BIOCHEMICAL STUDIES ON CYTIDINE DEAMINASE AND ADENOSINE DEAMINASE

A Dissertation

Presented to the

Faculty of the Department of Biophysical Sciences

College of Natural Sciences and Mathematics

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

by

Sang He Lee

May 1976

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ABSTRACT

Rhodium(II) acetate has been found to inhibit cytidine deaminase and deoxycytidine kinase in Swiss albino and BDF_1 mouse, respectively, *in vitro*. The phosphorylation of ara-C in L1210/S cells *in vivo* was slightly increased by this compound. A portion of the synergistic effect of ara-C and rhodium(II) acetate in the treatment of L1210/S cells bearing mice may partly be due to this slight increase in phosphorylation of ara-C observed.

DHMPR (1,6-dihydro-6-hydroxymethylpurine riboside) has been found to decrease the deamination of ara-A in L1210/S cells *in vitro*. This compound has not shown any effects on phosphorylation and deamination of ara-A in L1210/S cells *in vitro* and *in vivo*, respectively. The loss of the inhibitory activity of DHMPR against adenosine deaminase *in vivo* suggests the breakdown of this compound in the *in vivo* environment.

The compound 2'-deoxycoformycin has been found to be a potent inhibitor of adenosine deaminase partially purified from BDF_1 mouse brain. The mouse injected with this compound intraperitoneally showed about 68% inhibition of brain adenosine deaminase within 15 minutes after the injection and the inhibition lasted more than 24 hours. Combination therapy of ara-A and 2'-deoxycoformycin in BDF_1 mice bearing CNS L1210/S tumor showed a significant increase in the life span of these mice.

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INTRODUCTION

Analogs of purine and pyrimidine nucleosides have received considerable attention in many laboratories concerned with the development of chemotherapeutic agents for malignancies, viruses, and tropical parasites. Although a number of these compounds have been found useful in nonneoplastic disorders (1,2) the primary stimulus behind this area of research has been the hope of identifying new antineoplastic agents.

Among these, ara-A and ara-C have been the mainstays in the study of purine and pyrimidine nucleoside analogs. Ara-A, which had originally been synthesized as an antitumor agent (3), has highly selective activity against the DNA viruses herpes and vaccinia, and in cell culture. This

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Abbreviations used are: ara-A,9-β-D-arabinofuranosyladenine; ara-C,1-β-D-arabinofuranosylcytosine; ara-Hx,9-β-D-arabinofuranosylhypoxanthine; L1210/S and L1210/ara-C, L1210 cells respectively, sensitive and resistant to inhibition by ara-C; ara-AMP,9-β-D-arabinofuranosyladenine-5'-monophosphate; ara-ATP,9-β-D-arabinofuranosyladenine-5'-monophosphate; ara-CDP,1-β-D-arabinofuranosylcytosine-5'-diphosphate; ara-CTP, 1-β-D-arabinofuranosylcytosine-5'-triphosphate; ara-U,1-β-D-arabinofuranosyluracil; UTP,1-β-D-ribofuranosyluracil-5'-triphosphate; dCMP,1-β-D-deoxyribofuranosylcytosine-5'-monophosphate; DHMPR,1,6-dihydro-6-hydroxymethylpurine riboside; CMC,carboxymethylcellulose.

nucleoside analog also has been shown to have antitumor activity in some experimental tumor system (4,5) and is currently being tested clinically as a chemotherapeutic agent against cancer in human (6).

The precise mechanism of action of ara-A has not been completely elucidated. In 1966, however, York and LePage demonstrated that the triphosphate derivative of this analog, ara-ATP, inhibited DNA polymerase (7) and, using ^{14}C-labeled ara-A, that radioactivity was incorporated into the adenine and guanine of RNA.

Ara-A is rapidly deaminated by adenosine deaminase to ara-Hx, a compound of lower potency (8) requiring high doses of ara-A when the compound is given systematically.

Since the first report of ara-C on the antitumor and antileukemic activity in mice in 1961, ara-C has been a drug of choice in the treatment of acute myeloblastic and lymphoblastic leukemia in human adults (9). Ara-C, which is schedule dependent, can "cure" mice with leukemic cells given intraperitoneally and intracranially indicating that this compound can cross the blood-brain barrier (10). Ara-C has also been demonstrated to inhibit the proliferation of certain DNA viruses in mammalian cells (11). The nucleotide form of ara-C, ara-CTP, has been shown to be a potent inhibitor of the reverse transcriptase of RNA tumor viruses (12).

The mechanism of action of ara-C is fairly well elucidated. In dividing cells ara-C, which enters the cells

by a nucleoside transport system (13), is phosphorylated to ara-CMP, ara-CDP, and ara-CTP by deoxycytidine kinase, deoxycytidylate kinase, and nucleoside diphosphokinase, respectively (14,15,16). Ara-CTP is a potent inhibitor of mammalian DNA polymerase (17,18) and apparently inhibits DNA semiconservative replication. This inhibition of DNA synthesis by ara-C produces an eventual inhibition of RNA and protein synthesis (19). Mammalian DNA polymerase can catalyze the incorporation of ara-CTP into DNA and the incorporation of this analog into DNA produces termination of polydeoxynucleotide chain growth (20).

In mammals ara-C is rapidly deaminated to ara-U, a noninhibitory metabolite (21), by cytidine deaminase (22). It is possible to obtain relatively high intracellular level of ara-CTP because this analog is a weak feedback inhibitor (23) of deoxycytidine kinase and a weak allosteric activator of dCMP deaminase (24).

The most common problem with any antineoplastic agent seems to be development of drug resistance. The various possible mechanisms by which mammalian cells manifest resistance to the inhibitory effects of ara-A and ara-C are (1) an increase in deaminase activity (25) or high levels of natural deaminase activity in either host or tumor cells, (2) a decrease in kinase activity (26), (3) or modification of DNA polymerase (27).

The present studies were carried out in order to

determine the biochemical properties and/or improve the efficacy of ara-A and ara-C in the treatment of neoplasia with compounds known to have either a synergistic effect with ara-C (rhodium(II) acetate) (28) or inhibitory activity against adenosine deaminase (DHMPR and 2'-deoxycoformycin) (29,30).

EXPERIMENTAL METHODS AND MATERIALS

Animals, Cells, and Cell-free Extracts

Leukemia-1210 (L1210/S) cells were a gift from Dr. D. H. W. Ho of M. D. Anderson Hospital and Tumor Institute, Houston, Texas. The cells were carried in female DBA/2 mice (Texas Inbred Mice Co., Houston, Texas) implanted intraperitoneally with 1×10^6 cells. The cell preparations were carried out as follows: Ascitic fluid of tumor bearing mice was delivered into chilled tubes, and centrifuged at approximately 1,500 x g for two minutes on an IEC International Clinical Centrifuge. The packed cells were resuspended in 3 volumes of ice-cold distilled water for 30 seconds to lyse the red blood cells, then 3 volumes of 1.8% NaCl solution was added and mixed thoroughly. After centrifugation of this suspension the cells were washed once in 0.9% saline. The packed cell pellet contained 1.13×10^9 cells/ml as determined by dilution and counting with a Coulter Counter.

Cell-free extracts were prepared as follows: Pellets were uniformly mixed with 5 volumes of 0.05M Tris buffer (pH 7.4) and the suspensions frozen (-80°C) and thawed rapidly (40°C water bath) three times. After centrifugation for 45 minutes at 45,000 x g the supernatant fractions were used immediately in the enzyme assay or stored at -20°C.

Purification of Cytidine Deaminase

The procedure for the purification of cytidine deaminase was that of Tomchick et al. with little modification (31). A typical preparation is described below. Kidneys were removed from 200 female Swiss albino mice (weighing around 20 grams). The removed tissues (80 grams) were homogenized in cold 0.154M KCl (1:3.3 w/v) with a Potter-Elvehjem homogenizer fitted with a Teflon pestle. The 30% homogenate was centrifuged for 60 minutes at 78,000 x g (Beckman L3-50 Ultracentrifuge). The resulting supernatant (162 ml) was heated at 60°C for 8 minutes then the heat-denatured mixture was cooled and centrifuged for 15 minutes at 17,300 x g (Beckman J-21). Subsequent steps were carried out either in a cold room at 4°C or in an ice bucket. The supernatant (146 ml) was treated with solid ammonium sulfate. The concentration was raised from 0 to 30% saturation by the addition of 25.7 grams of ammonium sulfate. After 30 minutes the mixture was centrifuged for 15 minutes at 17,000 x g and then 9.0 grams of ammonium sulfate was added to the supernatant to make 40% saturation. After increasing the percentage of saturation from 40 to 50% by the addition of 9.2 grams of ammonium sulfate, the mixture was centrifuged, and the resulting pellet was dispersed in 0.02M potassium phosphate buffer (pH 7.5). This suspension was dialyzed against 7 liters of 0.02M potassium phosphate buffer (pH 7.5) for 2 hours. Alumina gel C_γ (100 mg of gel per 10 mg of

protein) was added to the solution and the mixture was stirred for 15 minutes. Centrifugation of the mixture for 10 minutes at 2,000 x g (Beckman J-21 Ultracentrifuge) yielded 10.4 ml of supernatant which was dialyzed for 3 hours against 3 liters of 0.02M sodium acetate buffer (pH 4.5). The precipitate was removed by centrifugation for 10 minutes at 2,000 x g and the supernatant was stored in test tubes as 0.5 ml aliquots at -20°C.

Determination of Protein for the Purification of Cytidine Deaminase

The procedure of Lowry et al. (32) was used for protein determinations. Crystalline bovine serum albumin was used as the standard.

Cytidine Deaminase Assay

The analytical determination of the enzyme activity was carried out by the method described by Tomchick et al. (31) with minor modification. Cytidine and ara-C were used as the substrates for the measurement of specific activity and kinetic study, respectively. Briefly, each reaction tube contained 1.3 ml of 0.05M sodium phosphate buffer (pH 7.0), 0.1 ml of enzyme, and 0.1 ml of substrate. The incubation mixture was preincubated for 10 minutes before the addition of the substrate, after which the incubation was allowed to proceed for 30 minutes at 37°C. The reaction was then stopped by the addition of 1.5 ml of ice cold 10%

trichloroacetic acid. After the removal of precipitated protein by centrifugation the absorbance of the clear solution was measured at 290 nm with a Beckman Model 25 spectrophotometer.

