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Inactivation and Reactivation of Intracellular S-Adenosylhomocysteinase in the Presence of Nucleoside Analogues in Rat Hepatocytes[1]

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ABSTRACT

Several nucleoside analogues, some of which are potent inactivators of isolated *S*-adenosylhomocysteinase (AdoHcyase), were tested with respect to their effect on intracellular AdoHcyase and *S*-adenosylhomocysteine (AdoHcy) catabolism in intact rat hepatocytes. Among the analogues tested, 9-β-D-arabinofuranosyladenine and 9-β-D-arabinofuranosyl-3-deazaadenine were the most potent inactivators of intracellular AdoHcyase. Compounds like 2-chloroadenosine and carbocyclic adenosine are extremely efficient inactivators of the isolated enzyme, but these nucleosides exerted only a limited effect on the enzyme in intact hepatocytes. Only a moderate effect was observed with 2-chloro-3-deazaadenosine and 2′-deoxyadenosine, and a high concentration of 5′-deoxy-5′-methylthioadenosine was required to inactivate the enzyme. There was a correlation between inactivation and accumulation of AdoHcy. Carbocyclic-3-deazaadenosine caused no inactivation of AdoHcyase in liver cells but caused a massive accumulation of AdoHcy, suggesting that this analogue functions as a reversible inhibitor of the enzyme. Residual enzyme activity was observed with all the nucleoside analogues tested. The intracellular enzyme inactivated in the presence of 2′-deoxyadenosine and 9-β-D-arabinofuranosyladenine was readily recovered when the cells were transferred to fresh medium containing adenosine deaminase, but reactivation of the enzyme activity after treatment of the cells with 2-chloroadenosine and 5′-deoxy-5′-methylthioadenosine was also observed. The inactivation of intracellular enzyme induced by 2-chloro-3-deazaadenosine, 9-β-D-arabinofuranosyl-3-deazaadenine, and carbocyclic adenosine was essentially irreversible under the conditions of the experiments. Factors determining the effect of nucleoside analogues on intracellular AdoHcyase are discussed.

INTRODUCTION

Several nucleoside analogues have oncostatic and antiviral properties (33). The enzyme AdoHcyase,[3] which is responsible for the metabolic degradation of the endogenous transmethylase inhibitor AdoHcy, may be an important target enzyme for some of these compounds (6, 17). A nucleoside analogue may serve as either a substrate, an inhibitor, or an inactivator (irreversible inhibitor) of AdoHcyase (34). This has been demonstrated for purified enzyme and for the enzyme in intact cells (34).

Inactivation of purified AdoHcyase was first demonstrated with ara-A and 2′-Ado by Hershfield (17, 19). Several other adenosine analogues, including 2-Cl-Ado, C-Ado (1, 7), and adenosine dialdehyde (5), also inactivate isolated AdoHcyase. The kinetics of the inactivation suggest that these compounds function as suicide substrates of the enzyme (5, 7). These nucleoside analogues, except adenosine dialdehyde (5), induce an irreversible inhibition of AdoHcyase *in vitro* (7, 21, 34).

ara-A is a potent inactivator of AdoHcyase both *in vitro* (12, 17, 20) and *in vivo* (9, 13, 15, 16). AdoHcyase in whole cells and tissues exposed to ara-A gradually recovers, even in the presence of an inhibitor of protein synthesis. This suggests that the intracellular ara-A-enzyme complex is reactivated (9, 14, 16).

In the present paper, the following points are addressed. (*a*) To what extent do inactivators of isolated AdoHcyase block AdoHcy catabolism in intact cells? (*b*) Is the inactivation of AdoHcyase induced by nucleoside analogues other than ara-A reversible in the intact cell?

MATERIALS AND METHODS

Chemicals. AdoHcy, DL-homocysteine, adenosine, 2′-dAdo, 2-Cl-Ado, MTA, ara-A, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, collagenase (type 1), 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; 2-Cl-c^3-Ado, c^3-ara-A, C-Ado, and C-c^3-Ado were synthesized by methods described previously (26, 28, 31). [8-^{14}C]Adenosine (0.59 ci/mmol) was purchased from the Radiochemical Centre, Amersham, England.

