10 min. This observation indicated that the concentrations of AT-T and AT*-T do not change significantly during the determination. This is consistent with the very low values obtained for k_d^T(app), which indicate that the total complex concentration would decrease by much less than 1% during assay, an amount which would not be kinetically significant. Further, if deacylation is rate limiting, one of two conditions must apply. Either k_d is approximately equivalent to k_d' or k_{acyl} is small such that the conversion of AT-T to AT*-T is not large enough to significantly alter the value of k_d^T(app). In other words, if one of these conditions were not met, k_d^T-(app) would vary as the concentration of AT*-T increased due to acylation.

The experimental results which are summarized in Table I suggested that acylation is rate limiting for the release of thrombin via deacylation. For this reason, k_d^T(app) was converted to k_d^T throughout the text by dividing k_d^T(app) by the concentration of AT-T (eq A5). The value of k_d^T at any pH will therefore be the sum of k_d and k_{acyl} as shown in eq A7:

$$k_d^T = k_d + k_{acyl} \quad \text{(A7)}$$

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Adenosine Analogues as Substrates and Inhibitors of *S*-Adenosylhomocysteine Hydrolase†

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ABSTRACT: In the reaction adenosine + L-homocysteine ⇌ *S*-adenosyl-L-homocysteine, catalyzed by *S*-adenosylhomocysteine hydrolase from beef liver (EC 3.3.1.1), 11 nucleosides are able to substitute for adenosine to generate their corresponding *S*-nucleosidylhomocysteine congeners: 3-deazaadenosine, 2-aza-3-deazaadenosine, nebularine (purine ribonucleoside), formycin, N^6-methyladenosine, 8-azaadenosine, adenosine N^1-oxide, pyrazomycin, 8-aminoadenosine, inosine, and the carbocyclic analogue of adenosine [(±)-aristeromycin]. *S*-Adenosylhomocysteine hydrolase from lupin seeds is able to utilize all of these nucleosides except inosine to synthesize analogues of *S*-adenosylhomocysteine. There is no correlation between the ability of these nucleotides to function as substrates and their inhibitory potencies, except in the case of 3-deazaadenosine. The carbocyclic analogue of adenosine is the most potent inhibitor of *S*-adenosylhomocysteine hydrolase with a K_i of 5×10^{-9} M. When incubated with 3T3-L1 fibroblasts, the carbocyclic analogue of adenosine caused a 10-fold increase in the cellular concentration of *S*-adenosylhomocysteine. The cellular generation of *S*-2-aza-3-deazaadenosylhomocysteine was observed when 3T3-L1 fibroblasts were incubated with 2-aza-3-deazaadenosine.

S-Adenosylhomocysteine (AdoHcy)[1] is a product in all of the biological methylations in which *S*-adenosylmethionine (AdoMet) serves as the methyl donor. AdoHcy hydrolase (EC 3.3.1.1) catalyzes the hydrolysis of AdoHcy in eukaryotes in a reversible reaction first described by de la Haba & Cantoni (1959).

$$\text{AdoHcy} + H_2O \rightleftharpoons \text{Ado} + \text{Hcy}$$

The equilibrium of the reaction favors the synthesis of AdoHcy, but physiologically the reaction proceeds in the hydrolytic direction because Ado and Hcy are efficiently removed by further metabolism (Cantoni & Chiang, 1980). In mammalian tissues, Ado can be deaminated to inosine by Ado deaminase (EC 3.5.4.4) or be phosphorylated by Ado kinase (EC 2.7.1.20) to AMP; in plants, Ado is hydrolyzed to adenine and D-ribose (EC 3.2.2.7). The role of Ado and 2′-deoxy-Ado

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[1] Abbreviations used: AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; Ado, adenosine; Hcy, L-homocysteine; Ara-A, 9-β-D-arabinofuranosyladenine; C-Ado, carbocyclic analogue of adenosine [(±)-aristeromycin]; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; NucHcy, an analogue of *S*-nucleosidylhomocysteine; NucMet, an analogue of *S*-nucleosidylmethionine.

in mammalian cells has been reviewed recently (Fox & Kelley, 1978). The Hcy produced is either remethylated to methionine or converted to cystathionine by condensation with serine (Finkelstein et al., 1974; Mudd & Poole, 1975).