Deoxycytidine Kinase Assay:

a. Column Preparation for Fractionation of Ara-C Metabolites

Prior to column packing, Dowex 2 x 8 (200-400 mesh, chloride form) was converted to the hydroxide form with 1.0M NaOH, washed with distilled water until neutral and then converted to the chloride form with 3.0M HCl. The resin was washed with distilled water until all the acid was removed. A water slurry of the resin was introduced into a 0.5 cm diameter glass column. The resin was washed from the side of the column with distilled water and was adjusted to a height of 7 cm.

b. Separation of Ara-C and Its Metabolites

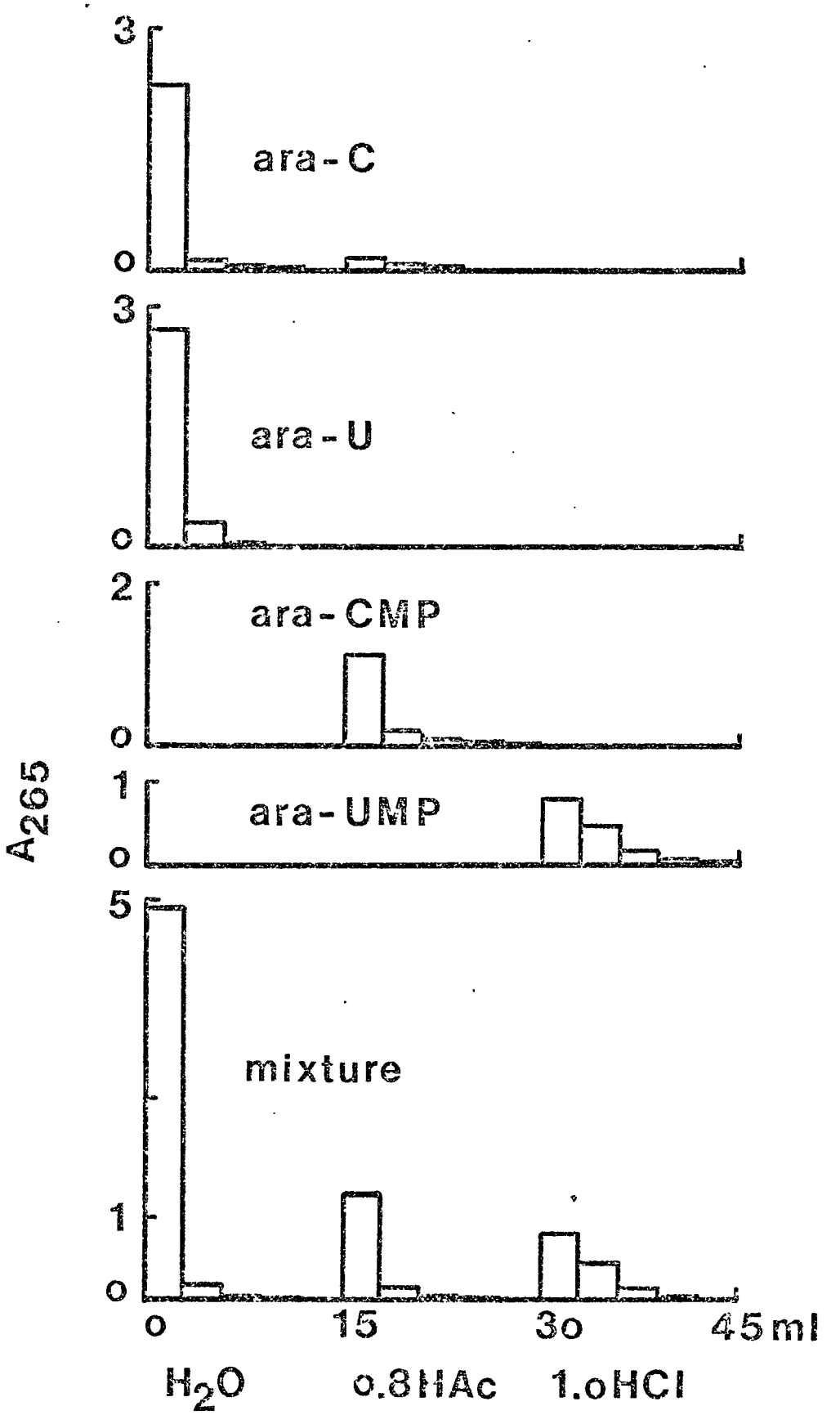
Aqueous solutions (3.0 ml each) of ara-C, ara-U, ara-CMP, ara-UMP (200 µg each) or their mixture in the same final concentrations were applied to Dowex 2 x 8 columns and eluted stepwise with water, 0.8M acetic acid, and 1.0M hydrochloric acid. The absorbance was measured at 265 nm. Elution profiles are shown in Figure 1.

c. Deoxycytidine Kinase Assay with Whole Cell Preparations

Each reaction tube contained 0.8 ml of cell suspension in

Figure 1

Separation of Ara-C, Ara-U, Ara-CMP, and Ara-UMP by Anionic Exchange Column Chromatography


3
ara-C
0
3
ara-U
0
2
ara-CMP
0
1
ara-UMP
0
A265
5
mixture
1
0
0
15
30
45 ml
H2O
0.8 HAc
1.0 HCl

Krebs-Ringer phosphate buffer supplemented with 10.8mM glucose, 0.1 ml of Krebs-Ringer phosphate buffer supplemented with 10.8mM glucose, and 0.1 ml of substrate (ara-C-^{3}H, 40 mCi/mmole). L1210/S cells (1 x 10^8 cells) were incubated with 50 μM ara-C-^{3}H in Krebs-Ringer phosphate buffer supplemented with 10.8mM glucose for 15 minutes at 37°C after 5 minutes preincubation. The reaction was stopped by chilling and then centrifuged for 2 minutes at 1,500 x g. The cells were washed two times with cold physiological saline, extracted with 1.2M perchloric acid, and heated for 15 minutes at 100°C to convert di- and tri- to monophosphate (33). After neutralizing the extract with 2M KOH and the removal of $KClO_4$ by centrifugation, ara-CMP was separated from ara-UMP by fractionation in Dowex 2 x 8 columns. The radioactivity of aliquots was counted in a Packard Tri-carb Liquid Scintillation Spectrometer (Model 3380) using Aquasol (New England Nuclear) as the scintillation solution.

d. Deoxycytidine Kinase Assay with Cell-free Extracts

The assay was a modification of a method previously described (34). Briefly, each reaction tube contained 0.1 ml of cell-free extracts (0.1 mg protein), 0.1 ml of 50mM Tris-HCl buffer (pH 7.4), 0.1 ml of 3mM $MgCl_2 \cdot 6H_2O$, 0.1 ml of 3mM UTP, and 0.1 ml of substrate (ara-C-^{3}H, 20 mCi/mmole). The reaction tubes were incubated at 37°C for 25 minutes and the reaction was stopped by immersing in a boiling water

bath for 2 minutes. The reaction mixture was treated with 1.2M perchloric acid, heated for 15 minutes at 100°C, and 2M KOH was added as previously described. After the removal of $KClO_4$ by centrifugation, aliquots of the supernatant were applied to Dowex 2 x 8 columns. The radioactivity of aliquots of eluate was counted as previously described.

Adenosine Deaminase (Calf Intestinal Mucosa) Assay

The substrate concentration of enzyme activity were determined by optical means (35). Each reaction tube contained 0.1 ml of adenosine deaminase (0.2 μg), 1.0 ml of substrate (ara-A) in 0.05M sodium phosphate buffer (pH 7.5), and 0.1 ml of water or inhibitor dissolved in water. The reaction was allowed to occur for 5 minutes after the addition of the substrate and the negative change of optical density was measured at 265 nm with Beckman Model 25 spectrophotometer.

Adenosine Deaminase (L1210/S Cells) Assay

The assay was a modification of a method of LePage *et al*. (36). Each reaction tube contained 0.3 ml of Krebs-Ringer phosphate buffer, 0.2 ml of substrate (ara-A-2-^{3}H, 2 mCi/mmole), and 1 x 10^8 L1210/S cells.

The incubation was allowed to proceed for 15 minutes at 37°C, stopped by the addition of 7M perchloric acid, neutralized with 7M KOH, and centrifuged for 5 minutes at 1,500 x g to remove the $KClO_4$ precipitate. Aliquots of the

supernatant were spotted on 3MM Whatman chromatographic paper prespotted with 30 µg of ara-Hx as a carrier. The chromatograms were developed for 6 hours in an aqueous solvent containing 5% $Na_2HPO_4 \cdot 7H_2O$. The radioactive ara-Hx spots were visualized under an ultraviolet lamp, cut out, eluted with 0.1M HCl, and counted using aquasol as the scintillation fluid.

Adenosine Deaminase (Mouse Brain) Assay

Female BDF_1 mice brains were homogenized (20% w/v) in Krebs-Ringer phosphate buffer and then centrifuged for 60 minutes at 15,000 x g. The supernatant solution was used as an enzyme source (37). Each reaction tube contained 0.5 ml of the supernatant (4-5 mg of protein per ml), and 0.2 ml of substrate (ara-A-2-^{3}H, 2 mCi/mmole). Protein concentration was determined by ultraviolet absorption at 280 nm by the method of Warburg and Christian (38). The incubation was allowed to proceed for 30 minutes at 37°C. The rest of the assay procedure was the same as for the L1210/S adenosine deaminase assay.

Phosphorylation of Ara-A (L1210/S Cells) Assay

The assay was a modification of a method of Breitman (39). Each reaction tube contained 0.2 ml of Krebs-Ringer phosphate buffer supplemented with 10mM glucose, 0.15 ml of substrate (ara-A-2-^{3}H, 2 mCi/mmole), 1 x 10^8 L1210/S cells, 3.5µM 2'-deoxycoformycin, and the inhibitor. The

incubation was allowed to proceed for 15 minutes at 37°C and stopped by immersing the tubes in a boiling water bath for 2 minutes. The cells were washed with 0.9% saline for 4 times and the nucleotides were extracted with 7M perchloric acid. Aliquots of the extract were adsorbed on DEAE-cellulose discs, washed 5 times with water and one time with 70% ethanol, eluted with 0.1M NaOH in scintillation counting vial, and counted using aquasol as the scintillation cocktail as previously described.

Survival Studies

Female BDF_1 mice differing in weight by not more than 1 gram from an average weight of 20 grams were divided into groups of 6 mice each and implanted intracranially with 2.5×10^4 cells in 0.05 ml of physiological saline. Drugs or saline (1.0 ml/mouse) was administered intraperitoneally on days 1, 4, and 7. The day of death was recorded and the average life span and standard deviation were computed.

RESULTS

Inhibition of Deamination of Ara-C by Rhodium(II) Acetate

It was reported that rhodium(II) acetate (Figure 2) acts synergistically with ara-C to inhibit the L1210/S tumor in mice (28). To determine the biochemical mode of the action of rhodium(II) acetate, cytidine deaminase, which is known to rapidly deaminate ara-C to ara-U, a noninhibitory metabolite (21), was purified from Swiss albino mouse kidneys and the inhibitory activity of this compound on the enzyme was studied. Detailed procedures for the purification of cytidine deaminase and the assay methods are described in the Experimental Methods and Materials section. Table 1 shows the summary of the enzyme purification which was 35 fold. The most effective purification steps were ammonium sulfate precipitation and alumina gel adsorption. This partially purified cytidine deaminase was used following substrate specificity and inhibition kinetic studies. Table 2 shows that mouse kidney cytidine deaminase deaminates cytosine nucleoside analogs only and that this enzyme has minimal activity for the pyrimidine base or its nucleotides in accord with the findings of Tomchick et al. (31). The rate of deamination for 2'-deoxycytidine and ara-C was about one-half and one-fourth the rate, respectively, for cytidine.