Preparation of Isolated Rat Hepatocytes. Hepatocytes were prepared by a slight modification (29) of the collagenase perfusion method described by Berry and Friend (4). Cell viability, determined by the dye exclusion test (29), was higher than 90% at the start of the experiments.

Incubation Conditions. The hepatocytes were incubated in an isotonic salt solution, consisting of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.4, bovine serum albumin, glucose, salts, and antibiotics, as described in a previous publication (15). The temperature was 37°.

Extraction of AdoHcyase from Intact Cells. The hepatocytes were incubated in the presence of potential inactivators of AdoHcyase, and were separated from the medium by centrifugation of 250-μl aliquots through oil. The pellet was immediately resuspended and homogenized in ice-cold buffer containing homocysteine. Under these conditions, no further inactivation of the enzyme takes place after cellular disrupture (15).

Assay for AdoHcyase Activity in Cell Extracts. To remove nucleosides which might inhibit AdoHcyase in the extract from liver cells, 2 procedures were used.

1. Samples of 150 μl were immediately applied to Sephadex G-25 columns (0.5 x 10 cm) equilibrated with 100 mM potassium phosphate buffer, pH 7.0, and the protein fraction, which appeared in the void volume, was assayed for AdoHcyase activity as described elsewhere (16, 36).

2. The cellular extracts were supplemented with dextran-coated charcoal (Norit, 5 mg/ml; and dextran 0.5 mg/ml), and incubated for 20 min

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[3] The abbreviations used are: AdoHcyase, *S*-adenosylhomocysteine hydrolase, EC 3.3.1.1; AdoHcy, *S*-adenosylhomocysteine; dCF, 2′-deoxycoformycin; ara-A, 9-β-D-arabinofuranosyladenine; c^3-ara-A, 9-β-D-arabinofuranosyl-3-deazaadenine; 2-Cl-Ado, 2-chloroadenosine; 2-Cl-c^3-Ado, 2-chloro-3-deazaadenosine; C-Ado, carbocyclic adenosine; C-c^3-Ado, carbocyclic 3-deazaadenosine; 2′-dAdo, 2′-deoxyadenosine; MTA, 5′-deoxy-5′-methylthioadenosine.

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at 0–4°. The charcoal was then removed by centrifugation, and the supernatant assayed for AdoHcyase activity.

Neither gel filtration nor treatment with charcoal affected the AdoHcyase activity in cells not exposed to adenosine analogues (data not shown).

Determination of Cellular Content of AdoHcy and AdoMet. AdoHcy and AdoMet in perchloric acid extract were determined by high-pressure liquid chromatography on a Partisil 10 SCX column. AdoHcy was also determined by reversed-phase liquid chromatography on a 3-μm ODS Hypersil column. Details have been given in a previous publication (16).

Determination of Cellular Content of ara-A and Carbocyclic Adenosine. The amount of ara-A or carbocyclic adenosine associated with the hepatocytes was determined in cells exposed to these analogues. To avoid interference from nucleosides present in the extracellular medium, the cells were separated from the medium by centrifugation through oil. The oil and the medium were removed by suction, and the pellet homogenized in 0.8 N perchloric acid, which was neutralized to pH 7.0 (15, 16).

The neutralized extracts were analyzed by reverse-phase liquid chromatography, by a slight modification of an isocratic system described earlier (16). To separate C-Ado and ara-A from adenosine and other UV-absorbing material, the column was eluted with 100 mM ammonium formate, pH 3.5:15 mM potassium acetate, pH 4.5:14% methanol, 12.5:15.5:36.

RESULTS

Removal of Nucleoside Analogues from the Cellular Extract. The inactivation of intracellular AdoHcyase in the presence of nucleoside analogues was determined by measuring the enzyme activity in the cellular extract. Because most inactivators of AdoHcyase also function as competitive inhibitors of the enzyme (7, 34), the extracts from cells treated with adenosine analogues were subjected to gel filtration or treated with charcoal to remove compounds which reversibly inhibit the enzyme. The enzyme activity in extracts so treated increased by less than 40%. Gel filtration and adsorption to charcoal gave identical results (data not shown).

