enzyme activity was also found in liver cells and several tissues exposed to ara-A (5–7) and lymphocytes incubated with 2′-deoxyadenosine (25). This phenomenon may be explained by metabolic consumption of the inactivator followed by reactivation of the enzyme. Alter-

[5] J. S. Schanche and P. M. Veland, unpublished results.

natively, the residual enzyme activity may be related to protection of intracellular AdoHcy hydrolase by metabolites (5, 25). The results obtained with (*S*)DHPA and D-eritadenine, which are neither deaminated nor phosphorylated (13, 19, 20), indicate that a small portion of intracellular AdoHcy hydrolase is secluded from the action of adenosine analogues.

The present paper shows that D-eritadenine, (*S*)DHPA, and L-eritadenine are inhibitors of intracellular AdoHcy hydrolase and block the metabolic degradation of AdoHcy (Figs. 2 and 3). The conclusion that a particular compound is an inhibitor of cellular AdoHcy catabolism must be founded on experiments with intact cells and/or whole animals, since some nucleoside analogues, including 2-chloroadenosine and aristeromycin (8), are extremely effective inhibitors of the isolated enzyme, but exert only a small effect on AdoHcy catabolism in intact cells (26).[4] This suggests that some inhibitors of isolated AdoHcy hydrolase do not reach the intracellular target enzyme, either because of rapid metabolism or inefficient transport. In addition, cellular factors may prevent inactivation/inhibition of AdoHcy hydrolase. It has been demonstrated that inactivation of the isolated enzyme in the presence of some purines (27, 28) is affected by the presence of inorganic phosphate. Furthermore, adenine nucleotides, which induce an irreversible inhibition of the enzyme *in vitro* (18, 27), are probably not effective under the conditions existing in the cell.

The inactivation of AdoHcy hydrolase induced by exposing intact cells to ara-A (18) or 2′-deoxyadenosine (25) is reversible. In several tissues of mice and liver of rats treated with ara-A, reactivation of AdoHcy hydrolase resulted in total recovery of the enzyme activity within 10 hr (6, 7). However, no reactivation was observed with cells exposed to D-eritadenine and other aliphatic adenine analogues (Fig. 3). In fact, the inactivation process continued even after cells exposed to low level of D-eritadenine were transferred to a fresh medium (Fig. 3). This observation may be explained by the metabolic stability of the aliphatic adenine analogues (13, 19, 20). Lack of reactivation may contribute to the effectiveness of these compounds *in vivo*.

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