Figure 2

Rhodium(II) Acetate

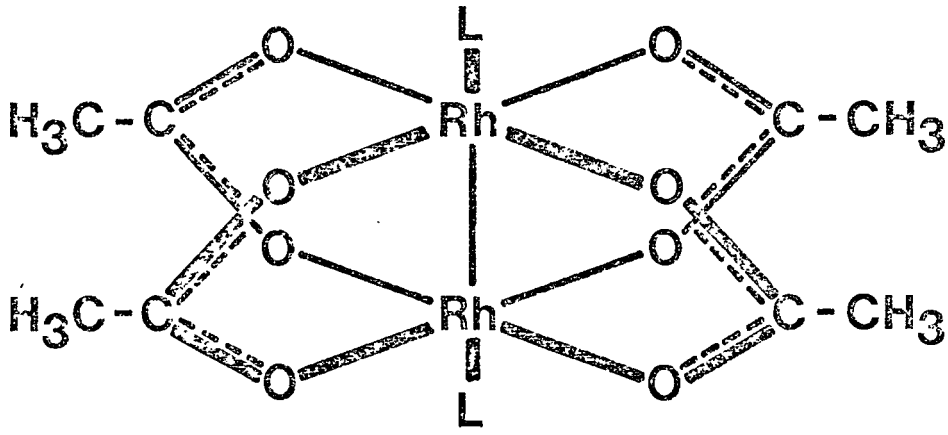

L
O
O
H3C - C
Rh
C - CH3
O
O
O
O
H3C - C
Rh
C - CH3
O
O
L

Table 1

Summary of the Purification of Cytidine Deaminase from Swiss Albino Mouse Kidney[a]

Procedure	Total Volume ml	Total Units	Protein mg/ml	Specific Activity nm/mg/min	Relative Purity
Whole homogenate	250	15826	32.8	1.9	1
Supernatant	182	9414	22.2	2.3	1.2
Heat denaturation	130	8580	13.2	5.0	2.5
$(NH_4)_2SO_4$ fraction	15	6968	19.0	24.5	12.6
Dialysis (phosphate buff.)	15	6952	16.0	29.0	15.0
Alumina gel C_γ	10	2867	10.0	46.8	24.2
Dialysis (acetate buff.)	9	4254	6.7	66.8	35.6

[a]The purification and assay procedure are described under Experimental Methods and Materials.

Table 2

Substrate Specificity of Cytidine Deaminase[a]

Compounds[b]	Relative Rate
Cytidine	100[c]
2'-Deoxycytidine	59
Arabinosylcytosine	27
Cytosine	0
2'-Deoxycytidine 5'-monophosphate	3
Cytidine 5'-triphosphate	3

[a]The assay procedure is described under Experimental Methods and Materials (each reaction mixture contained 200 μg of protein).

[b]The concentration of each substrate was 2×10^{-4}M.

[c]Deaminase activity is expressed in terms of the most active substrate, cytidine, which is arbitrarily assigned a value of 100.

In the study of inhibition of cytidine deminase by rhodium(II) acetate, shown in Table 3, the enzyme (134 μg of protein) and rhodium(II) acetate were incubated for 30 minutes at 37°C after 10 minutes preincubation before the addition of the substrate, ara-C (2×10^{-4}M). The degree of inhibition of the enzyme was dependent on the concentration of rhodium(II) acetate. The 50% inhibitory concentration of rhodium(II) acetate was found to be around 7×10^{-5}M. When a Hill plot of these data (Figure 3) was employed to see how many binding sites are involved of rhodium(II) acetate and also to determine the possible cooperativity there was a unit slope to the curve. A Lineweaver-Burk plot, shown in Figure 4, was employed to observe the mode of inhibition of ara-C deamination by rhodium(II) acetate. Ara-C, the concentration of which ranged from 1.25×10^{-4}M to 5×10^{-4}M, was incubated for 30 minutes at 37°C with rhodium(II) acetate at 1×10^{-4}M which gives approximately 50% inhibition of cytidine deaminase. Least squares analysis was used to fit the best lines to the data points. The apparent Km for ara-C was decreased 2.5 times for the control when rhodium(II) acetate was added. The plot showed that rhodium(II) acetate gave an inhibition of the mixed type.

The Effect of Rhodium(II) Acetate on Phosphorylation of Ara-C

In dividing cells ara-C is phosphorylated to ara-CMP, ara-CDP, and ara-CTP (14,15,16); ara-CTP is the major form

Table 3

Inhibition of Cytidine Deaminase[a] by Rhodium(II) Acetate[b]

Concentration of rhodium(II) acetate(M)	ara-C,[c] nmoles deaminated	% of control
0	82.8	100
1×10^{-5}	75.0	91
5×10^{-5}	53.3	64
1×10^{-4}	33.0	40
2.5×10^{-4}	10.0	12
5×10^{-4}	0	0

[a]The assay procedure is described under Experimental Methods and Materials (each reaction mixture contained 134 μg of protein).

[b]The reaction mixture was preincubated with rhodium(II) acetate for 10 minutes before the addition of the substrate, ara-C.

[c]The concentration of the substrate was 2×10^{-4}M.

Figure 3

Hill Plot of the Binding of Rhodium(II) Acetate to Cytidine Deaminase

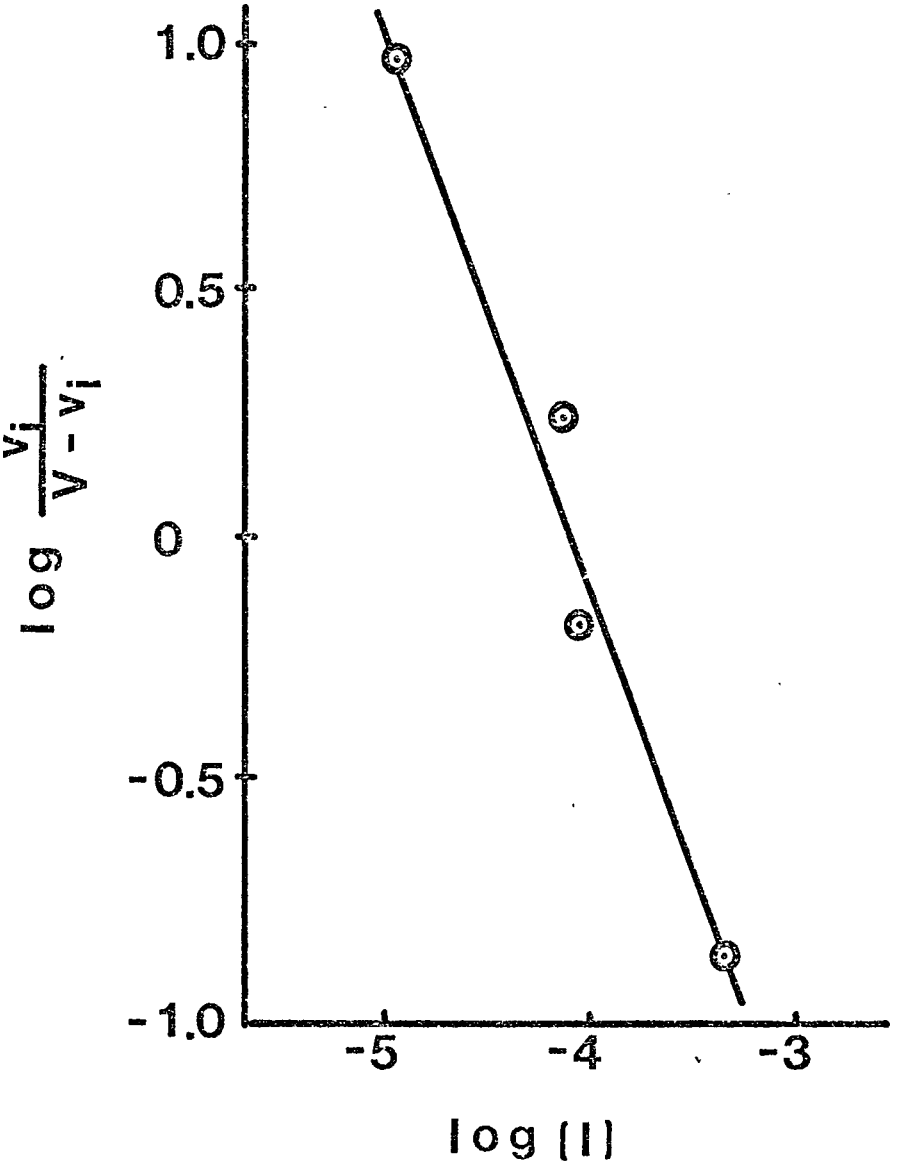
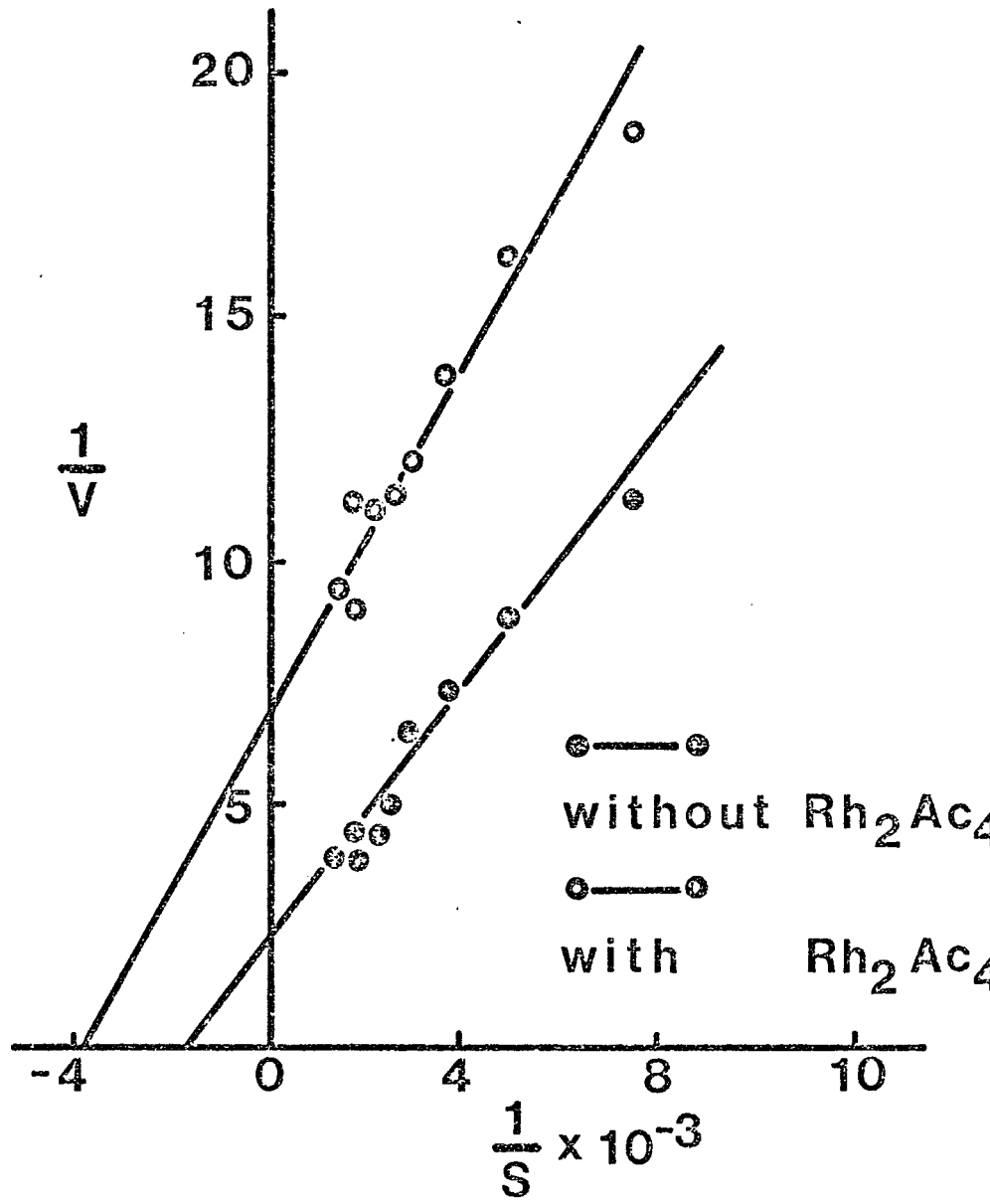

1.0
0.5
0
-0.5
-1.0
-5
-4
-3
log [I]
log $\frac{v_i}{V - v_i}$

Figure 4

Lineweaver-Burk Plot of Rhodium(II) Acetate Inhibition of Cytidine Deaminase

Cytidine deaminase and ara-C were incubated in 0.05M sodium phosphate buffer(pH 7.0) for 30 minutes at 37°C with or without rhodium(II) acetate(1×10^{-4}M).