Inactivation of AdoHcyase. AdoHcyase in rat hepatocytes was inactivated in a time- and dose-dependent manner when either ara-A, c^3-ara-A, 2-Cl-c^3-Ado, 2-Cl-Ado, 2′-dAdo, C-Ado, or MTA was added to the cell suspension. ara-A and c^3-ara-A were most potent in this respect (Chart 1). The inactivation did not proceed to completion, and residual enzyme activity was observed at all concentrations of these nucleoside analogues. No inactivation of the enzyme was observed in cells exposed to C-c^3-Ado (Chart 1). The adenosine deaminase inhibitor, dCF (2) (1 μM) did not increase the rate or extent of the inactivation process significantly in the presence of these analogues (data not shown).

Cellular Content of AdoHcy and AdoMet. The amount of AdoHcy increased markedly in cells exposed to ara-A and c^3-ara-A. 2-Cl-c^3-Ado, 2′-dAdo, and MTA showed a response characterized by an increase in AdoHcy, and then a decrease was observed. In the presence of 2-Cl-Ado, a transient increase was followed by a marked decline to amounts below that found in nontreated cells. C-Ado induced a moderate increase in the amount of AdoHcy, whereas a massive accumulation of AdoHcy was observed in cells exposed to C-c^3-Ado (Chart 1). dCF did not or did only slightly enhance the effect of the nucleoside analogues on the AdoHcy content in isolated hepatocytes (data not shown).

The amount of AdoMet increased about 2-fold in cells exposed to nucleoside analogues blocking AdoHcy catabolism, and a correlation between elevation of AdoHcy and AdoMet was observed. In cells treated with 2-Cl-Ado and 2-Cl-c^3-Ado, there was an increase in AdoMet followed by a marked decline (data not shown).

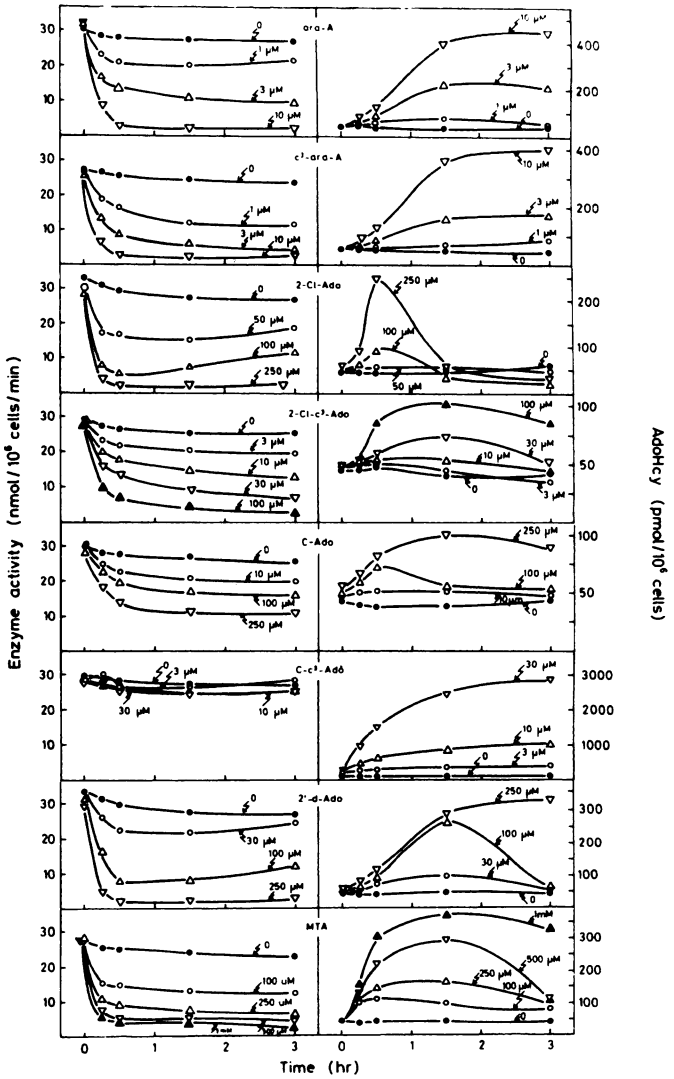

Chart 1. Inactivation of AdoHcyase and accumulation of AdoHcy in hepatocytes exposed to various nucleoside analogues. Hepatocytes (6 × 10^6 cells/ml) were incubated in the presence of increasing concentrations of various nucleoside analogues. *Left*, rate of inactivation of the intracellular enzyme; *right*, accumulation of AdoHcy in the hepatocytes under the same conditions. A typical experiment is shown.