AdoHcy is a potent inhibitor of biological methylations, and it is generally thought that the ratio AdoMet/AdoHcy is important in the control of biological methylations (Cantoni et al., 1979). Recently, purified mammalian and plant AdoHcy hydrolases have been shown to form novel *S*-nucleosidylhomocysteines (NucHcy's) from adenosine analogues and Hcy (Chiang et al., 1977; Richards et al., 1978; Guranowski & Pawełkiewicz, 1977). The ability of the enzyme to form novel congeners of AdoHcy has also been shown in cellular systems and in vivo. Biosynthesis of large amounts of 3-deaza-AdoHcy was demonstrated in isolated rat hepatocytes (chiang et al., 1977), chicken embryo cells (Bader et al., 1978), rat liver (Chiang & Cantoni, 1979), and neutrophils (Chiang et al., 1979) after the administration of 3-deaza-Ado. After injection of N^6-methyl-Ado into mice, the appearance of *S*-N^6-methyladenosylhomocysteine in the liver was observed (Hoffman, 1978).

By the use of 3-deaza-Ado, it has been possible to manipulate the in vivo and in vitro ratio of AdoMet/NucHcy (Chiang et al., 1977, 1979; Bader et al., 1978; Chiang & Cantoni, 1979) and exercise a degree of control over certain methylation reactions (Cantoni et al., 1979; Chiang et al., 1977). 3-Deaza-Ado exerts its many biological and biochemical potencies through its inhibition of AdoHcy hydrolase and the simultaneous formation of 3-deaza-AdoHcy (Chiang et al., 1977, 1978, 1979, 1980; Richards et al., 1978; Bader et al., 1978; Chiang & Cantoni, 1979; Leonard et al., 1978; Im et al., 1979; Cantoni et al., 1979; Trager et al., 1980; Phyall et al., 1981). The potential use of analogues of adenosine to achieve perturbation of biological methylations is thus well illustrated by 3-deaza-Ado, which unlike adenosine owes its metabolic stability to its resistance to adenosine deaminase (Ikehara & Fukin, 1974; Chiang et al., 1977) and adenosine kinase (Miller et al., 1979).

An interesting development of the concept of AdoHcy hydrolase as a biological target is the observation that Ado, 3-deaza-Ado, 2′-deoxy-Ado, and Ara-A can irreversibly inactivate the enzyme in the absence of Hcy or AdoHcy (Chiang et al., 1979; Hershfield, 1979). We have found that a large number of nucleosides can inactivate AdoHcy hydrolase irreversibly (Chiang et al., 1981). We present here comparative studies on the ability of 11 of these nucleosides to serve as both alternative substrates for and competitive inhibitors of AdoHcy hydrolase from beef liver and lupin seeds.

Materials and Methods

Enzyme Purification. Beef liver AdoHcy hydrolase was prepared by crystallization as previously described (Richards et al., 1978), except the hydroxylapatite step was carried out with a linear gradient of 8–150 mM phosphate (pH 7.6), 5 mM dithiothreitol, and 1 mM EDTA. AdoHcy hydrolase from lupin (*Lupinus luteus*) seeds was purified as described previously (Guranowski & Pawełkiewicz, 1977). Ado deaminase from intestinal mucosa was purchased from Sigma. Ado kinase was isolated from lupin seeds as described (Guranowski, 1979).

Enzyme Assays. To estimate the initial velocities of the synthesis of NucHcy by AdoHcy hydrolase, an incubation mixture in 50 μL contained 50 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol, 10 mM DL-[^{35}S]Hcy, 1 mM nucleoside, and enzyme diluted appropriately in 50 mM Tris-HCl, pH 8.0, containing bovine serum albumin, 1 mg/mL. The incubation was at 37 °C, and 15-μL aliquots were transferred at intervals to 5 μL of 0.15 M HCl to stop the reaction; 10 μL of the resultant mixture was spotted on chromatographic sheets (Merck) coated with cellulose plus fluorescent indicator, followed by 2 μL of 2 mM standard solution of the corresponding NucHcy (added to the same spot). The chromatogram was developed in 5% Na_2HPO_4 for 90 min, and after visualization under ultraviolet lamp, the spots of NucHcy were cut and counted. In this chromatographic system, Hcy migrates closely to the front and is therefore effectively separated from analogues of NucHcy (Table I).