20
15
10
5
1/V
-4
0
4
8
10
1/S x 10^-3
without Rh2Ac4
with Rh2Ac4

of the antimetabolite in the cell (40,41). The S-phase specificity of ara-C is mainly due to an increase in deoxycytidine kinase activity (23), an enzyme found primarily in proliferating cells (14) which catalyzes the phosphorylation of ara-C to ara-CMP (14,42). Since rhodium(II) acetate turned out to be an inhibitor of cytidine deaminase, the treatment of L1210/S cells or cell-free extracts with this compound should increase the phosphorylation of ara-C provided that rhodium(II) acetate is not an inhibitor of deoxycytidine kinase. The results of such an experiment are shown in Table 4. L1210/S cells suspended in Krebs-Ringer phosphate buffer supplemented with 10.8mM glucose were incubated for 15 minutes at 37°C in the presence of 50μM ara-C and various concentrations of rhodium(II) acetate ranging from 1×10^{-5}M to 5×10^{-4}M. The data indicate that rhodium(II) acetate at these concentrations had no effect on the phosphorylation of ara-C; that is, that rhodium(II) acetate is not an inhibitor of deoxycytidine kinase, or that this compound minimally penetrates the cell membrane. In order to clarify this point, similar experiments with cell-free extracts were carried out. L1210/S cell-free extracts were incubated for 25 minutes at 37°C in the presence of 100μM ara-C, 3mM $MgCl_2$, 3mM UTP (34), 50mM Tris buffer, and rhodium(II) acetate at 1×10^{-4}M or 5×10^{-4}M. In this experiment the phosphorylation of ara-C was found to be inhibited by rhodium(II)

Table 4

The Effect of Rhodium(II) Acetate on Phosphorylation of Ara-C in L1210/S Cells[a] in vitro

Concentration of rhodium(II) acetate(M)	nmoles of ara-CMP formed by 1×10^8 cells	% of control
0	10.8	100
1×10^{-5}	10.6	99
1×10^{-4}	10.2	95
5×10^{-4}	10.5	97

[a]The assay procedure is described under Experimental Methods and Materials.

1×10^8 cells from BDF_1 mice were incubated with 50μM ara-C-^{3}H (40 mCi/mmole) in Krebs-Ringer phosphate buffer supplemented with 10.8mM glucose with or without rhodium(II) acetate.

acetate in a concentration and preincubation time-dependent manner as shown in Table 5. The 100% inhibitory concentration of rhodium(II) acetate after 5 minutes preincubation with this compound before the addition of the substrate was found to be 5×10^{-4}M. When ara-C was added to the reaction mixture, which had been preincubated with 1×10^{-4}M rhodium(II) acetate for 5 minutes, the inhibition of the phosphorylation was about 95% of the control while the non-preincubation group with the same rhodium(II) acetate concentration showed only 40% of inhibition of the control. The results of these experiments can rule out the possibility that rhodium(II) acetate has no inhibitory activity against deoxycytidine kinase; that is, that rhodium(II) acetate does not penetrate the cell membrane. The phosphorylation of ara-U, however, was found to be inhibited by rhodium(II) acetate as shown in Table 6, when L1210/S cells were incubated in the presence of 50μM ara-C and rhodium(II) acetate. The 50% inhibitory concentration of rhodium(II) acetate was found to be 5×10^{-4}M.

The Effects of Rhodium(II) Acetate on in vivo Phosphorylation and Deamination of Ara-C

When mice bearing L1210/S tumors were treated *in vivo* with rhodium(II) acetate in 0.9% saline, the degree of phosphorylation of ara-C was found to be slightly increased by rhodium(II) acetate as shown in Table 7. There was no significant differences of phosphorylation between 20 mg/kg and

Table 5

The Effect of Rhodium(II) Acetate on Deoxycytidine Kinase Activity[a] of Cell-free Extracts from L1210/S Cells

Concentration of rhodium(II) acetate(M)	nmoles of nucleotides of ara-C formed per 0.1 mg of protein	% of control
0	1.448	100
1×10^{-4} [b]	0.067	4.6
1×10^{-4} [c]	0.885	61.1
5×10^{-4} [b]	0.005	0.3
5×10^{-4} [c]	0.015	10.2

[a]The assay procedure is described under Experimental Methods and Materials.

[b]5 minutes preincubation with rhodium(II) acetate

[c]No preincubation with rhodium(II) acetate

Cell-free extracts (0.1 mg of protein) were incubated with 100μM ara-C-^{3}H (20 mCi/mmole), 3mM $MgCl_2$, 3mM UTP, 50mM Tris buffer (pH 7.4) with or without rhodium(II) acetate for 25 minutes at 37°C.

Table 6

The Effect of Rhodium(II) Acetate on Phosphorylation of Ara-U in L1210/S Cells[a] in vitro

Concentration of rhodium(II) acetate(M)	nmoles of ara-UMP formed by 1×10^8 cells	% of control
0	0.90	100
1×10^{-5}	0.88	99
1×10^{-4}	0.71	79
5×10^{-4}	0.48	53

[a]The assay procedure is described under Experimental Methods and Materials.

1×10^8 cells from BDF_1 mice were incubated with 50μM ara-C-^{3}H (40 mCi/mmole) in Krebs-Ringer phosphate buffer supplemented with 10.8mM glucose with or without rhodium(II) acetate.

Table 7

The Effects of Rhodium(II) Acetate on Phosphorylation and Deamination of Ara-C[a] in BDF_1 Mice Bearing L1210/S Cells

	pmoles of ara-CMP + ara-UMP formed per 1 x 10^8 cells	% ara-CMP	% ara-UMP
Experimental[b] group	496.8	56.9	43.1
Control group	511.5	51.7	48.3
Experimental[c] group	520.0	54.1	45.9
Control group	533.6	49.7	50.3

[a]The assay procedure is described under Experimental Methods and Materials.

[b]20 mg/kg rhodium(II) acetate was given one hour before the injection of ara-C.

[c]24 mg/kg rhodium(II) acetate was given right after the injection of ara-C.

Female BDF_1 mice with 6 day implants of 5 x 10^5 L1210/S cells were injected introperitoneally with rhodium(II) acetate in 0.9% saline or 0.9% saline alone. The mice were treated intraperitoneally with 1μmole of ara-C-^{3}H (5 mCi/mmole) and sacrificed 30 minutes later. Radioactivity of the nucleotides was determined in the cells which were washed four times with 0.9% saline.

24 mg/kg injection of rhodium(II) acetate. Since this compound has been reported to be unstable in vivo (43), two time intervals between ara-C and rhodium(II) acetate injections were studied. No significant differences of phosphorylation between simultaneous injection of these two compounds or one hour delayed injections of ara-C could be observed.

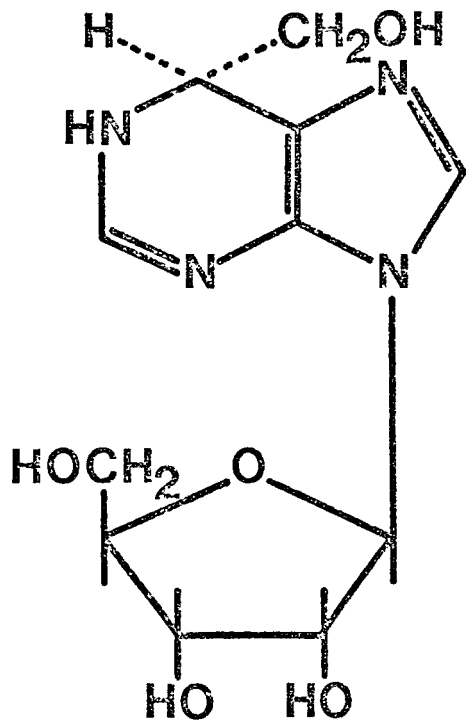
Inhibition of Deamination of Ara-A by 1,6-dihydro-6-hydroxymethylpurine riboside (DHMPR)

The distribution of deaminases in mammalian cells differ for each of the two nucleosides, cytidine and adenosine. The murine L1210/S tumor is responsive to ara-C and has little capacity for deamination of ara-C. Also, ara-C has been found to be the most effective single agent in the treatment of leukemia of adults (9). L1210/S, however, is unresponsive to ara-A, because it has very high levels of adenosine deaminase (44,45). Since it has been demonstrated that there is lack of cross-resistance between ara-C and ara-A (46), a suitable inhibitor for the deamination of ara-A would provide alternative therapy.

One of the adenosine deaminase inhibitors studied to improve the efficacy of ara-A in the treatment of neoplasia (L1210/S) was DHMPR (Figure 5). This compound was first described as a transition state analog (and a potent inhibitor of adenosine deaminase with an apparent dissociation constant for calf duodenum adenosine deaminase of

Figure 5

1,6-dihydro-6-hydroxymethylpurine riboside (DHMPR)


H
CH2OH
HN
N
N
N
HOCH2
O
HO
HO

7.6×10^{-7}M) by Wolfenden (29) and was reported recently to be an intermediate in the chemical synthesis of coformycin by Ohno et al. (47). DHMPR was prepared and identified by Dr. E. Sherwood using the method of Evans and Wolfenden (29). The mass spectra, NMR, and ultraviolet spectra indicated that the compound synthesized by Dr. E. Sherwood was identical with the one that Evans and Wolfenden studied.

In the study of inhibition of adenosine deaminase by DHMPR, shown in Table 8, 0.2 μg of calf intestinal mucosa adenosine deaminase (Sigma type I) was put in 1.2 ml cuvettes and 5 minutes later the change in optical density at 265 nm was measured along with the substrate (ara-A, 3.7×10^{-5}M) and DHMPR by Beckman spectrophotometer. The inhibition of the adenosine deaminase was shown to concentration dependent and the 50% inhibitory concentration of DHMPR was found to be around 1×10^{-6}M. A Lineweaver-Burk plot for DHMPR inhibition of ara-A deamination by calf intestinal mucosa adenosine deaminase is shown in Figure 6. In this experiment, the concentration of DHMPR was 1.05×10^{-6}M which gives approximately 50% inhibition of 0.2 μg of calf intestinal mucosa adenosine deaminase and ara-A concentration was ranged from 1.8×10^{-5}M to 9.2×10^{-5}M. Least squares analysis was used to fit the best lines to the data points. The plot showed that DHMPR gave an inhibition of competitive type. A Hill plot was employed, shown in Figure 7, to see whether a critical site on the enzyme

Table 8

Inhibition of Adenosine Deaminase (Calf Intestinal Mucosa)[a] by DHMPR

Concentration of DHMPR (M)	Relative rate	% inhibition
0	100[b]	--
2.7×10^{-7}	73	27
5.3×10^{-7}	66	34
1.3×10^{-6}	42	58
4.0×10^{-6}	20	80
5.3×10^{-6}	18	82

[a]The assay procedure is described under Experimental Methods and Materials.