Cellular Content of ara-A and C-Ado. Hepatocytes were incubated with either ara-A (30 μM) or C-Ado (30 μM), and the amounts of these analogues associated with the cells and present in the medium were determined at various time points (Chart 2). The ara-A content of the hepatocytes increased rapidly to about 350 pmol/10^6 cells and then declined. In contrast, the C-Ado content reached only a low level (about 80 pmol/10^6 cells) and remained constant for the time of the experiment (3 hr) (Chart 2).

The amount of adenosine in the hepatocytes was not affected by treatment of the cells with ara-A or C-Ado (data not shown).

Recovery of AdoHcyase Activity in Intact Hepatocytes. We have shown previously that the AdoHcyase activity in hepato-

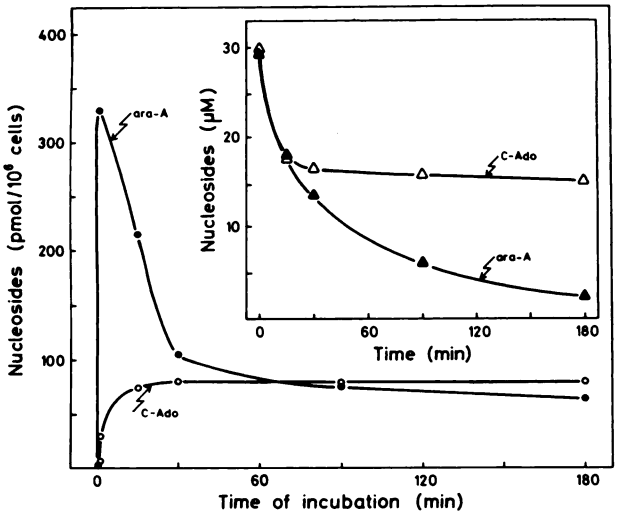

Chart 2. Association of ara-A and carbocyclic adenosine with hepatocytes. Hepatocytes (6×10^6 cells/ml) were incubated for increasing period of time with ara-A or carbocyclic adenosine, at initial 30 μM concentrations. The amount of ara-A (●) or carbocyclic adenosine (○) associated with the cells is shown. *Inset*, amount of these compounds remaining in the extracellular medium. A typical experiment is shown.

cytes and some cultured cells exposed to ara-A gradually recovers when the cells were transferred to a medium containing adenosine deaminase (14). In the present work, hepatocytes were incubated for 30 min in the presence of various nucleoside analogues, washed, and then resuspended in a medium supplement with adenosine deaminase. The recovery of enzyme activity was extensive in the cells treated with the naturally occurring nucleoside, 2′-deoxyadenosine (8-fold increase during 4 hr), and was somewhat lower for cells treated with ara-A (4-fold increase). The increase in AdoHcyase activity of cells exposed to 2-Cl-Ado and MTA was relatively low (2- to 3-fold increase), whereas no reactivation was observed with cells treated with 2-Cl-c³-Ado, C-Ado, and c³-ara-A. With c³-ara-A and C-Ado, the inactivation continued even after the cells were transferred to a fresh medium (Chart 3).

Cellular Content of AdoHcy. The amount of AdoHcy decreased rapidly during recovery of the AdoHcyase activity in cells exposed to 2′-dAdo, ara-A, and 2-Cl-Ado. In cells treated with c³-ara-A, 2-Cl-c³-Ado, C-Ado, and MTA, the decrease in cellular AdoHcy was less pronounced (Chart 3). Export of AdoHcy (15) was observed with cells containing high levels of AdoHcy (data not shown), and this obviously contributed to the decrease in cellular AdoHcy content (Chart 3).

The AdoHcy content of the cells at the start of the reactivation experiment (Chart 3) did not exactly match the amount of AdoHcy in the cells exposed to inhibitors, as shown in Chart 1. This is related to a somewhat different AdoHcy response from one preparation of hepatocytes to another (up to 4-fold variation in AdoHcy content in cells treated in the same way). Furthermore, the rapid decline in the amount of AdoHcy in cells exposed to some analogues immediately after transfer to a fresh medium makes the assessment of AdoHcy content at the start of the reactivation difficult.