Table I: R_f Values of Adenosine Analogues and Their *S*-Nucleosidylhomocysteines Synthesized by Beef Liver AdoHcy Hydrolase

	R_f values			
	system A[a]		system B[b]	
nucleoside	nucleoside	NucHcy	nucleoside	NucHcy
adenosine	0.43	0.53	0.53	0.23
3-deaza-adenosine	0.41	0.48	0.42	0.12
2-aza-3-deaza-adenosine	0.45	0.52	0.40	0.12
nebularine	0.71	0.71	0.50	0.24
formycin	0.50	0.58	0.47	0.17
N^6-methyl-adenosine	0.52	0.60	0.59	0.30
8-azaadenosine	0.58	0.65	0.65	0.35
adenosine N^1-oxide	0.69	0.74	0.33	0.15
pyrazomycin	0.75	0.78	0.54	0.24
8-amino-adenosine	0.26	0.33	0.48	0.14
inosine	0.69	0.73	0.42	0.17
carboxylic adenosine	0.40	0.40	0.43	0.20

[a] System A: cellulose plates developed in 5% Na_2HPO_4. R_f for homocysteine in system A is 0.9. [b] System B: silica gel plates developed in 1-butanol/acetic acid/water (12:3:5).

To obtain solutions of standard samples of NucHcy, the incubation mixture contained 50 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol, 10 mM DL-Hcy, 2 mM of the appropriate nucleoside, and AdoHcy hydrolase from beef liver (0.5 mg/mL). The incubation was carried out for 16 h at 37 °C and was terminated by heating for 2 min at 100 °C. The denatured protein was removed by filtration and the solution stored at −20 °C. The effectiveness of synthesis of NucHcy by this method was checked by chromatography of the samples on silica gel plates (Table I).

Enzyme Inhibition Assays. The inhibitory potencies of the nucleosides were assayed with 2 μM [U-^{14}C]Ado in a final volume of 50 μL containing 50 mM Bicine, pH 8.3, 2 mM dithiothreitol, 10 mM DL-Hcy, and the appropriate nucleoside. The reaction was started by the addition of AdoHcy hydrolase from either source, and after 2.5, 5, and 7.5 min, 15-μL aliquots of the incubation mixture were transferred to 5 μL of 0.15 N HCl; 10 μL of each resultant mixture was spotted onto aluminum chromatographic sheets coated with silica gel plus fluorescent indicator. The chromatograms were developed for 60 min in propan-2-ol/ethyl acetate/ammonia/water (23:27:4:4 v/v) (Guranowski & Pawełkiewicz, 1977). The spots containing [^{14}C]AdoHcy were cut out and counted. Kinetic parameters were determined by Dixon plots (Dixon, 1953) and, where necessary, by the method of Cleland (1963).

Materials. 3-Deaza-Ado was synthesized as described previously (Montgomery et al., 1977), 2-aza-3-deaza-Ado according to the method of Carbon (1960), and the carbocyclic

Table II: Relative Velocities of Enzymatic Synthesis of *S*-Nucleosidylhomocysteines Catalyzed by Beef Liver and Lupin Seed AdoHcy Hydrolases[a]

substrates	relative velocity (%) beef liver AdoHcy hydrolase	lupin seed AdoHcy hydrolase
adenosine	100	100
3-deazaadenosine	165	104
2-aza-3-deazaadenosine	113	35.8
nebularine	34.3	48.5
formycin	18.1	17.5
N^6-methyladenosine	15.9	0.6
8-azaadenosine	8.6	1.3
adenosine N^1-oxide	5.4	3.0
pyrazomycin	5.0	2.2
8-aminoadenosine	4.2	2.8
inosine	1.5	0.0
carbocyclic adenosine	0.1	0.3

[a] Assayed at 10 mM DL-[^{35}S]Hcy and 1 mM nucleoside.

analogue of adenosine (C-Ado) by the method of Shealy & Clayton (1969). Pyrazomycin was supplied by E. Lilly Laboratory (Indianapolis, IN); nebularine (purine ribonucleoside), 8-aza-Ado, 8-amino-Ado, N^6-methyl-Ado, N^6-dimethyl-Ado, Ara-A, 2-chloro-Ado, 8-bromo-Ado, 6-mercaptopurine ribonucleoside, 2′-deoxy-Ado, and tubercidin were from Chemalog (South Plainfield, NJ); adenine xylonucleoside was a gift of Dr. R. I. Glazer (National Cancer Institute, Bethesda, MD); the rest of the reagents were of the highest purity available commercially.