[b]Deaminase activity is expressed in terms of zero concentration of DHMPR which is arbitrarily assigned a value of 100.

0.2 μg of calf intestinal mucosa adenosine deaminase (sigma type I) was put in 1.2 ml cuvette and 5 minutes later the negative change of optical density at 265 nm was measured in the presence of 3.7×10^{-5}M ara-A.

Figure 6

Lineweaver-Burk Plot for DHMPR Inhibition of Adenosine Deaminase

Reaction mixture contained 0.2 μg calf intestinal mucosa adenosine deaminase, 1.05×10^{-6}M DHMPR in 0.05M sodium phosphate buffer (pH 7.5), and ara-A. The negative change of the optical density at 265 nm was measured 5 minutes after the addition of ara-A at room temperature by Beckman spectrophotometer.

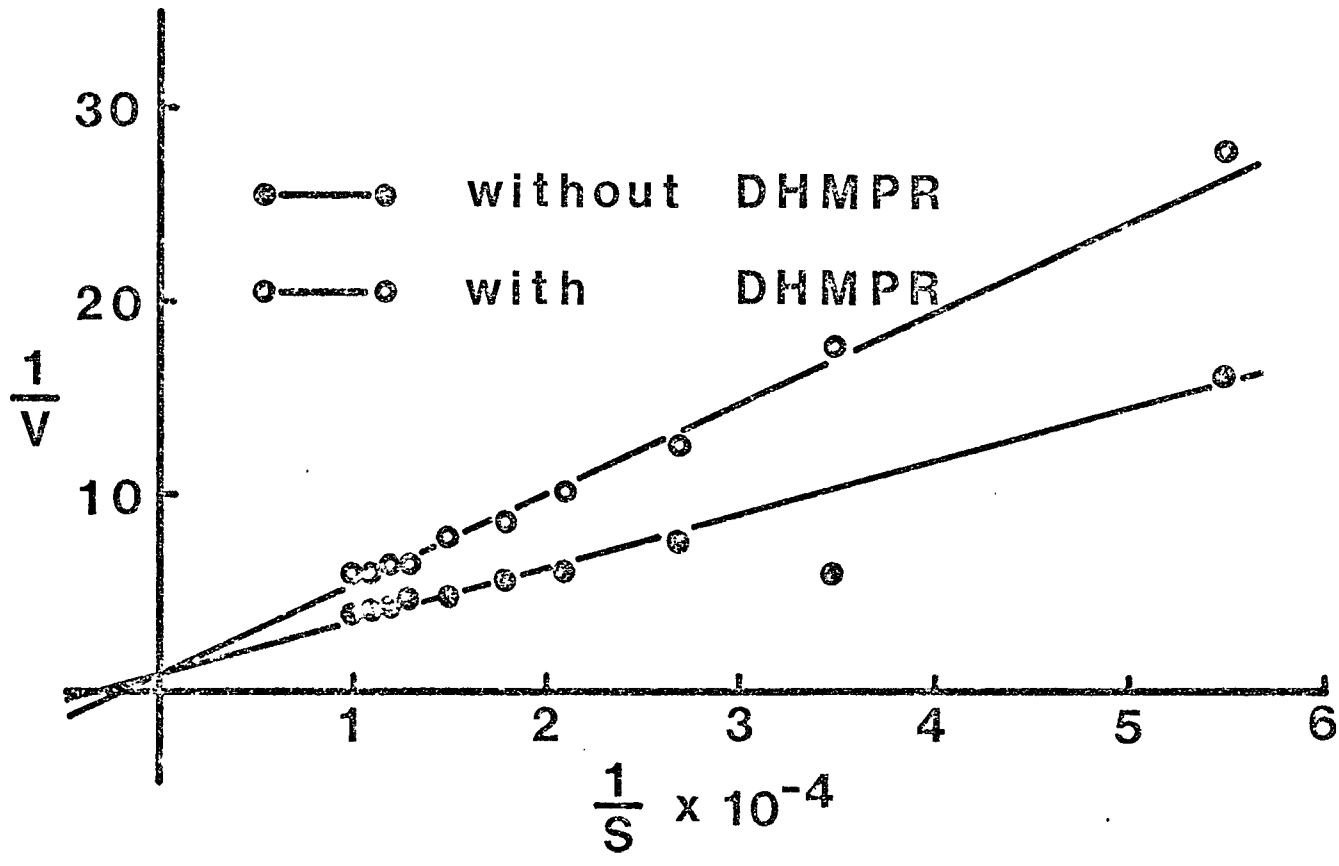
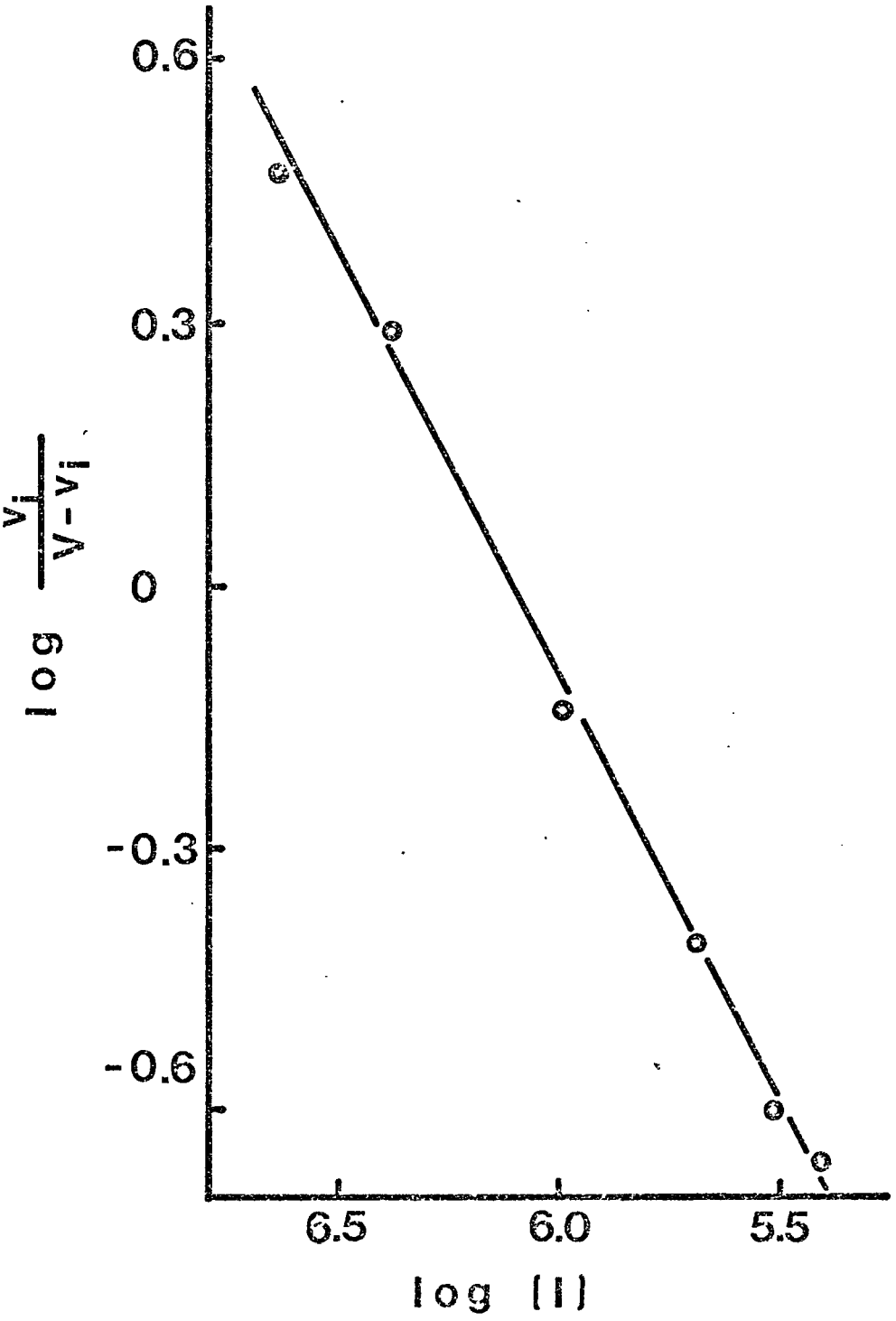

30
20
10
1/V
without DHMPR
with DHMPR
1
2
3
4
5
6
1/S x 10⁻⁴

Figure 7

Hill Plot of the Binding of DHMPR to Adenosine Deaminase


0.6
0.3
0
-0.3
-0.6
log $\frac{v_i}{V-v_i}$
6.5
6.0
5.5
log [I]

was involved in binding of DHMPR. The Hill plot in this study has a slope of one. When L1210/S cells suspended in Krebs-Ringer phosphate buffer of 0.8mM ara-A and various concentrations of DHMPR, shown in Table 9, the inhibition was concentration dependent manner and the 50% inhibitory concentration of DHMPR was found to be 8.3×10^{-6}M. *In vivo* duration of the inhibition of adenosine deaminase of L1210/S cells in female BDF_1 by DHMPR was also studied. BDF_1 mice (two mice each group) were injected intraperitoneally with 20 mg/kg of DHMPR (nontoxic concentration determined by toxicity test) in 0.2 ml of 0.9% saline, 5 days after the implants of 5×10^5 L1210/S cells. Every 4 hours, L1210/S cells were collected from the mice, homogenized with a Potter-Elvehjem homogenizer, centrifuged (2 minutes at 1,500 x g), and adenosine deaminase activity was measured as described previously. The data, shown in Table 10, indicate that at this concentration of DHMPR there was no inhibition at all during a 24 hour period. When 100 mg/kg of this compound (toxic level) was injected, there was 22% inhibition of ara-Hx formation of the control group after one hour of the injection.

There is a report that DHMPR seems to be easily oxidized to 6-hydroxymethylpurine ribonucleoside (29) whose apparent dissociation constant for calf duodenum adenosine deaminase is 12 fold higher than that of DHMPR. To answer the question of whether the *in vivo* noninhibitory activity

Table 9

Inhibition of Adenosine Deaminase in L1210/S Cells[a] by DHMPR

Concentration of DHMPR(M)	nmoles of ara-Hx formed per 1 x 10^8 cells per 15 minutes	% inhibition
0	441	--
8.3×10^{-6}	225	49
1.7×10^{-5}	218	51
3.3×10^{-5}	169	62
5.0×10^{-5}	134	70
6.6×10^{-5}	125	72

[a]The assay procedure is described under Experimental Methods and Materials.

1 x 10^8 L1210/S cells from BDF_1 mice were incubated with 0.8mM of ara-A-2-^{3}H (2 mCi/mmole) for 15 minutes at 37°C in Krebs-Ringer phosphate buffer with or without DHMPR.

Table 10

Duration of Inhibition of Adenosine Deaminase[a] in L1210/S Cells from BDF_1 Mice Injected with DHMPR[b]

Hours	nmoles of ara-Hx formed per 1×10^8 cells per 15 minutes
0	358
4	403
8	393
12	390
16	406
20	374
24	385
0[c]	313
1	244

[a]The assay procedure is described under Experimental Methods and Materials.

[b]Each mouse was injected with 20 mg/kg of DHMPR in 0.9% NaCl solution (0.2 ml, i.p.).

[c]Each mouse was injected with 100 mg/kg of DHMPR in 0.9% NaCl solution (0.2 ml, i.p.).