DISCUSSION

Most nucleoside analogues investigated in the present work are irreversible inactivators of AdoHcyase (7, 19, 20, 34), and C-

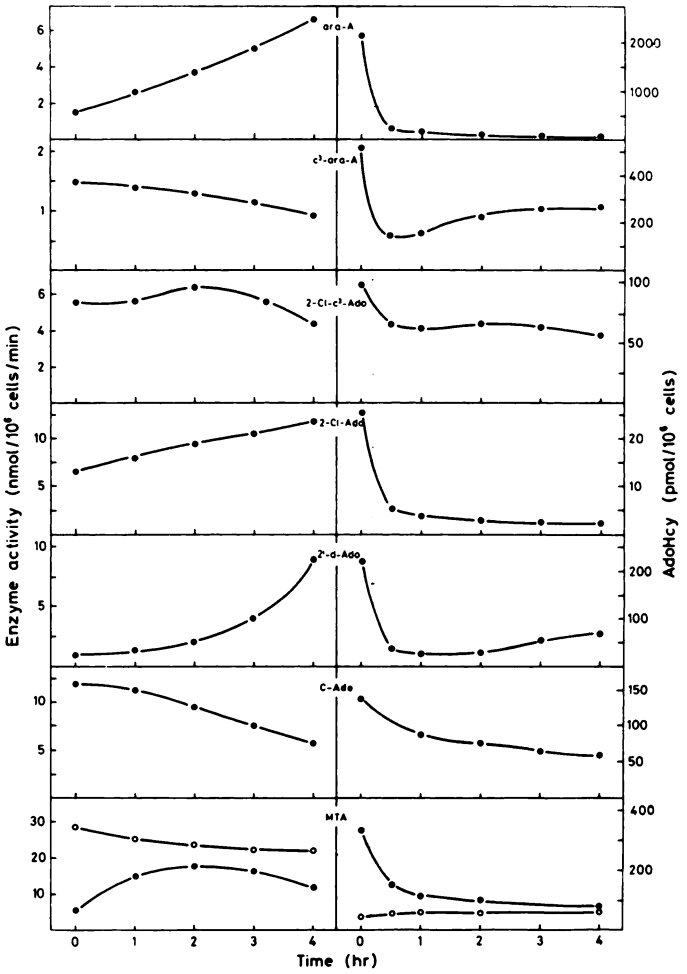

Chart 3. Recovery of AdoHcyase activity and AdoHcy content in cells treated with various nucleoside analogues. Hepatocytes (6×10^6 cells/ml) were incubated for 30 min with one of the following compounds: ara-A (30 μM), c³-ara-A (30 μM), 2-Cl-c³-Ado (100 μM), 2-Cl-Ado (100 μM), 2′-dAdo (500 μM), C-Ado (200 μM), MTA (1 mM), or no inactivator (○, *bottom*). The cells were then centrifuged and resuspended in a fresh medium containing adenosine deaminase (16 units/ml), described in the text. *Left*, AdoHcyase activity; *right*, amount of AdoHcy associated with the cells. A typical experiment is shown.

Ado and 2-Cl-Ado are the most potent inactivators of purified enzyme yet discovered (7). One general conclusion that can be derived from our data is that potent inactivators of isolated AdoHcyase do not always inactivate intracellular AdoHcyase or induce massive accumulation of AdoHcy.

Most compounds tested here inactivate isolated AdoHcyase according to first-order kinetics, suggesting that the inactivation proceeds from a reversible nucleoside-enzyme complex. Furthermore, the enzyme is protected against inactivation in the presence of adenosine or AdoHcy (7, 19, 34). These nucleosides are active site-directed agents, and may be classified as type A inactivators, according to Chiang *et al.* (7).

The mechanism of inactivation of the enzyme by certain nucleosides has been studied. ara-A inactivates purified AdoHcyase according to a mechanism involving irreversible reduction of enzyme-bound NAD^+ (13, 20), but other molecular events may take place as well (20). *In vitro* inactivation of AdoHcyase according to first-order kinetics, but not involving NAD^+ reduction, has been demonstrated with 3′-deoxyadenosine (6, 7, 20). In contrast to ara-A, 3′-deoxyadenosine seems to be an inefficient

inactivator of AdoHcyase in the intact cell (11). However, the fact that the inactivation of the isolated enzyme in the presence of C-Ado is associated with NAD^+ reduction (1) shows that a mechanism involving this process does not ensure effectiveness *in vivo*.