Determination of Intracellular Concentrations of AdoMet, AdoHcy, and NucHcy. Cellular concentrations of AdoMet, AdoHcy, and NucHcy were determined by high-performance liquid chromatography with a VYDAC cation-exchange column (Chiang & Cantoni, 1979; Hoffman, 1975). A subline of mouse embryo fibroblasts, 3T3-L1, was cultured in 75-cm^2 Falcon tissue culture flasks for 5 days with 15 mL of [^{35}S]-methionine (30.1 μCi) supplemented by Dulbeccos–Vogt's modified Eagles medium containing 10% fetal calf serum. The medium was changed every other day, and also on the fifth day before the incubation with nucleosides. Each flask contained about 15 million cells at the time of incubation with nucleosides. After incubation with nucleosides, the medium was removed by suction, and after the medium was washed quickly with 15 mL of phosphate-buffered saline, the cells were scraped off by a rubber policeman with two 1-mL applications of phosphate-buffered saline. Two flasks of each series of cells were combined and added to 0.5 mL of 50% sulfosalicyclic acid and were frozen at −20 °C until ready for use. The sulfosalicyclic acid extract was thawed and centrifuged at 20000 *g* for 10 min to determine the levels of metabolites. The supernatant was next applied onto a VYDAC cation column, which was calibrated with standards of the metabolites. After the column was washed with 120 mL of 0.01 M ammonium formate, pH 4.0, elution was started by a 100-mL linear gradient of 0.01–0.8 M ammonium formate, pH 4.0. With this column, 2-aza-3-deaza-AdoHcy is eluted 10 fractions (1.5 mL/fraction) behind AdoHcy; 3-deaza-AdoHcy is 14 fractions behind, and C-Ado-Hcy is 12 fractions behind AdoHcy.

Results

Substrate Specificities. (1) Beef Liver AdoHcy Hydrolase. Eleven of the nucleosides tested (Figure 1) are able to substitute for adenosine in the reaction catalyzed by AdoHcy hydrolase from beef liver to yield the corresponding NucHcy congeners (Table II). These novel congeners could be separated from the parent compounds by thin-layer chromatography (Table I). At 1 mM concentrations, the best substrate for the liver AdoHcy hydrolase is 3-deaza-Ado, followed by 2-aza-3-deaza-Ado. In spite of the absence of the 6-amino group, nebularine (purine ribonucleoside) is 30% as active as adenosine as a substrate. Formycin and N^6-methyl-Ado are poor substrates whereas 8-aza-Ado, Ado N^1-oxide, pyrazomycin, and 8-amino-Ado are only marginally active (10% or less). Unexpectedly, inosine can also function as a substrate, albeit only about 1.5% of that of Ado. The nucleoside with the lowest activity as a substrate is C-Ado. The K_m values for inosine and Ado were determined by the method of Cleland (1963) and were 1.9 ± 0.3 and 1.8 ± 0.3 μM, respectively. The V_{max} for Ado is 40 times higher than that for inosine.