1×10^8 L1210/S cells from DHMPR injected mice were incubated with 0.8mM of ara-A-2-^{3}H (2 mCi/mmole) for 15 minutes at 37°C in Krebs-Ringer phosphate buffer.

of DHMPR can be abolished by an antioxidant, an identical experiment in which 10mM ascorbic acid instead of 0.9% saline was used as the solvent was performed. The data, shown in Table 11, indicate that this concentration of ascorbic acid did not improve the in vivo inhibitory activity of DHMPR against L1210/S adenosine deaminase.

The Effect of DHMPR on Phosphorylation of Ara-A

Enzymatic studies have suggested that inhibitory effects of ara-A on DNA synthesis and cell viability may result from the inhibition of DNA polymerase (48) and possibly from the inhibition of ribonucleotide reductase by ara-ADP and ara-ATP (49). Ara-ATP also serves as a substrate for a DNA polymerase (50). Even though DHMPR did not show the in vivo inhibition of adenosine deaminase in BDF_1 mice, the study of in vitro effects of DHMPR on the metabolism of ara-A would be interesting, utilizing this compound as an experimental tool. L1210/S cells suspended in Krebs-Ringer phosphate buffer solution supplemented with 10mM glucose were incubated for 15 minutes at 37°C in the presence of 0.8mM ara-A, 3.5μM 2'deoxycoformycin, and various concentrations of DHMPR. Data, shown in Table 12, show that at these concentrations, DHMPR does not have any effect on the phosphorylation of ara-A. Also, this concentration of 2'-deoxycoformycin has no effect on the phosphorylation of ara-A (A. P. Kimball, personal communication).

Table 11

Duration of Inhibition of Adenosine Deaminase[a] in L1210/S Cells from BDF_1 Mice Injected with DHMPR[b] in Ascorbic Acid Solution

Hours	nmoles of ara-Hx formed per 1×10^8 cells per 15 minutes
0	301
4	319
8	331
12	333
16	341
20	314
24	318

[a]The assay procedure is described under Experimental Methods and Materials.

[b]Each mouse was injected with 20 mg/kg of DHMPR in 10mM ascorbic acid solution (0.2 ml, i.p.).

1×10^8 cells from DHMPR injected mice were incubated with 0.8mM of ara-A-2-^{3}H (2 mCi/mmole) for 15 minutes at 37°C in Krebs-Ringer phosphate buffer.

Table 12

The Effect of DHMPR on the Phosphorylation of Ara-A in L1210/S Cells

Concentration of DHMPR(M)	nmoles of nucleotides of ara-A formed per 1 x 10^8 cells per 15 minutes	% inhibition
0	165.2	--
8.9×10^{-4}	139.6	15
8.9×10^{-5}	137.0	17
8.9×10^{-6}	171.6	0

[a]The assay procedure is described under Experimental Methods and Materials.

1 x 10^8 L1210/S cells from BDF_1 mice were incubated with 0.8mM ara-A-2-^3H (2 mCi/mmole) for 15 minutes at 37°C in the presence of 3.5μM 2'-deoxycoformycin and DHMPR in Krebs-Ringer phosphate media supplemented with 10mM glucose.

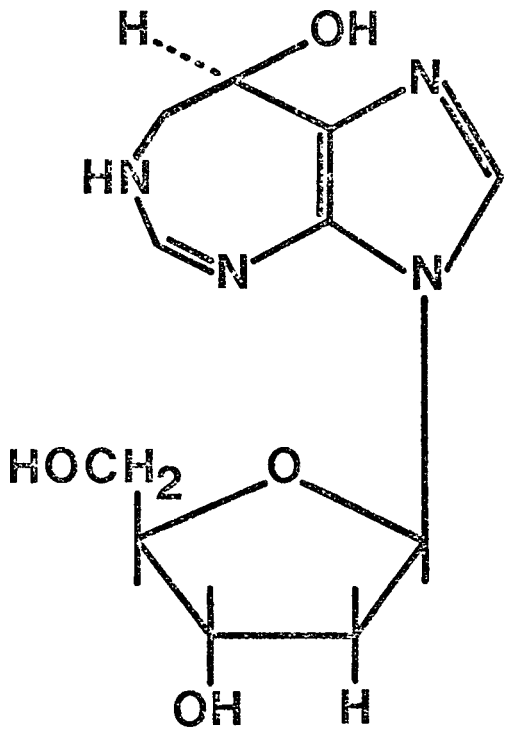
Inhibition of Adenosine Deaminase by 2'-deoxycoformycin

Recently, a potent adenosine deaminase inhibitor became available (30) and was identified as 2'-deoxycoformycin (Figure 8). Because of the unusually strong inhibition caused by this compound it has not been possible by the application of standard procedures for the study of enzyme inhibitors to determine with precision the Ki value of this compound. Recently, Cha et al. studied a series of tight-binding inhibitors and their kinetic behavior (51,52) and estimated that the Ki value of coformycin, which is the analog of 2'-deoxycoformycin and displays some of the features of a transition state inhibitor as described by Lienhard (53), is in the range of 1×10^{-10}M to 1×10^{-11}M (52). The preliminary study of 2'-deoxycoformycin showed that the Ki value of this compound is in more or less the same range as that of coformycin. Kimball et al. reported that there was a significant synergistic effect when ara-A was used with 2'-deoxycoformycin in the treatment of mice bearing ascitic L1210/S or L1210/ara-C cells (46,54). Some of the biochemical and survival studies with female BDF_1 mouse brain adenosine deaminase and BDF_1 mice with CNS L1210/S with combinations of ara-A and 2'-deoxycoformycin were carried out to see the possible synergistic effects of these two compounds against the mouse CNS tumor.

The important point between tight-binding inhibitors and enzymes under ordinary experimental conditions is the

Figure 8

2'-deoxycoformycin


H
OH
N
HN
N
N
HOCH2
O
OH
H

prolonged non-steady state phase (52,55). To observe the possibility of this effect of 2'-deoxycoformycin on partially purified adenosine deaminase from BDF_1 mouse brain (see Experimental Methods and Materials), the enzyme was preincubated with 1.2×10^{-6}M and 5×10^{-7}M 2'-deoxycoformycin for up to 40 minutes before the addition of 6.9×10^{-4}M ara-A. Data, shown in Figure 9, show that the non-steady state phase between 2'-deoxycoformycin and the adenosine deaminase is remarkably long and there is no significant difference of the activity of this compound on the adenosine deaminase in terms of inhibition at these two inhibitor concentrations. When partially purified brain adenosine deaminase (2.3 mg of protein) was preincubated with various concentrations of 2'-deoxycoformycin for 60 minutes at 37°C and incubated for 30 minutes after the addition of 0.7mM ara-A (Table 13), the 100% inhibitory concentration of 2'-deoxycoformycin was found to be approximately 5.2×10^{-9}M. A plot from these data, v_o/v_i vs 2'-deoxycoformycin concentration, shown in Figure 10, indicates that the I_{50} of 2'-deoxycoformycin for 2.3 mg of partially purified female BDF_1 mouse brain adenosine deaminase is 1.1×10^{-10}M. In an identical experiment in which 5.2×10^{-12}M 2'-deoxycoformycin was used, shown in Figure 11, the inhibition was approximately proportional to the amount of the protein, indicating a "titration" of the adenosine deaminase. The data, shown in Table 13, show a Hill coefficient of

Figure 9

Inhibition of Adenosine Deaminase by 2'-deoxycoformycin

Partially purified adenosine deaminase in Krebs-Ringer phosphate buffer and ara-A-2-^{3}H (6.9×10^{-4}M, 2 mCi/mmole) were incubated for 30 minutes at 37°C.

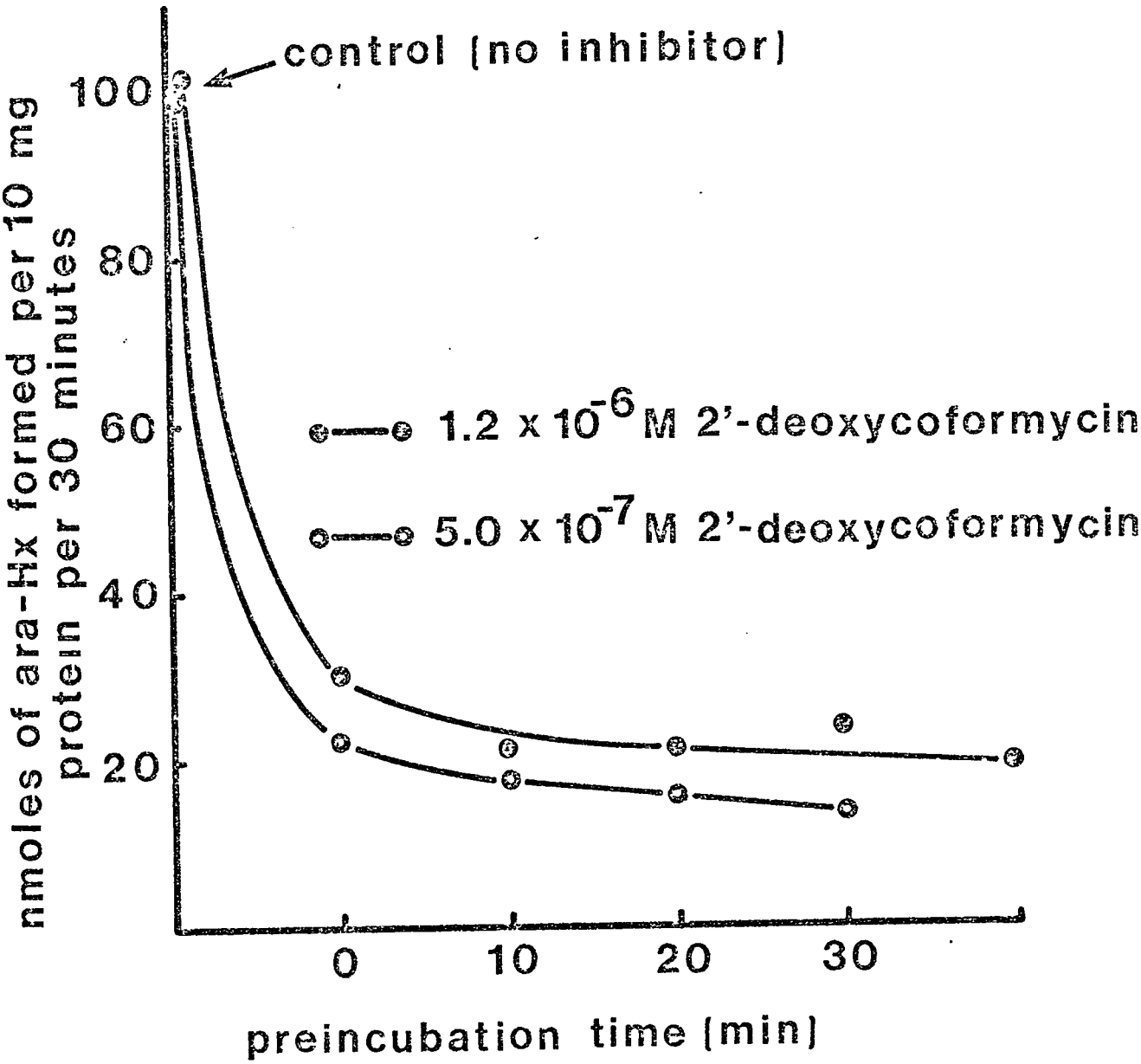

control (no inhibitor)
100
80
60
40
20
nmoles of ara-Hx formed per 10 mg protein per 30 minutes
1.2 x 10⁻⁶ M 2'-deoxycoformycin
5.0 x 10⁻⁷ M 2'-deoxycoformycin
0
10
20
30
preincubation time (min)

Table 13

Inhibition of Partially Purified BDF_1 Mouse Brain Adenosine Deaminase[a] by 2'-deoxycoformycin

Concentration of 2'-deoxycoformycin (M)	nmoles of ara-Hx formed per 10 mg protein per 30 minutes	% inhibition
0	103.2	--
5.2×10^{-9}	0	100
5.2×10^{-10}	12.2	90.1
2.6×10^{-10}	30.2	71.4
5.2×10^{-11}	58.0	44.1
5.2×10^{-12}	94.0	9
5.2×10^{-13}	101.4	2

[a]The assay procedure is described under Experimental Methods and Materials.