Interactions of nucleoside analogues with AdoHcyase may be counteracted by rapid degradation of these compounds. Adenosine deaminase inhibitors, like dCF (2), potentiate the long-term effects of ara-A and related compounds on AdoHcy catabolism in whole animals (9, 16), but dCF does not enhance the effect of several nucleoside analogues on AdoHcy catabolism in isolated hepatocytes (this paper, data not shown). This finding suggests that deamination of the nucleoside analogues (22, 24) tested did not limit their effect on AdoHcy metabolism in these cells. Besides, some analogues, including c^3-ara-A,[4] 2-Cl-c^3-Ado,[4] and MTA (30) are not deaminated.

The amount of intracellular nucleoside available to AdoHcyase may also be reduced by efficient phosphorylation to the corresponding nucleotides. To determine whether C-Ado gains access to AdoHcyase or is ineffective in the intracellular compartment, the amount of this analogue taken up by the cells was determined and compared with the uptake of ara-A (Chart 2). The amount of C-Ado associated with the liver cells was low relative to the ara-A content in these cells under the same conditions (Chart 2). This may account for the low effectiveness of C-Ado in the intact cell (Chart 1). Extensive phosphorylation of this analogue (3) may explain the low concentration of this nucleoside in the hepatocytes (Chart 2).

The adenosine analogue, 2-Cl-Ado, is taken up by intact cells to the same extent as adenosine (32). Furthermore, 2-Cl-Ado is a poor substrate of adenosine kinase, and 2-Cl-c^3-Ado is probably not phosphorylated [since c^3-Ado itself is not (25)]. Thus, the relatively small effect on AdoHcyase and AdoHcy metabolism is observed in the presence of 2-Cl-Ado (38) and 2-Cl-c^3-Ado (Chart 1) cannot be explained by slow uptake or rapid metabolism of these compounds.

2-Cl-Ado shows a response, characterized by a transient increase in AdoHcy, followed by a decrease to levels below that found in nontreated cells (Chart 1). The low cellular content of AdoHcy and AdoMet observed after prolonged treatment with high concentrations of 2-Cl-Ado may be related to perturbation of AdoMet and AdoHcy formation, or to cell damage (23) causing leakage of cellular constituents.

MTA, a product of polyamine biosynthesis (37), is a mediocre inactivator of purified AdoHcyase from various sources (10, 19, 37). AdoHcyase inactivation has been assigned a possible role in the mechanism of action of this naturally occurring nucleoside (10). However, high concentrations of exogenous MTA were required to block the AdoHcy catabolism in isolated hepatocytes (Chart 1). This finding does not support the possibility that biological effects (37) of MTA are mediated by AdoHcyase.

C-c^3-Ado induced a massive accumulation of AdoHcy in rat hepatocytes, but no inactivation of AdoHcyase in the cell (Chart 1). This finding is in accordance with the finding that C-c^3-Ado is an extremely potent competitive inhibitor of isolated AdoHcyase (27). No inactivation of the intracellular enzyme in the presence of this nucleoside suggests that the inactivation observed with other nucleoside analogues (Chart 1) is not caused by increase in cellular AdoHcy or inhibition of metabolic flux of endogenous AdoHcy through the AdoHcyase pathway.

AdoHcyase inactivated by ara-A in the intact cell (15) and in several tissues in whole animals (9, 16) gradually recovers even in the presence of an inhibitor of protein synthesis. The enzyme activity reaches almost pretreatment levels within 10 hr (16). This finding has been explained by an intracellular mechanism reactivating AdoHcyase. The so-called reactivation process is enhanced by supplementing the extracellular medium with adenosine deaminase, and inhibited by the adenosine deaminase inhibitor, dCF (14, 16). In the present paper, it is shown that the AdoHcyase activity also recovers in hepatocytes exposed to inactivators other than ara-A (Chart 3). It is noteworthy that the degree of recovery was particularly high in hepatocytes exposed to the naturally occurring nucleoside, 2′-dAdo (Chart 3).