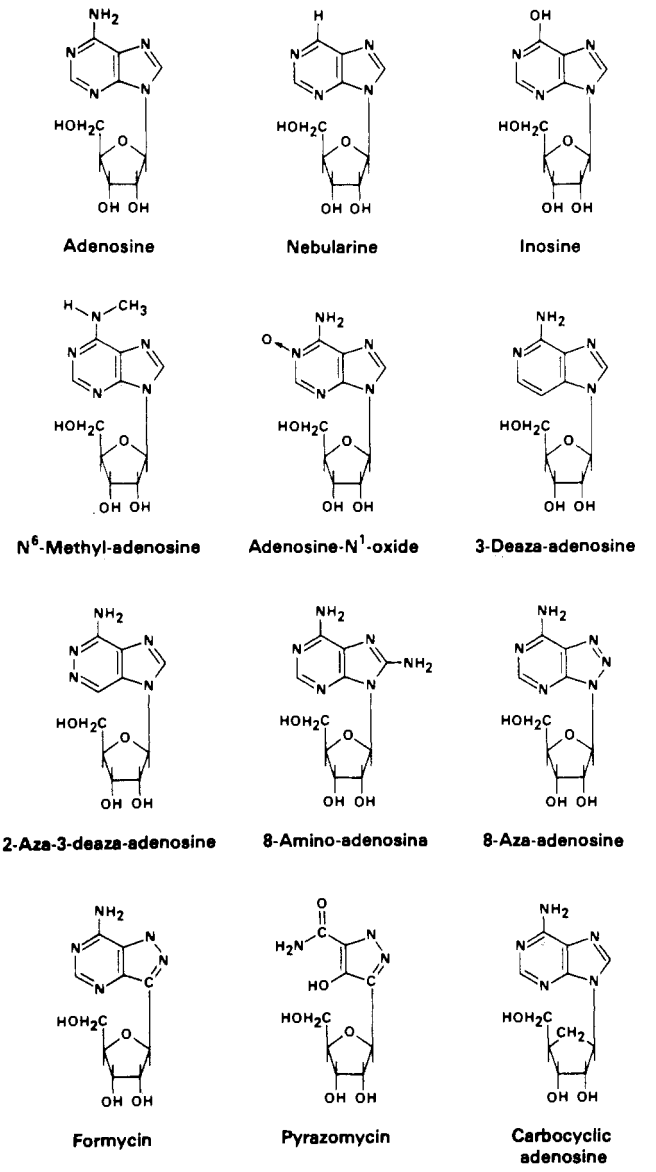

FIGURE 1: Chemical structures of adenosine analogues.

(2) Lupin Seed AdoHcy Hydrolase. In general, AdoHcy hydrolase from lupin seeds is less tolerant of changes in the adenosine molecule (Table II). In contrast to the enzyme from liver, AdoHcy hydrolase from lupin seeds shows comparable rates for both 3-deaza-Ado and Ado, and the catalytic rate for 2-aza-3-deaza-Ado is only about 36% of that of Ado. The rest of the nucleosides have about the same substrate effectiveness as that observed for liver AdoHcy hydrolase, except that inosine is not a substrate at all and N^6-methyl-Ado is a very poor substrate.

Table III: K_i Values of Competitive Inhibitors of the Enzymatic Synthesis of *S*-Adenosylhomocysteine Catalyzed by Beef Liver and Lupin Seed AdoHcy Hydrolase

inhibitor	K_i (μM) beef liver AdoHcy hydrolase	K_i (μM) lupin seed AdoHcy hydrolase
carbocyclic adenosine	0.005	0.005
3-deazaadenosine	4	1
8-aminoadenosine	15	14
8-azaadenosine	190	200
N^6-methyladenosine	190	250
formycin	280	390
2-aza-3-deazaadenosine	340	340
inosine	980	1100
nebularine	1390	1460
adenosine N^1-oxide	1800	1450
pyrazomycin	9870	10700

The following nucleosides were tested and found not to be substrates for AdoHcy hydrolase from either liver or lupin seeds: Ara-A, adenine xylonucleoside, N^6-dimethyl-Ado, 8-bromo-Ado, 6-mercaptopurine ribonucleoside, tubercidin, and 2′-deoxyadenosine.

Nucleosides as Competitive Inhibitors of AdoHcy Hydrolase from Liver and Lupin Seeds. Table III shows that C-Ado, in sharp contrast to its ineffectiveness as a substrate, is the most potent competitive inhibitor found so far, far surpassing 3-deaza-Ado. The K_i for C-Ado is about 5×10^{-9} M for the enzyme from either source and is 3 orders of magnitude lower than those for 3-deaza-Ado, the K_i's of which are 4 and 1 μM for the AdoHcy hydrolase from liver and plant, respectively. The K_i for 3-deaza-Ado as an inhibitor of liver AdoHcy hydrolase is very close to the one reported earlier, which is 3 μM when assayed in the hydrolytic direction (Chiang et al., 1978). The third most potent inhibitor is 8-amino-Ado. 2-Aza-3-deaza-Ado, despite its ability to serve as a substrate, is rather a mediocre inhibitor compared to 3-deaza-Ado. The least effective inhibitor is pyrazomycin. It is interesting that the K_i values for each nucleoside are very similar for the enzyme from either source.