Partially purified adenosine deaminase was incubated for 30 minutes with 0.7mM ara-A-2-^{3}H (2 mCi/mmole) in Krebs-Ringer phosphate buffer after 60 minutes preincubation with or without 2'-deoxycoformycin at 37°C.

Figure 10

Determination of I_{50}

A plot of v_o/v_i vs 2'-deoxycoformycin concentration (2.3 mg of protein from BDF_1 mouse brain supernatant was used).

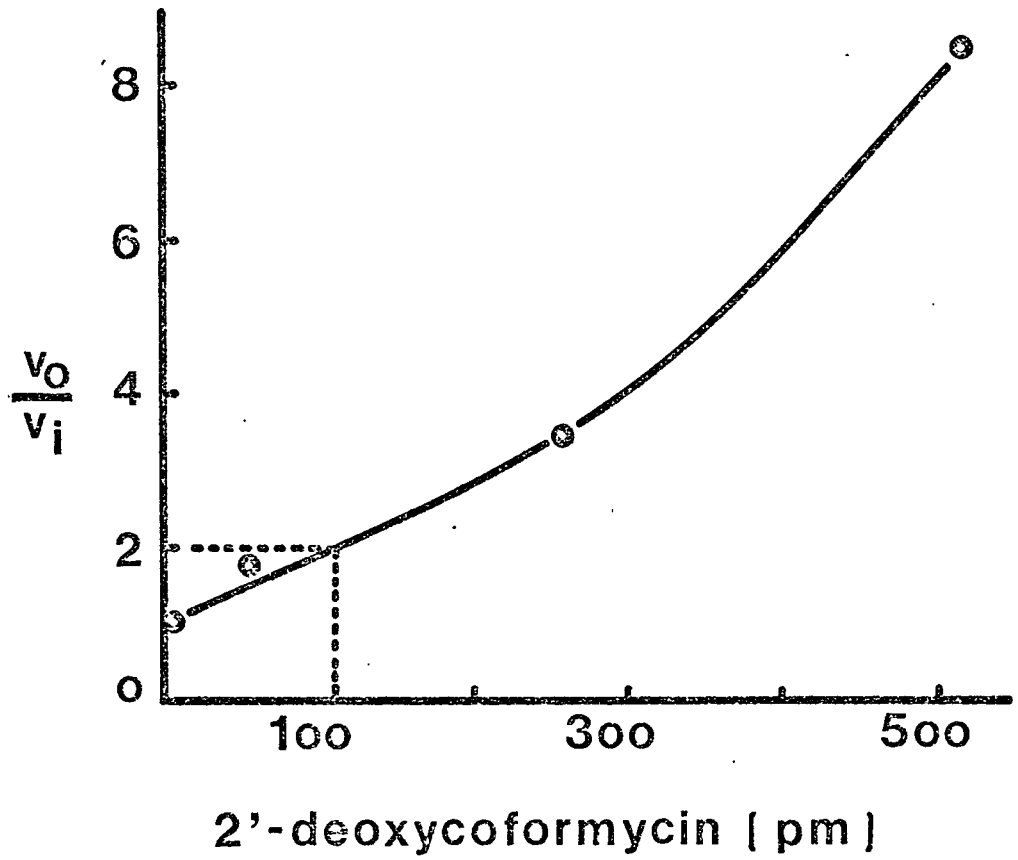
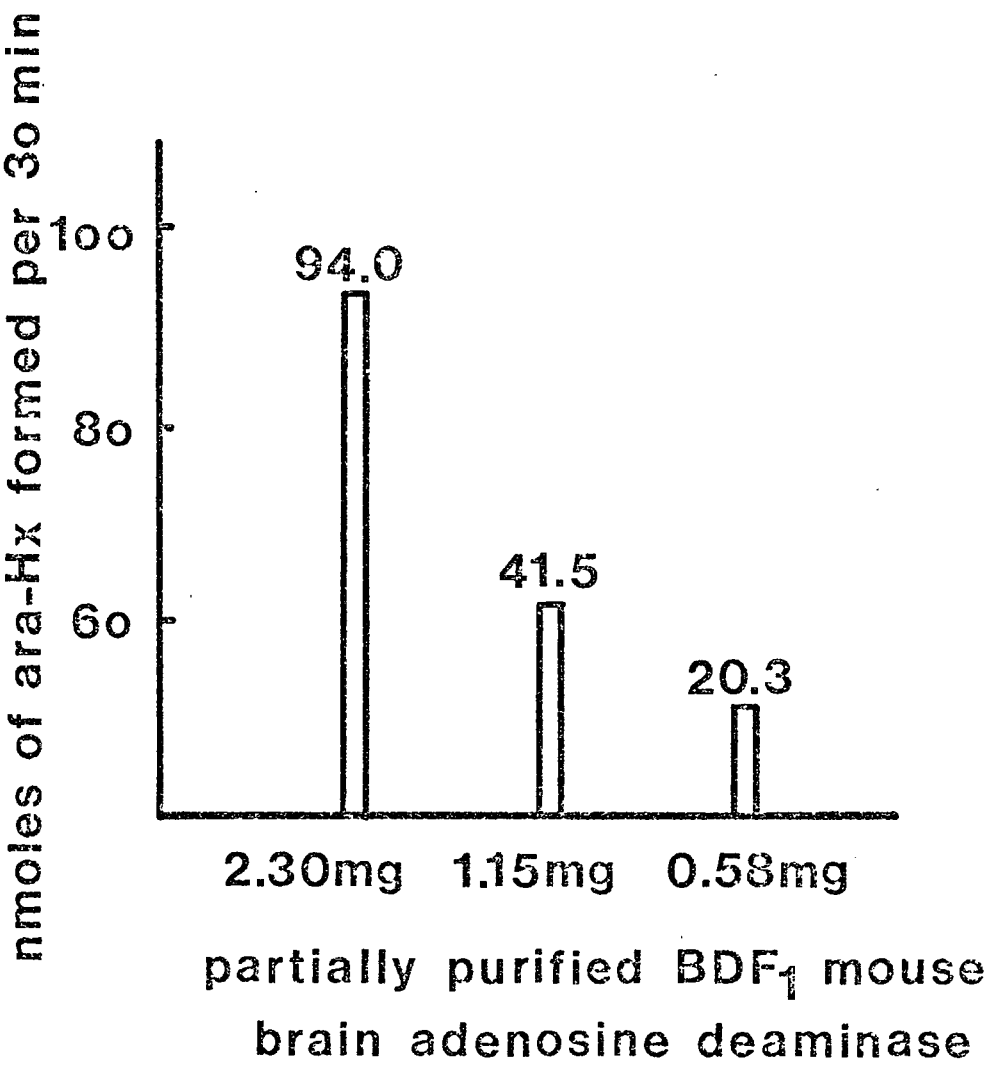

8
6
4
2
0
$\frac{V_o}{V_i}$
100
300
500
2'-deoxycoformycin [pm]

Figure 11

Inhibition of Partially Purified BDF_1 Mouse Brain Adenosine Deaminase by 2'-deoxycoformycin

Partially purified adenosine deaminase was incubated for 30 minutes with 0.7mM ara-A-2-^{3}H (2 mCi/mmole) in Krebs-Ringer phosphate buffer after 60 minutes preincubation at 37°C with 5.2 x 10^{-12}M 2'-deoxycoformycin.


nmoles of ara-Hx formed per 30 min
100
80
60
94.0
41.5
20.3
2.30mg
1.15mg
0.58mg
partially purified BDF1 mouse
brain adenosine deaminase

approximately one indicating that the titration is stoichiometric and on a one-to-one basis between 2'-deoxycoformycin and the adenosine deaminase.

Female BDF_1 mice (two mice each group) were injected intraperitoneally with 1 mg/kg, 0.5 mg/kg, and 0.25 mg/kg of 2'-deoxycoformycin in 0.2 ml of physiological saline to see the in vivo duration of inhibition of brain adenosine deaminase. At 0.25, 1, 3, 6, 10, 24, and 27 hours after the injection, the brain was excised after cervical dislocation, homogenized with a Potter-Elvehjem homogenizer, centrifuged (2 minutes at 1,500 x g), and the adenosine deaminase activity was measured. As indicated in Table 14, at these concentrations of 2'-deoxycoformycin, the inhibition was effective within 15 minutes and the inhibition lasted for at least 27 hours.

Survival Studies

A series of experiments was performed to test the effects of ara-A and 2'-deoxycoformycin in the therapy of mouse CNS L1210/S tumor. These data are presented in Table 15. Combination therapy of ara-A (50 mg/kg, 8 times daily on days 1 and 4) and 2'-deoxycoformycin (1 mg/kg, once daily on days 1 and 4) gave the highest T/C of 169. Since 1 mg/kg of 2'-deoxycoformycin (once daily on days 1 and 4) injection to CNS L1210/S tumor bearing mice gaving a lower T/C than that of control group, 0.5 mg/kg of

Table 14

Duration of Inhibition of Adenosine Deaminase of BDF_1 Mouse Brain[a] Injected with 2'-deoxycoformycin, i.p.

	Hours	nmoles of ara-Hx formed per 10 mg of protein	% inhibition
Exp[b]	0.25	6.9	62
	1.0	5.9	67
	3.0	6.2	66
	6.0	6.4	65
	10.0	8.1	55
	24.0	8.0	56
	27.0	5.5	70
	control	18.1	--
Exp[c]	0.25	4.3	74
	1.0	4.7	72
	3.0	4.4	74
	6.0	3.9	77
	10.0	4.0	76
	24.0	5.0	70
	27.0	17.4	--
	control	16.8	--

Table 14 (continued)

	Hours	nmoles of ara-Hx formed per 10 mg of protein	% inhibition
Exp[d]	0.25	9.0	69
	1.0	10.3	64
	3.0	15.3	47
	6.0	9.9	65
	10.0	10.7	63
	24.0	10.8	62
	27.0	11.6	59
	control	28.6	--

[a]The assay procedure is described under Experimental Methods and Materials.

[b]Each mouse was injected with 1 mg/kg of 2'-deoxycoformycin in 0.9% saline (0.2 ml, i.p.).

[c]Each mouse was injected with 0.5 mg/kg of 2'-deoxycoformycin in 0.9% saline (0.2 ml, i.p.).

[d]Each mouse was injected with 0.25 mg/kg of 2'-deoxycoformycin in 0.9% saline (0.2 ml, i.p.).

After cervical dislocation brains were excised, homogenized with a Potter-Elvehjem homogenizer, centrifuged (2 minutes at 1,500 x g), and 0.5 ml of the supernatant was incubated with ara-A-2-^{3}H (8.4×10^{-4}M, 2 mCi/mmole) for 30 minutes at 37°C in Krebs-Ringer phosphate buffer.