The AdoHcyase activity in hepatocytes treated with 2-Cl-Ado and MTA recovered to a certain extent (Chart 3). This finding argues against the possibility (14) that deamination of the nucleoside analogue is essential for the reactivation process, because neither 2-Cl-Ado (24) nor MTA (30) is a substrate of adenosine deaminase. However, metabolic degradation of MTA by MTA phosphorylase (30) may lead to a rapid decrease of the amount of intracellular MTA.

The inactivation of intracellular AdoHcyase induced by c^3-ara-A, 2-Cl-c^3-Ado, and C-Ado was not reversible under the conditions of the experiments. This may be explained by the fact that the processes underlying the reactivation cannot handle the complexes between these analogues and the enzyme. Alternatively, metabolic stability of a nucleoside analogue (*i.e.*, c^3-ara-A[4]), or a slow release of the nucleoside (C-Ado) from a metabolic precursor, the corresponding nucleotide, may cause a continuous trapping of newly reactivated enzyme.

Cellular factors may alter the behavior of AdoHcyase in the presence of nucleoside analogues. It has been shown that inorganic phosphate affects the response of the purified enzyme to purines like adenine, adenine nucleotides (35), ara-A, and inosine (18). Furthermore, the inactive enzyme formed in the presence of adenosine dialdehyde is reactivated *in vitro* upon dialysis against Tris buffer, but not phosphate buffer (5). Further experiments with purified AdoHcyase should be carried out to identify possible cellular factors modulating the response of the enzyme to nucleoside analogues. Such data might give insight into the mechanism(s) behind the reactivation process.

Recent reports have demonstrated that mammalian AdoHcyase has 2 classes of adenosine binding sites residing on nonequivalent pairs of subunits (1, 8). Two sites participate in the catalytic cycle, whereas the other 2 sites do not, but bind adenosine and 2′-deoxyadenosine (1) and may function as allosteric sites *in vivo*. Nucleoside analogues may interact with the catalytic sites, or with the allosteric sites, or both. Therefore, it is conceivable that rather complex molecular events, including site-to-site interactions, may underly the inactivation as well as the reactivation of intracellular AdoHcyase.

In conclusion, the ability of adenosine analogues to inactivate AdoHcyase and thereby induce the elevation of AdoHcy in intact cells have been related to several other biological properties of these compounds, including the function of these nucleosides as inactivators of isolated AdoHcyase, and as substrates of adenosine-metabolizing enzymes. Some of these data are summarized in Table 1 and show that a poor correlation exists between the ability of nucleoside analogues to inactivate Ado-

[4] J. A. Montgomery and P. W. Allen, unpublished results.

Table 1

Summary of some biological properties of adenosine analogues

Compound	Inactivation of AdoHcyase[a]		Reactivation of cellular Ado-Hcyase	Substrate for		Cellular content of	
	Isolated	Cellular		Adenosine deaminase	Adenosine kinase	AdoHcy[b]	AdoMet
ara-A	Potent	2	Extensive	Good	Poor[c]	400 (10)[d]	↑
c^3-ara-A	Weak	1	No	NS[e]	NS[e]	400 (10)	↑
2-Cl-c^3-Ado	Potent	10	No	NS[e]	NS[e]	100 (100)	↑↓
2-Cl-Ado	Potent	75	Moderate	Poor	Poor	250 (250)[f]	↑↓
2′-dAdo	Fair	80	Extensive	Good	Poor[c]	320 (250)	↑
C-Ado	Potent[g]	100[g]	No	Fair	Good	100 (250)	↑
C-c^3-Ado	No[f]	No[f]		NS[e]	NS[e]	850 (10)	↑
MTA	Fair	100	Moderate	NS[e]	NS[e]	350 (1000)	↑

[a] Approximate concentration (μM) for 50% inactivation at 3 hr.
[b] pmol/10^6 cells at 1.5 hr.
[c] Fair for deoxycytidine kinase.
[d] Numbers in parentheses, concentration of inhibitor (μM).
[e] NS, not a substrate.
[f] At time 0.5 hr.
[g] Reversible inhibitor.

Hcyase in cell-free systems and in the intact cell. Furthermore, neither the inactivation nor the reactivation of the intracellular enzyme shows a strict correlation with the function of the analogues as substrates for adenosine deaminase and adenosine kinase. Therefore, identifying a particular nucleoside analogue as an inactivator of AdoHcyase should be based on *in vivo* experiments.

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