The previous report that 9-(*erythro*-2-hydroxy-3-nonyl)-adenine and 2′-deoxycoformycin (Cantoni et al., 1979; Chiang et al., 1979) could inhibit AdoHcy hydrolase cannot be confirmed.

Effects of Some Nucleosides on Cellular Concentrations of AdoMet, AdoHcy, and NucHcy. Fibroblasts (3T3-L1) were cultured with L-[^{35}S]methionine-supplemented medium for 5 days to achieve isotopic equilibrium, and then the cells were exposed to C-Ado, 3-deaza-Ado, and 2-aza-3-deaza-Ado, 10 μM of each for 2 h. As expected from the inhibitory potencies, C-Ado caused the largest increase (10-fold) in the cellular concentration of AdoHcy followed by 3-deaza-Ado, which caused an 8-fold increase (Table IV). 2-Aza-3-deaza-Ado did not affect the cellular concentration of AdoHcy. Large concentrations of the novel congeners 3-deaza-AdoHcy and 2-aza-3-deaza-AdoHcy were observed in the cells incubated with 3-deaza-Ado or 2-aza-3-deaza-Ado, respectively. There appeared to be a drop in the concentration of AdoMet when the cells were incubated with C-Ado, and this might be caused by the phosphorylation of C-Ado to its triphosphate derivative (Suhadolnik, 1970), which may be inhibitory toward AdoMet synthetase (EC 2.5.1.6). Nucleoside triphosphates derived from some uncommon nucleosides have been shown to inhibit AdoMet synthetase (EC 2.5.1.6) from mammalian cells (Glazer & Peale, 1978). The addition of 2′-deoxycoformycin, and irreversible inhibitor of adenosine deaminase (John & Adamson, 1976; Lapi & Cohen, 1977), to the incubation medium to prevent the deamination of C-Ado neither changed the concentration of AdoHcy or AdoMet in the cells treated with C-Ado nor formed any detectable level of C-AdoHcy. The overall pattern of the ^{35}S-labeled metabolites in the 3T3-L1 fibroblasts in response to an inhibitor or substrate is similar to that of chick embryo fibroblasts reported previously (Bader et al., 1978).

Table IV: Distribution of ^{35}S-Labeled Nucleosidyl Metabolites in 3T3-L1 Fibroblasts after Incubation with Adenosine Analogues[a]

analogue	distribution [pmol/(30 × 10^6 cells)] AdoHcy	NucHcy	AdoMet
none	40		3200
carbocyclic adenosine	410		2300
3-deazaadenosine	330	1500	3900
2-aza-3-deazaadenosine	40	400	3300

[a] Fibroblasts were incubated with the adenosine analogues (10 μM) for 2 h. Experimental details given under Materials and Methods.

Discussion

There are two principal methods to perturb biological methylations via modulation of the catalytic activity of AdoHcy hydrolase, leading to an attendant change in the ratio of AdoMet/NucHcy which is probably a reflection of the degree of control of methylation reactions. The first is to provide the cells with alternative substrates, capitalizing on the ability of AdoHcy hydrolase to utilize a large variety of nucleosides, as shown by the present investigation (Table II), thus raising the cellular level of NucHcy(s), since the equilibrium of the enzymatic reaction favors synthesis. The second method is to build up a large pool of AdoHcy by inhibiting the hydrolysis of the natural substrate AdoHcy by AdoHcy hydrolase through the use of nucleoside inhibitors, such as those listed in Table III. By the use of either or both of these two methods, NucHcy levels can be manipulated in cells (Table IV).

When rats were injected with 3-deaza-Ado, a reduction in the ratio of AdoMet/NucHcy in their livers resulted, and a number of perturbations of methylation reactions were observed (Chiang & Cantoni, 1979; Chiang et al., 1980): (a) a drastic reduction in the methylation of phospholipids, (b) a reduction in the biosynthesis of liver creatine, and (c) a decrease in the urinary excretion 3-methoxy-4-hydroxymandelic acid. These effects are ascribed to the inhibition of methylation reactions by an increase in AdoHcy and the formation of 3-deaza-AdoHcy (Chiang & Cantoni, 1979).