Table 15

Treatment of CNS L1210/S Tumor Bearing Female BDF_1 Mice with Ara-A and 2'-deoxycoformycin

Exp. No.	No. of Mice	Treatment	Median Survival Days	T/C
1	6	control - 0.9% saline, 1 ml/mouse	8.0 ± 0.5	100
	6	ara-A, 50 mg/kg, q3H, 8x on days 1,4	8.5 ± 0.3	106
	6	ara-A, 50 mg/kg, q3H, 8x on days 1,4; 2'-dCF, 1 mg/kg	13.5 ± 0.8	169
2	6	control - 1% CMC in 0.9% saline, 0.5 ml/mouse	9.0 ± 0.5	100
	6	ara-A, 50 mg/kg, q3H, 8x on days 1,4,7	10.0 ± 0.3	111
	6	ara-A, 50 mg/kg, q3H, 8x on days 1,4,7; 2'-dCF, 0.5 mg/kg	12.0 ± 1.0	133
3	6	control - 0.9% saline, 1 ml/mouse	9.0 ± 0.7	100
	6	2'-deoxycoformycin	8.5 ± 0.5	94
	6	ara-A, 25 mg/kg, q3H, 8x on days 1,4,7; 2'-dCF, 1 mg/kg	11.0 ± 0.6	122

Table 15 (continued)

Exp. No.	No. of Mice	Treatment	Median Survival Days	T/C
4	6	control - 0.9% saline, 1 ml/mouse	10.0 ± 0.7	100
	6	ara-A, 45 mg/kg, q3H, 8x on days 1,4,7; 2'-dCF, 0.5 mg/kg	12.5 ± 0.7	125
	6	ara-A, 30 mg/kg, q3H, 8x on days 1,4,7; 2'-dCF, 0.5 mg/kg	11.0 ± 1.0	110

L1210/S cells were transplanted in BDF_1 mice, intracranially, 2.5×10^4 cells/mouse. The day of tumor transplantation is designated day 0. 2'-deoxycoformycin was given only once daily one hour prior to the first ara-A injection.

2'-deoxycoformycin (once daily on days 1, 4, and 7), which has been reported to be a nontoxic level (46) was given. The T/C of this treatment, however, was lower than that of 1 mg/kg treatment by about 21%. Any treatment of ara-A whose amount was less than 50 mg/kg (8 times daily on days 1 and 4) gave a T/C lower than 169.

DISCUSSION

Ara-C which has been known to be a drug of choice in the treatment of acute leukemia (9) was reported to act synergistically with rhodium(II) acetate to inhibit the growth of the L1210/S tumor in mice (28). The studies reported here have shown that rhodium(II) acetate inhibits ara-C deamination. Lineweaver-Burk and a Hill plots of the inhibition of cytidine deaminase indicate that rhodium(II) acetate binds to the enzyme in the vicinity of the catalytic center on a one-to-one molar basis. This inhibition of cytidine deaminase by rhodium(II) acetate was expected since mammalian cytidine deaminase has been reported to be inhibited by sulfhydryl reagents such as p-chloromercuribenzoate (31). Rhodium(II) acetate has two axial ligand positions which are available for ligand formation with electron-donating groups. The actual binding of rhodium(II) acetate to sulfhydryl group of this enzyme was not studied. Because of the insensitivity of mammalian deoxycytidine kinase to sulfhydryl group inhibitors (34) and the structural unsimilarity of the rhodium(II) acetate to cytidine analogs, the inhibition of phosphorylation of ara-C by rhodium(II) acetate was somewhat unexpected. The findings of noninhibitory and inhibitory activity of rhodium(II)

acetate on *in vitro* phosphorylation of ara-C when L1210/S cells and cell-free extracts from L1210/S cells were used, respectively, raised the question of the penetration of rhodium(II) acetate through the cell membrane. The inhibition of the *in vitro* phosphorylation of ara-U by rhodium(II) acetate in L1210/S cells, however, suggests the possibility of the permeability of this compound to the cell membrane, which is not surprising since this compound has a net charge of zero.

A small portion of the synergistic effect of rhodium(II) acetate and ara-C in L1210/S cell bearing BDF_1 mice may be due to a slight net decrease of deamination and a slight net increase of phosphorylation of ara-C *in vivo*. It was also reported that rhodium(II) acetate had inhibitory effects on DNA synthesis (56). The *in vivo* instability of rhodium(II) acetate (43) would make this compound a less potent inhibitor of cytidine deaminase.

Ara-A is an effective inhibitor of cell growth at a relatively high concentration, i.e., about 1×10^{-4}M (57,58) but the inhibition of mammalian DNA polymerases by ara-ATP has a Ki of 1×10^{-6}M (59). This suggests that the conversion of ara-A in cellular systems to ara-ATP is relatively inefficient. This is due, in large part, to the extensive deamination of ara-A and ara-Hx mainly in the cells or tissues showing high DNA synthesis and intense mitotic activity.

As was expected, adenosine deaminase in L1210/S cells as well as the purified calf intestinal enzyme were inhibited by DHMPR. Lineweaver-Burk and a Hill plots of the inhibition of purified intestinal mucosa adenosine deaminase indicate that DHMPR binds to the enzyme in the catalytic center on a one-to-one basis. The inhibition of calf intestinal mucosa adenosine deaminase by this compound showed a Ki of 1.5×10^{-6}M and the Km was 3×10^{-4}M when ara-A was used as the substrate. These values are about one order of magnitude higher than those reported (29) where calf duodenum adenosine deaminase and adenosine were the enzyme and the substrate, respectively. Because there seems to be no permeability problem when whole L1210/S cells were used in vitro, the failure to observe the in vivo inhibition of deamination of ara-A in BDF_1 mice bearing L1210/S cells by DHMPR suggests an in vivo instability of this compound. Since the proposed oxidation product of DHMPR, 6-hydroxy-methylpurine ribonucleoside, still has inhibitory activity againat calf duodenum adenosine deaminase with an apparent dissociation constant of 9.4×10^{-6}M (29) and an antioxidant, ascorbic acid, did not increase the in vivo inhibitory activity against this enzyme at all, the in vivo instability of this compound is presumably not due to oxidation. Even though there was no effect on phosphorylation observed by DHMPR in vitro, the in vivo animal use of this compound on adenosine deaminase is not feasible unless the mechanism of

breakdown of this compound is found.

The inhibition pattern of 2'-deoxycoformycin against mouse brain adenosine deaminase is similar to that of coformycin, another tight-binding inhibitor of adenosine deaminase; the inhibitory activity of 2'-deoxycoformycin increased with the increase of preincubation time to a certain extent. This effect of preincubation is somewhat unexpected if the concentration of an inhibitor is the only determinant of inhibition under a given experimental condition. Upon the observation of the slow binding of some transition state analogs, Cha and Wolfenden (52,55) hypothesized that there occurred a reorganization of the structure of the enzyme-inhibitor complex and a setting of this complex into a "locked" position. One possible suggestion for this slow-binding is that it might be due to the extremely low concentration of the potent inhibitor required (1×10^{-9}M - 1×10^{-11}M) compared with the majority of inhibitors where larger amounts are needed compared with the amount of enzyme taken to produce a significant inhibition. With 2'-deoxycoformycin concentration of 5×10^{-7}M and 1.2×10^{-6}M, which are 100 and 200-fold higher concentration, respectively, than required for a 100% inhibition of partially purified mouse brain adenosine deaminase, they showed increasing inhibitory activity with increasing preincubation time. The finding that these two high inhibitor concentrations do not give 100% inhibition of the enzyme even after 30 minutes

preincubation explains that the slow-binding is not due only to low concentrations of the inhibitor supporting Cha and Wolfenden's hypothesis.

The findings of *in vivo* fast inhibition (average of 68% inhibition within 15 minutes) of mouse brain adenosine deaminase, and the duration of inhibition of up to 27 hours after the injection without significant change is somewhat surprising in comparison with the *in vitro* studies. Recently, Ma *et al*. reported that human brain has mainly the C-form of adenosine deaminase (60). The fast *in vivo* inhibition observed may indicate that there may be differences in the affinity between 2'-deoxycoformycin and the different forms of adenosine deaminase.

Schaefer reported that analogs of adenosine with a 2'-hydroxyl group bind to the enzyme more tightly than ones without an oxygen atom at the 2' position, and also, the compounds become weaker inhibitors as the 6 position of the purine ring become more highly substituted (61). This increases the size of the inhibitor molecules. The inhibitory activity of 2'-deoxycoformycin, which was reported to be 2 to 3-fold more active than coformycin (62) and which has a larger ring than adenosine, may come mainly from the puckered 7 membered ring (63).

Human pharmacological studies have shown that ara-A is rapidly deaminated to its hypoxanthine derivative. In contrast to ara-C where antitumor and antiviral activity is

limited to the parent compound, both ara-A and its hypoxanthine derivative, ara-Hx, have antiviral activity (64). The findings of the increase of survival time of BDF_1 mice bearing L1210/S cells in combination therapy with ara-A and 2'-deoxycoformycin and a lack of response to ara-A alone suggest that this tumor line may not have ara-Hx utilizing systems. Since adenosine deaminase has a higher Km and Vm than adenosine kinase, it can be speculated that frequent injections of ara-A at a lower dosages may give greater survival times of tumor bearing mice. In the presence of a potent adenosine deaminase inhibitor the intracellular concentration of ara-A may not need to be maintained at 1×10^{-4}M in order to obtain the inhibitory ara-ATP concentration. This might solve another problem of utilizing ara-A as an antitumor and antiviral agent that of its low solubility in physiological saline which is not more than 2 μmoles/ml at room temperature.

SUMMARY

Studies with rhodium(II) acetate show that the compound can inhibit not only the cytidine deaminase partially purified from Swiss albino mice kidneys but also the deoxycytidine kinase in L1210/S cells *in vitro*. BDF_1 mice injected with rhodium(II) acetate show a slight increase in net phosphorylation and a slight decrease in net deamination of ara-C in L1210/S cells *in vivo*. This slight increase of phosphorylation of ara-C *in vivo* may not be enough to explain the synergistic effect observed in combination therapy of ara-C and rhodium(II) acetate in BDF_1 mice bearing L1210/S tumors.

Studies with DHMPR show that the compound inhibits *in vitro* mouse adenosine deaminase but not adenosine kinase in L1210/S cells *in vitro*. The studies of deamination of ara-A with DHMPR in L1210/S bearing BDF_1 mice show that this compound does not inhibit adenosine deaminase suggesting that this compound is labile in the *in vivo* environment. The noninhibitory product produced *in vivo* does not seem to be the oxidation product of DHMPR.

Studies with 2'-deoxycoformycin show that this is a potent inhibitor of partially purified BDF_1 mouse brain adenosine deaminase with an I_{50} of 100pM when 2.3 mg of

protein was used. BDF_1 mouse brain from mice injected intraperitoneally with nontoxic concentration of this compound (0.25 mg/kg) shows that about a 70% inhibition of the adenosine deaminase occurs within 15 minutes after the injection and that the inhibition lasts more than 24 hours. In vitro studies of partially purified mouse brain adenosine deaminase with this compound show the characteristic of tight-binding inhibitors; a prolonged non-steady state phase and an inhibition approximately proportional to the amount of protein used.

Survival studies of BDF_1 mice implanted with L1210/S intracranially show significant increase in life span when ara-A are used in combination with 2'-deoxycoformycin. The group treated with ara-A alone showed no increase in life span.

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