The biological effects of 3-deaza-Ado are varied and interesting. It can act as a potent antiviral agent (Bader et al., 1978; Chiang et al., 1978) and exhibits an in vitro antimalarial effect, which can be potentiated by Hcy-thiolactone (Trager et al., 1980). Chemotaxis by neutrophils (Chiang et al., 1979; Schiffmann et al., 1979), phagocytosis by macrophages (Leonard et al., 1978), and lymphocyte-mediated cytolysis (Zimmerman et al., 1978) are inhibited by 3-deaza-Ado. The use of 3-deaza-Ado as a potential hypotensive agent has been suggested (Phyall et al., 1981). Finally, Thompson et al. (1979) have shown that 3-deaza-Ado and other inhibitors of AdoHcy hydrolase can inhibit, rapidly and reversibly, synaptic responses between retinal neurons and muscle fibers in culture. A large number of nucleosides can serve as substrates for AdoHcy hydrolase. These nucleosides serve as Michaelis-type

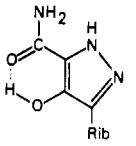

FIGURE 2: Proposed intramolecular hydrogen bond for pyrazomycin.

competitive inhibitors of the enzyme and are also capable of inactivating the enzyme irreversibly (Chiang et al., 1981). Of the 11 compounds tested here, 6 are natural nucleosides found in various organisms (Suhadolnik, 1970): C-Ado [(±)-aristeromycin], N^6-methyl-Ado, formycin, nebularine (purine ribonucleoside), pyrazomycin, and inosine. It is conceivable that some of these nucleosides exert part of their biological activities, such as antiviral, antibacterial, or cytotoxic activity (Suhadolnik, 1970), by acting either as alternative substrates of AdoHcy hydrolase, thus generating NucHcy's, or as competitive or irreversible inhibitors of the enzyme (Chiang et al., 1981). The inhibitory potencies of some of the NucyHcy analogues toward various methylases have been compared (Borchardt, 1975; Borchardt et al., 1976; Pugh et al., 1977).

In terms of in vivo or in vitro elucidation of the hierarchy of biochemical methylations (Chiang & Cantoni, 1979; Cantoni & Chiang, 1980), the usefulness of these natural nucleosides is probably limited because they undergo phosphorylation or deamination (Suhadolnik, 1970). Moreover, Zimmerman et al. (1979) have shown that a large number of nucleosides can undergo metabolic conversion to their NucMet analogues.

At first glance, it is rather surprising that the monocyclic compound pyrazomycin can function as a substrate and also as an inhibitor, but upon closer examination it is clear that an intramolecular hydrogen bond can form between the hydroxyl group of the pyrazole ring and the carbonyl group of the carboxyamide, yielding a conformation resembling a 6-aminopurine riboside (Figure 2). Nebularine can function as a substrate with 30–50% efficiency relative to Ado (Table II), showing that the 6-amino group is not essential for recognition and synthesis by AdoHcy hydrolase. The inability of N^6-dimethyl-Ado to function as a substrate is probably due to steric hindrance of the 6 position; N^6-methyl-Ado possesses less steric hindrance and can be tolerated by AdoHcy hydrolase. The deletion of the nitrogen atom at the 3 position yields two good substrates, 3-deaza-Ado and 2-aza-3-deaza-Ado, both of which are resistant to deamination and phosphorylation. The 8-substituted analogues on the whole are poor substrates, and this may reflect a lack of bulk tolerance by the enzyme for groups at the 8 position. Since AdoHcy hydrolase from beef liver can synthesize *S*-inosylhomocysteine (Tables I and II), this enzyme is probably responsible for the accumulation of some amount of *S*-inosylhomocysteine in neuroblastoma cells (Crooks et al., 1979).

In sharp contrast to its poor ability to serve as substrate, C-Ado, with a K_i of 5×10^{-9} M, is the most potent inhibitor of the enzyme found so far, indicating the necessity for the oxygen of the ribofuranose ring for the enzymatic reaction, but not for binding to the active site. With the exception of 3-deaza-Ado, there is no apparent correlation between substrate ability and inhibitory potencies for the rest of the nucleosides tested.

The close similarities between substrate specificities, sensitivities toward nucleoside inhibitors, and their subunit molecular weights [55 000 for AdoHcy hydrolase from lupin seeds (Guranowski & Pawełkiewicz, 1977) and 50 000–60 000 for AdoHcy hydrolase from beef liver (Richards et al., 1978; Palmer & Abeles, 1979)] indicate that the active sites of AdoHcy hydrolase from these two sources may be very much alike.

The inhibition data presented herein may provide a basis for the design of nucleoside analogues with greater specificity and inhibitory potencies for AdoHcy hydrolase.

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Effects of Self-Association of Ornithine Aminotransferase on Its Physicochemical Characteristics†

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ABSTRACT: Previous work in this laboratory [e.g., Peraino, C., Bunville, L. G., & Tahmisian, T. N. (1969) *J. Biol. Chem. 244*, 2241–2249, and Morris, J. E., Peraino, C., & Strayer, D. (1974) *Proc. Soc. Exp. Biol. Med. 147*, 707–709] has shown that the molecular weight of ornithine aminotransferase (OAT) is concentration dependent. In the present study this property of OAT was further characterized by using sedimentation equilibrium centrifugation to determine the molecular weight of OAT in a range of enzyme concentrations. It was shown that OAT aggregates in a two-stage process as its concentration increases. The first stage involves the association of enzymatically active monomers into trimers, with association of the trimers into higher order aggregates occurring in the second stage. Decreasing the pH or raising the ionic strength enhances aggregation, while raising the pH inhibits aggregation; however, the two-stage nature of the aggregation process was not affected by changes in pH and ionic strength. Kinetic analyses of purified enzyme showed that aggregation results in an increase in the K_m for both substrates with the V_{max} remaining constant, indicating that aggregation of monomers sterically hinders substrate binding. Increased K_m values were also obtained for OAT sequestered in mitochondria from rats fed a high-protein diet to increase mitochondrial OAT levels. The higher K_m values suggest that the elevation of OAT in vivo is accompanied by aggregation of the enzyme within the mitochondrion. We propose that the aggregation-dependent increase of K_m in vivo has adaptive value in that it spares ornithine for use in the urea cycle.

Ornithine aminotransferase (EC 2.6.1.13) catalyzes the transfer of the amino group of ornithine to α-ketoglutarate, producing glutamic acid and glutamic semialdehyde. In rat liver, ornithine aminotransferase (OAT)[1] is localized in the mitochondria (Peraino & Pitot, 1963). Although it has been proposed (Herzfeld & Knox, 1968; Volpe et al., 1969) that OAT generates ornithine for use in the urea cycle, considerable evidence (Strecker, 1965; Peraino, 1972; Morris & Peraino, 1976; McGivan et al., 1977) suggests that OAT reduces urea cycle activity by catabolizing ornithine.

Considerable disagreement exists in the reported values for the molecular weight of OAT. Peraino et al. (1969) determined a molecular weight of 132 000. Several investigators (Matsuzawa et al., 1968; Yip & Collins, 1971; Kalita et al., 1976) reported molecular weights from 160 000 to 180 000. Sanada et al. (1976) concluded that there are two forms of OAT: form I with a molecular weight of 177 000 and form II with a molecular weight of 105 000. Evidence that the observed molecular weight of OAT is concentration dependent was presented by Peraino et al. (1969) and Morris et al. (1974), suggesting that OAT undergoes aggregation. In the present study, we have examined the stages of OAT aggregation and assessed the effects of changes in pH and ionic strength on the aggregation process. In addition, we investigated the effects of aggregation on the kinetic properties of the enzyme.

Materials and Methods

Preparation of OAT and Assays of Activity. OAT was purified from rat (*Rattus norvegicus albinus*) liver as described by Peraino et al. (1969) and Morris et al. (1974), except that the pH precipitation step was omitted. Two different procedures were used in assaying the enzyme. One was a single

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[1] Abbreviations used: OAT, ornithine aminotransferase; GDH, glutamate dehydrogenase; EDTA, ethylenediaminetetraacetic acid; NaDodSO$_4$, sodium dodecyl sulfate; OTC, ornithine transcarbamoylase; K_m, Michaelis constant; V_{max}, maximum velocity.

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